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Contributor contact details

(* = main contact)

Editor

Dr Øyvind Lie
Director of National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: oyvind.lie@nifes.no

Chapter 1

Professor J. Tuomisto*
Department of Environmental Health
KTL (National Public Health Institute)
PO Box 95
FI-70701 Kuopio
Finland
E-mail: jouko.tuomisto@ktl.fi

Dr L. Frøyland
National Institute of Nutrition and Seafood Research (NIFES)
PO Box 2029
Nordnes
N-5817 Bergen
Norway
E-mail: livar.froyland@nifes.no

Chapter 2

Dr Anne-Katrine Lundebye* and Dr Marc H. G. Berntssen
National Institute of Nutrition and Seafood Research (NIFES)
PO Box 2029
Nordnes
N-5817 Bergen
Norway
E-mail:
anne-katrine.haldorsen@nifes.no
marc.berntssen@nifes.no

Chapter 3

D. Little*, G. Milwain and C. Price
Institute of Aquaculture
University of Stirling
Stirling
Stirlingshire
Scotland FK9 4LA
UK
E-mail: d.c.little@stir.ac.uk

Chapter 4

Dr B. Lunestad*
National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: blu@nifes.no

Dr O. Samuelsen
Fish Disease Group
Institute of Marine Research
P.O. Box 1870
Nordnes
5817 Bergen
Norway
E-mail: ole.samuelsen@imr.no

Chapter 5

Dr R. Reimschuessel
VMD
US Food and Drug Administration (USFDA)
Office of Research
8401 Muirkirk Road
Laurel, MD 20708
USA
E-mail: rreimsch@cvm.fda.gov
renate.reimschuessel@fda.hhs.gov

Chapter 6

Professor H. Sørum
Norwegian School of Veterinary Science
Department of Food Safety and Infection Biology
Section for Microbiology, Immunology and Parasitology
P.O. Box 8146 Dep.
NO-0033 Oslo
Norway
E-mail: henning.sorum@veths.no

Chapter 7

Dr Ø. Bergh* and Dr A. H. Nerland
Institute of Marine Research
P.O. Box 1870
Nordnes
5817 Bergen
Norway
E-mail: oivind.bergh@imr.no
audun.nerland@imr.no

Chapter 8

Dr F. J. Gatesoupe
INRA – Ifremer
UMR1067 Nutrition Aquaculture and Genomics
Ifremer
BP 70
F-29280 Plouzané
France
E-mail: joel.gatesoupe@ifremer.fr

Chapter 9

Dr M. Espe
National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: marit.espe@nifes.no

Chapter 10

Dr T. Gjedrem
Akvaforsk
Institute of Aquaculture Research
P.O. Box 5010
1432 Ås
Norway
E-mail: trygve.gjedrem@nofima.no

Chapter 11

Dr J. G. Bell
Institute of Aquaculture
University of Stirling
Stirling
Scotland FK9 4LA
UK
E-mail: g.j.bell@stir.ac.uk

Chapter 12

Dr S. J. Kaushik*
INRA
UMR1067 Nutrition Aquaculture and Genomics
Pôle d'hydrobiologie
CD918
64310 St-Pée-sur-Nivelle
France
E-mail: kaushik@st-pee.inra.fr

Dr G. Hemre
National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: gro-ingunn.hemre@nifes.no

Chapter 13

Professor R. Hardy
University of Idaho
3059F National Fish Hatchery Road
Hagerman, ID 83332
USA
E-mail: rhardy@uidaho.edu

Chapter 14

Dr J. Luten*
Nofima Marine
Muninbakken 9-13
P.O. Box 6122
9291 Tromsø
Norway
E-mail: joop.luten@nofima.no

E. Schram
Wageningen IMARES
P.O. Box 68
1970 AB IJmuiden
The Netherlands
E-mail: edward.schram@wur.nl

Professor E. Elvevoll
Institute for Marine Biotechnology
University of Tromsø
Tromsø
Norway
E-mail: edel.elvevolle@nfh.uit.no

Chapter 15

Dr Rune Waagbø
National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: rune.waagbo@nifes.no

Chapter 16

Dr B. Lunestad*
National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: blu@nifes.no

Dr J. T. Rosnes
Norconserv AS
Seafood Processing Research
Niels Juelsgate 50
P.O. Box 327
4002 Stavanger
Norway
E-mail: jtr@norconserv.no

Chapter 17

Dr A. Levsen* and Dr B. Lunestad
National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: arne.levsen@nifes.no

Professor B. Berland
Department of Biology
University of Bergen
Bergen
Norway

Chapter 18

Dr S. Davies
School of Biological Sciences
University of Plymouth
Drake Circus
Plymouth PL4 8AA
UK
E-mail: S.Davies@plymouth.ac.uk

Chapter 19

Dr Z. Papp
Research Institute for Fisheries Aquaculture and Irrigation
P.O. Box 47
5541 Szarvas
Hungary
E-mail: gyongy@haki.hu

Chapter 20

Dr Børge Damsgård
Nofima Marine
Muninbakken 9-13
P.O. Box 6122
9291 Tromsø
Norway
E-mail: borge.damsgaard@ nofima.no

Chapter 21

Dr M. Jahncke* and Dr M. Schwarz
Virginia Seafood AREC
Virginia Tech
102 South King Street
Hampton, VA 23669
USA
E-mail: mjahncke@vt.edu
mschwarz@vt.edu

Chapter 22

Dr A. Maage*, H. Hove and K. Julshamn
National Institute of Nutrition and Seafood Research (NIFES)
P.O. Box 2029
Nordnes
5817 Bergen
Norway
E-mail: ama@nifes.no

Chapter 23

Dr M. Lees* and F. Thomas
Eurofins Scientific Analytics
B.P. 42301
44323 Nantes
France
E-mail: MicheleLees@eurofins.com

Chapter 24

Ms Pendi Najran
Food Standards Agency (FSA)
Labelling, Standards and Allergy Division
Aviation House
125 Kingsway
London WC2B 6NH
UK
E-mail: pendi.najran@foodstandards.gsi.gov.uk

1

Introduction

T. Børresen, Technical University of Denmark, Denmark

1.1 Introduction

While most consumers perceive seafood as healthy and nutritious, there is a lack of a thorough understanding, enabling comparison across Europe, of aspects that determine the levels of seafood consumption, such as consumers' motives, barriers, quality perception and information requirements. It is well known that a diet containing seafood, and in particular omega 3 fatty acids, will reduce the occurrence of cardiovascular diseases, but less is known about the other health benefits of a seafood diet. Some consumers are concerned about seafood safety, as some products may contain contaminants that lead to illness or components which could possibly lead to long-term negative effects on health. It is therefore important to reduce these obstacles so that consumers can obtain all the benefits of seafood consumption.

The objective of this book is to provide an overview of the most recent research into understanding the consumers' attitudes toward seafood, the essential factors relating to seafood nutrition, safety and eating quality, as well as the opportunities for new supplies presented by aquaculture. The opportunity to compile this overview was very timely as the lead authors were the coordinators and project leaders of the Integrated Research Project SEAFOODplus. The overall approach to the research for this project is illustrated in Fig. 1.1, in which the seafood production chain is shown. A fork-to-farm consideration was applied, starting with the factors of importance for consumers' health and well-being, and working backwards in the chain, seeking the best raw materials and processing conditions. This leads to seafood products with optimal features with regard to nutrition, safety and eating quality.

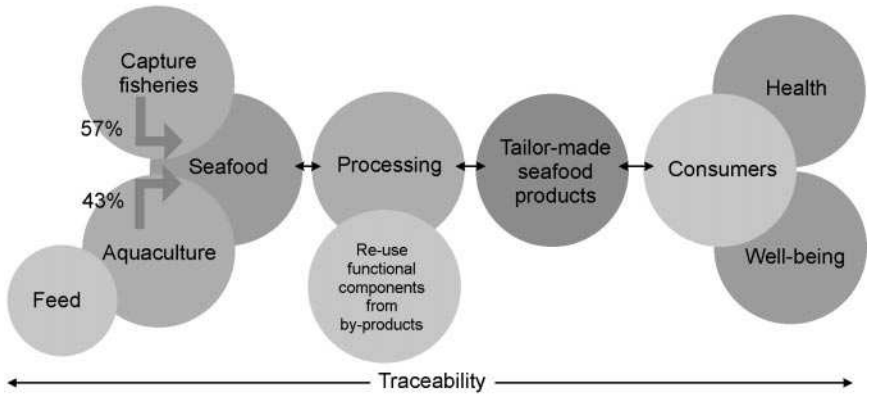

Fig. 1.1 The overall approach for research within SEAFOODplus. Percentages for amounts of fish originating from capture fisheries and from aquaculture are given by FAO statistics 2006 (FAO, 2007) and are likely to shift to higher percentages originating from aquaculture in the future.

The book is divided into parts and chapters reflecting this approach, starting with studies of how to gain a better understanding of consumer perceptions of seafood and how messages about seafood nutrition and safety can best be communicated to them. Seafood consumption is balanced between positive health messages and frequently observed statements about the potential risks from environmental contaminants, particularly in oily fish. This is unfortunate, as oily fish are also the species that contain most of the health-positive omega 3 fatty acids. It should be emphasised that the negative statements concern *potential* risks, as most of the experimental background for recommending reduced seafood intake results from animal studies, often with exaggerated doses of the different components. Obviously, intervention studies with contaminants cannot be performed on human beings, and until now few epidemiological studies have allowed firm conclusions to be drawn. However, some evidence is gathering to show that consumers who have ingested large amounts of seafood with high contaminant levels are actually performing better in health tests than comparable groups with a lower seafood intake, in spite of the accumulated higher doses of contaminants ingested. It thus seems that a seafood diet counteracts the potential negative effects, which would be real if only the contaminants themselves were ingested. A study by Mozzafarian and Rimms (2006) leads to the simple conclusion that the benefits of fish intake exceed the potential risks.

The message that seafood may contain harmful substances is continuously broadcast by the media and seems to come across to the public in a simplified form, resulting in the information being understood as ‘fish is harmful’. Michael T. Morrissey at Oregon State University expresses his concerns about the situation in an editorial called ‘Misinformation’ (Morrissey, 2003), in which he warns that over-complex messages transmitted by, e.g. medical doctors, are interpreted as ‘the consumer should not eat seafood’.

In the SEAFOODplus safety projects, the focus was on the real safety risks which may occur as a consequence of contamination by viruses or bacteria. If the incidence of direct illnesses caused by viral or bacterial infections or by the toxins produced by bacteria could be eliminated, consumers would be safer when having a seafood meal. Combined with a production chain with full traceability, this would give consumers the basis for the most credible seafood products.

1.2 Structure of the book

Throughout the book, reference is made to project work carried out in SEAFOODplus, reflecting the different projects, in most cases giving only the acronym of the specific project in question. In order to give a better picture of all the projects of SEAFOODplus and to explain what lies behind each acronym, the following overview of projects and objectives is included, and it is indicated in which parts of the book the different projects are being presented.

Part I: Consumers and seafood
The fact that seafood consumption seems to be dropping in spite of general knowledge that seafood is healthy is being addressed in baseline studies and research, revealing consumer motives and barriers to seafood consumption across Europe. The lack of knowledge about consumers' preferential behaviour, demands for information, and the impact and effectiveness of health, safety and ethical messages relating to seafood, is also addressed. Finally, there is discussion leading towards a better understanding of how the sensory-quality attributes of seafood are perceived by consumers.

Project CONSUMERSURVEY: *Seafood Consumption – Explaining attitudes, preferences and eating habits across consumer segments in Europe*
The objective was to develop an integrated approach towards explaining seafood consumption covering two areas: consumers' choice of food and consumers' choice of seafood. Important levels of analysis covered by this project include motives and barriers to seafood consumption, cross-cultural variations in Europe, attitudes and preferences in relation to seafood, and last but not least, how these aspects are linked to lifestyles, perceived health and well-being from a consumer's point of view.

Project SEAFOODSENSE: *Improved seafood sensory quality for the consumer*
The objective was to develop and apply consumer-oriented Seafood Sensory Quality Models that will enable the seafood industry to improve the eating quality of seafood available to consumers, encourage increased seafood consumption, and in so doing, contribute to improved consumer health.

Project SEA-INFOCOM: *Seafood Information and Communication – Assessment of consumers' needs for seafood information and the development of effective seafood communication*
The objective was to assess consumers' needs for information about seafood and develop effective communication about seafood, relating to traceability, health, safety and ethical issues.

Project CONSUMEREVALUATE: *Consumer evaluation and willingness to buy convenience and tailor-made seafood products*
The objective was to explore and explain consumers' preferences, evaluation and buying behaviour relating to convenience and tailor-made seafood products.

Part II: The health benefits of seafood

The relevance of seafood in the diet to diminish the increased incidence of nutrition-related chronic diseases (cardiovascular, cancer and inflammatory) was addressed in SEAFOODplus by performing dietary intervention and epidemiological studies in areas where seafood may contribute to reducing or preventing the development of such diseases. Another focus area was the health of young populations, to treat obesity, prevent the development of osteoporosis and specifically focus on the high rate of postpartum depression observed in women giving birth.

Project FISHGASTRO: *Gastro-intestinal health with special emphasis on the reduction of the risk of colon cancer and inflammatory bowel disease*
The objective was to clarify to what extent fish consumption improves the health of the gastrointestinal tract, what aspects of fish are important in this respect, and what are the mechanisms of protection.

Project YOUNG: *Health of young European families and fish consumption*
The objective was to increase knowledge about the nutritional effects of fish constituents, to promote health and prevent diseases in young European families.

Project METAHEART: *Metabolism of n-3 fatty acids and heart disease*
The objectives were to provide proof about the major protective effect of seafood against the risk of heart disease; to unravel its underlying mechanism; to study the potential of different dietary n-3 fatty acids; and to determine how the conversion and metabolism of n-3 fatty acids in the human body are controlled and how they can be modulated by other dietary factors. Furthermore, this project investigated whether and how dietary n-3 fatty acids can prevent cardiac arrhythmia and related heart disease risk, and which specific n-3 fatty acids in seafood and other foods may be responsible for this effect.

Part III: Ensuring seafood safety
Among the clearest food safety risks connected with seafood consumption are diseases caused by viral shellfish poisoning and the continuing occurrence of histamine poisoning in Europe. These topics were selected as relevant research areas, as well as studies of how contamination with the pathogenic bacteria, such as *Vibrio* species, can be controlled.

Project REFHEPA: *Development of standard reference methods for Hepatitis A virus and Norovirus in bivalve molluscan shellfish*
The objective was to develop sensitive, quantitative and standardised ISO (International Standards Organisation) polymerase chain reaction (PCR)-based methods for the detection of Hepatitis A virus and Norovirus in bivalve molluscan shellfish.

Project REDRISK: *Reduction of risk in shellfish harvesting areas*
The objectives were to identify pollution sources and the conditions responsible for microbial contamination in shellfisheries, and to determine their impact on viral contamination in shellfisheries. This will provide a framework for the development of a preventative strategy to reduce the virus risk associated with shellfish, by using a risk management approach in shellfish harvesting areas, based on HACCP principles. A preventative strategy of this kind will reduce the virus risk associated with the consumption of bivalve molluscan shellfish for the European consumer.

Project SEABAC: *Enhanced assessment of bacterial-associated contamination*
The objective was to develop standardised techniques to detect and characterise pathogenic Vibrios. This will facilitate future assessment of the health risks posed to European consumers by these organisms.

Project BIOCOM: *Biogenic amines in seafood – assessment and management of consumer exposure*
The objective was to provide data that will reduce European consumers' intake of biogenic amines from seafood and reduce the incidence of histamine fish poisoning (HFP).

Part IV: Seafood from source to consumer product
In order to retain the intrinsic qualities of seafood, it is necessary to consider the whole production chain. Special attention is needed to obtain tailor-made products with satisfying eating characteristics such as taste and texture. Emphasis was placed on the prevention of contamination with pathogens during the production of perishable seafood convenience products. Another challenge was to exploit the health-promoting compounds contained in fractions that today are considered as by-products.

Project CONSUMERPRODUCTS: *Consumer-driven development of innovative tailor-made seafood products, with functional components of plant or marine origin, to improve the health of consumers*
The objectives were to develop innovative, functional seafood products from both capture (under-utilised) and farmed fish, containing health-promoting compounds aimed at the improvement of intestinal health and lipid metabolism, as well as the potential prevention of cancer.

Project PROPEPHEALTH: *High-added value functional seafood products for human health from seafood by-products by innovative mild processing*
The objectives were to screen, map and recover 'new' health-beneficial compounds from seafood by-products by advanced mild refining processes; to develop 'new' bioactive (functional) seafood ingredients; and to use these novel ingredients, either directly in the food industry, or in the CONSUMER-PRODUCTS project for the development of new, functional seafood products, accepted by the target consumers.

Project HURDLETECH: *Hurdle technology, including minimal processing, to ensure the quality and safety of convenience seafood*
The objective was to ensure the safety and quality of convenience seafood products.

Project LIPIDTEXT: *Preventing seafood lipid oxidation and texture softening to maintain healthy components and quality of seafood*
The objectives were to secure and maintain the high sensory quality (colour, flavour, texture parameters) and nutritional value (high level of anti-oxidants, n-3 lipids, and low levels of potentially toxic oxidation products) of seafood products, including fresh and frozen fish fillets, fish-based products and fish oil-enriched systems.

Part V: Seafood from aquaculture
Intensive production of seafood from aquaculture presents both opportunities and potential environmental impacts. In order to meet consumer needs and expectations of healthy, high-quality seafood, the full potential of farmed fish has to be developed for a diversity of species reared in sustainable and environmentally friendly systems. Quality and ethical factors were addressed in studies investigating genomic and physiological traits, as well as husbandry practices and slaughtering methods for a wide range of European farmed fish, including freshwater species.

Project BIOQUAL: *Physiology and genetics of seafood quality traits*
The objectives were to establish novel endocrinological, physiological and genetic tools in order to identify quality traits in finfish aquaculture; to apply these to fish-fed novel diets; to lay the foundations for the establishment of high-

throughput protein-array technology to assess muscle quality and produce gene microarrays, to aid in broodstock selection based on quality traits.

Project ETHIQUAL: *Ethical quality traits in farmed fish – the role of husbandry practices and aquaculture production systems*
The objective was to examine how husbandry practice, aquaculture systems and pre-slaughter conditions contribute to the flesh quality and ethical quality of finfish seafood.

Part VI: Seafood traceability to ensure consumer confidence
In today's production systems valuable information is lost, leading to products for which documented authenticity cannot be provided. It is thus a challenge to implement validated traceability systems, agreed and accepted by all the players in the production chain.

Project METHODS: *Methodology*
The objectives were to define the vocabulary for the 'shall elements' in the existing Tracefish standard so that it can be easily used in the whole fishery industry in practice; to add and define new elements of information from the results of other research areas in the SEAFOODplus project; and to develop a Good Traceability Practice (GTP) guideline 'manual'.

Project IMPLEM: *Implementation*
The objectives were to study current information flow in case chains; to specify what changes are needed in each link in order to ensure that traceability is in place; to test, evaluate and make suggestions for the improvement of advanced technology for global batch identification and data catch; and to integrate data captured with advanced technology into traceability software with functionality for data storage and transmission.

Project VALID: *Validation*
The objectives were to validate the traceability systems developed and implemented in different fish production chains across Europe; and to validate the traceability data coming from the chains by testing different tools, such as authenticity methods and other specially adapted tools and methods for the validation process.

Although the research undertaken in each of the projects is described in the specific chapters, there was considerable integration between the different projects. This is highlighted throughout the book and collaboration between the researchers in the various disciplines is explained.

1.3 Future trends

Based on the results emerging from SEAFOODplus, new avenues for research can be indicated and new directions given for the further development of the seafood sector. As already mentioned, media misinformation to the general public makes it difficult for consumers to take full benefit of seafood diets. Observations made in the consumer studies show that consumers do not place very much trust in media information, and it leads to confusion. Medical doctors, however, come at the top of the list of information sources that consumers *do* trust. If, for example, a negative message about consuming seafood originating from the media is channelled through a medical doctor, the effect is transformed into something that the consumer trusts. A new strategy could thus be formulated, targeting medical doctors to inform consumers about the health-beneficial effects of seafood diets.

Evidence has been gathered in SEAFOODplus showing that it is not only omega 3 fatty acids that have positive health effects; other components, including the proteins, also have positive effects. In intervention studies with hypocaloric diets for weight reduction, it has been shown that such diets containing lean or oily fish lead to greater weight loss than a control diet, and the effect is more pronounced in men than in women. These are observational studies and the mechanisms behind the effects should be studied further to understand how seafood can be better used in the fight to reduce overweight and obesity.

Other components, such as selenium and taurine, are also known to be present in high amounts in fish, but vary among fish species and tissues. A new concept has been investigated in SEAFOODplus, in which farmed fish is being used as a carrier for important trace elements. It has been shown that selenium can be enriched in plants from soil, and these can be included in the fish feed to produce farmed fish containing consistently high levels of selenium. In future, further work should be carried out to demonstrate the health effects and validity of such a concept to deliver necessary nutrients through seafood diets.

Another promising development concerns functional seafood. Functional food is an area that is consistently expanding, and for seafood, new combinations have been tried in SEAFOODplus, showing that it is possible to add, e.g. plant fibre by-product fractions containing natural antioxidants, to restructured fish products, controlling oxidation and adding fibre components to the product. It is thus possible to utilise nutritionally valuable components from by-products. The by-product fractions from fish processing also contain many valuable components, and the screening for effects, e.g. within the pharmaceutical sector, as done in SEAFOODplus, is only the start of a trend that will expand considerably in the future.

Some of the studies in the human nutrition area of SEAFOODplus have shown that depression in women after giving birth may be considerably reduced if they are given a seafood diet through pregnancy. This is an example of how seafood may impact on human psychology. Other studies have also revealed that

seafood may have a pronounced effect on the development of brain functions. It is believed that further studies in this area will be very rewarding, particularly relating to very early development and through childhood.

1.4 References

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2

Environmental contaminants in farmed fish and potential consequences for seafood safety

M. H. G. Berntssen and A.-K. Lundebye, National Institute of Nutrition and Seafood Research (NIFES), Norway

2.1 Introduction

The presence of environmental contaminants in farmed fish has received increased attention in recent years due to concerns regarding seafood safety. Since food safety issues in farmed fish have predominantly been related to fish feed, this chapter will provide an insight into the main groups of chemical compounds in marine feed ingredients and farmed fish that pose a potential threat to human health. In general, two main groups of environmental contaminants will be addressed, namely persistent organic pollutants (POPs) and metals. A brief background will be given on chemical characterisation, environmental sources, and relevance for consumer safety of selected environmental contaminants. Emphasis will be made on the specific chemical forms and behaviour of these contaminants in the marine system, occurrence in fish feed ingredients, their dietary transfer to farmed fish, and approaches to control and reduce these components in farmed fish. Legislation related to these environmental pollutants in feed ingredients and fish feed, and fish and seafood as food products will also be addressed.

Chemical food contaminants can be defined as components that are not intended to be present in food, and can be potential harmfully to the consumer when ingested at sufficiently high levels (Watson, 2001). The non-intentional introduction and non-functionality of these contaminants separates them from other potentially harmful chemicals in food such as colouring agents and micronutrient additives. The term ‘contamination’ itself does not indicate the degree

to which a food component is a potential threat to human health. This will depend on several factors such as toxicity of the component (which is amongst other factors related to its chemical form), its concentration in feed, the transfer from feed to edible tissue (bioavailability), and the amount of the food product consumed. Chemical contaminants can be introduced at most stages of food production (Watson, 2001).

Food can be contaminated during processing (e.g., poly aromatic hydrocarbons from smoking of fish products), by packaging (migration from plastic to food product) or farming practice (e.g., contamination of feed ingredients with pesticides). Global pollution of the aquatic environment is an important source of chemical contamination of wild fish and consequently marine fish feed ingredients and farmed fish. This is particularly relevant for persistent pollutants that readily biomagnify in the aquatic food chain and that can also be found at relatively high concentrations in oily fish species that are caught for industrial purposes such as the production of fish feed ingredients. Characteristics of global environmental pollutants:

- often unintentional introduction to the environment and food chain
- often poorly degraded (persistent in the environment)
- can be acutely toxic at high doses and
- can give chronic effects after long-term exposure to low doses.

Central to the environmental contamination of fish are the processes of bioaccumulation and bioconcentration. Several definitions exist for these well used terminologies. Generally, bioaccumulation describes the transfer of contaminants from all routes of exposure, i.e. water, sediment and food. Bioconcentration is the transfer of contaminants from the water. Biomagnification describes the increase in the concentration of a certain contaminant from one level in the food chain to the next trophic level. Persistent lipid soluble contaminants, such as dioxins and polychlorinated biphenyls (PCBs) readily bioaccumulate into fatty tissue of fish and biomagnify up the food chain. Oily fish generally have substantially higher concentrations than lean fish due to the lipophilicity of these contaminants. The concentration of contaminants in wild fish also depends on other factors such as seasonal variation, geographic distribution, trophic position, age (life stage) and size of the fish (e.g., NORA, 2003; Fisk *et al.*, 1998).

Not all potentially hazardous components present in fish result from environmental pollution. The aquatic ecosystem contains relatively high background levels of certain metals such as mercury compared to the terrestrial environment (Fitzgerald and Mason, 1997).

2.2 Environmental contaminants and marine feed ingredients

Commercially formulated fish feeds have traditionally had relatively high contents of marine feed ingredients such as fish meal and fish oil. Fish oils are a

Table 2.1 Levels of metals (arsenic, cadmium and mercury) and dioxins in fish meal, fish oil and vegetable oil used as feed ingredients in fish feed (after Måge *et al.*, 2006)

Feed ingredient	Arsenic (mg/kg)	Cadmium (mg/kg)	Mercury (mg/kg)	Dioxins (sum PCDD/F, ng WHO-TEQ/kg)
Fish meal	($n = 16$)	($n = 16$)	($n = 16$)	($n = 8$)
mean	6.0	0.44	0.14	0.46
min.–max.	(1.8–13)	(0.14–1.36)	(0.04–0.23)	(0.12–0.73)
Fish oil	($n = 10$)	($n = 8$)	($n = 8$)	($n = 6$)
mean	8.9	<0.03	<0.02	2.7
min.–max.	(5.6–10.8)			(0.26–5.2)
Vegetable oil	($n = 12$)	($n = 8$)	($n = 8$)	($n = 5$)
mean	0.34	<0.03	<0.02	0.34
min.–max.	(<0.01–1.6)			(0.12–1.1)

major source of POPs for fish species raised on 'high energy diets' (high levels of fat), whereas fish meal is often an important additional source of POPs for fish species farmed on less high energy feed. The level of inclusion of fish meal and oil in fish feeds varies among the species of fish which are currently farmed. Feed for Atlantic salmon (*Salmo salar*) typically contains 35–47% fish meal and 25–33% fish oil, whereas seabream (*Sparus auratus*) feed has approximately 40–45% fish meal and 15–20% fish oil, and carp (*Cyprinus carpio*) feed usually contains 20–25% fish meal and 5–10% fish oil. Marine feed ingredients are generally derived from pelagic fisheries, and fish oils are considered the main source of persistant organic pollutants (POPs) in farmed fish (e.g., WHO, 1999; Jacobs *et al.*, 2002; Easton *et al.*, 2002; Carlson and Hites, 2005). There are large seasonal differences in the degree of background POP contamination of fish oils and consequently the level of POPs in farmed fish. Fish oil from pelagic fish species caught in the North Atlantic ocean in winter have considerably lower levels of POPs (e.g., dioxins and PCBs) than fish oils obtained from fish caught in the spring. During early spring the lipid content decreases in the fish, consequently the concentration of dioxins and PCBs increases in the extracted oil (NORA, 2003). POP levels in fish oils also depend on factors such as fish species, age, or geographical origin (EC, 2000; NORA, 2003). Fish oils from pelagic fish species of Pacific Ocean origin generally have lower dioxin and to a lesser degree also lower PCB levels than fish oils from the Atlantic Ocean (EC, 2000). In contrast to marine oils, vegetable oils in general have considerably lower concentrations of POPs (Table 2.1).

2.3 Organic contaminants

Organic pollutants are compounds with a carbon structure. Most of these components have been or still are man-made (anthropogenic), produced by industry

for a wide range of applications (e.g., flame retardance of textiles, pesticides to protect crops, PCB in plastics, etc.). Some are accidentally formed as by-products in industrial processes or by natural processes such as forest fires or volcanic activity (e.g., dioxins). In addition to being persistent, these POPs are typically fat soluble, semi-volatile and some are potentially toxic at low doses. Persistent organic pollutants are often globally dispersed and readily accumulate along the aquatic food chain, and they are often found at elevated levels in oily fish species or oils obtained from fish. The following section will give a brief introduction on chemical characterisation, environmental sources, and relevance to human safety of selected POPs that are important with regard to food safety of farmed fish. These include dioxins, PCBs, brominated flame retardants, and organochlorine pesticides.

2.3.1 Dioxins

Dioxins is the generic term given to two groups of chlorinated hydrocarbons namely polychlorinated dibenzo-para dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) (Fig. 2.1). These groups have a very similar basic structure, with a total of 210 different forms (or congeners) depending on the position or number (between 1 and 8) of chlorine atoms. The position of chlorine atoms is important with regard to the toxicity and persistence of the different congeners. Seventeen of the congeners are considered to be particularly toxic, of which tetra chlorinated dibenzo dioxin (TCDD) with four chlorine atoms in the most outward positions (2,3,7,8) is considered to be one of the most toxic, together with the penta chlorinated 1,2,3,7,8 PCDD. The other congeners have been assigned toxic equivalency factors (TEF) that expresses their toxicity relative to 2,3,7,8 TCDD. The concentration of dioxins is expressed as toxic equivalents (TEQ), where a toxic equivalency factor (TEF) is applied to the concentration of the individual congeners and summed to generate the total TEQ in a mixture of the 17 congeners. The TEFs established in 1997 by the World Health Organisation have been most widely used for human risk assessment (Van den Berg *et al.*, 1998) by government bodies; these TEFs were re-evaluated in 2005 (Van den Berg *et al.*, 2006) (Table 2.2).

Toxic responses to dioxin exposure are mediated through binding to a specific cytoplasmic receptor called the aryl hydrocarbon (Ah) receptor. The binding of dioxins to the Ah-receptor affects a range of fundamental biochemical processes. Dioxins can disrupt normal homeostatic processes that regulate cellular growth

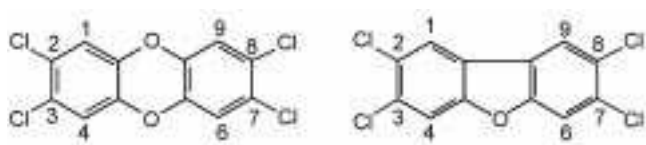

Fig. 2.1 Chemical structure of polychlorinated dibenzo-para dioxins (PCDD) and the polychlorinated dibenzofurans (PCDF). To the left is 2,3,7,8 tetra CDD and to the right is 2,3,7,8 tetra CDF.

Table 2.2 Current and re-evaluated toxic equivalency factors (TEFs) established by World Health Organisation (WHO) for polychlorinated dibenzo-para dioxins (PCDD) and the polychlorinated dibenzofurans (PCDF) and non-ortho and mono-ortho dioxin-like PCBs (DL-PCBs). The 1997 TEF values apply in current EU legislation. (Source: Van den Berg *et al*., 2006)

PCDD/PCDF	WHO-TEF 2005	WHO-TEF 1997	Dioxin-like PCB	WHO-TEF 2005	WHO-TEF 1997
PCDD			*Non-ortho DL-PCBs*		
2,3,7,8-tetra-CDD	1	1	3,3′,4,4′,5 penta-CB (PCB 126)	0.1	0.1
1,2,3,7,8 penta-CDD	1	1	3,3′,4,4′,5,5′ hexa-CB (PCB 169)	0.03	0.01
1,2,3,4,7,8-hexa-CDD	0.1	0.1	3,4,4′,5 tetra-CB (PCB 81)	0.0003	0.0001
1,2,3,6,7,8-hexa-CDD	0.1	0.1	3,3′,4,4′-tetra-CB (PCB 77)	0.0001	0.0001
1,2,3,7,8,9-hexa-CDD	0.1	0.1	*Mono-ortho DL-PCBs*		
1,2,3,4,6,7,8-heptaCDD	0.01	0.01	2,3,3′,4,4′-penta-CB (PCB 105)	0.00003	0.0001
1,2,3,4,6,7,8,9-octa-CDD	0.0003	0.0001	2,3,4,4′,5-penta-CB (PCB 114)	0.00003	0.0005
PCDF			2,3′,4,4′,5-penta-CB (PCB 118)	0.00003	0.0001
2,3,7,8-TCDF	0.1	0.1	2′,3,4,4′,5-penta-CB (PCB 123)	0.00003	0.0001
1,2,3,7,8-penta-CDF	0.03	0.05	2,3,3′,4,4′,5-hexa-CB (PCB 156)	0.00003	0.0005
2,3,4,7,8-penta-CDF	0.3	0.5	2,3,3′,4,4′,5′-hexa-CB (PCB 157)	0.00003	0.0005
1,2,3,4,7,8-hexa-CDF	0.1	0.1	2,3′,4,4′,5,5′-hexa-CB (PCB 167)	0.00003	0.00001
1,2,3,6,7,8-hexa-CDF	0.1	0.1	2,3,3′,4,4′,5,5′-hepta-CB (PCB 189)	0.00003	0.0001
2,3,4,6,7,8-hexa-CDF	0.1	0.1			
1,2,3,7,8,9-hexa-CDF	0.1	0.1			
1,2,3,4,6,7,8-hepta-CDF	0.01	0.01			
1,2,3,4,7,8,9-hepta-CDF	0.01	0.01			
1,2,3,4,6,7,8,9-octa-CDF	0.0003	0.0001			

and differentiation. These disruptions cause a variety of toxicities and pathologies (e.g., Mandal, 2005). Chronic exposure to elevated dioxin concentrations can cause cancer, reproductive disorders and foetal deformities. Dioxin exposure can also cause liver damage, and have negative effects on the immune and nervous system.

Dioxins are not industrially produced as is the case with certain other POPs such as brominated flame retardants (see below); however, they are unwanted by-products of a wide range of industrial processes including: magnesium, aluminium and nickel production, paper manufacturing (bleaching of pulp), oil refining and the manufacture of industrial and agricultural chemicals. They also result from various combustion processes with high temperatures such as chemical and municipal incinerators. Dioxins are produced when mixtures of hydrocarbons and chlorine are exposed to high temperatures. They are very persistent chemicals and are highly lipid soluble. Dioxins are widely dispersed in the environment and are present in most food products, particularly in fat-rich foods including milk, meat and fish.

The SCF established a tolerable weekly intake for dioxins (including dioxin-like PCBs) of 14 pg WHO-TEQ per kg body weight for adults, JECFA has set a provisional monthly intake of dioxins and dioxin-like PCBs of 35 pg WHO-TEQ, whereas the WHO has set a tolerable daily intake of 1–4 pg WHO-TEQ/kg body weight. The EU has established maximum limits for dioxins (total TEQ for the 17 congeners with TEFs) and for the sum of dioxins and dioxin-like PCBs (total TEQ for the 29 congeners with TEFs) in fish feed, fish oil, fish protein hydrolysates containing more than 20% fat, and fish, other aquatic animals, their products and by-products (with the exception of fish oil), and fish oil. These limits are given in Table 2.3 taken from EC (2006a). Legislation on the maximum levels for PCCD/Fs and dioxin-like PCBs (DL-PCB, see section below) are based on the WHO-TEFS from 1997. The maximum permitted level (in WHO-TEQ) for dioxins alone, and the sum of dioxins and dioxin-like PCBs in muscle meat

Table 2.3 Overview of upper limits for dioxins (PCDD/F) and dioxins + dioxin-like PCBs (PCDD/F-PCB) in different feed ingredients, fish feed and fish (after EC, 2006a)

Products intended for animal feed	Max content in feeding stuff (12% moisture) ng WHO-PCDD/F-TEQ/kg	Max content in feeding stuff (12% moisture) ng WHO-PCDD/F-PCB-TEQ/kg
Fish oil	6	24
Fish, other aquatic animals their products and by-products with the exception of fish oil and fish protein hydrolysates >20% fat	1.25	4.5
Fish protein hydrolysates >20% fat	2.25	11
Feed for fish	2.25	7

from fish for human consumption are 4 and 8 pg WHO-TEQ/g fresh weight respectively (EC, 2006b), with the exception of muscle meat from eel (for which the maximum levels are 4 and 12 pg WHO-TEQ/g fresh weight respectively).

2.3.2 Polychlorinated biphenyls

Polychlorinated biphenyls (PCB) are, like dioxins, organochlorine compounds. They consist of two phenyl rings connected with a carbon bond. Depending on the number and position of chlorine atoms a total of 209 different chemicals can theoretically exist (Fig. 2.2). PCBs have been widely used in complex mixtures with different technical properties. Some PCB congeners are very similar in structure to dioxins and exert 'dioxin-like' biological effects. These 12 PCB congeners are called dioxin-like PCBs (DL-PCB) and have been assigned toxic equivalent factors (TEF) which express their potential toxicity compared to the most toxic dioxin (Table 2.2). The 12 dioxin-like PCBs include four non-ortho and eight mono-ortho PCBs. The dioxin-like PCBs with chlorine atoms in the outer ring (para and meta) positions and not in the inner ring (ortho) position (Fig. 2.2) are most similar to 2,3,7,8-tetra-CDD and are called non-ortho dioxin-like PCBs. The other dioxin-like PCBs have not more than one chlorine atom in the inner (ortho) position in addition to chlorine atoms in the outer (para and meta) positions, and these are the mono-ortho PCBs.

Like dioxins, PCBs are ubiquitous in the environment and they are very persistent and lipid soluble. They are not readily degraded by chemical oxidation or bacterial action. PCBs are primarily man-made and accumulate in both animals and sediment. Contamination of the aquatic environment has occurred from direct discharge of waste containing PCBs into rivers, lakes and coastal areas in addition to atmospheric deposition into large water systems. PCBs were used world-wide, especially in industrially developed countries. In contrast to dioxins, they have been produced for a certain purpose. Production began in the early 1930s and they were widely used as technical mixtures, for example as heat exchange and hydraulic fluids, dielectric fluids in transformers and capacitors and in the manufacture of paints, plastics, adhesives and flame retardants. In the early 1970s it became evident that chronic exposure to PCBs could be hazardous both to humans and to the environment. This led to a ban on

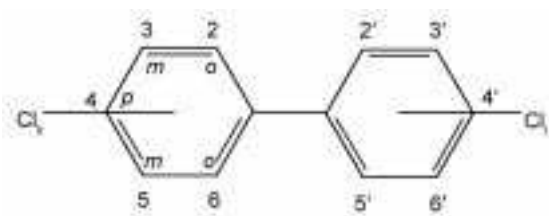

Fig. 2.2 Basic chemical structure of polychlorinated biphenyls (PCBs) where k+l can be between 1 and 8 chlorine atoms. Numbers indicate positions of chlorine atoms, *o* = ortho position, *m* = meta position, and *p* = para position.

the production of PCBs in 1977 in the USA, closely followed by other industrially developed nations.

Chemical identification of the PCB congeners which contribute most to the toxicity of PCB mixtures has led to focus on those that elicit 'dioxin-like' responses. The dioxin-like PCBs bind to Ah-receptors and have similar toxic effects to 2,3,7,8-TCDD. The non-dioxin-like PCBs bind weakly to the Ah-receptor and hence do not exert dioxin-like toxicity. The effect of non-dioxin-like PCBs are hard to distinguish from dioxin-like PCBs because both are often present in technical PCB mixtures. Moreover, technical PCB mixtures may also contain small amounts of dioxins or dioxin-like components. The toxicological effects of technical PCB mixtures are therefore similar to those of dioxins and include effects on liver, thyroid, immune function, reproduction and behaviour, and carcinogenicity. Individual exposures to non-dioxin-like PCB congener have been show to cause effects on the thyroid, liver and brain biochemistry, oestrogenicity, immunotoxicity, reproduction and, typically, neurodevelopment (EC, 2005a). Maximum limits have been established in the EU for dioxin-like PCB in feed and food (see dioxin section). Although several EU countries have national maximum levels for indicator PCBs, such as PCB-7 (the seven congeners that are most typically present in seafood, and include one dioxin-like PCB), no EU legislation currently exists on non-dioxin-like PCBs in food or feed ingredients.

2.3.3 Polybrominated flame retardants

The brominated flame retardants (BFRs) represent a group of chemicals with a basic structure of aromatic rings that are brominated, as opposed to chlorinated, as it the case for dioxins and PCBs. The major BFRs used in industry are tetrabromodisphenol A (TBBPA), hexa bromocyclododecane (HBCD), and polybrominated diphenyl ether mixtures (PBDEs). Whereas TBBPA is used as a single compound, HBCD and especially PBDE are used in complex technical mixtures. HBCD is used in a mixture of at least three isomers (α, β, χ) of which the α-isomer is predominantly present in fish. For PBDEs there are theoretically 209 different congeners (each named according to the number of attached bromine atoms and their position (Fig. 2.3)).

The nomenclature of PBDEs is similar to that of PCBs. PBDE congener groups are often classified after the number of bromine atoms in a PBDE, for

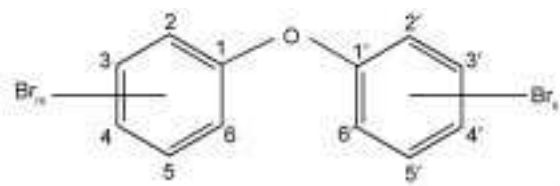

Fig. 2.3 Basic structure of a polybrominated diphenyl ether (PBDE) where m + n can be between 1 and 10 bromine atoms. Numbers indicate positions of bromine atoms.

example a PBDE with three bromines is called a tri-BDE and a PBDE with six bromines a hexa-BDE. As for PCBs, they are persistent and highly fat soluble and seem to accumulate in the aquatic food chain. PBDE congeners are used in three commercially available products, namely the PentaBDE, OctaBDE and DecaPBDE mixtures. The commercial mixtures often contain several congener groups. Lower brominated PBDE congeners (tri-to hexaBDE), which are present in the commercial penta- and octa-mixtures, are persistent and accumulate in the environment. Higher brominated PBDE congeners (hepta to deca), which are used in the octa-mixtures, are less persistent and can be broken down to lower brominated congeners. The DecaPBDE mixture is mainly composed of only deca-BDE congener (PBDE-209), which is not stable in the environment and is also debrominated to lower brominated congeners. Some of the predominant PBDE congeners found in marine feed ingredients and farmed fish are tri-BDE-28, tetra-BDE-47, penta-BDE-99, penta-BDE-100, hexa-BDE-153, hexa-BDE-154, and hepta-BDE-183. This chapter will concentrate on the PBDEs.

Brominated flame retardants are chemicals that are industrially produced to reduce the risk and spread of fire. Brominated flame retardants are found in a variety of common products from fabrics containing polyurethane foams, to plastic in items such as televisions, computers, cars, and construction materials. Although BFRs are considered 'new' contaminants, they were detected as possible contaminants in fish as far back as 1981. The increasing PBDE concentrations found in human breast milk from around 1970 to around 1998 has led to increasing concern for these POPs as an environmental threat to human health (Noren and Meironyte, 2000). Food of animal origin is one of the exposure routes for BFRs. Food with high fat content, e.g. oily fish, are the main contributors to dietary exposure as is the case for PCBs. Other possible routes are inhalation of indoor air (e.g., rooms with electronic equipment that contains flame retardants) and skin contact (textiles that are impregnated with flame retardants). PBDE is not considered either genotoxic or carcinogenic to humans (EU, 2000, 2002, 2003), neither does PBDE exert Ah-like properties and previously reported Ah-mediated effects might be the result of dioxin-like impurities (Van den Berg *et al.*, 2006). PBDE affects the thyroid hormones, causes hepatic and thyroidal histopathological changes, and causes neurotoxic/behavioural effects. The most important non-lethal toxic effect of PBDE, however, seems to be their ability to disrupt the thyroid hormones T_3 and T_4. These hormones are important in regulating metabolism and development in most organisms. Currently, no international legislation exists for safe levels of BFR in food, although national (UK) tolerable daily intakes (TDI) for some BFR exist.

2.3.4 Organochlorine pesticides

Pesticides are a group of synthetic chemicals that are produced for the sole purpose of controlling unwanted organisms that can adversely affect public health or damage food crops. One group of pesticides, the organochlorines, includes some of the most well-known environmental contaminants of aquatic

systems (e.g., DDT, aldrin/dieldrin, endrin, toxaphene, chlordane, heptachlor, hexachlorocyclohexane, and mirex). These pesticides are persistent and lipophilic and hence tend to biomagnify similar to other organochlorines such as, for example, PCBs (Blus, 1995). Several of these pesticides have also been shown to be a potential threat to human health. Other pesticide groups, such as organophosphates or carbamates, are readily metabolised or excreted and do not biomagnify in the marine food chain. Organochlorine pesticides can be grouped in five major groups based their on chemical structure (Blus, 1995), which includes:

- DDT and its analogues
- hexachlorocyclohexane (HCH)
- cyclodienes
- toxaphene and related chemicals
- mirex and chlordecone structures.

All of these groups have several related compounds, isoforms and/or breakdown products. Organochlorine pesticides include many different compounds with a wide variety of chemical structures. DDT is an example of a well-known pesticide with many different chemical structures. Chemical analogues of dichlorodiphenyltrichloroethane (DDT) include methoxychlor, ethylan, and chlorobenzilate. In addition, DDT itself has two isoforms (o,p′ and p,p′ DDT) with two predominant metabolites for each isoform (o,p′ and p,p′ dichlorodiphenyldichloroethylene (DDE) and tetrachlorodiphenylethane (DDD)) (Fig. 2.4). Hexachlorocyclohexane has eight steric isomers including the well-known

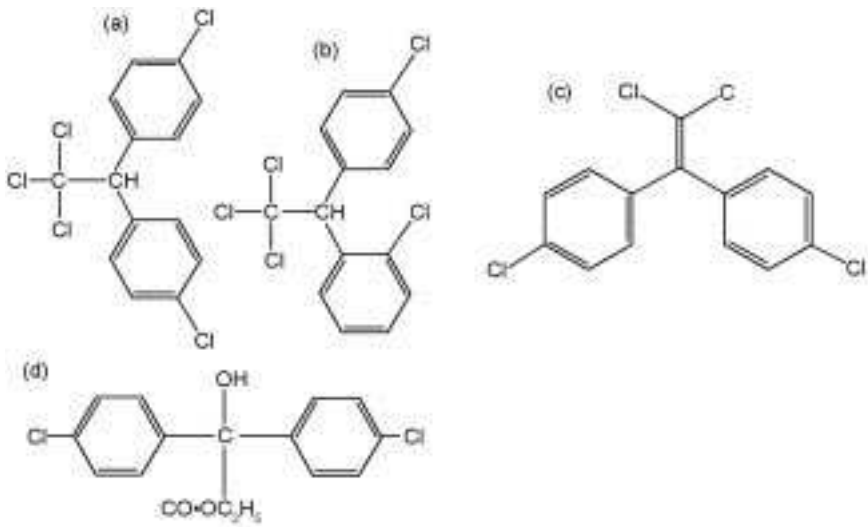

Fig. 2.4 Chemical structures of the two DDT isomer p,p′-dichlorodiphenyltrichloroethane (DDT) (a) and o,p′-dichlorodiphenyltrichloroethane (DDT) (b), the DDT metabolite p,p′ dichlorodiphenyldichloroethylene (p,p′DDE) (c), and the DDT analogue ethyl-dichlorobenzilate (chlorobenzilate) (d).

γ isomer, lindane. Cyclodienes include many known organochlorine pesticides such as aldrin, dieldrin/endrin, heptachlor, chlordane and endosulfan. Toxaphene (also known as camphechlor) is used in complex technical mixtures with at least 202 different compounds identified (EC, 2005b). Mirex has been introduced as a replacement for some cyclodienes as has chlordecone, and they are readily stored in the body (Blus, 1995).

The widespread use of persistent pesticides over several decades, for example DDT in mosquito control, has caused them to become ubiquitous environmental contaminants. Although the use of several organochlorine pesticides has been phased out in most of the world, they are still detectable in aquatic biota. Pesticides are developed to specifically control certain organisms such as insects, fungi, plants, snails and rodents. However, it is evident that pesticides can also have adverse effects on non-target organisms such as fish and humans, and have been shown to affect wild-life. Their primary adverse action on target animals is non-specific disruption of the central nervous system. The organochlorine pesticides are the most important group of pesticides with regard to background contamination of marine feed ingredients and most relevant with regard to the safety of farmed fish. The potential adverse effects in humans following consumption of environmentally contaminated fish are diverse. These include hormone disrupting effects (especially for lindane, DDT and DDE), negative effects on reproduction, immunotoxicity (e.g., toxaphene), neurological effects, and possible carcinogenic effects. Legislation in feed and feed ingredients exists in the EU for several persistent organochlorine pesticides that bioaccumulate in the food chain. The EU maximum limit for aldrin, dieldrin, toxaphene, chlordane, DDT, endosulfan, endrin, heptachlor, hexachlorobenzene and hexachlorocyclo-haxane in feeding stuffs are given in Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed. This directive also gives maximum limits for other undesirable substances including arsenic, lead, fluorine, mercury, nitrites, cadmium, mycotoxins and botanical impurities. The directive has been updated several times by the European Commission following risk assessments by the European Food Safety Authority.

2.4 Metals

Most metals are naturally present in the aquatic environment as a result of geological activity such as weathering of rocks, ore formation and volcanic activity. In geographical areas with specific geological activity (e.g., volcanic), high background levels of certain metals can be found. In addition to natural sources, metals may enter the environment from human activity (e.g., mining, smelting, discharge, battery waste). Metals can be present in either inorganic or organic forms. Organically bound metals are mainly present in the aquatic environment and are not predominant in the terrestrial environment. The chemical form or speciation of metals is important in terms of the potential

toxicity of the metal and its carry-over from feed to farmed fish. Feed ingredients can also be an important source for some metals in farmed fish. Most of these metals readily bind to sulphur (SH), phosphate (H_2PO_4) and carbonate (COOH) structures in proteins. As opposed to the liphophilic POPs, metals are often associated with the protein fraction of the fish. The marine protein fraction of fish feed (e.g., fish meal) is the main dietary source of metals in farmed fish; however, certain organic forms of the metalloid arsenic are present in fish oils. The following sections give more detailed information regarding selected metals (or metalloid in the case of arsenic), also known as non-essential elements, that are relevant for food safety.

2.4.1 Mercury

Mercury in the natural environment is found in many different chemical forms and can be roughly divided into the groups given in Table 2.4. In marine and freshwater ecosystems, inorganic mercury (Hg^{2+}) compounds may be converted into organic mercury, such as methylmercury (CH_3Hg^+), by bacterial processes (Fitzgerald and Mason, 1997). The most toxic forms of mercury are the organic forms and the dominant one in fish is methylmercury (also known as short chained alkyl mercury compound), whereas the inorganic forms are less toxic. Methylmercury bioaccumulates in organisms and is biomagnified up the food chain, subsequently high concentrations can be found in long-living, marine predators. Mercury levels in fish also depend on the size and age of the individual (e.g., Boudou and Ribeyre, 1985).

Although mercury is a natural part of the environment, anthropogenic introduction to the environment is widespread. Mercury is produced in mining and smelting of ores and is used in the production of chlorine and sodium hydroxide, thermometers, batteries, tooth-fillings, and as a catalyst in chemical processes and in certain fungicides and medicines.

Methylmercury is known to cause damage to the central nervous system and is teratogenic in mammals and humans. The chemical form of methylmercury in fish has been identified as methylmercury-cysteine (CH_3Hg-cyst), and is probably part of proteins (Harris *et al.*, 2003; Amlund *et al.*, 2007). The acute toxicity of the pure chemical methylmercury ion forms (such as CH_3HgCl) is higher than the methylmercury-cysteine form (Harris *et al.*, 2003; Oyama *et al.*, 2000).

Table 2.4 Chemical forms of mercury in the environment

Inorganic	
Hg^0	Metallic mercury
Hg_2^{2+}	Monovalent mercury
Hg^{2+}	Divalent mercury
Organic	
CH_3Hg^+	Short chained alkyl mercury compounds
$C_6H_5Hg_+$	Aryl and alkyl mercury compounds

The maximum permitted level of mercury in most fishery products in the EU is 0.5 mg/kg wet weight for most species, and 1 mg/kg for a limited list of fish species. The maximum limit for mercury in marine feed ingredients and in feed are 0.5 mg/kg and 0.1 mg/kg respectively (88% dry matter basis).

2.4.2 Arsenic

Arsenic concentrations found in terrestrial animals are lower compared to concentrations found in aquatic animals, and arsenic levels in freshwater fish are again lower than marine fish (Table 2.5). As opposed to the terrestrial system, many different chemical forms (species) of arsenic exist in the aquatic environ-

Table 2.5 Total arsenic concentrations in feral marine and freshwater fish species (μg As/g ww), and terrestrial animals (ng As/g ww). Please note the different units. The fish are either wild caught or bought at fish markets. Muscle tissue concentrations are presented as mean (minimum-maximum level) or ±SD, according to the data available in the corresponding reference. Sample size (*n*) is given when provided in the reference (after Amlund, 2005)

Species	Origin	Total arsenic μg/g ww	*n*	Ref.
Marine teleost				
Farmed Atlantic salmon (*Salmon salar*)		2.2 (1.8–3)	20	2
Blue whiting (*Gadus poutassou*)	North Sea	10.4	1[a]	1
Capelin (*Mallotus villosus*)	Barents Sea	1.6	2[a]	1
Small sand eel (*Ammodytes tobianus*)	North Sea	1.8	1[a]	1
Freshwater teleost				
Atlantic salmon (*Salmo salar*)	Norway	<0.05	3[a]	2
Bass (*Micropterus salmoides*)	USA	0.03 ± 0		3
Brown trout[b]	Czech Republic	0.12 ± 0.07	5	4
Yellow perch (*Perca flavescens*)	USA	0.05 ± 0.01	51	3
Terrestrial animals				
Cattle	Canada	7.8 (ng/g) ww		5
Pig	Canada	8.2 (ng/g)		5
	Spain	1.92–62.5 (ng/g)		6
Poultry	Canada	29.9 (ng/g)		5
	UK	0.004		7

[a] Pooled sample, [b] Species Latin name not given.
References: 1. Julshamn *et al.* (2004), 2. NIFES seafood data (www.nifes.no), 3. Burger *et al.* (2002), 4. Svobodová *et al.* (2002), 5. Dabeka *et al.* (1993), 6. Bordajandi *et al.* (2004), 7. MAFF (1999).

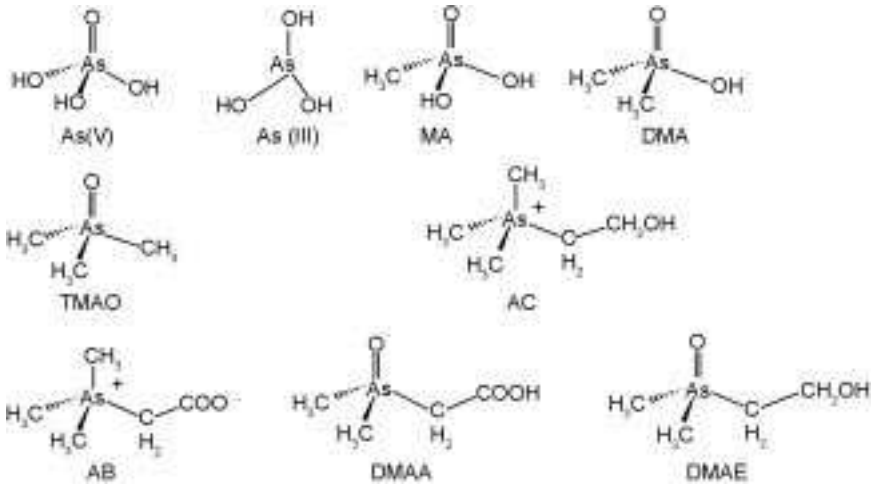

Fig. 2.5 Examples of some of the different chemical forms of inorganic arsenic (As V and III) and organic arsenicals which may be found in marine biota (Francesconi and Kuehnelt, 2004) (abbreviations: As(V) = arsenate, As(III) = arsenite, MA = methylarsonate, DMA = dimethylarsinate, AB = arsenobetaine, TMAO = trimethylarsine oxide, AC = arsenocholine, DMAA = dimethylarsinoylacetate, DMAE = dimethylarsinoylethanol.

ment. The chemical speciation in marine animals is considerably more complex than land animals. While only a few different chemical forms of arsenic have been found in terrestrial animals, more than 30 different water-soluble arsenic forms have been identified in marine fish (examples of some of the different chemical forms of arsenic are shown in Fig. 2.5), arsenobetaine ($(CH_3)_3As^+CH_2COO^-$) is the predominant form of arsenic in marine fish, but presumably also in freshwater fish (Francesconi and Edmonds, 1993, 1997) and consequently also in fish meal. Arsenic is one of the few elements that is found in relatively high concentrations in fish oils, up to 15 mg/kg, and fish oil can contribute considerably to the total arsenic levels in fish feeds (Sloth *et al.*, 2005). Lipid soluble arsenic forms are, like water soluble organoarsenicals, thought to consist of several organic forms commonly called arsenolipids (Francesconi and Kuehnelt, 2004; Schmeisser *et al.*, 2005). As opposed to the water extractable forms, little is known regarding the chemical forms of these arsenolipid compounds, their carry over from feed to fillet, or potential toxicity to mammals including humans.

The toxicity of arsenic greatly depends on the chemical form (Table 2.6). In contrast to mercury, organic forms of arsenic are considerably less toxic than inorganic forms (Shiomi, 1994), which have historically been used as a poison. In fish feed and marine feed ingredients the organic arsenic compounds, mainly arsenobetaine, are the dominant forms (Sloth *et al.*, 2005), whereas inorganic arsenic forms are dominant in terrestrial plants (Francesconi and Kuehnelt, 2002). Arsenic is naturally present in the environment; however, human activity, such as use of pesticides and wood preservatives, can cause environmental contamination with inorganic arsenic. The main dietary source of arsenic in

Table 2.6 Acute toxicity of different chemical forms of arsenic in mice and rats exposed to different arsenic forms

	LD_{50} values (mg/kg)*
As (III), arsenite	15–42
As (V), arsenate	20–800
MA, momomethylarsenate	700–1800
DMA, dimethylarsenate	1200–2600
AC, arsenocholine	6500
AB, arsenobetaine	>10000

*Dose that is lethal to 50% of the test organisms.
Compiled from Donohue and Abernathy (1999) and Shiomi (1994) after Amlund (2005).

humans is from seafood; however, this is not of concern from a food safety perspective since the predominant form is arsenobetaine which is considered non-toxic, as evident in Table 2.6. The International Agency for Research on Cancer (IARC) has classified inorganic arsenic as a human carcinogen, since it has been shown to cause skin and internal cancer. The Joint Expert Committee on Food Additives and Contaminants (JECFA) has set a provisional tolerable weekly intake (PTWI) for inorganic arsenic of 0.015 mg/kg body weight. There is no EU maximum level for arsenic in food; however, some countries have national limits. The upper limits for total arsenic in fish feed and marine feeding stuffs are 6 mg/kg and 15 mg/kg (88% dry matter); however, from a toxicological perspective it is recommended that the upper limit should be based on inorganic arsenic (EC, 2005c) in line with the PTWI.

2.4.3 Lead and cadmium

There are relatively few studies in the literature on the oral toxicity and toxicokinetics of lead in fish. Although lead exists in many different forms in marine and fresh waters, most of the lead found in fish is inorganic in nature, and is bound to proteins. Lead is used principally in the production of lead-acid batteries, solder, and alloys. Organolead compounds tetraethyl and tetramethyl lead have also been used extensively in petrol, although their use for these purposes in many countries is being phased out. Owing to the decreasing use of lead-containing additives in petrol the concentrations in the environment are declining (WHO, 1993b). Long-term adverse effects of non-lethal lead intoxication include damage to the nervous system, kidney and especially red blood cells (anaemia). JECFA has set a provisional tolerable weekly intake (PTWI) for lead of 0.025 mg/kg body weight. The maximum permitted level of lead in fish in the EU is 0.2 mg/kg wet weight for most species, and 0.4 mg/kg for a limited list of fish species (EC, 2001). The EU maximum levels for lead in crustaceans (excluding the brown meat of crab), bivalve molluscs and cephalopods (without viscera) are 0.5 mg/kg, 1.5 mg/kg and 1 mg/kg respectively (all on a wet weight basis).

Cadmium is bound to proteins that contain considerable numbers of sulphur groups (SH). Cadmium occurs naturally in the environment as a result of volcanic emissions. In addition, anthropogenic activity has increased the background levels of cadmium in soil, water and organisms. Cadmium is released to the environment in wastewater, and diffuse pollution is caused by contamination from fertilisers and local air pollution (WHO, 1993a). Industrial applications of cadmium include plastic manufacturing, batteries, pigments, stabilisers, plating and alloys. The uptake of cadmium from food is low, and as for lead this uptake decreases with increasing dietary calcium levels. Once taken up in the body, only a fraction is excreted and cadmium accumulates in organs such as liver and kidney. Sublethal toxic effects in humans include loss of kidney functions and related disturbance to calcium and phosphate metabolism, possibly resulting in bone damage. JECFA has set a provisional tolerable weekly intake (PTWI) for cadmium of 0.007 mg/kg body weight. The maximum permitted level of cadmium in fish in the EU is 0.05 mg/kg wet weight for most species, and 0.1 mg/kg for a limited list of fish species (EC, 2001). The EU maximum levels for cadmium in crustaceans (excluding the brown meat of crab and head and thorax of lobster and similar large crustaceans), bivalve molluscs and cephalopods (without viscera) are 0.5 mg/kg, 1 mg/kg and 1 mg/kg respectively (all on a wet weight basis, EC, 2001). Shellfish including the brown meat of lobster, however, can contain considerably higher levels of cadmium.

2.5 Carry-over of environmental contaminants to farmed fish

Dietary carry-over is a term used in food safety that describes the transfer of a feed contaminant to a food product (i.e., edible tissue of a farm animal). Dietary contaminants that accumulate or biomagnify in the edible part (predominantly fillet in the case of fish) are the most relevant with regard to food safety. Accumulation is the progressive increase in the concentration of a feed contaminant in an organism or tissue. Biomagnification normally refers to the increase of an environmental contaminant at the succeeding trophic levels. However, dietary contaminants are also said to biomagnify when the concentration in the fish exceeds that of the feed.

The relative dietary accumulation among environmental contaminants is often expressed as ‘retention’, ‘assimilation’, or ‘accumulation efficiency’, which is defined as the fraction of a contaminant in the consumed feed that is deposited in fish or fish tissue over time. Dietary accumulation occurs when the uptake exceeds the organism’s ability to remove the contaminant from the body. Dietary accumulation of environmental contaminants in fish can hence be modelled by the net function of uptake and elimination rates.

The uptake rate from food expresses the entry of a contaminant from the food into the fish, and can be described as the product of the uptake (absorption) efficiency from food and the feeding rate. Elimination is the removal of a contaminant from the body by processes such as biotransformation and/or

physico-chemical elimination. Other processes that can reduce the level of a contaminant in the fish include growth dilution and reproduction (Sijm *et al.*, 1992). In aquaculture reproduction is often of minor importance since fish are often harvested before sexual maturation occurs. In this chapter the carry-over of several feed contaminants will be compared. The relative carry-over will be addressed in terms of dietary accumulation (retention) and/or biomagnification in cultured fish species.

The carry-over of dietary inorganic forms of metals is generally lower than that of POPs or organic forms of metals. The distribution of these contaminants among organs, including muscle, also differs among the different contaminant groups (e.g., metals versus POPs) or chemical species of contaminants (e.g., inorganic or organic form of metals). For example, dietary methylmercury is much more efficiently accumulated in fish muscle compared to inorganic mercury. This section will give a brief overview over the dietary carry-over of environmental contaminants into the edible part of the fish. Levels of metals and POPs in feed and farmed fish are given in Chapter 22 (Monitoring and surveillance to improve farmed fish safety).

2.5.1 Dietary carry-over of persistent organic pollutants in farmed fish

Dietary accumulation of several POPs is related to the hydrophobicity of a particular POP (e.g., Fisk *et al.*, 1998). More hydrophobic organochlorines will have a greater tendency to accumulate from the feed than less hydrophobic organochlorines. Lipid soluble POPs readily accumulate in the lipid compartment of fish, and oily fish species have higher loads of these contaminants than lean fish. In order to compensate for differences in relative tissue lipid content among different fish species, levels of POP concentrations are commonly expressed on lipid basis. However, concentrations of POPs in fillet or food product (wet weight) basis are more relevant for human consumption and hence food safety assessment.

Concentrations of POPs in flesh samples of farmed fish have been demonstrated to be correlated with feed concentrations (Bell *et al.*, 2005; Lundebye *et al.*, 2004; Berntssen *et al.*, 2005; Isosaari *et al.*, 2005). Fish oil used in commercial fish feeds are the main source of POPs in farmed fish, and organochlorine levels in feed and farmed rainbow trout and Atlantic salmon increase with increasing fat inclusion in the feed during a production cycle (Karl *et al.*, 2003; Berntssen *et al.*, 2005). In farmed Atlantic salmon and rainbow trout (*Onchorhynchus mykiss*), the fat content in feed and fish gradually increases, resulting in the highest fat and POP levels in the market-sized fish (Karl *et al.*, 2003; Berntssen *et al.*, 2005). Farmed salmonids, such as Atlantic salmon and rainbow trout, have higher inclusions of fish oil in their feeds (and hence higher POP levels) compared to other farmed species such as sea bream (*Dicentrarchus labrax*) and carp (*Cyprinus carpio*). Furthermore, salmonids are oily fish causing higher accumulation of the lipid-soluble POPs in fillets of these farmed species compared to leaner species. Consequently studies on the carry-over of POPs

from feed to farmed fish have focused on oily fish species such as rainbow trout and Atlantic salmon.

In farmed oily fish, as in other farmed animals, it is believed that no steady state in POP levels will be reached during a normal product cycle, due to a continued growth and a relatively limited lifespan because of harvesting for consumption (e.g., EC, 2005a). In addition to the level of feed contamination, growth, feed utilisation and lipid deposition are other factors influencing the level of POPs in farmed fish. A reduced specific growth rate and feed conversion has been shown to increase the concentration of dioxins and PCBs in farmed Atlantic salmon (Berntssen *et al.*, 2005). Although POP levels are generally higher in oily fish species compared to leaner species, the direct relationship between lipid content and POP levels in farmed fish is inconclusive. No clear relationship between total PCB concentrations and total lipid content was observed in farmed Atlantic salmon (Carlson and Hites, 2005). Growth, feeding utilisation and lipid deposition often change in combination during a production cycle, making it difficult to singularly relate one of these factors with POP levels in a farmed fish.

Fish size, age, species, salinity and temperature, as well as duration and magnitude of exposure are important factors that have been shown to affect dietary carry-over of POPs in experimental studies (e.g., Opperhuizen and Sijm, 1990; Fisk *et al.*, 1998; Seubert and Kennedy, 2000). Many dietary studies are performed on small fish, or fish species that are not farmed, and involve relatively high exposures for short duration. Fewer studies involve consumer-sized farmed fish that were fed realistic POP levels over longer time, which is most relevant with regard to food safety. The following section will mainly address the dietary accumulation of selected POPs in slaughter-sized farmed fish species.

2.5.2 Dioxins and polychlorinated biphenyls

Dioxins (PCDD/Fs) are generally readily deposited in the muscle of both farmed rainbow trout and Atlantic salmon (Karl *et al.*, 2003; Lundebye *et al.*, 2004; Isosaari *et al.*, 2004). Approximately 29% of the consumed PCDD/Fs and 52% of the dioxin-like PCBs (DL-PCBs) are retained in the edible part of slaughter-sized cultured rainbow trout (Isosaari *et al.*, 2002; Karl *et al.*, 2003). The non-fillet part (viscera) of the fish has higher levels of PCDD/Fs and PCBs than the skinned fillet, which accumulates about 30% of the total PCDD/F and PCB content found in whole Atlantic salmon (Isosaari *et al.*, 2004). The seventeen PCDD/Fs and twelve dioxin-like PCBs congeners that are listed in Table 2.2 accumulate differently, depending on congener group and/or chlorine substitution pattern (Isosaari *et al.*, 2004). The retention of DL-PCBs is higher than that of PCDD/Fs, which causes relative higher levels of DL-PCBs compared to dioxins in muscle than feed. Farmed catfish (*Ictalurus punctatus*) that are fed diets containing considerably lower levels of lipids than salmon, have no preferential accumulation of the minor dioxin-like PCB fraction in the feed compared to other PCBs (Fiedler *et al.*, 1998).

The lower chlorinated (tetra- and penta-) PCDD/Fs, and more toxic, congeners accumulate more readily than the higher (hepta- and octa-) chlorinated congeners in whole Atlantic salmon. This causes a preferential enrichment of the more toxic PCDD/Fs in the fish (Isosaari *et al.*, 2004). Chlorine substitution patterns that are associated with a preferential accumulation among DL-PCBs, include non-ortho substitution (Isosaari *et al.*, 2004). Biomagnification seems to decrease with increasing chlorination among 160 PCB congeners in fillets of farmed salmon (Carlson and Hites, 2005). Several PCB congeners were shown to be biomagnified from commercial feed in the muscle of reared sea bass (*Dicentrarchus labrax*) although no clear trend was observed (Serrano *et al.*, 2003).

2.5.3 Polybrominated diphenyl ethers

The general retention of polybrominated diphenyl ethers (PBDEs) in whole fish was found to be high, and an average of 95% in total of fifteen PBDE congeners in feed accumulated in whole consumption-sized Atlantic salmon (Isosaari *et al.*, 2005). The skinned fillet accumulates 42–59% of the sum PBDE consumed (Isosaari *et al.*, 2005). Although PBDEs are organic contaminants with properties and nomenclature similar to PCBs, they have a different dietary carry-over compared to PCBs (52, 153, and 180), which have similar lipophilicity (Stapleton *et al.*, 2004a). Biotransformation of higher brominated PBDEs into lower brominated PBDE congeners is an important factor in the retention estimations of the PBDE congeners. The actual carry-over of low brominated BDEs such as tetra-BDE-47 is lower than reported due to the formation of new tetra-BDE from higher brominated PBDEs. The predominant congeners found in fish and marine feed ingredients result from direct uptake of these congeners as well as debromination (biotransformation) from higher brominated congeners (e.g., penta-BDE-99 and hepta-BDE-183) into lower brominated congeners (e.g., tetra-BDE-47 and hexa-BDE-154) (Stapleton *et al.*, 2004a,b; Tomy *et al.*, 2004).

2.5.4 Organochlorine pesticides

Several organochlorines have been reported in farmed salmon and feeds, including mirex, DDTs, chlordane isomers, toxaphene, endrin/dieldrin, aldrin, nonachlor isomers, heptachlorepoxide, hexachlorhexane (HCH), hexachlorbenzene (HCB), and endosulfan (Easton *et al.*, 2002; Hites *et al.*, 2004). Although waterborne exposures are well researched for most organochlorine pesticides, relatively little is known regarding dietary exposures and carry-over to the edible part of farmed fish, though several organochlorine pesticides from feed have been shown to accumulate in the edible part of fish. In commercially reared sea bream (*Dicentrarchus labrax*) biomagnification from feed to muscle of DDTs and HCB has been shown to occur (Serrano *et al.*, 2003). Mirex, HCH and HCB were found to biomagnify in juvenile rainbow trout (Fisk *et al.*, 1998), and HCH, heptachlorepoxide and DDTs rapidly accumulated in the carcass

(whole body minus liver and GI tract) of juvenile rainbow trout (Konwick *et al.*, 2006). In cultured Asian seabass (*Lates calcarifer*), 15% of DDT and its metabolites partitioned in muscle (Bayen *et al.*, 2005). In farmed rainbow trout, the dietary retention of sum chlordane (cis-trans and oxychlordane) and sum toxaphene in the edible part of consumption-sized fish was approximately 33 and 27%, respectively (Karl *et al.*, 2002). The dietary retention of DDE and dieldrin in whole large mouth bass (*Micropterus salmonids floridanus*) after prolonged exposure was approximately 20–25% and 32–35%, respectively, with lower levels in muscle compared to gonads and liver (Muller *et al.*, 2004, 2005).

Different pesticide isomers have variable carry-over from feed, and biotransformation and/or elimination plays an important role in the accumulation of pesticides in fish muscle. For example, clordane isomers accumulate differently in channel catfish, with a preferential accumulation of the *cis* form over the *trans* form, and biotransformation leads to formation and accumulation of the metabolite oxychlordane (Murphy and Gooch, 1995). Hepatic biotransformation is also important in the accumulation dynamics and formation of DDT metabolites that also readily accumulate (Bayen *et al.*, 2005).

2.5.5 Metals

Fish meal is the dominant source of metals in fish feed. The carry-over of inorganic metals to fillet is in generally much lower than that of POPs. Whereas several studies have been performed on waterborne exposures of metals, few relevant studies exist on dietary exposure in fish. The exposure pathway (water or diet) is of great importance for the internal distribution of metals in target tissues in fish. Dietary metals first accumulate in the intestine and subsequently circulate via the portal system to the liver before reaching other organs. Dietary metals are often preferentially accumulated in the liver (Campbell *et al.*, 2005), and accumulation in muscle tissue is relatively low and requires long term exposure compared to the other internal organs. An exception are the organic forms of metals and metalloids such as methylmercury and arsenobetaine that readily enter the fish and are preferentially deposited in the muscle tissue.

Important factors that determine the degree of tissue contamination after ingesting metal-contaminated feed include ingestion rate and bioavailability of the dietary metal (Schlekat *et al.*, 2005). In general the ingestion rate (% body weight per day) of metals is higher in juvenile fish than adult fish. Assessment of the assimilation efficiencies of a given diet-borne metal can vary considerably, and it is difficult to give a broad generalisation about the bioavailability among the different metals (Schlekat *et al.*, 2005), except for the aforementioned distinction between inorganic and organic forms of metal. The feed composition has a major influence on the digestion and release of metals from feed components in the intestinal lumen and subsequently on bioavailability. Association with organic ligands such as phytates or proteins and/or amino acids can affect the absorption of a metal over the intestinal tract. Micronutrient interactions, important in formulated commercial fish feeds which contain vitamin and

mineral supplementation, can also influence the intestinal absorption of metals. Most of the understanding of micronutrient-metal interactions comes from mammalian studies; however, similar mechanisms are likely to occur in fish. Cadmium (Cd), for example, is a mimic of calcium (Ca) and high calcium or calcium-regulating factors can therefore decrease the availability of Cd from feed, presumably by competition for transport carriers. Dietary Ca supplementation resulted in much lower chronic accumulation of dietary Cd in target tissues of rainbow trout (Franklin *et al.*, 2005). Other micronutrient conditions such as vitamin C and D, iron and zinc can affect the intestinal uptake and/or accumulation of cadmium (Rothe *et al.*, 1992). Fish fed on low iron diets accumulated more Cd in the liver via the gut (Cooper *et al.*, 2006). Also body lead (Pb) retention can be affected by interactions involving calcium and the vitamin D endocrine system (Fullmer, 1992), and increased dietary Ca levels can reduce lead body burdens in dietary Pb exposed fish (Alves and Wood, 2006). Furthermore, general biological condition such as feed intake, nutritional status, gut passage time and gut physiology are factors that contribute to the large inter- and intraspecies differences (Schlekat *et al.*, 2005), thus further contributing to large variations in bioavailability.

Few life cycle studies including consumption-sized farmed fish have been performed. In the following section the relative accumulation of selected metals will be addressed, in experimental studies on farmed fish species, although often not reared up to market size.

2.5.6 Mercury and arsenic

For mercury and arsenic the carry-over to the edible part of the fish strongly depends on the chemical speciation. Methylmercury and arsenobetaine are the dominant chemical forms in fish (see Table 2.4 and Section 2.4 for relative toxicity) that readily accumulate in muscle, which is a main organ of deposition. In contrast, dietary inorganic mercury and arsenic accumulates considerably less in the edible part of the fish compared to other organs such as intestine, liver and kidney.

Estimates for whole body accumulation efficiencies for dietary methylmercury in fish vary considerably among studies (from 95% to 10%) and depend on source (natural prey versus formulated feed), fish species, fish size (Philips and Gregory, 1979; Leaner and Mason, 2002; Wang and Wong, 2003), and dose and length of exposure (Lock, 1975). In general, accumulation efficiencies are higher at lower doses (Houck and Cech, 2004; Lock, 1975) and lower with increasing exposure time (Houck and Cech, 2004). Accumulation efficiencies in juvenile freshwater Atlantic salmon or rainbow trout that were fed low contaminated pellet for a prolonged period were 41–23% (Lock, 1975; Berntssen *et al.*, 2004). Muscle accounted for 87–90% of the whole body burden in juvenile Atlantic salmon, indicating that muscle is an important sink for dietary methylmercury deposition. The whole body accumulation efficiencies for inorganic mercury vary between 6 and 27% depending on formulated diets or live prey,

respectively (Berntssen *et al.*, 2004; Wang and Wong, 2003). Muscle accumulated between 86 and 18% of carcass (whole fish minus intestinal tract), with decreasing muscle retention at increasing dietary levels.

For arsenobetaine, a high retention (40–55%) in muscle tissue following oral (Francesconi *et al.*, 1989) and intraperitoneally (Shiomi *et al.*, 1996) administration has been reported for yellow-eyed mullet (*Aldrichetta forsteri*) and carp, respectively. However, muscle absorption efficiencies as low as 8 and 15% have also been reported for Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*), respectively that were exposed for a prolonged time (Amlund *et al.*, 2006). Arsenobetaine is considered to be stable in the body, meaning that arsenobetaine will not be broken down to more toxic arsenic forms. Diet-borne inorganic arsenic accumulates in the intestinal tract, liver and kidney but not in muscle (Pedlar *et al.*, 2002).

2.5.7 Lead and cadmium

Net lead (Pb) retention in whole juvenile rainbow trout is 1–5%, and seems to be concentration dependent. Gut and internal organs, especially bone, are the main sites for lead accumulation. Bone accounted for 38% of whole body burden and lowest accumulation was in the muscle that accounted for 12% of the body burden (Alves and Wood, 2006; Alves *et al.*, 2006). For cadmium (Cd) whole body retention of administered dose is in the same order of magnitude as lead with whole body retention <1–5% for rainbow trout and eel (Franklin *et al.*, 2005; Haesloop and Schirmer, 1985; Handy, 1992; Harrison and Klaverkamp, 1989). Like most inorganic metals, highest levels are found in kidney and liver, and lowest in muscle. In juvenile rainbow trout, muscle contributed 30% of whole body burden in control fish and 1% in fish fed high Cd-contaminated feed (Handy, 1992).

2.6 Reducing the level of environmental contaminants to farmed fish

Consumption of oily fish can be an important source of human exposure to environmental contaminants, especially persistent organic pollutants (POP) including PCDD/F, DL-PCB, and PBDE and organochlorine pesticides (Hites *et al.*, 2004). The levels of metals in farmed oily fish seem to be of lesser concern with regard to human food safety (Foran *et al.*, 2004). As opposed to feral fish, farmed oily fish can be tailored is such a way that the exposure to humans of these contaminants is reduced when consuming fish.

Several strategies are being developed on how to produce farmed oily fish low in POPs, by designing diets and optimising feeding strategies taking into account sustainable aquaculture and fish welfare. There are three main approaches that singularly or in combination can reduce the levels of POPs in fish feed and farmed fish. One is to select marine fish oils with relatively low

natural levels of dioxins. Besides seasonal variation, there is a large variation in fish oil PCDD/F and DL-PCB levels depending on factors such as fish species, age, or geographical origin. Another strategy is to substitute fish oil with alternative, terrestrial feed ingredients which contain lower levels of dioxins than fish oils. Vegetable oils have lower PCDD/F and DL-PCB levels than most commonly used fish oils, and substitution of fish oil with vegetable oil has great potential to reduce the level of dioxins in farmed salmon. Finally, several techniques exists that can remove POPs from fish oils without affecting the nutritional status of the oils:

- Selection of fish oils with natural lower background levels of POPs.
- Use of alternative feed ingredient with lower level of POPs.
- Removal of POPs from feed ingredients.

2.6.1 Selective use of fish oils

Farmed salmon on the global market reflect regional differences in the levels of POPs (Hites *et al.*, 2004), which is probably partly related to the use of local resources as fish ingredients. Salmon reared in Chile had a relatively lower level of POPs compared to Norwegian or Scottish farmed salmon, which could reflect the lower background levels found in Pacific fish oils compared to North Atlantic fish oils (see Section 2.2). Selective use of marine fish oils with naturally low levels of dioxins and dioxin-like PCBs, such as oil obtained from fish in the Pacific Ocean, has been reported to reduce the levels of dioxins, and to a lesser degree dioxin-like PCBs in farmed Atlantic salmon (Isosaari *et al.*, 2004; Lundebye *et al.*, 2004). Consumer-sized Atlantic salmon fed on diets based on fish oils of Pacific origin for a period of 30 weeks had a total-TEQ PCDD/F and DL-PCB level of 2.9 ng WHO-TEQ kg^{-1}ww, which was not lower than the typical level found in Norwegian farmed Atlantic salmon fillets on the market (approximately 2.5 ng WHO-TEQ kg^{-1} ww, Hites *et al.*, 2004). Data from monitoring studies include randomly sampled farmed fish that have been fed different commercial feeds, including different sources of fish oil but also inclusion of alternative feed resources.

2.6.2 Use of alternatives to fish oils

Substitution of marine oils with vegetable oils has been shown to be an effective approach to reduce levels of both dioxins and dioxin-like PCBs in fish feeds and Norwegian and Scottish farmed salmon (Bell *et al.*, 2005; Berntssen *et al.*, 2005). The full substitution of fish oil with vegetable oil gave a sum-TEQ PCDD/F and DL-PCB level that are substantially lower than the current level found in Norwegian farmed Atlantic salmon fillets on the market (Berntssen *et al.*, 2005). The use of plant oils can also introduce potentially new risks to food safety, such as pesticides that have been used on crops. Pesticide contamination of plant oil is very variable compared to the more stable background levels of persistent organochlorine pesticides in fish oils. Contamination of plant oils with

pesticides depends on direct application of a pesticide on a crop and/or processing, and not on general bioaccumulation as for fish oils. A future challenge by increased use of vegetable oils in fish farming, is reducing the level of important nutrients such as very long chain omega-3 polyunsaturated fatty acids (VLCn-3 PUFAs) (Bell *et al.*, 2005; Berntssen *et al.*, 2005). Clearly, there is a trade-off between reducing undesirable substances and maintaining the nutritional status when tailoring farmed fish that is low in contaminants by using vegetable oils in the diet. One approach to reconstitute the typical marine fatty acids in salmon fed on vegetable diets is to feed with a full fish oil diet during the last phase of salmon culture, until market size (finishing diet). Feeding fish oil diets to salmon previously fed on vegetable oil diets for six months nearly (80%) restored flesh VLCn-3 concentrations, while the dioxins and dioxin-like PCB concentrations were still 60% and 47% lower than salmon fed fish oil diets throughout the production cycle (Bell *et al.*, 2005).

The incorporation of vegetable-based feed ingredients, however, potentially exposes farmed fish to groups of environmental contaminants that may otherwise be of limited significance. One group of chemicals of particular relevance are the pesticides. Current EU legislation on pesticides in feed and feed ingredients includes persistent organochlorine pesticides such as DDT, chlordane isomers, toxaphene isomers, endrin/dieldrin, aldrin, nonachlor isomers, heptachlorepoxide, hexachlorhexane (HCH), hexachlorbenzene (HCB), and endosulfan isomers (EC, 2002). Most persistent organochlorine pesticides have been, or are in the process of, being phased out in Europe and the USA. However, they can still being used in tropical and subtropical regions on crops from which vegetable meal and oil are produced. Vegetable oils can also contain higher levels of poly aromatic hydrocarbons (PAHs) than fish oils due to technological processes such as direct fire drying of grain, oilseeds or vegetable oils.

2.6.3 Removal of persistent organic pollutants

Decontamination of fish oils by the technical removal of POPs while maintaining beneficial nutrient status (deKock *et al.*, 2004; Breivik and Thorstad, 2005), is a further option that may support the production of Atlantic salmon low in contaminants and high in health-promoting nutrients. Several techniques exists that can remove POPs from fish oils (deKock *et al.*, 2004; Breivik and Thorstad, 2005). These decontamination techniques should aim to remove as many POPs as possible as well as having a minimal effect on bioactive components in the fish oil. Active carbon effectively removes PCDD/F, the removal of DL-PCB is less efficient and PBDEs are virtually not removed at all (Maes *et al.*, 2005; Berntssen *et al.*, 2006; Oterhals *et al.*, 2007). Other techniques such a volatilisation have a higher efficiency for removing DL-PCB but to a lesser extent PCDD/F (Carbonelle *et al.*, 2006), and a total removal of these POPs requires a combination of active carbon and volatilisation technical procedures (Carbonelle *et al.*, 2006). Efficiency of removal of PCDD/F and DL-PCB or other contaminants by such a combined technique is unknown. A third related

technique, short path distillation supplemented with a patented working fluid, removes all groups of POPs including PCDD/F, DL-PCB, PBDE, toxaphene, hexachlorbenzene, and DDT (Breivik and Thorstad, 2005). Depending on the experimental conditions, short path distillation can potentially reduce the levels of lipid soluble nutrient such as vitamin D and E, but to a far lesser extent than the removal of POPs (Berntssen *et al.*, 2006). A last technique, molecular distillation, which is used in the human food industry, efficiently removes POPs such as DDTs; however, removal of DDTs was concurrent with removal of the fat soluble vitamin A (Julshamn *et al.*, 1973).

2.6.4 Reducing the metal content in fish feed

Fish meal is the main source of metals (e.g., Cd) in fish feed and substitution with alternative protein sources could cause a change of metal levels in commercial feeds. Commercially used plant meal blends (including wheat, wheat gluten, corn gluten and soy concentrate) can contain lower levels of metals (e.g., inorganic arsenic, cadmium, mercury and lead) than fish meals and increasing substitution of fish meal with plant meal could potentially lower the level of metals in feed. Use of alternative marine protein resources can increase metal levels in feed. Inclusion of squid viscera increased the dietary cadmium level in feed to Japanese seabass (*lateolabrax japonicus*) without causing elevated muscle levels after 2 months feeding (Mai *et al.*, 2006). Similarly, the use of krill in Atlantic salmon and cod feed gave increased metal (e.g., Cd) levels in feed, exceeding EU upper limits, but not in the fillet (Moren *et al.*, 2006).

2.7 Future trends

Environmental contamination includes a vast variety of contaminants. Important contaminants such as polyaromatic hydrocarbons (PAH) and brominated flame retardants such as HBCD and TBBA, for example, were not addressed in this chapter. Furthermore, new emerging environmental contaminants continue to be identified with increased levels being found in the biota such as polychlorinated naphthalene (PCN), polybrominated diphenyls (PBB), polybrominated dibenzo-p-dioxins and furans (PBDD/Fs), and perfluorinated compounds (PFCs) including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Knowledge regarding the potential toxicity of such contaminants is required from a food-chain perspective to assess the potential risks to the consumer and the environment.

2.8 Sources of further information and advice

Contaminants in foods is a subject covered by numerous textbooks; however, few are related to seafood in particular. With regard to the risks associated with

food consumption, assessments are conducted in both feed and food by international and national bodies. The United Nations and the World Health Organisation established the Joint Expert Committee on Food Additives (JECFA) to conduct risk assessments of food additives, and later also of contaminants (http://www.who.int/ipcs/food/jecfa/en/). The JECFA provides scientific advice to the Codex Alimentarius which sets global food standards (http://www.codexalimentarius.net/web/index_en.jsp). At a European level the European Food Safety Authority (www.efsa.europa.eu) assesses risks of contaminants in feed and food and produces scientific opinions including the proposal of acceptable daily intakes for additives and tolerable intakes for contaminants which are subsequently used by the European Commission and Parliament in risk management, such as setting maximum permitted levels for contaminants in feed and food (http://ec.europa.eu/food/food/foodlaw/principles/index_en.htm). Risk assessments on contaminants in feed are based on scientific reports and publications, and take into consideration animal welfare, human health (food safety) and potential risk to the environment.

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3

Pesticide contamination in farmed fish: assessing risks and reducing contamination

D. C. Little, G. K. Milwain and C. Price, University of Stirling, Scotland

3.1 Introduction

Modern food production increasingly relies on maintaining optimal conditions for specifically modified crops and animals. Best management practices include ensuring timely and accurate supply of nutrients and protection from adverse growing conditions including attacks from pests – a term that encompasses any organism that retards performance of the farmed product. Trends to more intensive and widespread use of pesticides and their association with potential public health and environmental impacts have become associated with most forms of modern food production, including aquaculture. Demands for increasingly stringent regulatory controls in the use of chemical pesticides are matched by demands for cheaper food. Consumer awareness, stimulated by concern over long-term health impacts and a competitive media, has grown rapidly along with expectations for safe food that also encompass a variety of other 'qualities' (i.e., good for the environment, not damaging to specific parts of the environment-birds, views, corals, dolphins, etc., World Bank, 2006, 2007).

In this chapter we firstly set out the use of conventional pesticides in terms of their wider application and use, and clarify the likely direct and indirect channels to contamination of fish that is consumed. The trends in pesticide use are then presented based on, again, their broader use in the surrounding environment as well as their specific application within aquaculture. Potentially hazardous practices relating to farming fish or any other product can be assessed to place the risks in perspective and this process is introduced as part of developing good

practice in a further section. The effects of pesticides as individual compounds and in combination, is considered from both the perspective of users of pesticides, i.e. occupational risks, and consumers. The issue of *perception* of risk is also considered since non-scientists that make up the majority of consumers inevitably assess risk from their own knowledge and values.

There is a large range of chemical and biological compounds that have pesticidal properties and their detection in the food chain is a matter of increasing scrutiny matched only by a rapidly advancing technological capacity to identify ever lower levels. An overview of approaches to assessing pesticide residues, both within farming environments and the fish themselves, is given. This takes the perspective that the modern ubiquity of chemical pesticides and their ability to move through aquatic environments makes monitoring of pesticides and their metabolites a critical part of quality food production.

Approaches to controlling or eradicating pesticide use are then evaluated in the light of current practice and theory. Opportunities for mitigation of the risks imposed by pesticide residues in farmed fish on human health are assessed based on medical evidence and current health advisories for different classes of consumers.

The underlying sustainability of pesticide use is examined in the context of integrated pest management. Modern trajectories of intensive aquaculture production systems, particularly their dependence on marine ecosystem derived fish byproducts as feeds are critiqued. This is a major route through which pesticides and other persistent organic chemicals contaminate farmed fish (FAO, 2007; World Bank, 2006). The substitution of such feeds with terrestrially derived alternatives and the risk for contamination such a strategy imposes are reviewed. Finally future trends and major researchable issues are suggested and sources of further information given.

3.2 Trends in pesticide use in fish farming and in the vicinity of fish farms

Pesticide use, by volume and diversity, continues to increase globally within food production generally, towards broader human activities as well as more specifically in the production of farmed aquatic products. The relative risks to the environment and humans associated with increased pesticide application in support of intensification of aquaculture needs to be considered along with the risks to aquaculture from pesticides used elsewhere. Both the health and productivity of the fish and impacts on consumer health and perceptions deserve attention. The importance of aquaculture in terms of supporting fish consumption has grown strongly in the last two decades as pressures on wild stocks and demand from increasingly urbanized populations has increased (Little and Bunting, 2005). This controlled production is based on a wide range of species and culture systems operating over a huge range in intensity and productivity but often still relatively geographically limited. Thus, almost the entire global

production of farmed Atlantic salmon occurs in four countries and one country, China, produces almost 70% of global farmed output (FAO, 2007). In such rapidly developing economies, the broader economic context is equally dynamic and typically agriculture is also changing and intensifying with consequent increased use of pesticides. Increased multiple use of water within watersheds and irrigation systems renders cross contamination more likely, particularly within clusters of value-added food production – both horticulture and aquaculture.

Before looking at trends in pesticide use, an appreciation is required of the terminology, categories and classes that distinguish pesticides in terms of their nature, functions and the levels of risk they pose. Pesticides, as with other chemical products such as disinfectants, fertilisers, hormones, anaesthetics and therapeutic drugs, are commonly used in aquaculture, but few are manufactured specifically for that purpose (Pillay, 1994). Pesticides can be natural or synthetic, appear in different forms and with a range of characteristics and modes of action. Pesticides have been classified into many categories according to their functions, properties and toxicities. With respect to their functions, there are many sub-categories where some of the more common ones include herbicides, insecticides, fungicides, rodenticides, molluscicides, nematicides and acaricides. In terms of numbers of active ingredients, herbicides dominate pesticide use (50%) compared to insecticides and fungicides (17% each). In addition to mainstream food production (agriculture, horticulture, aquaculture) pesticides are widely used in forestry, amenity use (e.g., parks, playing fields), in the preservation of built structures (industrial, domestic, etc.), manufactured products (e.g., paint, wallpaper pastes, masonry treatment), homes and gardens, food and commodity storage, animal husbandry, public hygiene and pest control, human medicines, and general aquatic and marine pest control (e.g., anti-fouling paints) (BMA, 1992). Despite their well documented negative effects, pesticides have had positive effects including increased crop and livestock yields, improved food safety, human health, quality and longevity of life, reduced drudgery, energy use and environmental degradation.

Their form, properties and concentrations amongst other factors, such as the nature of the environment and sensitivity of susceptible organisms, influence the nature and severity of their effects (BMA, 1992). The World Health Organisation (WHO) has classified pesticide hazard levels based on their formulation and acute toxicity to rats through oral and dermal exposure (Table 3.1). The WHO also provides a list of 15 types of pesticides based on their antidote properties; however, there are more than this and some pesticides fall within more than one category. Further information on the effects of these types of pesticides is provided later in the chapter.

In aquaculture, as with any type of plant or animal cultivation, pests can affect production efficiency where the environment, pathogens and the host are all influential. Pests affecting aquaculture are wide ranging in their type, nature and distribution and are often associated with specific environmental conditions. From extensive to more intensive aquaculture, these conditions are sometimes

Table 3.1 Pesticide classification and common effects

Pesticide group	Effects
Organophosphates	Disturbs peripheral nervous system (long-acting)
Carbamates	Disturbs peripheral nervous system (short-acting)
Organochlorines	Disturbs central nervous system (long-acting)
Pyrethroids	Irritant to eyes, skin and respiratory tract
Thiocarbamates	Irritant to eyes, skin and respiratory tract
Paraquat	Irritant to skin and upper respiratory tract, if enters blood stream (through skin or ingestion) causes lung and kidney failure

Source: Murphy (1997).

made more favourable for pests where host species are often less tolerant to pathogens, more concentrated, stressed, damaged and living in poorer conditions than their counterparts in the wild. The use of exotic species in aquaculture is widespread. This may result in greater sensitivity, and thus increased need for pesticides, since such species are likely to be more poorly adapted to pests in the new environment. Such introductions and transfers are also related to the spread of pests into new environments and are often associated with negative impacts. Interactions between aquaculture and pesticides are affected by the characteristics of the system, i.e. if they are land- or water-based systems. Whereas land-based systems such as ponds and tanks can be effectively isolated from the water source, such exclusion of cages and enclosures is problematic.

3.2.1 Direct use of pesticides in aquaculture

In aquaculture, pesticides are used directly to control organisms that affect stock health and productivity through physical damage and disease, compete for resources or impact negatively on the culture environment and system efficiency. In the prevention and treatment of damage to stock, pesticides can be administered in feed or as bath, dip or flush treatments. The types of pesticides used and method of application depends on the target organism itself, the nature of the pesticide and the cultured species. The most common methods of controlling ecto-parasites include bath, dip and flush treatments, whilst for internal parasites in-feed treatments are often most appropriate where in both cases pesticides have more direct access to the pathogens. Bath and dip treatments are most suitable for dosage control in tanks or in cages using tarps where sufficient oxygen levels can be maintained when water exchange with the external environment is reduced. Dip treatments over short periods are often preferred for brood stock that are more susceptible to stress during the process. In raceways and flow-through systems where water flow cannot easily be reduced and oxygen levels cannot be artificially maintained, flush treatments over longer periods are most appropriate (Pottinger and Day, 1999). The type of pesticide

used in such cases depends on different factors including its effectiveness against the pathogen, toxicity to the cultured product and stability in the medium towards its target organism. There is a range of substances with pesticidal properties that are commonly used in the treatment of internal and external parasites. Some examples include the use of copper sulphate and malachite green in the control of freshwater fungus, cypermethrins for the control of protozoans, organochlorines and organophosphates in the control of *Argulosis* sp. and Phenothiazine in-feed treatment for trematodes. Dipterex and Bromex-50 are also used in the control of trematodes whilst malathion is used for copepod infections in carps.

In addition to their pesticidal properties, some compounds have other impacts on system management, particularly with regard to maintaining adequate water quality. Thus quicklime (CaO) has been traditionally used to 'disinfect' earthen ponds often at the same time as sun-drying. Its powerful oxidizing pesticidal properties also act to correct pH. Mahua cake, a natural product (*Bassia longifolia*) widely used to disinfect nursery ponds in India acts both to kill off insects and larger zooplankton, predatory on newly released hatchlings, but also subsequently as an organic fertilizer. Net fouling organisms like algae, macrophytes, tunicates and bivalves that congregate and reduce water exchange in fish cages are also controlled with pesticides. For anti-fouling purposes cage nets are commonly washed, dried and dipped or soaked in viscous solutions with pesticidal properties, many being copper-based. For preventative action against plant and animals that compete for resources, in pond systems, pesticides are often sprayed in liquid form or applied in solid form on the pond bed after a period of drying. Copper sulphate is the most widely used herbicide, especially for algal control, although various herbicides can be used in controlling floating, emergent and submergent weeds (Le Jeune *et al.*, 2006).

Different pesticides can be used to control predatory and other competitive animals. In ponds, for example, phenols have been used to control polychaete worms in the Philippines, Bayluscide for snails, whilst tobacco dust has been effective against crabs. Some non-specific pesticides can have lethal effects on both animals and plants, including chlorinated hydrocarbons like DDT and endrin.

A specific process based on use of organophosphates has been developed and widely adopted to reduce predation and enhance availability of live first feeding organisms in carp nursery ponds. A concentration of around 1 ppm of dipterex or similar products removes larger competitive zooplankton (copepods, cladocerans) and predatory insect larvae allowing smaller rotifers, which are relatively insensitive to such compounds, to dominate. The rapid breakdown rates of such organophopshates allow the re-establishment of copepods and cladocerans after a few days which have then become the preferred food of the growing carp fry. Traditional products such as tea seed cake, a residue containing saponin (glycoside) derived from the processing of wild tea (*Camelia* sp.), calcium oxide (quicklime) and extracts containing rotenone (belonging to the isoflavones) from several tropical and sub-tropical plants, particularly of the genus *Lonchocarpus*

and *Derris*, are commonly used in shrimp culture as it has expanded rapidly around coastlines in Asia. These compounds have broad effectiveness with less persistent damaging effects on the environment (Chiayvareesajja *et al.*, 1997).

Although aquaculture is the fastest sector of food production, its relative contribution to food supplies remains modest as does its direct consumption of pesticides compared to the broader agriculture sector. It is indirect impacts of pesticide use on aquaculture, particularly given the rapid expansion of value added horticultural products most dependent on chemical crop protection, to which attention must be drawn. The trends in pesticide use in agriculture and the spatial relationship with the growth of aquaculture is now further assessed.

3.2.2 Pesticide use and indirect impacts on aquaculture

Pesticides can also enter aquaculture systems and products indirectly through both man-made and natural processes. Some common examples of this include agrochemical spray drift, run-off and seepage from agricultural land or urban industry outfall (e.g., tanneries, pharmaceuticals) into aquatic systems that contain or feed freshwater or marine culture systems. Pesticide entry to aquaculture systems through irrigation from groundwater reservoirs and precipitation are also of concern, particularly in pond and tank systems dependent on these resources in drier, warmer regions. Pesticides can therefore originate from point or diffuse sources and influence aquaculture directly or indirectly where their contact with the product may or may not be the intended.

Understanding trends in pesticides sales and consumption requires careful interpretation. Ideally, trends in pesticide production and use, by quantity and value are differentiated by region, pesticide type, level of toxicity and the sector which they target. Comprehensive datasets providing this information are lacking, although such a database is currently being developed by the Food and Agriculture Organisation (FAO) of the United Nations. Nevertheless, there is much information that gives insight on the topic. Most of the global trade in pesticides relates to their use in agriculture. A growing world population and increasing demand for food has placed higher demands on natural resources and created the need for more intensive and efficient food production systems. Modern pesticide development and manufacture has an integral place in the industrialization of food production. Particularly since the 1950s and the onset of the Green Revolution, pesticides have become major value-added products produced mainly in industrialized countries, although markets for them have grown most rapidly in recent years in developing countries, particularly in the Asia-Pacific region. Sales have continually increased, in some countries pesticide use has doubled every decade since 1945. By 2000, global pesticide consumption had exceeded 2.5 million tonnes comprising several hundred chemical formulations and with estimated annual sales in agriculture alone of US$30 billion. Consolidation of the pesticide industry is well advanced; by 2000, 75% of pesticide sales were being made by only seven companies mainly located in developed countries (EJF, 2002).

In an increasingly globalized economy, agrochemical companies have accessed and developed markets world-wide. Market saturation and increased regulation of chemical manufacture and use in the developed world have both factored in the expansion of new developing world markets. In the Asia-Pacific region alone, Monsanto has offices in 11 countries. Patent protection on pesticide products does not always exist in developing countries where demand is higher for cheaper products. Newer more advanced products are therefore mainly targeted at Western markets whilst broad-spectrum generic products are more available in developing nations. The FAO and WHO have expressed concern that over 30% of pesticides marketed in developing countries do not meet international quality standards (EJF, 2002). With weaker legislative control over hazardous products and pollutants in developing than developed countries, one view is that poorer nations have become a 'dumping ground' for obsolete and the most hazardous of pesticide products.

With regard to risks to aquaculture and consumers, these trends in pesticide production and sales are cause for concern given the higher rate of expansion in agriculture and aquaculture in developing nations (EJF, 2002). Since 1980, agricultural production has grown at 2.6% per year of which 79% of growth was attributed to developing countries, increasing their global share in agricultural GDP by 9%. Two-thirds of this growth is a result of transforming economies in Asia and productivity gains. Horticulture forms a major part of this growth in developing countries with fruit and vegetable production increasing by 3.6% and 5.5% a year respectively between 1980 and 2004, with developing countries in Asia, particularly China, leading the way. Relative to cereals, horticulture increases returns on land 10-fold and is a major contributor to income and employment (World Bank, 2007). However, being more management intensive, heavy capital investment and chemical inputs are now associated with horticulture and an increase in the levels of risks associated with pest outbreaks and price volatility. This type of agriculture alone accounts for 28% of global pesticide consumption (World Bank, 2007).

The accelerating proportion of global fish supplies from aquaculture accounted for 43% of global fish supply in 2004 but production and consumption is very unevenly distributed. More than 90% of world production occurs in developing countries, with Asia accounting for over 80% in quantity and value. Aquaculture employs over 12 million people in Asia and is important in sustaining livelihoods and income from foreign exchange, particularly in developing countries, which accounts for some 22% of world trade in fish (World Bank, 2006, 2007). The speed of change can be extremely rapid; in one relatively confined area (the Mekong Delta) of one country (Vietnam) increases in the river catfish (*Pangasius* spp) production have leapt from less than 200,000 MT to around 1 million MT in less than three years. An estimated 22% of agricultural space is used by aquaculture and intensive commercial horticulture has expanded rapidly in the same area (Nhan *et al.*, 2007). Global trends in food production and pesticide use therefore suggests greater risks to the environment and human health in the developing world where trends in pesticide

use and aquaculture growth are most rapid. Moreover severe water shortages (Pearce, 2006) are likely to further encourage uptake of integrated water management in which both crops and fish production share and reuse the same water further exacerbating risks of contamination.

3.3 Risks to human health of pesticides in aquaculture products

3.3.1 Nature of risks and awareness

Pesticides can be harmful to human health, in the short-term and long-term, depending largely on the nature and toxicity of the chemical, its concentration and the length and type of human exposure. Pesticides can therefore have chronic or acute effects, in which gross symptoms may or may not materialise. People are likely to be much more aware of acute impacts than the risks associated with chronic poisoning where the effects may not be immediately apparent but of greater long term significance. Additionally the nature of the risk may vary with the level and nature of exposure to the same pesticide or its metabolite (BMA, 1992). People's awareness and perception of such risks, that subsequently influences their actions, are therefore equally as important in determining the actual risks as the physical factors themselves (Palis *et al.*, 2006). Frequent occupational exposure can lead to high chronic or acute poisoning. Consumers generally have much lower risks, typically through incidental chronic poisoning. Aside from these scenarios pesticides have also been associated with intentional poisoning and suicides, particularly amongst those, such as farm workers, that most frequently use them (DANIDA, 2003). Further sources relating to the specific chronic and acute medical impacts on people are given in the Further reading section.

3.3.2 Assessing factors influencing human health risks

Studies that attempt to assess the specific health risk associated with pesticides contained in fish are often confounded by the presence of other chemicals. For example, a study of tilapia (*Oreochromis mossambicus*) found that risks from arsenic and selenium needed to be considered as much as from DDE, a metabolite of the chlorinated pesticide, DDT (Moreau *et al.*, 2007).

The specific risks of pesticides to human health are influenced by many factors. Risks from the consumption of pesticide contaminated products are greatly affected by such factors as the species consumed (particularly with regard to trophic level and feeding habit), the specific body parts consumed, and the level and toxicity of pesticides present in the environment and retention within the product. Product storage, preservation and preparation can also affect actual exposure and risk. Individual sensitivity to pesticides is also variable (World Bank, 2006).

Risks from occupational pesticide use directly in aquaculture or allied food production has many influences. These include the efficiency and safety

associated with the application equipment used, the type of protective equipment and risk prevention measures taken, the nature of the pesticide products in terms of quality, packaging and labelling, and applicators' appreciation of the risks and appropriate mitigation measures (DANIDA, 2003; Dasgupta *et al.*, 2007). Literature on these issues are extensive; however, some common findings include farmers' use of pesticide application equipment that is not efficient from both economic and safety perspectives (Milwain *et al.*, 2006). Many users also lack suitably effective protective measures or have none in place, whilst many fail to employ other risk-reducing measures (Dasgupta *et al.*, 2007). Many pesticide products are also adulterated, have poor safety features such as lack of childproof caps, and are of poor quality and lack appropriate instructions for their use and health and safety advice. This can range from being not present or inaccurate, being presented in a language foreign to the country of use, or incorporating signs and symbols that users do not comprehend (Galt, 2007; EJF, 2002; Edworthy *et al.*, 2004; Milwain *et al.*, 2003). Although such circumstances are more common in developing countries, some of these problems, including limited access to health and safety information are also apparent in wealthier nations (Damalas *et al.*, 2006a; Flocks *et al.*, 2007). Users with limited education and less access to suitable information additionally are at higher risk from pesticides through lack of appreciation of the risks involved. Employees within the agricultural and aquaculture sectors are also often poorer and studies have shown that those who are more economically dependent are more likely to overuse pesticides to reduce financial risk and are less likely to acknowledge scientific evidence and warnings when judging pesticide-related health risks (Dasgupta *et al.*, 2007; Vaughan and Dunton, 2007; Peradeniya University, 2003a). These factors can encourage pesticide overuse, increased exposure and health risks to users in both developed and less developed country contexts (Dasgupta *et al.*, 2007; Galt, 2007). In recent surveys of farmers in Greece and over 800 farmers in Bangladesh, in each case almost half were overusing pesticides (Damalas *et al.*, 2006a; Dasgupta *et al.*, 2007). Almost all of the Greek farmers surveyed viewed pesticides as a guarantee for higher product yields and quality (Damalas *et al.*, 2006b). Studies of farming communities in Sri Lanka have revealed similar outcomes with use of pesticide cocktails, being more prominent amongst the poorest farmers attempting to reduce financial risk (Peradeniya University, 2003a).

3.3.3 Perception of risks

With regard to the risks associated with the consumption of contaminated products or occupational use of pesticides mentioned above, one major overarching factor is stakeholders' perceptions of the level of risks posed to them. This is important as these beliefs can have significant influence over aquaculture practices, trade and the actions or lack of them that stakeholders take in protecting themselves.

Firstly, considering consumers' perceptions of risk, these often vary depending on the type and source of information they have, their level of education and

experience with the issues at hand. The food industry has no obligation to provide information on what pesticides fish products have been treated with, thus leaving a vacuum in consumer awareness. It could therefore be argued that people such as those employed in fish culture are more familiar with the production process, chemical treatments and food safety risks than more distant and less knowledgeable consumers. Many developed and increasingly developing countries, have procedures to legislate and monitor health risks from pesticides in food; however, often the only reassurance of product safety consumers receive comes from product labelling and certification that indicate products as safe such as 'pesticide free' or 'organic' (DANIDA, 2003; Magkos *et al.*, 2003). Often such products are more expensive than conventionally produced products that incorporate pesticide use, raising the issue of consumers' affordability and willingness to pay for reducing associated health risks and the actual cost-benefits. Worse-off and less educated consumers are therefore potentially most vulnerable to this source of health risk. However, studies have shown that in some cases the health benefits from consuming certified safer foods over conventionally produced foods are not greater and that the additional costs outweigh the benefits (Magkos *et al.*, 2003).

Nevertheless, there are also cases where despite awareness by consumers of potential health hazards and ability to pay for safer products, they still choose not to purchase them. This scenario may result where potential risks apparently fail to manifest themselves and consumers remain sceptical, as perhaps with risks from chronic poisoning. On consumer perception of risk, the source, type and method of the information they receive can be influential. Food safety facts based on scientific research are often confined to business and regulatory circles. In many cases consumers are ill-informed, with the media being their only source of information, where risks are often over-exaggerated in dramatic fashion (Tucker *et al.*, 2006). Consumers' perceptions of risks have been shown to vary with the nature of the risk and their association with acute illness; however, consumer trust in the systems in place to protect their health is also influential (Knight and Warland, 2005; Saba and Messina, 2003). Consumer perception of risk has been shown to be higher where they perceive to have low personal control and there is a high societal responsibility and control in protecting the public (Miles and Frewer, 2003). These factors can have significant consequences on aquaculture business, trade and consumer health by changing consumer perception of risk and their actions. An example might be the highlighting of excessive pesticide residues in farmed salmon, increasing consumers' preference for wild salmon, which itself may pose higher health risks from toxins from its natural environment (Bell and Waagbø, 2008).

Such factors can also influence pesticide users' perception of risks from occupational exposure to pesticides. Studies have shown farmworkers in many countries to have low levels of awareness of pesticide exposure and prevention (Rao *et al.*, 2007). As mentioned these factors include users' education, literacy levels, the nature of the warnings and advice provided to them and their experience of the effects of pesticides on their own and other users' health

(Damalas *et al.*, 2006a). In countries where women have significant input to farming, yet are more restricted than men in their access to education, they have been shown to be at higher risk (Atreya, 2007). Although the risks from handling of pesticides is generally deemed higher than exposure from food, as with consumers, perceptions of chronic risks are often underestimated, particularly where symptoms fail to manifest themselves or appear minor in nature (Dasgupta *et al.*, 2007). Studies in the Philippines have shown farmers to perceive illness as inability to function, pesticides as medicines not poisons and exposure to occur only through inhalation and ingestion. Additionally they placed higher value on minor illnesses at the cost of exposure to pesticides (Palis *et al.*, 2006). As a result, those employed in applying pesticides, often the most marginalised, youngest and poorest in society are most vulnerable to the consequences of pesticide exposure, yet are also the ones least likely to have access to health care (Arcury and Quandt, 2007; Ntow *et al.*, 2006). However, there are cases where pesticide users, from having acknowledged the risks associated with spraying pesticides fail to reveal this in practice by taking mitigation measures. Studies of farming communities engaged in both horticulture and fish production in Thailand and Sri Lanka have shown that despite farmers' widespread recognition of health risks and protective measures required, few utilize them (AIT, 2003; Peradeniya University, 2003b). In such cases, protective measures are not taken due to their unavailability, high costs, uncomfortable nature, being time consuming to use and sometimes not being necessary (Damalas *et al.*, 2006b; Isin and Yildirim, 2007).

3.4 Detecting pesticide residues in farmed fish

3.4.1 Detecting pesticides

The process of detecting pesticides within aquaculture systems is as complicated as the pesticide compounds themselves. The extensive range of synthetic and naturally occurring pesticide compounds, as well as the numerous potential combinations in which they are applied, provides a complex analytical and logistical challenge in order to detect, quantify and monitor residue levels between different crops, systems and geographical areas.

There is widespread acceptance that, especially for the more persistent compounds such as the organochlorines, the potential exists for these compounds to make their way into the food chain and to be accumulated across trophic levels through consumption. Numerous studies have provided supporting evidence; for example in ospreys, shrimp (Robinson *et al.*, 2002), clams (Harino *et al.*, 2006) and rainbow trout. Therefore a relatively high degree of importance has been placed on detecting and monitoring those compounds that are applied within and surrounding farming systems. Due to the integration of farming systems in many parts of the world, pesticides applied for agricultural systems have the potential to make their way into aquaculture systems (Porte and Albaiges, 2002).

Analytical methodologies for the detection of pesticides are very often highly compound specific, and the number and range of active ingredients extensive,

therefore the detection of a particular compound often requires some level of a prediction of its existence in order for the test to detect it. This further complicates the situation associated with trying to detect pesticides which may have entered a farming system from elsewhere as the compound specific analysis may not be carried out for that particular compound, and screening for all the possible compounds can be very labour and capital intensive and therefore not suitable for the majority of monitoring/screening applications.

The most common analytical techniques involve chromatographic analysis carried out with HPLC and GC applications (van den Hoff and van Zoonen, 1999). A significant challenge within these respective methodologies involves the extraction of the pesticide compounds from the material in which they are occur or are thought to occur (e.g., water, fish tissue, soil, etc.).

Another significant analytical challenge arises when compounds are used together or occur in combination, as depending on the chemical group of the compounds in question, a number of synergistic effects are possible, thus the complexity of pesticide mixtures is of increasing concern and an active area of research (Faust *et al.*, 2000; Adam *et al.*, 2006; Key *et al.*, 2007; Macek, 1975). It has also been observed that 'inert' ingredients in pesticide formulations may be biologically or chemically active, further complicating assessment of overall impacts of their use (Cox and Surgan, 2006). Methodologies and the sensitivity of these respective methodologies are constantly evolving and improving (van den Hoff and van Zoonen, 1999).

3.4.2 Assessing residue concentrations and ecological effects

A pesticide's capacity to harm fish and other aquatic animals is largely a function of its toxicity, the exposure time, dose rate, and persistence in the environment.

Detection of pesticides through direct sampling can be achieved by collecting samples of the required medium (water, soil fish, etc.), for aquaculture systems determining the concentration of pesticides in water can be used as an indicator of the residues that would be present within the cultured organisms through exposure modeling. Depending on the biology of the cultured species and the culture conditions an assessment can be made as to the exposure and risks associated with the given level of contamination. Then direct analysis of the cultured organism can then provide validation of the modeled and predicted residue levels in that organism.

Another method for assessing the effects of certain concentrations of specific pesticides is to perform single species toxicity tests to determine the lethal concentrations and/or lethal dose which causes mortality in 50% of the population (LC50 and LD50 respectively) and the effective concentration (EC50) at which 50 percent of individuals show some effect as a result of the concentration present (Key *et al.*, 2007). These toxicological indicators can be useful in assessing the potential impacts of a given concentration detected in the field; however, caution must be applied when extrapolating this data as single

species toxicity tests are often conducted in laboratory conditions and therefore in isolation from the other abiotic and biotic factors that would normally be occurring at a field level scenario (Cairns, 1983).

A progression from the single species toxicity tests, conducted in the laboratory, to a more realistic field assessment are *in situ* bioassays. *In situ* bioassays involve placing an indicator species (a species that is normally present within the site, and one that shows some degree of sensitivity to the compounds being tested) into a cage or holding area within the body of water being tested, the cage must allow normal movement or water flowing through and should as closely mimic the 'normal/outside' environment as possible. The concentrations are then tested in the surrounding water body and the numbers of organisms in the bioassay, counted and recorded over time.

Whilst *in situ* bioassays are a step closer towards a realistic assessment of the effects of certain concentrations on specific organisms, they do still involve some degree of manipulation of the actual outside conditions (Kimball and Levin, 1985). Acclimatisation of the assayed organisms is an issue, as is the selection of suitable sensitive indicator species. In areas of high and prolonged exposure to pesticide residues suitable indicators may have already been excluded from the ecosystem and as such alternative organisms may not be indicative of the exposure situation.

Microcosm and mesocosm experiments provide an additional monitoring and assessment tool whereby the effects of certain compounds and concentrations are monitored over time, within a fixed body or water in which a 'natural' range of organisms are present. The population distributions and abundances are then recorded over time in relation to the different concentrations present in each experiment scenario. However, in order to truly assess the interactive impacts of the respective compounds toxicity, then ecosystem level testing is required (Kimball and Levin, 1985).

3.4.3 Exposure routes of fish to pesticides

Fish can be exposed to pesticides, their residues and metabolites from a variety of sources. Firstly pesticides may be applied intentionally to the fish for the purpose of disease treatments, for example the application of Cypermethrin bath treatments for the controlling of sea lice in salmon cages, or for the treatment of white spot in aquarium fish (Bodensteiner *et al.*, 2000).

Another route involves the inclusion of pesticides into the fish's diet, either intentionally, for example in-feed treatments containing avermectin and emamectin benzoate, or unintentionally through exposure to residues such as endosulphan in the raw materials of the formulated feed (Glover *et al.*, 2007).

Pesticides may also be applied for the purpose of pond preparation, for example the application of some organophosphates to kill copepods and other insects prior to the stocking of fish. Herbicides may also be applied to the periphery of ponds in order to control vegetation, these may also be applied to control aquatic macrophytes in the culture water itself (Chakroff, 1976).

Another input may be from the drift of pesticides into water bodies such as non-target areas. This input is particularly applicable where terrestrial crops and aquaculture coexist in close proximity, and a portion of the intentional application drifts into an aquaculture system. It has been estimated that often less than 0.1% of the pesticides applied to crops reaches the target pest (Pimentel and Levitan, 1986). Therefore a high proportion of the dose applied enters non-target areas, either directly as drift, through surface run off, or entering the groundwater and also accumulating in soils.

Therefore residues can be translocated between different compartments of the ecosystem predominantly through the movement of water, from one area to another. This occurs either through the surface waters or by percolating through groundwater to aquifers.

The translocation of water is often intentional in pumped and gravity-fed irrigation systems both within and between farms. Hydraulic connections between fish culture and crops inevitably lead to translocation of residues into non target areas where other organisms are being cultured for consumption (Pimentel and Levitan, 1986).

The issue of groundwater contamination is pronounced, for example extensive monitoring of groundwater quality carried out over several years has revealed pesticide contamination of aquifers in North America and Europe (Barbash *et al.*, 2001; Kolpin *et al.*, 1998; EEA, 1999; Gilliom *et al.*, 2006; IFEN, 2004). Measurements were recorded that were in excess of the European drinking-water limitations, $0.1\,\mathrm{mg\,L^{-1}}$ per substance except for aldrin, dieldrin and heptachlor, $0.03\,\mathrm{mg\,L^{-1}}$.

When assessing the risks to human health of exposure of farmed fish to pesticide residues, the major links to people need consideration. For example the occurrence of pesticide residues in ornamental fish is less of a risk to human health than pesticide residues in farmed fish intended for human consumption. Similarly fish that have been cultured for the purpose of brood-stock or for any purpose other than for direct human consumption would pose lower risks.

3.4.4 Occurrence and assessment of residues in farmed fish

The majority of pesticide compounds are lipophilic and as such the fish flesh and skin are a good indicator of recent exposure levels and the liver an indicator of historical exposure (Pyka and Miszczyk, 2005). However, the binding of these compounds and their metabolites onto lipids within the fish tissue make the issue of extraction and cleanup a complex one (Pyka and Miszczyk, 2005). A variety of cleanup methodologies now exist in which pesticide residues can be extracted from the matrix in which they are bound (Tekel and Hatrik, 1996; Lino and da Silveira, 1997).

Numerous biomarkers and indicator species have been developed to assess the impacts on, and monitor pesticide residues in, the environment (Maroni *et al.*, 2000). For example, freshwater mussels have been used as a biological monitor of residue levels (Bedford *et al.*, 1968), and similarly clams have been

used to the same effect (Hartley and Johnston, 1983) as has the use of AChE as a biomarker as previously discussed above. For example, organophosphorus insecticides (OPs) produce toxicity by inhibiting the cholinesterase enzymes in the nervous system and the monitoring of acetylcholinesterase (AChE) inhibition has been widely used in terrestrial and freshwater aquatic systems as an indicator of OP exposure and effects (Ranjbar *et al.*, 2002).

In order to minimize the human exposure of pesticides, maximum residue levels (MRLs) have been developed for certain crop types (including fish) with the intention of placing limits on the concentration of pesticide residues that can be found in any given food type (Dornseiffen and van Eck, 2000). MRLs are devised from a series of complex risk assessments and are then set by individual governments and thus vary from country to country depending on the level of regulation (Low *et al.*, 2004). In setting these limits, consideration is given to the recommended Acceptable Daily Intakes (ADIs) to different food stuffs derived from studies of likely consumption levels of each of the respective food stuffs and their typical levels of contamination (Nasreddine and Parent-Massin, 2002). In this regard pesticides that have more harmful effects and higher residence times are typically subjected to more severe restrictions.

The setting of limits on pesticide residues in aquaculture products and the environment is one way of reducing the risks associated with their presence; however, this requires robust legislation and regulation, monitoring and enforcement which varies widely between regions and countries.

3.5 Controlling pesticide use in aquaculture

3.5.1 Technical and management approaches

Various mitigation measures can and have been adopted, with various degrees of success, in reducing the need for and risks posed by pesticide interactions within aquaculture. Within partially or completely recirculating aquaculture systems, various mechanical, biological, chemical and radioactive water purification combined with good husbandry has been successful in reducing the entry and propagation of pathogens that would otherwise only been treatable with pesticides (Sarkar *et al.*, 2007). However where aquaculture systems are more open to the wider environment, such as in cages or in some cases in pond based culture, the control of pathogens can be more difficult. In some instances, natural predators of external parasites such as cleaner fish (e.g., wrasse) can be used in conjunction with salmon farmed in cages as a means of biological pest control (Pillay, 1994). Also the recent pressure on the development of less harmful compounds has lead to decreased environmental and health costs associated with the control of pests and pathogens in aquaculture systems, although issues such as toxicity of mixtures, metabolites and synergistic effects needs to be more fully understood before any of these compounds can be considered as safe.

These risks are all the greater in Asia because of the tendency for high value horticulture and aquaculture to co-locate in high potential irrigated areas, lower

controls of pesticide use and broader range of older more toxic compounds in use. The dynamism and wide diversity of cultured aquatic species, including many exotics, in this region also encourages movement and introductions, both of which increase risk from pests and pathogens. However, the efficacy of pesticide use is often relatively poor in aquatic systems and, compared to terrestrial agriculture quarantine treatments and protocols, are relatively underdeveloped. Trends toward best practice and organic management elsewhere in food production typically control, reduce or eliminate pesticide use in food production. This may be practically difficult given the open nature of many aquaculture systems. Hence producers of organic Atlantic salmon in net cages are regularly given derogations to use chemical anti-helminths such as cypermethrin against salmon lice on the grounds of animal welfare.

In contrast, pest problems are relatively less problematic in aquaculture than terrestrial agriculture systems but this probably reflects a more recent history of intensive aquaculture in most contexts. The potential for breaking the cycle of pests through simple drainage and drying of aquatic systems – the equivalent of fallow in conventional farming, is also an important advantage of managing pests in aquatic farming.

3.5.2 Pesticide control-market incentives

With the increasing scale of global food demand, intensity in food production and use of such pesticides, greater awareness and concern is directed at food hygiene and its safety for human consumption. The result of these concerns have been consumers' increasing demands for healthier food, with guarantees for purchases and increasingly stricter regulatory measures governing the food industry and trade in food products, including those derived from aquaculture. The market for organic and pesticide free products has increased and corresponding certification and labelling are now associated with many products, including those derived from aquaculture, where restrictions are placed on the use of pesticides. The growth in consumer demand for healthier products and the regulatory stringency governing the food industry has been greater in more developed wealthier countries than poorer nations, although growth is increasing in the latter. This would appear to be linked with the increased cost of producing and buying certified healthier foods over other foods, the cost of effective enforcement of regulations, the level of public awareness through education of such health risks, economic development and consumers' value of risk in comparison with other livelihood constraining factors. It is understandable that the amount consumers are willing to pay for such products varies with the importance they place on reducing these risks in the context of their overall livelihoods (World Bank, 2006). The obvious resulting scenario is that worse-off citizens living in poorer, less developed countries, in theory are at higher risk of pesticide contamination from local aquaculture products than the better-off people that live in wealthier nations. Additionally, in terms of trade, those in the aquaculture business in poorer nations are generally more restricted in the global

sale of their produce than their counterparts in more developed countries. Therefore overall, the prospect of conducting an international trading aquaculture business that attempts to mitigate human health risks from pesticides in aquaculture products, appears to favour better-off people in more developed wealthier countries than the worse-off in poorer, developing nations (FAO, 2007).

3.5.3 Control of pesticide use in aquaculture products

Regulation

Advances in the control of pesticide risks to the environment and humans from pesticide links with aquaculture have developed through scientific research and greater awareness of their fate and effects. As a result, various regulatory measures, that have been discussed, have emerged to minimize these risks. Whilst in theory these tools should be conducive to trade, the environment and human health needs, the standards that are set, robustness of legislation, regulation, monitoring and enforcement often vary between different countries. This is seen, for example, in cases where certain pesticides are banned in some countries but not in others. In some cases where they are banned, they are still in use as stockpiles, are run down or they are imported illegally, for example in the continued use of tributyltin (TBT) as an anti-foulant (FAO, 2007; Harino *et al.*, 2006; DANIDA, 2003). Where international trade is concerned, these differences can create trade imbalances that influence risks to consumers' health and the livelihoods of those that are dependent to any degree on these industries (FAO, 2007). Therefore, despite their positive effects in protecting consumers' health, there are drawbacks with regard to other livelihood factors.

Management

In examination of other control measures of pesticides in aquaculture and derived products, each source of interaction is addressed in turn. Starting with the control of pests that affect stock itself there are various ways in which the need for hazardous pesticides can be reduced. Through brood-stock management, breeding programmes, genetic engineering and advances in diets, the resistance of stock to certain pathogens can be improved. In addition, within self-contained, flow-through or partly re-circulated aquaculture systems the creation of more favourable culture conditions through oxygenation, water filtration and purification (mechanical, biological, chemical or radioactive), good husbandry and other measures to isolate stock from potential sources of contamination can reduce pathogen populations and aid stock tolerance. However, where aquaculture systems are more open to the wider environment, such as in cage or in some cases pond culture, control of pathogens can be more difficult. In some instances, natural predators of parasites can take the place of pesticides. Polyculture is a means of natural pest control and developing sustainable aquaculture where certain species, such as bivalves are able to concentrate and accumulate pathogenic micro-organisms and chemical

substances in polluted waters, thus reducing the need for and presence of pesticides. In adapting polyculture technology, Chinese, Vietnamese and Bangladeshi farmers have extended fish culture into their rice paddies, to the benefit of both fish and rice production. Irrigated rice fields are particularly suitable for nursing of carp and tilapia over short periods at relatively low densities compared to alternative systems; this results in large valuable juveniles for on-growing and the high value of the fish crop encourages the reduction or elimination of pesticides on the rice crop. Technically this could be emulated in millions of hectares of rice-growing areas of Asia and Africa (World Bank, 2006).

In order to reduce pesticide use in associated agriculture and ameliorate impacts on aquaculture, Good Agricultural Practice (GAP) and Integrated Pest Management (IPM), concepts have been developed, implemented and more widely promoted over the years. These have had variable results but assessed impacts of many projects have found overall positive impacts of training in IPM. Where fish production in ricefields has been introduced as part of farmer field schools promoting IPM pesticide has been eliminated among producers and reduced significantly even among their neighbours.

Aquaculture development is fuelling the development of new classes of synthetic and often highly selective pesticides with high efficacy and quicker degradation times that reduce environmental and human risks. For example, research concerning the use of the now widely used pesticide, emamectin benzoate ('SLICE'), have shown that at working concentration used in the treatment of sea lice, the active ingredient has negligible impact on sensitive zooplankton species that form the basis of marine food chains, thus reducing risks to humans through bioaccumulation. In addition to the use of more environmental and consumer friendly pesticides, such risks are also being reduced through more efficient chemical treatment approaches. In open aquaculture systems the use of tarps in bath treatments allows containment of pesticides during treatments thus reducing the loading required and amount released to the environment. In such instances, the reduction of water exchange between the culture and wider environment requires artificial control of life supporting parameters such as oxygen levels. Modelling of water and sediment concentrations is now routine for assessing impacts of such pesticide use in intensive aquaculture. The complexity of sample collection in the dynamic marine environment associated with coastal cage culture, requires constant revision and development of methodologies to ensure the most accurate possible situation assessment with regard to the monitoring of the dissipation of these compounds post treatment.

In the case of the use of pesticides as anti-foulants, other measures are currently being researched, in line with more stringent legislation, that include the use of natural substances such as tannins and natural marine biocides that are less toxic and have promising results. Additionally, recent research has also revealed the effectiveness of some 'new technology' cage materials' anti-fouling properties by way of reducing the ability of spat to settle and propagate (Hyperlast, 2003; Linder, 1992). Biofilm formation, a prerequisite for macro-algae and macro-fauna

colonisation, first comprises an overlapping sequence of events starting with the development of a conditioning layer, bacterial settlement and extra-cellular polymeric substance (EPS) (Corner *et al.*, 2007). Experiments have shown the use of some non-toxic, long chain, high molecular weight polymer coatings with polar and apolar blocks, applied to nets, to have some effectiveness against some bio-fouling organisms, but particular limited effectiveness on macroalgae, being able to perforate the coating (Schram, 2003). Such measures that have been mentioned so far to reduce pesticide use are just part of codes and guidance on best management practice (BMP) that are being promoted in the industry with many benefits (World Bank, 2006). Often, the benefits of pest and disease control are more holistically effective in areas of intense aquaculture operations where BMPs are acknowledged and practiced by all aquaculturists. As a result, many of these BMPs require aquaculture management on a macro-scale requiring agencies and industry groups to employ local area management initiatives with legislative and regulatory support. An example of such a management initiative includes synchronized farm treatments for sea lice infestations within management zones, which has proven beneficial in suppressing their widespread propagation (Fish Site, 2007; Treasurer and Grant, 1997). In consideration of the interaction between agriculture and aquaculture, such macro-management practices have also been incorporated into farming with regard to pesticide use. In addition, buffer zones between the two industries have also been successful in reducing leaching of agrochemicals into aquatic systems. The incorporation of buffer zones of natural vegetation such as wetlands has proven useful in reducing emissions of pesticides to water bodies (Kohler *et al.*, 2004).

In principle each of these mechanisms have benefits and constraints and many require knowledge and skill to be implemented effectively; however, they have been documented to work most effectively when the most suitable methods are used together as a comprehensive management plan by farmers with these attributes who are willing and able to do so.

Effective control of pesticide interaction with aquaculture requires appreciation of the industry, dynamics of pest problems and consideration of the wider environmental influences. Different methods of pesticide regulation and control are being exercised by means of both penalties and incentives, with varying outcomes for the environment, trade and livelihoods. However, other measures including the use of alternative, less harmful means of pest control and good management practices in aquaculture and other influential industries, are having some degree of success.

3.6 Pesticides in fish feeds

3.6.1 Occurrence of pesticides in raw feed materials

The growth of the aquaculture industry has been accompanied by increasing demands on raw materials for formulated aquaculture feeds. As has been previously discussed, bioaccumulation, biomagnification and bioconcentration

occurs when persistent compounds and their metabolites are consumed with some degree of regularity, therefore as potentially toxic substances become more and more present in all compartments of the ecosystem, then the risk of these substances being translocated is increased.

This trend can be seen for a range of different contaminants. Firstly PCBs and dioxins, which the evidence suggests are first being bioconcentrated within the wild fish stocks, then concentrated again when captured and refined for fish meals and fish oils, as a component of fish food (Serrano *et al.*, 2003; Jacobs *et al.*, 2002).

Secondly, and perhaps more pertinently for this chapter, is the increasing occurrence of pesticide residues and their metabolites within the raw materials incorporated into aquaculture feeds. Pesticide residues in feeds can originate from both terrestrially derived feed ingredients such as soya, corn and rice, and also from bioaccumulation within fish meal and fish oil components (Easton *et al.*, 2002). However, due to the respective intensity of pesticide application to agricultural crops in relation to aquacultural ones, the majority of the risks related to inputs of feed ingredients contaminated with pesticide residues originate within the agricultural sector.

Given the wide-reaching fate pathways of pesticides and their metabolites, as well as their extensive use within modern mono-crop agriculture, there appears to be a conflict of interests; on the one hand the justifiable movement away from fishery derived fish meal and fish oil contributions, to what is widely perceived as more sustainable resources, such as alternative plant-based feed ingredients. However, this plant production often requires intensive crop production in order to meet the demand for plant derived protein and lipid sources. Modern trends in intensive crop production, towards an increasing reliance on pesticides for pest control, suggest the importance of pesticide residues entering the food chain through inclusion in aquaculture feeds.

Evidence suggests that terrestrial, plant-based alternatives are nutritionally adequate (Stubhaug *et al.*, 2005) and can lower the inclusion and subsequent impact of toxic components such as polychlorinated biphenyls (PCBs) and dioxins associated with fish derived meals and oils (Berntssen *et al.*, 2005). It has also been widely recognized that both fish meals and fish oils contain significant levels of pesticide contaminants especially the organochlorines (Maule *et al.*, 2007), for example endosulfan (1,4,5,6,7,7-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3,-benzodioxathiepin-3-oxide), which has been shown to persist in vegetable material which is then subsequently incorporated into fish feeds (Glover *et al.*, 2007). Endosulfan metabolite levels as high as $90\,\mu g\,kg^{-1}$ have been measured in olive oil (Rubio *et al.*, 2006), which, if incorporated into fish feeds, would exceed the European regulatory limit of $0.05\,\mu g\,kg^{-1}$ (EC, 2002). This could implicate the diet as a possible source of endosulfan toxicity, and could potentially limit the development of certain sustainable fish feeds (Glover *et al.*, 2007). Other sources of pesticide contamination involve the use of pesticides during the storage of raw feed ingredients, i.e. to reduce losses to pests which consume the respective

ingredient prior to its inclusion in an aquaculture feed. Rodenticides, fungicides and anti-microbial pesticides amongst the most prevalent.

3.7 Future trends

The further development of global trade in aquaculture products will require continued improvements in traceability and adoption of Good Management Practices by producers. Increasingly any aquaculture enterprise targeting high value markets, whether located in developing or developed economies, will be monitored by third party certifiers for which absence of pesticide contamination will be an essential aspect.

As with any standards, setting such monitoring will be a moving target. The stringency of standards can be expected to continue to increase as analytical capacity to monitor the growing number of compounds develops. The trend to reductions in the detectable levels of pesticide compounds and their metabolites and cost of testing can be expected to fall in line with demand for such analysis. Further reductions in the relative proportions of fish-derived ingredients in aqua feeds are likely partly driven by the need to reduce residue levels. This trend will be accelerated by a relative surge in production of omnivorous and herbivorous species, such as catfish and tilapia, for which lower cost feeds are an important part of their comparative advantage.

These trends will be driven by public expectations for safe food and the market competing to serve such demand. Both government and private sector investment in alternative pest control approaches can be expected to accelerate but to be constrained by competing demands for land, labour and water. Control of hazardous pesticide stocks and production capacity will become an important issue of governance. Such products are likely to remain in common use among poorer producers and markets if cost effective alternatives are not made available.

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4

Veterinary drug use in aquaculture

B. T. Lunestad, National Institute of Nutrition and Seafood Research (NIFES), Norway and O. Samuelsen, Institute of Marine Research, Norway

4.1 Introduction

Like all other living species, fish are subject to a wide range of different diseases, some of which are of infectious aetiology. Infectious diseases are a consciously present challenge in aquaculture, and may cause economic losses as well as problems associated with animal welfare. Fish in aquaculture may encounter infections by viruses, micro-organisms such as fungi and bacteria, or by internal and external parasites.

Several viral diseases cause problems in aquaculture on a world-wide basis, but since there is virtually no available treatment for such infections, they do not contribute to consumption of therapeutic agents in aquaculture.

Diseases of fish caused by fungi, such as saprolegniose (*Saprolegnia* sp.) are easily observable, and have thus been well known since ancient times. Fungi are mainly a problem in the freshwater phase, and may necessitate antifungal treatment of fish eggs or fry.

More than 100 bacterial species have been associated with diseases in freshwater and marine fish farming. World-wide, the greatest economic losses have been caused by *Aeromonas hydrophila*, *A. salmonicida*, *Flavobacterium* sp., *Flexibacter* sp., *Tenacibaculum* spp., *Edwardsiella tarda*, *Photobacterium damselae* subspecies *piscicida* (*Pasteurella piscicida*), *Renibacterium salmoninarum*, *Streptococcus* sp. *Vibrio anguillarum* and *Yersinia ruckeri*. The most severe bacterial diseases encountered in temperate water aquaculture have been furunculosis (*Aeromonas salmonicida*), cold water vibriosis (*Vibrio salmonicida*), vibriosis (*Listonella anguillarum*), yersiniosis (*Yersinia ruckeri*) and several external infections mediated by bacteria such as *Moritella viscosa*

(former *Vibrio viscosus*), *Flavobacterium* sp. and *Flexibacter* sp. (Lillehaug *et al*., 2003). Winter ulcer (caused by *M. viscosa*) is the infectious disease causing highest losses in salmon farming in recent years in Norway. In lobster farming gaffkaemia caused by the bacterium *Aerococcus viridans* has been a serious threat.

In the seawater phase of aquaculture in temperate waters, the parasites that most often require drug treatments are sea lice such as the salmon lice (*Lepeotphtheirus salmonis*) and tape worms (cestodes). For these parasite infections several therapeutic agents are applied on a regular basis.

Since effective vaccines against several of the most important bacterial diseases have been introduced, viruses and parasites, especially sea lice are at present the most troublesome diseases for farmed fish in temperate waters. In connection with such vaccination as well as other handling of fish, anaesthetic agents are routinely used.

In this chapter the properties of and consumption patterns for antimicrobial agents, antiparasitic agents and anaesthetics used in aquaculture will be discussed. Furthermore, the seafood safety aspects and environmental considerations for such agents will be discussed.

4.2 Commonly-used veterinary drugs and routes of administration

4.2.1 Antibacterial agents

The use of antibacterial agents in aquaculture is limited by several practical constraints. In theory almost all treatments can be administered by injection, as is otherwise common in veterinary practice. However, individual injection of fish is costly in time and labour, requiring fish to be caught and anaesthetised with the inevitable associated stress. Therefore, injection is only used in the treatment of fish with high individual value such as broodstock, or ornamental fish. For invertebrates in aquaculture, the parenteral route is most relevant to treat, for example, a valuable lobster stock for gaffkaemia. Bath treatment, although easy to carry out with agents of high water solubility, is restricted to recirculating systems or tanks of limited size and is therefore used mainly in the treatment of small fish. Bath treatment has shown high efficacy in treating fish suffering from both systemic infections and skin or gill infections. Important, however, is the ability of cations in the seawater to complex with certain antibacterial agents such as oxytetracycline and flumequine and thereby inactivate and reduce the ability for the fish to absorb the substances. The dose and treatment regimen is therefore very different in freshwater compared with seawater. A higher dose and extended time of treatment is usually required in seawater. Oral administration permits treatment of large quantities of adult fish relatively easily at minor cost in labour and has become the prime route of medication to fish. Nonetheless, there are also limitations to oral administration. The absorption from the gut may be inadequate, the drug may be unpalatable

and the palatability may differ from species to species. Both hierarchical behaviour and the fact that diseased fish often show reduced feeding may result in uneven distribution of the antibacterial agent in the stock. Therefore it is important that therapeutic regimes be designed to maximise the efficacy and thereby minimise the risk of the development of resistant pathogens. Ideally, therapy should only be carried out when the target pathogen and its drug sensitivities have been properly identified. Speculative chemoprophylaxis to prevent the possible outbreak of disease, or the use of antimicrobials as growth promoters should not be employed in the aquatic environment, which is an ideal one for the development and spread of drug resistance.

The antibacterial agent is administered in the form of medicated food pellets. The drug is either coated on the outside of the pellets or incorporated in the pellet by mixing the drug with the food ingredients prior to the pelleting process.

4.2.2 Antiparasitic agents

Drugs against both ectoparasites and endoparasites are today available for oral administration as medicated food pellets. However, equally important as orally administered drugs is bath treatments. Previously, the most common method employed involved moving the fish into a mobile pen that was surrounded by a tarpaulin. The pen could be moved around a fish farm allowing for reuse of the treatment solution. To reduce stress from crowding, the treatment pen was oxygenated. After use the pen was towed into open sea before releasing the chemical. The use of a mobile pen is, however, labour intensive and has largely been replaced by tarpaulins or skirt as described below. Using a tarpaulin, the cage to be treated is drawn up to reduce the cage volume and then surrounded by a tarpaulin before the approved amount of the compound in use is added to the cage. Oxygen diffusers are placed in the cage to maintain adequate oxygen levels and to aid the mixing of the compound into the water. Where the conditions are favourable, cages to be treated are surrounded by a skirt rather than being completely enclosed. After treatment the tarpaulin is removed and the chemical is allowed to disperse into the sea.

4.2.3 Anaesthetic agents

Anaesthetic agents used in large-scale aquaculture in connection with handling as vaccination are administered as baths.

4.3 Drugs used in aquaculture: antibacterial agents

The most important antibacterial, antiparasitic or anaesthetic agents used in aquaculture will be described in more detail in the following sections.

Antibacterial agents are substances that kill or inhibit the growth of bacteria. These substances may be naturally produced by bacteria or fungi (antibiotics),

synthesised (chemotherapeutics) or made by a combination of these processes (semi-synthetic agents). Antibiotics have existed for many years and may have played a role as defence or competition mechanisms for the producing organism. Antibacterial agents for clinical purposes are used extensively on a global basis in the prevention and treatment of bacterial diseases among humans, animals and even plants.

Several antimicrobial agents have found application in the treatment of bacterial diseases of fish. The most important groups in this respect are the tetracyclines, quinolones, sulphonamides, aminopyrimidines and amphenicols. The main agents from each of these groups are described below. The structural formula for the antibacterial agents is given in Fig. 4.1.

It should be noted that the pharmacokinetic data presented in this section is not a result of a uniform experimental set-up and a direct comparison of the data must therefore be made with caution.

4.3.1 Tetracyclines

The development of the tetracycline antibiotics was the result of a systematic screening of soil specimens collected from many parts of the world for antibiotic-producing micro-organisms. A number of tetracycline varieties are currently in use in veterinary and human medicine. Chlortetracycline, the first isolated component in this group, was introduced in 1948, and oxytetracycline (OTC) was introduced in human medicine shortly after. Both OTC and chlortetracycline are antibiotic substances naturally produced by the actinomycetes *Streptomyces rimosus* and *Streptomyces aureofaciens*. Tetracyclines in general are broad-spectrum antibiotics, thus having an effect on a wide range of both gram-positive and gram-negative bacteria and some protozoa. As for other bacteriostatic agents, only multiplying micro-organisms are affected. Tetracyclines inhibit bacterial protein synthesis and the site of action is therefore the bacterial ribosome. The bacterial ribosome is smaller (70S) than the eukaryotic ribosome (80S), and is composed of 50S and 30S subunits as compared to 60S and 40S subunits for the eukaryotic ribosome. This difference explains why eukaryotic ribosomes are not particularly affected by tetracyclines. In the bacterial cell, tetracyclines bind principally to the 30S subunit of the bacterial ribosomes. They appear to prevent access of aminoacyl t-RNA to the acceptor site on the m-RNA-ribosome complex, which prevents the addition of amino acids to the growing peptide chain. OTC was examined for potential use in aquaculture shortly after its introduction into human medicine. In 1951, OTC was found to be effective against ulcer disease caused by *Haemophilus piscium* in brook trout (*Salvelinus fontinalis*). This made OTC rapidly become the drug of choice in the treatment of several bacterial diseases in fish.

Most of the tetracyclines are incompletely absorbed from the gastrointestinal tract in humans. The absorption of an oral dose of oxytetracycline is, for example, approximately 60% when the stomach is empty (Sande and Mandell, 1990). In fish, the absorption of OTC is generally very low and examples of

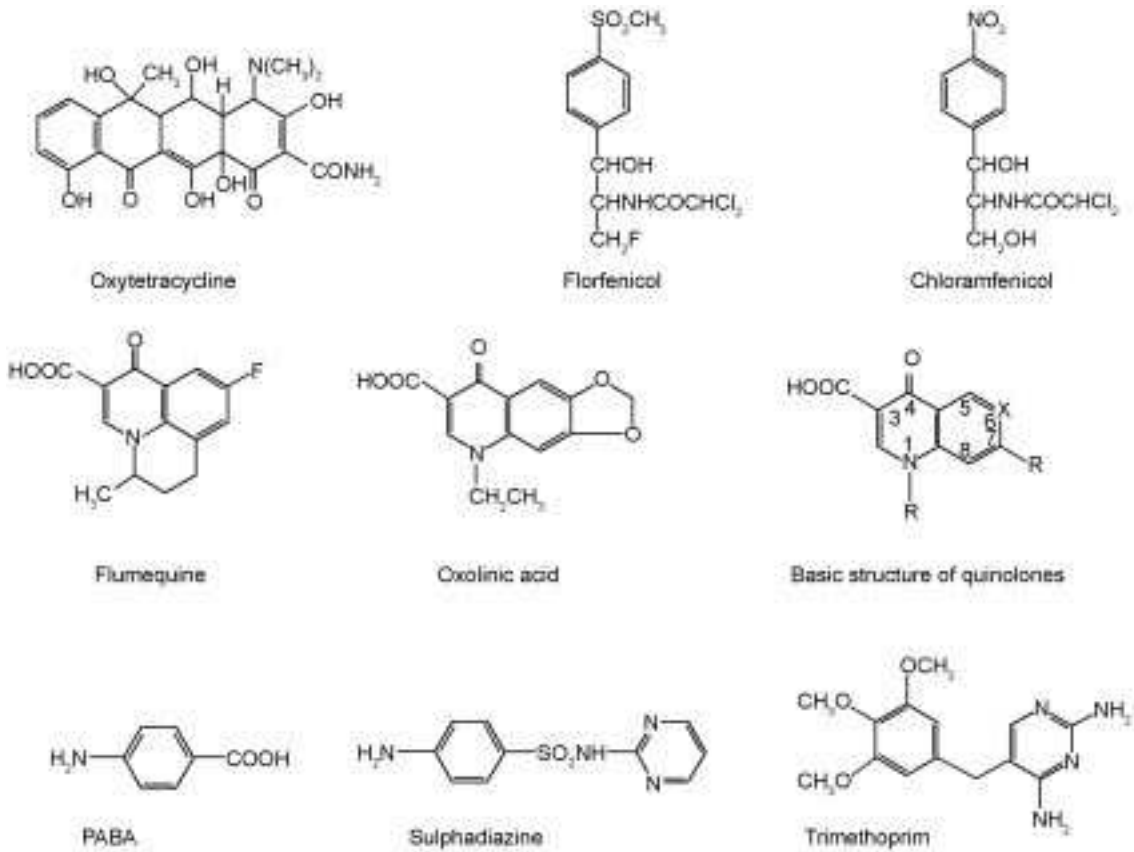

Fig. 4.1 Structural formula for common antibacterial agents in aquaculture.

bioavailabilities are 1.0–1.7% in channel catfish (*Ictalurus punctatus*) (Plakas *et al.*, 1988), 0.6% in common carp (*Cyprinus carpio*) (Grondel *et al.*, 1987), 7–9% in rainbow trout (Cravedi *et al.*, 1987), 3.2–7.3% in Arctic char (*Salvelinus alpinus* L.) (Haug and Hals, 2000), 9% in gilthead sea bream (*Sparus aurata*) (Rigos *et al.*, 2003), 22% in sea bass (*Cicentrarchus labrax* L.) (Rigos *et al.*, 2004) and 2% in Atlantic salmon (Elema *et al.*, 1996). Oxytetracycline is known to make chelates with di- or trivalent cations like calcium, magnesium, beryllium, iron and aluminium (Lunestad and Goksøyr, 1990). When such ions occur in the diet they significantly impair the absorption of oxytetracycline from the intestine (Sande and Mandell, 1990). Furthermore, marine teleosts are hypo-osmotic and must drink seawater to compensate for loss of water. Seawater with a salinity of 3.2% contains 54 mM Mg^{2+} and 10 mM Ca^{2+}. Due to this poor absorption, the standard dose recommended for treatment of fish is five to ten times higher than doses commonly used in veterinary practice, giving an increased environmental load of this agent.

The elimination of OTC in fish is temperature dependent, generally slow and with corresponding long withdrawal periods. In Atlantic salmon held in seawater the half-life at a temperature of 8 ºC was determined to 50 h (Elema *et al.*, 1996) whereas half-lives of 69 and 10 h at temperatures of 13.5 and 22 ºC respectively were found in sea bass (Rigos *et al.*, 2002a). The elimination is faster in seawater than in freshwater.

Even though resistance to tetracyclines has been observed in bacteria isolated before these agents came into common use (Hughes and Datta, 1983), the frequency of resistant strains has increased dramatically after the introduction of these agents in antimicrobial therapy (Levy, 1988). At present, resistance to tetracyclines is among the most common among all bacterial species of clinical relevance. In addition to development of resistance to the drug itself, bacteria exposed to tetracyclines often develop co-resistance to other and chemically unrelated antimicrobial agents (Levy, 1986; Lewin, 1991). There are several reports in the literature of rapidly emerging plasmid-mediated resistance against oxytetracycline in fish pathogens as well. Fortunately, the frequency of resistance has been greatly reduced during recent years following a period with no or very little use of the drug in Norway.

4.3.2 Quinolones

The quinolones are analogues of the earlier developed antibacterial agent nalidixic acid, originally isolated as a by-product in chloroquine synthesis, thus being an indirect result of anti-malarial research. The quinolones are completely synthetic and no similar products have so far been identified as products of any living organisms (Wolfson *et al.*, 1989).

Quinolones are bactericidal broad-spectra antibacterial agents. All quinolones share the basic structure shown in Fig. 4.1. Substitution at position 1, 3 and 4 appear to be essential for the antibacterial activity of quinolones and substitutions at position 6, 7 and 8 seem to enhance the activity. Fluorination

at position 6 increases the antibacterial potency and the activity against gram-positive bacteria. Quinolones enter the bacterial cell by passive diffusion through water-filled protein channels (porines) in the outer membrane and intracellular inhibit bacterial growth by interfering with the enzyme DNA-gyrase. This enzyme is essential in the coiling and uncoiling of bacterial DNA, and in the process of packing the DNA molecule within the cell, thus terminating normal DNA synthesis. Nalidixic acid, the prototype quinolone was introduced into human medicine in the early 1960s. However, today this drug has no significance because of its limited therapeutic utility and the rapid development of bacterial resistance (Wolfson *et al.*, 1989).

The two most used quinolones in aquaculture are oxolinic acid and flumequine. Owing to their low minimum inhibitory concentration (MIC) value for most susceptible fish pathogens and effective systemic distribution in fish when administered orally via medicated feed, oxolinic acid and flumequine have been used extensively to treat systemic bacterial infections in fish (Martinsen, 1993). They are effective against all common bacterial infections such as furunculosis, atypical furunculosis, classical vibriosis, cold-water vibriosis and yersinosis. The drugs are given via medicated feed, by injection for brood fish and by bath for smaller fish. Since flumequine and oxolinic acid both are weak acids and tend to complex especially with magnesium, the pH and the concentration of divalent cations in the intestine of the fish are important factors influencing absorption. The bioavailability of orally administered flumequine and oxolinic acid is therefore reduced in seawater, similar to what is observed for OTC. For the same reasons the absorption from seawater, applying bath treatment, is dramatically lower than from fresh water with a favourable pH value. High drug concentrations in the bath and extended exposure are therefore necessary to achieve therapeutic concentrations in the seawater fish. A major pathway for excretion of quinolones in fish is via the bile into the intestine. This process is also found to be more rapid in seawater than in freshwater. The pharmacokinetic properties of the quinolones in fish have recently been reviewed (Samuelsen, 2006).

Quinolone resistance has become a major problem in areas with extended use of the drugs in aquaculture. However, there are no indications of development of a bacterial plasmid-mediated resistance towards the quinolones.

4.3.3 Oxolinic acid

Oxolinic acid was first described in 1968 and is effective against gram-negative bacteria including many fish pathogens. Therefore, oxolinic acid has found broad use in Norwegian aquaculture since its introduction in 1987 and has to a large extent replaced drugs like OTC.

The bioavailability of oxolinic acid when given in feed to seawater fish varies and is low to moderate for both cold and temperate water species. In Atlantic salmon the bioavailability ranges from 21.4 to 40% (Rogstad *et al.*, 1993; Martinsen and Horsberg, 1995; Samuelsen *et al.*, 2000) with corresponding values

of 15% in halibut (Samuelsen and Ervik, 1999), 55% in cod (Samuelsen *et al.*, 2003a), 14% in gilthead sea bream (Rigos *et al.*, 2002b) and 28% in turbot (Poher and Blanc, 1998). In freshwater species, however, bioavailabilities of over 90% were measured in channel catfish and rainbow trout (Kleinow *et al.*, 1994).

Elimination of oxolinic acid varies with species, salinity and temperature. In Atlantic salmon elimination is rapid with half-lives ranging from 10 to 20 h (Martinsen, 1993) whereas in other cold water species like cod and halibut the elimination is considerably slower with plasma elimination half-lives of 84 and 52 h, respectively (Samuelsen and Ervik, 1999; Samuelsen *et al.*, 2003a). Between temperate-water species the variation is also large with elimination half-lives of 55 h in sea bass, 20 h in turbot and 12 h in gilthead sea bream (Poher and Blanc, 1998; Rigos *et al.*, 2002a,b). In rainbow trout held in fresh water the elimination is six times slower than in rainbow trout held in seawater and under otherwise identical experimental conditions. Reported half-lives for fish held in fresh water are 69 and 81 h respectively for rainbow trout and channel catfish (Treves-Brown, 2000).

4.3.4 Flumequine

Flumequine contains fluorine in position 6 and is a so-called second generation quinolone. The fluorination increases the antibacterial potency and activity against gram-positive bacteria.

In general the bioavailability in fish is higher for flumequine than for oxolinic acid. In Atlantic salmon the bioavailability ranges from 38 to 47% (Martinsen, 1993) with corresponding values of 31% in halibut (Samuelsen and Ervik, 1997), 65% in cod (Hansen and Horsberg, 2000) and 59% in turbot (Hansen and Horsberg, 1999).

Elimination is similar to oxolinic acid with the slowest elimination in cod and halibut (75 and 43 h respectively) and 34 h in turbot and approximately 20 h in Atlantic salmon. In sea bass, however, flumequine is rapidly eliminated with a half-life of 11 h (Rigos *et al.*, 2002c). In fresh water species elimination half-lives of 60 and 104 h were found in African catfish (*Clarias gariepinus*) and common carp (*Cyprinus carpio*) respectively (van der Heijden *et al.*, 1994).

4.3.5 Sulphonamides and aminopyrimidines

The sulphonamides is a large group of purely synthetic antibacterials that have been used against bacterial diseases in fish since 1948 when sulfamerazine was introduced in the USA (Alderman, 1988). They are structurally similar to para-amino benzoic acid (PABA) (Fig. 4.1) and act by competing with PABA for the enzyme dihydropteroate synthetase thus preventing the synthesis of bacterial folic acid, which is important in the bacterial nucleic and amino acid synthesis.

Treatments with sulphonamides may be combined with a 2,4 diamino-pyrimidine (trimethoprim or ormethoprim). Trimethoprim and ormethoprim are fully synthetic drugs and potent inhibitors of bacterial dihydrofolate reductase,

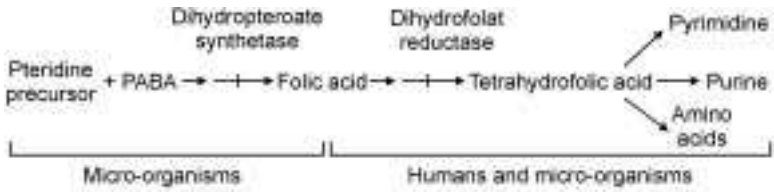

Fig. 4.2 Mode of action of sulphonamides and aminopyrimidines.

and in combination with a sulphonamide a synergistic antibacterial activity is achieved. A combination of sulphadimethoxine and ormethoprim in a 5 to 1 ratio has been given the trade name Romet®, whereas sulfadiazine in combination with trimethoprim (5:1) is called Tribrissen®. Both Romet® and Tribrissen® are widely used in veterinary medicine. Romet® and Tribrissen® have both proved effective in the treatment of enteric redmouth disease, furunculosis, vibriosis and enteric septicaemia (Alderman, 1988). These combinations are also called potentiated sulphonamides and the synergistic effect is a result of its inhibition of two sequential steps in the synthesis of tetrahydrofolic acid. The sulphonamide inhibits the incorporation of PABA into folic acid and trimethoprim and ormethoprim prevents reduction of dihydrofolate to tetrahydrofolate. The mode of action of sulphonamides and aminopyrimidines are shown in Fig. 4.2.

The bioavailabilities of trimethoprim and ormethoprim in fish are moderate to high ranging from 52% for ormethoprim in channel catfish to 85% in Atlantic salmon and 100% for trimethoprim in Atlantic salmon (Treves-Brown, 2000). The sulphonamides have moderate to low bioavailability in fish ranging from 16% to 46% (Treves-Brown, 2000). Sulphonamides are intensively metabolised in fish to their N4 acetyl derivatives that is found mainly in the bile.

The individual elimination of sulphonamides varies but is in general fairly rapid with plasma half-lives between 10 and 25 h. Elimination of both trimethoprim and ormethoprim is similar with half-lives of 20 to 25 h depending on species. An interesting effect of trimethoprim and ormethoprim is the large build-up of these drugs in the kidney and other melanin rich tissues. Although the use of potentiated sulphonamides reduce the change of resistance development, plasmid mediated resistance against both sulphonamides and aminopyrimidines have emerged following intensive use of these drugs (Treves-Brown, 2000).

4.3.6 Florfenicol

Florfenicol is a semi-synthetic antibacterial agent with chemical structure and spectrum of antibacterial activity similar to thiamphenicol. Both florfenicol and thiamphenicol are chloramphenicol analogues where the p-nitro group on the aromatic ring is substituted with a sulfonylmethyl group. Florfenicol and thiamphenicol are not associated with the toxic side effects as have been shown for chloramphenicol. Bacteria resistant to chloramphenicol and thiamphenicol

due to the production of the drug inactivating enzyme acetyltransferase are susceptible to florfenicol (Burka *et al.*, 1997). Florfenicol binds to the bacterial 50S ribosomal subunit and inhibits protein synthesis at the peptidyltransferase step. *In vitro* investigations with florfenicol have demonstrated potent activity against several bacteria pathogenic to fish. *In vivo* efficacy against furunculosis in Atlantic salmon and classical vibriosis in cod has been demonstrated (Nordmo *et al.*, 1998; Samuelsen and Bergh, 2004). *Yersinia ruckeri*, however, seem to have a natural resistance against this agent (Burka *et al.*, 1997).

Florfenicol is well absorbed in all fish species tested and possesses bioavailabilities of 91 and 100% in cod and Atlantic salmon, respectively (Horsberg *et al.*, 1996; Samuelsen *et al.*, 2003b). Elimination of florfenicol from plasma of cod is slow with a half-life of 43 h compared with Atlantic salmon with a half-life of 12 and 14 h (Horsberg *et al.*, 1996; Samuelsen *et al.*, 2003b). The metabolite florfenicol-amine is described as a major metabolite in Atlantic salmon, whereas in cod this metabolite was not detected in any quantitative amount. It is suggested that the lack of this metabolic pathway may contribute to the slower elimination in cod compared to Atlantic salmon.

Bacterial resistance towards florfenicol may be mediated both by chromosomal genes and plasmids. The magnitude of resistance among fish pathogens in general is not known, but it has been reported that despite widespread use, resistance to florfenicol does not seem to occur frequently (Michel *et al.*, 2003).

4.3.7 Resistant bacteria in farmed fish

In all situations where antibacterial agents are applied, such as in hospitals, in the society during non-hospitalised treatment, in animal farms or during treatment of pet animals, bacterial resistance has proved to be a problem (Teale, 2002). Many authors are concerned about the negative effects due to the use of antibiotics in agriculture and aquaculture (Bogaard and Stobberingh, 2000; Willis, 2000; Russel, 2002; Teale, 2002). Infections mediated by bacteria may necessitate the use of antibiotics in aquaculture. Such agents are today applied on a world-wide basis in the farming of fish and other aquatic organisms. Since only a few countries provide reliable and open statistics on drugs used for fish farming, information on types and the exact amounts of therapeutic agents used in aquaculture throughout the world is not easily obtainable (Burka *et al*., 1997).

Human diseases caused by multi-resistant micro-organisms have so far not been reported to be linked directly to consumption of seafood. However, an epidemic outbreak of cholera in Ecuador with multi-drug resistant *Vibrio cholerae* indicates an influence from aquaculture (Weber *et al*., 1994).

4.4 Antiparasitic agents

Agents against parasites are commonly divided into ecto- and endoparasitic substances. The prefix *ecto* means external and refers to parasites on skin or

gills, whereas *endo* means internal, and refers to parasites in the gastro-intestinal system of the fish. The structural formula for the antiparasitic agents is given in Fig. 4.3.

4.4.1 Ectoparasitic agents

Ectoparasitic agents may be applied in baths (acetylcholinesterase inhibitors, pyrethrines and pyrethroids) or by addition to medicated feed (avermectins and chitin synthetase inhibitors).

4.4.2 Acetylcholinesterase inhibitors

Acetylcholinesterase is an enzyme that specifically cleaves acetylcholine to acetate and choline. It is located both in the pre- and post-synaptic nerve terminal, bound to the membrane. An acetylcholinesterase inhibitor indirectly provides a cholinergic action by prolonging the lifetime of acetylcholine produced at the cholinergic nerve endings. This results in the accumulation of acetylcholine in the synaptic space. One among several effects of this accumulation is paralysis of motor function (skeletal muscles) and convulsion.

Organophosphates bind covalently to a serine OH group at the active site of the acetylcholinesterase molecule. When this occur the enzyme is permanently inactivated and unless a reactivator like pralidoxime (PAM), a synthetic pyridinium compound which can reactivate inhibited acetylcholinesterase by displacing the organophosphate, is administered, restoration of the activity require synthesis of new enzyme molecules. If a reactivator is not administered, the phosphorylated enzyme release one of the alkyl groups attached to the organophosphate. This process is called ageing and once occurred makes it impossible for the reactivators to brake the covalent bond between the enzyme and the organophosphate. Therefore, the reactivator must be administered before ageing takes place. Some of the organophosphates, which age in seconds, are extremely toxic and were developed for military use as nerve agents.

Organophosphates are administered by bath. Former compounds in use were trichlorfon (Neguvon) and dichlorvos (Nuvan). These are no longer licensed in Norway. At present the only organophosphate in use is azametiphos. Azametiphos is effective against adult and pre-adult lice whereas the larval stages are unaffected. Resistance to organophosphates has developed and requires an increase in dose and exposure time to achieve acceptable results.

4.4.3 Pyrethrines and pyrethroids

The pyrethroid insecticides have their origin in the natural insecticide pyrethrum, an extract of *Chrysanthemum* sp. The extract contains six esters, pyrethrines, all with insecticidal properties. The naturally occurring pyrethrines degrade quickly when exposed to light, and synthetic analogues have been developed (pyrethroids) to enhance both insecticidal activity and reduce the

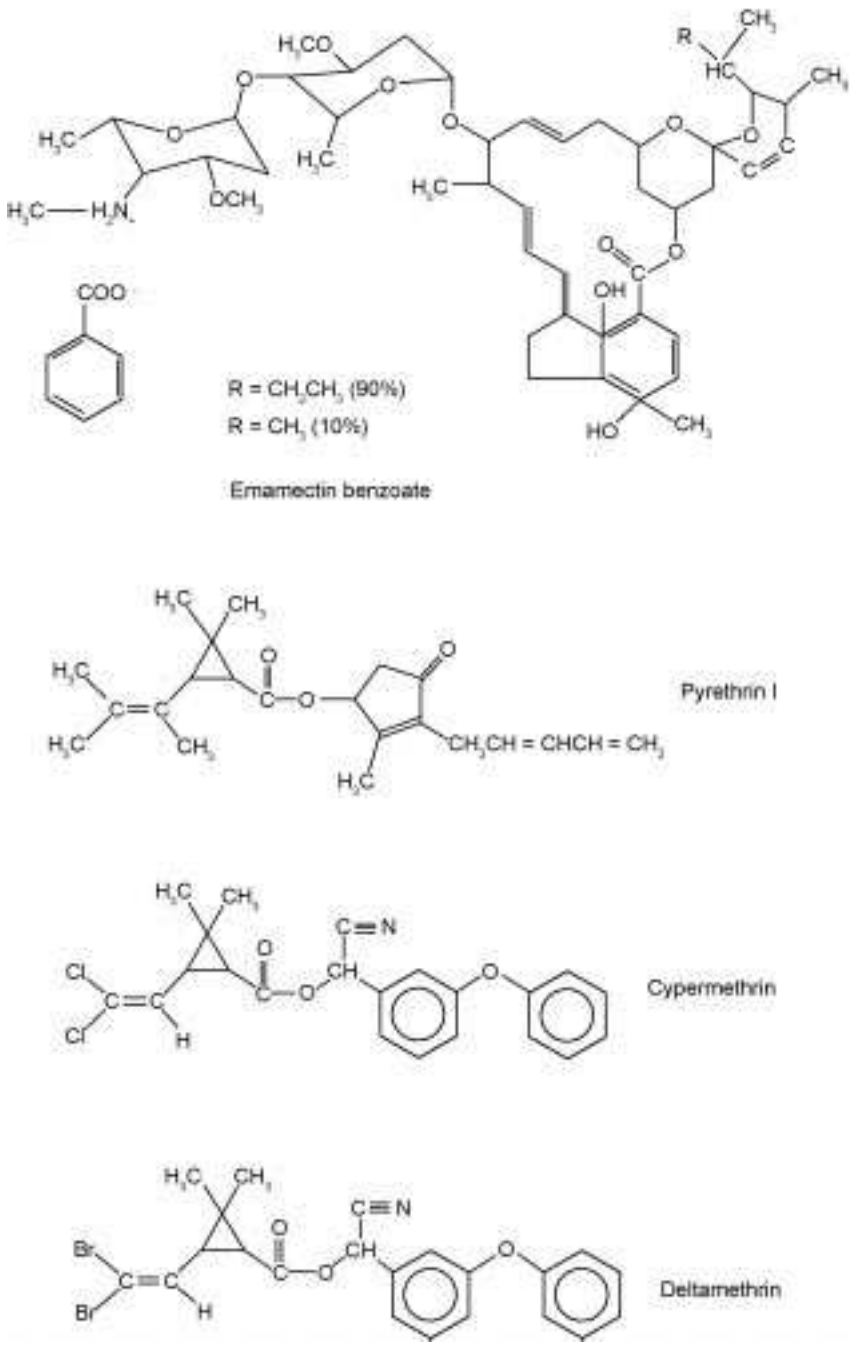

Fig. 4.3 Structural formula for common parasitic agents (ecto- and endo-) in aquaculture.

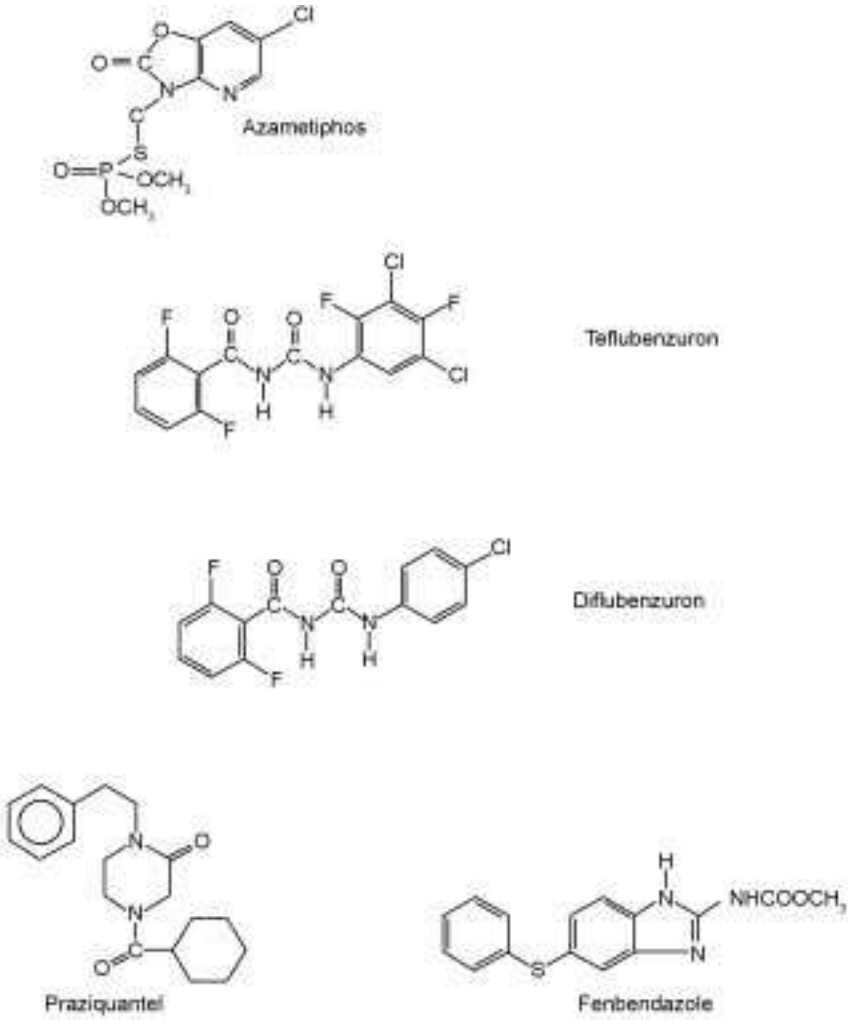

Fig. 4.3 (continued)

photolability. The two most common pyrethroids are deltamethrin and cypermethrin (Fig. 4.3). The primary mechanism of action of pyrethroids has been considered to be interference with the sodium channels in the nerve membrane by prolongation of the open phase. This results in tremors and convulsions due to hyperexcitation and eventually paralysis due to total blocking of neural activity caused by complete depolarisation of the membrane.

A second proposed mechanism of action is inhibition of normal chloride channel function at the neuro-transmitter gamma-amino-butyric acid receptor (GABA) – ionophore complex. Excess entry of chloride ions hyperpolarises the cell making it more difficult to depolarise and a subsequent reduction in neural

excitability and finally paralysis of the parasite. Furthermore, it has been hypothesised that the observed neural action of pyrethroids can also be related to interference with the calcium regulation in the cell. Direct effects on the release of neurotransmitters have been observed, by blocking of the calcium channels.

The pyrethroids are relatively safe for mammals due to lower receptor sensitivity and poor absorption from the gut. In fish, however, the safety margin is less. The pyrethroids have a broader spectrum of activity than the organo-phosphates since chalimus, pre-adult and adult stages of the lice are affected. The pyrethroids in use today are cypermethrin and deltamethrin. Pyrethroids are administered by bath using an emulsifier due to low water solubility. Pyrethrum is administered using a layer of oil on the surface of the water in which the drug is dissolved. The fish must pass the oily layer in order for the drug to have effect either by active jumping or in association with handling.

4.4.4 Avermectins

Originally isolated from the actinomycete, *Streptomyces avermitilis*, the avermectins represent a most recent developed group of insecticides. The mechanism of action in invertebrates, as described in the nematode *Caenorhabditis elegans*, is by binding to glutamate-gated chloride channels leading to an influx of chloride ions, thus giving a hyperpolarised cell. A second mechanism of action of avermectins is by increasing the production of the inhibitory neuro-transmitter GABA at nerve endings and prolonging the binding of GABA to the receptor. This gives the same effect as described above, hence increased chloride influx and hyperpolarised cells. In invertebrates, avermectins act on muscle cells and synapses in the peripheral nervous system, causing paralysis and eventually death of the parasite. In mammals, however, the toxic effect is low, since the avermectins do not cross the mammalian blood brain barrier, thus do not affect GABA-mediated neurones situated in the central nervous system (CNS). In fish, the blood brain barrier is not as impermeable as in mammals and CNS depression and deaths have been reported in salmon using ivermectin at therapeutic doses. There is no marked authorisation for the use of ivermectin in fish anywhere in the world, since the original patentees have stated that they do not intend to make any application for aquaculture use. Thus, the avermectin used in fish farming is emamectin benzoate under the trade name Slice. Emamectin benzoate is highly effective against pre-adult and adult lice and prevents the maturation of chalimus to motile stages. Given orally incorporated in feed emamectin gives protection for reinfestation for an extended period compared to the bath-administered compounds.

4.4.5 Chitin synthetase inhibitors

While the pyrethroids, organo-phosphorus compounds and avermectins all act on the nervous system of the parasite, the benzyl-ureas have an entirely different mode of action.

A polymer is a substance made up of a great number of small units called monomers joined together in a regular way. Chitin is a polymer where the monomer is D-glucose-amine and where the enzyme chitin synthetase is involved in the polymerisation process. Chitin is a major part of the exoskeleton (cuticle) of insects and crustaceans. The orally administered delousing agents Ektobann Vet. (teflubenzuron) and Lepsidon Vet (diflubenzuron) act by inhibiting chitin synthetase, thereby interfering with the formation of cuticle, thus exerting an effect at the moulting stage in the life cycle of exposed organisms. Chitin synthetase inhibitors have effects on all stages except adult lice that already have formed their exoskeleton. Diflubenzuron is moderately absorbed from the gut of the fish with a bioavailability of 31%. The drug enters the lice via the blood and skin of the fish.

The labour associated with oral treatment is far less then applying bath treatment. Therefore, a synchronised delousing of several fish farms in a defined geographic area is easier to obtain using an orally administered agent. The environmental effect is, however, more comprehensive for both avermectins and benzyl-ureas due to large stability in marine sediments and high toxicity to crustaceans with effect ranging from direct mortality to indirect behavioural responses (Selvik *et al.*, 2002).

4.4.6 Endoparasitic agents

Since endoparasites are by definition found in the stomach or intestine of fish, agents against such parasites are only given orally.

4.4.7 Praziquantel and the benzimidazoles

Praziquantel is rapidly and reversibly absorbed by the parasite. It is believed to have two primary and immediate actions in susceptible organisms. At the lowest therapeutic concentration the drug causes increased muscular activity followed by contraction and spastic paralysis. It is probably this effect that causes the parasite to lose their attachment to the host tissue. At a higher but still therapeutic concentration, praziquantel induces vacuolisation and vesiculation of the parasite tegument. This leads to osmotic and nutritional imbalance and release of the contents of the parasite, followed by activation of the host defence mechanisms and finally destruction of the worm.

The effect of praziquantel may be explained, at the molecular level, by increased membrane permeability to certain mono- and divalent cations, particularly calcium. Substituted benzimidazoles is a group of compounds with similar molecular structures. Some of these are among the most potent chemotherapeutic agents known with complete larvicidal activity. They have, however, a relatively low mammalian toxicity and lack activity towards other microorganisms.

The primary mechanism of action of benzimidazoles is unknown but they are believed to inhibit the synthesis of micro-tubuli by inhibiting the polymerisation

of tubulin. Micro-tubuli are used for intracellular transport. In addition, benzimidazoles inhibit the uptake of external glucose, a major nutrient for the parasite (nematodes) and the secretion of acetylcholinesterase leading to paralysis and dislodgement of the parasite (cestodes). The benzimidazoles in use are fenbendazole, medbendazole and albendazole.

4.5 Anaesthetic agents

Anaesthetics are used primarily for immobilisation of fish and more rarely for surgery. The loss of sensation is important for the reduction of stress during procedures like transport handling, vaccination and blood sampling. These agents are only administered as baths. The structural formula for the anaesthetic agents is given in Fig. 4.4.

The anaesthetics used in aquaculture are generally classified as local anaesthetics, although in fish they largely affect the CNS. The mechanism of action is by a specific blocking of the sodium channels in the neurones and a potentiation of the GABA action on chloride entry into the neurone.

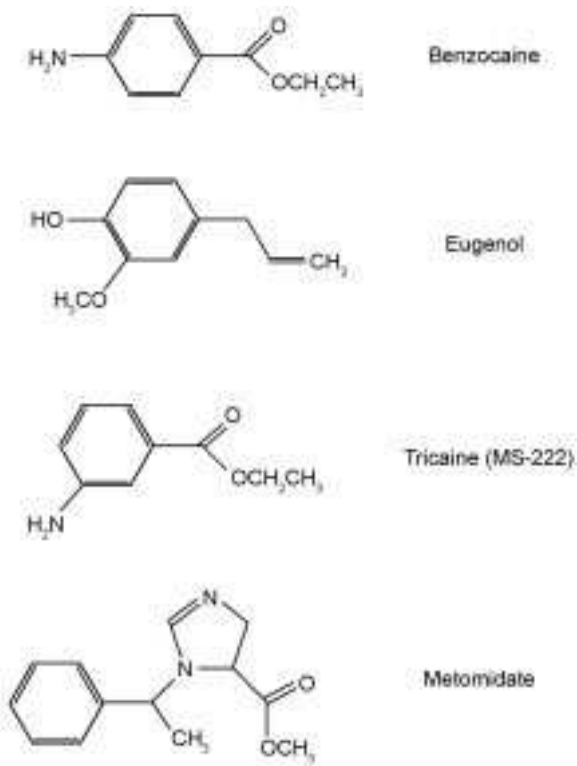

Fig. 4.4 Structural formula for common anaesthetic agents in aquaculture.

Stages of anaesthesia identified in fish according to Treves-Brown (2000).

1. Light sedation – Slight loss of reactivity to external stimuli, equilibrium normal.
2. Deep sedation – Total loss of reactivity to external stimuli except strong pressure; slight increase in opercular ventilation rate, equilibrium normal.
3. Partial loss of equilibrium – Partial loss of muscle tone, erratic swimming; reaction only to strong tactile and vibrational stimuli.
4. Total loss of equilibrium – Total loss of muscle tone and equilibrium; rapid opercular ventilation (slow with some agents); reaction to only deep pressure stimuli.
5. Loss of reflex reactivity – Total loss of reactivity; opercular movements very shallow, heart rate very slow.
6. Medullary collapse – Opercular movements cease immediately after gasping, followed by cardiac arrest.

Stages 1 and 2 are used to reduce stress during transport whereas handling normally requires stage 4 anaesthetic.

The anaesthetics depresses different parts of the central nervous system sequentially. As the concentration increases some parts are depressed before others and affected successively:

1. The cerebral cortex – leading to analgesia and some sedation.
2. The basal ganglia and cerebellum – leading to delirium and excitement.
3. The spinal cord – producing surgical anaesthesia.
4. The medulla – causing vaso-motor paralysis and death.

An ideal anaesthetic for fish may fulfil these criteria:

- Handleability (stage 4) in approximately 3 minutes.
- Short recovery, from 5 to 10 minutes.
- Large therapeutic index.
- No or minor effect on physiological and/or behavioural parameters.
- Fast metabolism and excretion to avoid excessive withdrawal period.
- Can be used repetitively.
- Inexpensive.

4.5.1 Benzocaine

Benzocaine is colourless crystals or a white crystalline powder and is very sparingly soluble in water. Therefore it is initially dissolved in ethanol or acetone. Mechanism of action is by a specific blocking of the sodium channels in the neurone.

4.5.2 Tricaine or MS-222

Tricaine is a white powder with both a high lipid and water solubility. It is a very common drug to use and in many countries, tricaine is the only anaesthetic with

a marked authorisation for fish. Mechanism of action is as for benzocaine. Benzocaine and tricaine are quickly metabolised and excreted mainly via the gills and kidney.

4.5.3 Metomidate

Metomidate is a hypnoticum rather than an anaesthetic and possesses virtually no analgesic action. Mechanism of action is by a specific blocking of the sodium channels in the neurones and a potentiation of the GABA action on chloride entry into the neurone and thereby hyperpolarises the cell making it more difficult to exitise. It is useful for sedation but not for surgery. Since metomidate blocks the cortisol response to adrenocorticotrophic hormone (ACHT), prolonged use of metomidate may be stressful even though the endocrine response (increased plasma cortisol), normally used as a measure of stress, is negative.

4.5.4 Eugenol

Eugenol is a colourless or pale yellow liquid extracted from cloves and has been used as an analgesic in dental practice. In fish farming this substance is known under the trade name AQUI-S. Eugenol is insoluble in water and therefore AQUI-S is a mixture of eugenol and the solubilising agent polysorbate (Tween 80). It is used mainly during harvest to eliminate bruising, improve carcass quality and better bleeding out and is also useful for general husbandry operations.

4.6 Antifungal agents

The fungal diseases of importance in aquaculture are of superficial nature, thus the drugs against such diseases are administered by baths for eggs and smaller fish, or as local application on affected areas of larger fish. The structural formula for the antifungal agents is given in Fig. 4.5.

4.6.1 Malachite green

Malachite green is a synthetic organic dye with antifungal activity. In fish farming this agent has been in use for a long time but is now largely out of use due to its mutagenic properties.

4.6.2 Bronopol

Bronopol (Pyseze) is at present the main substance for treatment of fungal infections. It is used both as a prophylactic and in the treatment of salmonids and salmonid eggs. The mechanism of action is not fully understood but it is believed to be inhibition of thiol-containing dehydrogenating enzymes bound to

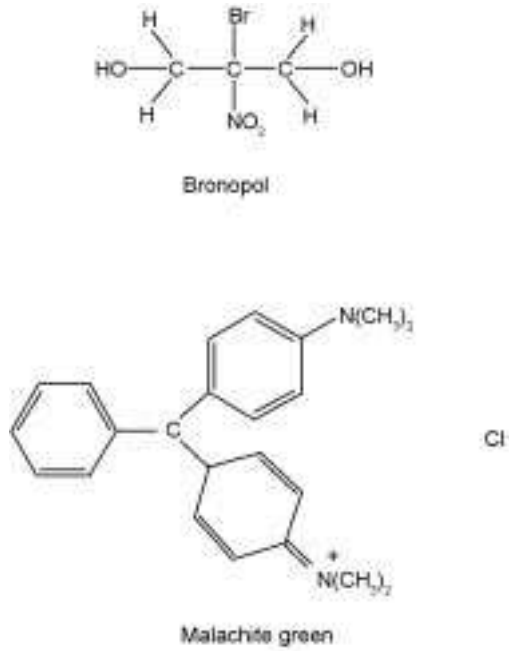

Fig. 4.5 Structural formula for common antifungal agents in aquaculture.

biological membranes. This leads to leakage, osmotic problems and finally a collapse of the cell occur. Bronopol is metabolised to propylenglycol and further to lactate and pyruvate.

4.7 Trends in drug use in aquaculture

Information on types and exact amounts of therapeutic agents used in aquaculture throughout the world is not easily obtainable. Only some countries provide reliable and open statistics on drug used for fish farming. As an example Norway has since the mid 1980s elaborated and published detailed statistics on drug consumption for farmed species (Grave *et al.*, 1999, 2004; Lillehaug *et al*., 2003; Lunestad and Grave, 2005). An overview of the consumption of antibacterial, antiparasitic and anaesthetic agents and the increase in fish production in the period from 1980 to 2006 is given in Fig. 4.6.

The Norwegian aquaculture industry has experienced a substantial growth during the latest decades. The production of farmed fish in 1980 was 7500 metric tonnes, as the corresponding figure for 2006 was approximately 700 000 metric tonnes. This gives an average annual growth rate in production of aproximately 25%. The main species in Norwegian aquaculture is Atlantic salmon (*Salmo salar*) which represented 626 000 tonnes sold during 2006. The same year the corresponding figure for Rainbow trout (*Salmo gairdneri*) was

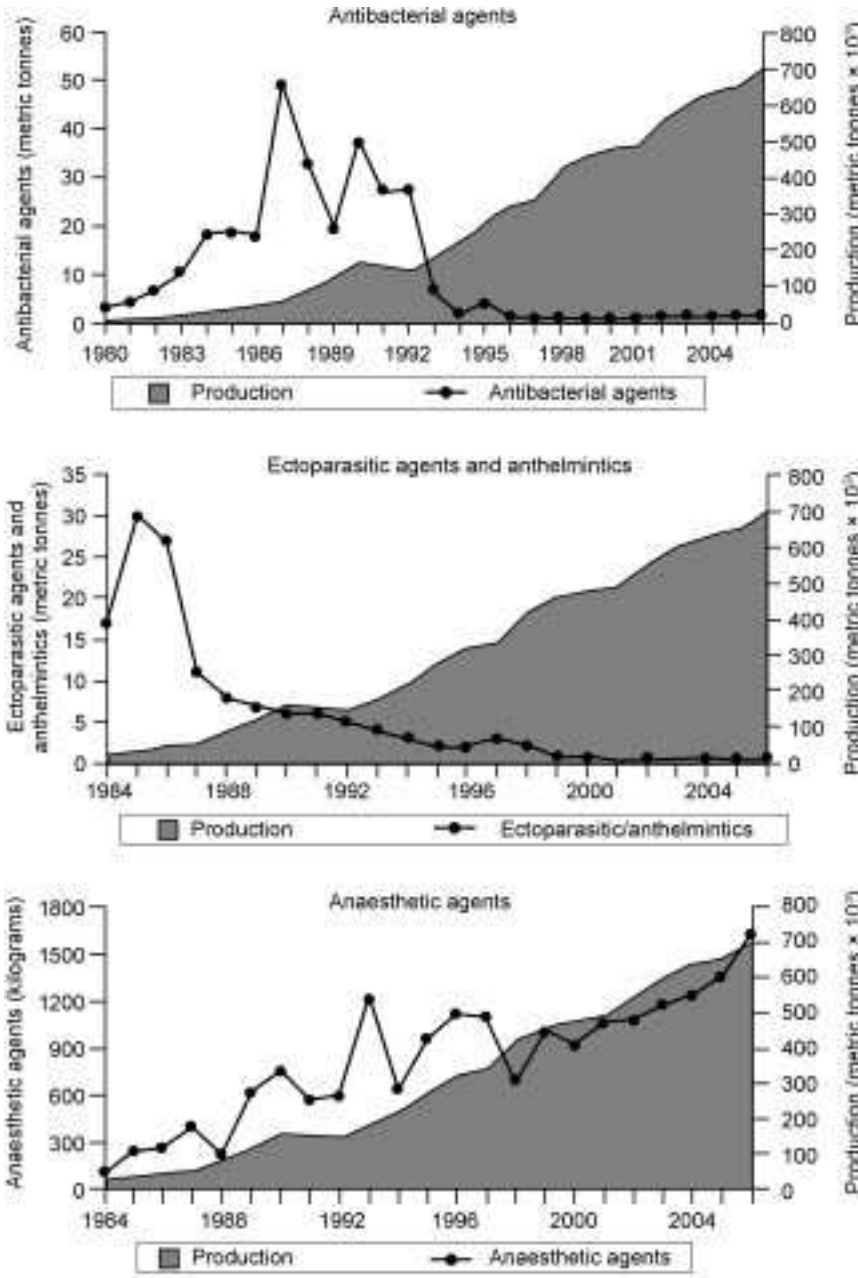

Fig. 4.6 Consumption of therapeutic agents for fish in Norway.

62 000 tonnes, cod (*Gadus morhua*) 11 087 tonnes, Atlantic char (*Salvelinus alpinus*) 881 tonnes, halibut (*Hippoglossus hippoglossus*) 1185 tonnes. In addition smaller volumes of catfish (*Anarhichas lupus*), turbot (*Psetta maxima*), coal fish (*Pollachius virens*) and European eel (*Anguilla anguilla*) with a total of 2798 tons were produced that year (Source: Directorate of Fisheries, Bergen, Norway, www.fiskeridir.no).

The amount of antibacterial agents used in Norwegian aquaculture in 2006 was only a fraction of the amount applied in treatments of fish in 1987. The reason for this substantial reduction in drug consumption are the introduction of effective vaccines, selection of more optimal farm sites and a general improvement of farm hygiene. In 2006 the following amounts of antibacterial agents were used (kg pure substance): oxolinic acid (1119 kg), florfenicol (302 kg) and flumequine (7 kg). These agents were administered orally as medicated fish feed pellets. In addition the antifungal agent bronopol (492 kg) were used in 2006 in bath treatments of eggs and fryes. When comparing the total consumption of antibacterial agents for fish, other livestock animals and humans in Norway, there has been a profound change in recent years. In 1987 such agents used for fish represented more than half of the total consumption, whereas in 2006 the corresponding consumption was only some few percent.

The following anti lice agents were used in 2006: cypermethrin (49 kg), deltamethrin (23 kg) and emamectine (60 kg). For internal parasites belonging to the cestode group, fenbendazole (27 kg) and praziquantel (145 kg) were applied this year.

Due to the increased efforts put on preventative health measures involving vaccination, the amount of anaesthetic agents used in Norway increased concomitantly with the increased production of fish. In 2006 the following agents were used: benzocaine (400 kg, approximate value) and metacaine (1216 kg). Updated statistics are available at http://www.fhi.no/.

4.7.1 Legislation, monitoring and controlling drug use in aquaculture

It has for long been recognised that the use of therapeutic agents in aquaculture may give negative environmental effects as well as having potential implications for human health. Possible effects on the human health of drug residues in seafood organisms include toxic effects, allergic reactions, effect on the normal gut flora and increased resistance of pathogens of concern for humans (McDermott *et al.*, 2002).

Several national and international regulations have been elaborated to approve drugs and secure safe aquaculture products (Alderman and Hastings, 1998). In some countries, however, regulations may exist but are not effectively enforced, whereas in other countries no regulation regimes are found at all (WHO, 1999; Cabello, 2006). In cases where no regulations exist, the farmer may use any drug that could be purchased.

In existing regulations a main element is the establishment of scientifically based withdrawal periods. These withdrawal periods are defined as the

minimum time to elapse between final treatment and harvest of the organism in question, and are specific for each drug. Since the elimination of the majority of drugs in fish is highly temperature dependent, the length of the withdrawal period is set according to the seawater temperature found where the fish is held. At low temperatures the withdrawal period is generally substantially longer then it would be at higher temperatures. For each substance the period to reach acceptable residue level should be prolonged with an extra safety margin in case of individual fish to fish variations in elimination.

The Codex Alimentarius is a global institution initiated by the UN and affiliated under the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) (http://www.codexalimentarius.net). The main aim of The Codex Alimentarius is to protect the health of the consumer and to facilitate fair trade by the elaboration of common international food standards. At the international level, The Codex Committee on Residues of Veterinary Drugs in Foods has the responsibility of setting acceptable limits to concentrations of drugs in foods for human consumption, including farmed fish. These acceptable concentrations are expressed as the so-called Maximum Residue Limits (MRL) and are based on a careful toxicological evaluation of the substance, combined with information on food consumption patterns. Consumption of food having drug residues below the MRL should by large safety margins not pose a health threat to the consumer. The MRLs are given for each substance either for all food producing species or for groups of related species such as salmonids.

Drug residues in fish are also covered by the EC legislation. In addition to legislation on production of safe food in general terms, several directives deal with drugs specifically. Of special relevance in this context are the directives EC 2377/90 1990 and 96/23/EC.

The directive EC 2377/90 describes a Community procedure for the establishment of MRLs for veterinary medicinal products in food stuffs of animal origin. The setting of MRLs is aided by the European Medicines Agency (EMEA) (http://www.emea.eu.int/). EMEA co-ordinates the evaluation and supervision of medicinal products throughout the European Union, and performs the scientific evaluation of drugs to be used in aquaculture. Information on specific dugs may be found on the web page of EMEA (http://www.emea.europa.eu/index/indexv1.htm).

According to directive 96/23/EC each member country should initiate and run annual surveillance for a number of foreign substances in all food-producing species. At least one sample for each 100 tonnes produced should be examined, and samples should be collected according to specified plans taking into account the geographic production pattern of each country. The components specified in Directive 96/23 includes legally applied drugs, drugs that are not permitted as hormones and other growth promoters, organic and inorganic pollutants, mycotoxins and certain dyes. Each member country has to submit reports on their activity on an annual basis. EC Directive 96/23 represents an important and standardised means of drug residue control among the members of the EC.

A surveillance system for drug usage and control in Norwegian aquaculture was first initiated in 1987 (Bangen *et al.*, 1994). This system has been further developed over the years, and may work as a model system. The main elements of this control system are mandatory official licensing for fish farming, restricted prescription of drugs by veterinarians or fish health biologists, a compulsory reporting system for information from feed mills, pharmacies and slaughtering facilities, combined with analytical control for drug residues.

Methods applied in the detection of drug residues in fish, shellfish or crustaceans are based on microbiological, immunological or chemical principles. The microbiological methods are used in routine detections of antibacterial substances, and are based on the growth inhibiting capability these substances have on bacteria. Different test bacteria that are specifically sensitive to relevant antibacterial agents are inoculated on optimal media together with a part of the tissue to be examined. If the sample contains residues of any growth-inhibiting substance, a zone without growth may be observed after incubation. Examination of fish for drug residues has shown that residue levels in liver samples are higher than muscle samples from the same individual, thus liver samples are preferably applied in these examinations. The microbiological method is able to detect a range of antimicrobial substances, but the sensitivity is not high. Positive or uncertain findings from microbiological methods should be verified using more sensitive and specific chemical methods. Several methods involving immunological principles are also commonly used for detection of drug residues, one of which is the Charm test. A range of chemical methods are available and currently applied in the examination of drug residues. The analytical principles involved are separation by liquid or gas chromatography combined with various detectors (MS, UV, Fluorescence, FID, EC). Compared to the microbiological methods, the chemical methods detect drug residues in much lower concentrations and in addition are more specific.

4.7.2 Environmental aspects of drugs in fish farming

Considerable attention has been paid to environmental effects of fish farming activities. Such effects may be classified as follows: (a) genetic interference with wild populations caused by escaped fish, (b) release of organic material as faeces or uneaten food, (c) spread of pathogenic micro-organisms and parasites, and (d) the release of bioactive components such as antimicrobials or antiparasitic agents.

Therapeutic agents used in aquaculture given via the food are disseminated in the environment in different ways as outlined in Fig. 4.7. Some of the drugs given to fish may end up in the wild fauna in the vicinity of the aquaculture plants. Several studies have shown traces of antibacterial agents in fish, crabs and blue mussels during and after medication of fish (Ervik *et al.*, 1994).

Diseased fish may be treated using food pellets coated with or containing the desired agent. When the pellets are thrown into the water **(a)**, fragments containing the antibacterial agent may fall off or drugs may be released from the

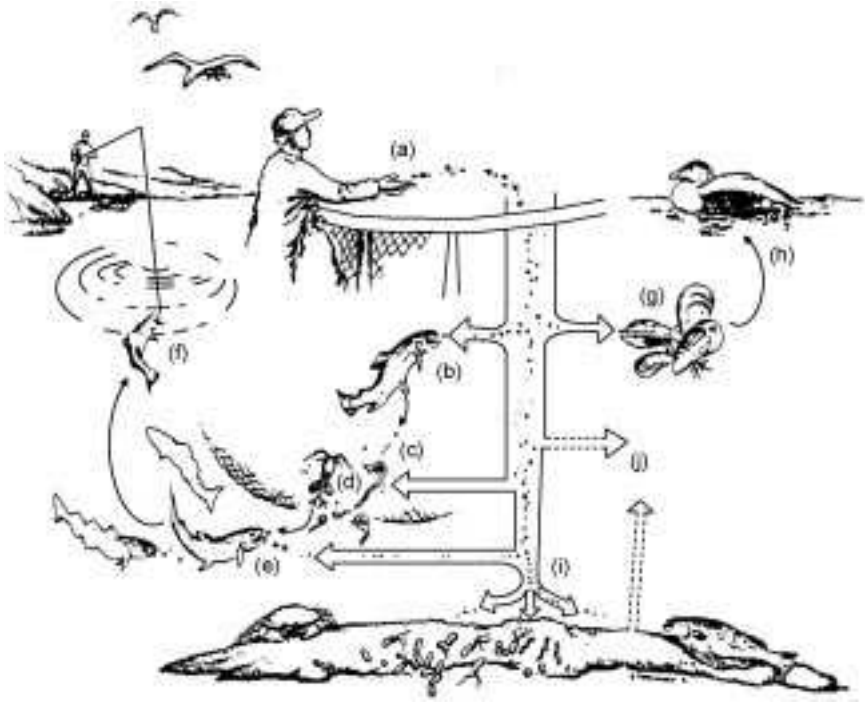

Fig. 4.7 Overview of how a therapeutic agent used in the treatment of farmed fish may be distributed in the living and non-living part of the surrounding environment. (Illustration: S. Mortensen.)

pellet in a dissolved form. Depending on the substance, dissolved drugs may be chemically or microbiologically degraded or diluted in the water masses. Some dissolved drugs can also be absorbed by organisms directly from the water. Provided a satisfactory appetite, a varying part of the medicine will reach the fish under treatment **(b)**. However, an effective treatment is often obstructed due to the reduced appetite of the diseased organisms. Of the drugs entering the gastrointestinal system of the fish a major part will not be absorbed, but excreted into the surrounding water masses together with faecal material **(c)**. Fragments released directly from the pellets or faecal particles containing antibacterial substances may subsequently be ingested by pelagic filter-feeding organisms such as copepods or appendicularians **(d)**. Wild fish and crustaceans found in the vicinity of an aquaculture facility during medication will be exposed to antibacterial agents if they eat excess food pellets or filter feeders which have previously ingested particles loaded with antibacterial agents **(e)**. If fish or crustaceans are harvested, antibacterial agents may reach human consumers **(f)**. Particles containing antibacterial agents can also be taken up by sessile filter feeders such as mussels **(g)** that may be predated by birds **(h)**. Medicated food pellets not eaten by the organisms in or outside the aquaculture facility, will ultimately reach the bottom deposits underneath the net pens **(i)**. Some of the

antibacterial agents deposited will be degraded by micro-organisms, but others are very stable and will only disappear from the sediments by diffusion to the surrounding water masses **(j)**. Antibacterial agents released from the food pellets or washed out from the sediments may also be inactivated by chemical or photochemical reactions taking place in the water.

When investigating possible effects of antibacterial agents on the environment, the persistence of the substances in question must be taken into consideration. In studies with drug stability in seawater, some agents seems to be stable whereas others are degraded mainly by the influence of naturally occurring light (Lunestad *et al.*, 1995). When exposed to underwater light intensities oxytetracycline, furazolidone and to a certain extent flumequine are decomposed, whereas oxolinic acid, sulfadiazine, sulphadimethoxine, trimethoprim and ormethoprime have been shown to be stable for at least 21 days.

A substantial part of excess therapeutics administered via food pellets ends up in the sediments beneath the fish cages. Under experimental conditions differences in stability among the different drugs has been observed (Samuelsen *et al.*, 1994). For instance oxytetracycline, oxolinic acid, flumequine, and sulphdiazine are found to be rather stable, sulphadimethoxine is partially unstable, whereas ormethoprim and trimethoprim are unstable.

Possible environmental effects of therapeutic agent introduced by aquaculture may include toxic effects on non-target organisms, reduction or alterations of microbial flora in sediments and enhanced development of bacterial drug resistance.

Marine sediments naturally harbour a high number of bacteria. Aquaculture sediments are reported to harbour between 3.0×10^8 to 1.4×10^{10} bacteria per gram wet sediment (Husevåg *et al.*, 1991). When adding antibacterial agents to sediments, a conservation of the sediment is obtained as the bacterial degradation activity is reduced. The normal mineralisation of the organic material is reversibly obstructed resulting in a more rapid accumulation of organic deposits underneath fish cages (Kupka Hansen *et al.*, 1992).

One of the main concerns in connection with antibacterial agents in aquaculture has been the possible development of bacterial resistance. The development of bacterial resistance in marine aquaculture sediments has been studied both in experimental and field investigation. In sediments with added antimicrobials the bacteria showed a very rapid development of resistance towards the drugs in question, in some cases the resistant bacteria in affected sediments constituted more than half of the culturable bacteria present. When examining sediments at abandoned fish farm sites, an increase in the level of resistance to some antibacterial agents was detected (Husevåg *et al.,* 1991). Such findings indicate that there are long-term effects of the introduction of antibacterial agents to sediments, or that other factors associated with aquaculture favour the growth of resistant bacteria or induce development of resistance among bacteria already present.

The importance of horizontal gene transfer in natural environments has been emphasised for many years (Levy, 1986; Trevors *et al.*, 1987; Genther *et al.*, 1988;

Coughter and Stewart, 1989; Jeffrey *et al.*, 1990; Stewart and Sinigalliano, 1990). In this respect special concern has been given to the possibility for transfer of resistance genes from bacteria in the environment to bacteria pathogenic to fish or humans (Sørum, 2006). Conjugal transfer of resistance plasmids from *Vibrio anguillarum* to *Escherichia coli* (Sandaa *et al*., 1992) and from *V. anguillarum* to *V. cholerae* (Nakajima *et al*., 1983) has actually been demonstrated. There are also several examples of transfer of resistant bacteria or genes coding for resistance from animals to humans via food (Bogaard and Stobberingh, 2000; McDermot *et al.*, 2002; Willis, 2000; Russel, 2002; Teale, 2002).

It has been shown that the resistance of pathogenic bacteria in humans may be partly caused by the application of antibiotics in agriculture. For this, evidence has been reviewed by several authors (Kruse, 1994; Willis, 2000; Sørum and L'Abée-Lund, 2002; Teale, 2002). Antimicrobials used in aquaculture may play a part in the development of resistance in bacteria of human health concern (Cabello, 2006; Akinbowale *et al.*, 2006); however, the magnitude of this problem is not easily assessable (Smith *et al.*, 1994; Alderman and Hastings, 1998).

4.8 Future trends

The absence of harmful residues of drugs in farmed fish is one of several key issues in providing safe products. On a global basis much is still to be done in providing a well regulated and transparent system for drug application and residue control.

Considerable success has been obtained with vaccination against bacterial diseases encountered in fish farming. To reduce the amount of antibacterial agents used, further research and development of vaccines should be undertaken.

More sensitive and discriminative methods for detection of drug residues in farmed fish will be developed and taken into use.

4.9 Sources of further information and advice

Information on specific dugs may be found on the web page of the European Medicines Agency (EMEA) (http://www.emea.europa.eu/index/indexv1.htm).
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5

Assessing the human health implications of new veterinary drugs used in fish farming

R. Reimschuessel, US Food and Drug Administration (USFDA), USA

5.1 Introduction

'So what's new?' Shakespeare's Prospero in The Tempest had it right when he said 'Tis new to thee'. Any discussion of 'new' drugs being used in fish farming, must first question the concept of '*new*'. Are these compounds truly '*new*' or is *aquaculture use* actually the '*new*' factor? Because of the small market share for drug sponsors, it is rather uncommon for drugs to be developed exclusively for aquaculture use. The majority of 'new' drugs for aquaculture have been used either in mammalian medicine (both human and animal) or in environmental pest management. For example, the major classes of antimicrobials used in many countries to treat bacterial diseases of fish, such as tetracyclines, macrolides, sulfa drugs and fluoroquinolones have all been used in terrestrial farm animals. Similarly, most drugs for treating fish parasites are substances that are used to manage mammalian parasites or invertebrate pests in plant agriculture and have had their toxicity evaluated in a variety of species. Further, terrestrial farm animal anthelminitics, such as the benzimidazoles, piperazines and macrocyclic lactones (ivermectin) have also been used in human medicine, thus their toxicity for humans has been established.

As a result of these mainstream veterinary or human medical uses, there often exists a fair amount of data on the toxicity of these potential fish drugs in both humans and animals. One of the premier sources of information is the on-line searchable data network sponsored by the United States National Library of Medicine, 'TOXNET'. Another excellent source of information is the United

States Agency for Toxic Substances and Disease Registry (ATSDR) website. There are also multiple United States Environmental Protection Agency (USEPA) sites which describe the toxicity of pesticides and other products that enter the environment. Worldwide there are also many sites which can be searched for toxicity data (Table 5.1). In addition, there are a number of on-line resources that provide information regarding drug metabolism and potential carcinogenicity (Erhardt, 2003; Keller *et al.*, 1998; Reimschuessel *et al.*, 2005; Van Eeckhout *et al.*, 1998; Yan and Sadee, 2000) (Table 5.2).

5.2 The regulation of veterinary drug use

Veterinary drug use, in most developed countries, is regulated by government agencies (EMEA, http://www.emea.eu.int/exlinks/world.htm, Schnick, 1999; Schnick *et al.*, 1999; Tera, http://www.tera.org/links/). These agencies often interact with global organizations such as the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) which coordinate internationally recognized standards and codes of practice, FAO (http://www.fao.org/Legal/prs-ol/lpo38.pdf). It is not the purpose of this chapter to detail the veterinary drug approval processes in the entire world. Suffice it to say that generally, regulatory agencies consider efficacy and safety in the animal to be treated (target animal) as well as potential public health effects. Of concern are potential adverse health reactions that could arise from consuming animals containing drug residues. In addition, injurious health effects can also occur from contact exposure to the drug while treating animals or to drugs already released into the environment.

In general, before any drug can be approved for use in animals intended for human consumption, a drug's sponsor must submit data to the regulatory agency detailing the drug's metabolism, toxicology test results and residue levels in the target animal and/or laboratory animals. Such studies usually include information on the drug's toxicokinetic properties, absorption, distribution, metabolism and excretion. This data must come from valid, well designed studies, often requiring adherence to internationally recognized protocols and are usually conducted under quite rigorous experimental circumstances. For example, in the United States, such studies must be conducted under 'Good Laboratory Practices' conditions (CFR-21 1999 pt 58). The information obtained from these studies is analyzed by the regulatory agencies to ensure that harmful residues will not be present in edible animal products. That process usually involves some type of risk assessment, consisting of hazard identification and characterization, exposure assessment and risk characterization (FAO/WHO, 2006a).

5.3 Measuring drug residues in foods

Chemical residues have been reported in many food animals, including fish (Anonymous, 2003; Nhiem *et al.*, 2006; UK Food Standards Agency, 1999,

Table 5.1 Some toxicology data resources

Agency	Website name	Link
US-GS	Acute Toxicity Database	http://www.cerc.usgs.gov/data/acute/acute.html
US-NLM	TOXNET	http://toxnet.nlm.nih.gov//
US-NLM	PubChem Substance	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=pcsubstance
US-EPA	Substance Registry System	http://www.epa.gov/srs/
US-EPA	Envirofacts Master Chemical Integrator (EMCI)	http://www.epa.gov/enviro/html/emci/index.html
US-EPA	New Chemicals Program	http://www.epa.gov/oppt/newchems/pubs/invntory.htm
US-EPA	Pesticide Product Information System	http://www.epa.gov/opppmsd1/PPISdata/index.html
US-EPA	Ecotox Database	http://cfpub.epa.gov/ecotox/
US-Dept of Labor	Occupational Safety and Health Administration	http://www.osha.gov/dts/chemicalsampling/data/CH_242600.html
CDC-ASTDR	Agency for Toxic Substances and Disease Registry	http://www.atsdr.cdc.gov/
California-EPA	Office of Environmental Health Hazard Assessment	http://www.oehha.ca.gov/risk/ChemicalDB/index.asp
NPIRS Purdue	National Pesticide Information Retrieval System	http://ppis.ceris.purdue.edu/htbin/epaprod.com
Health Canada	Health Canada	http://www.hc-sc.gc.ca/dhp-mps/index-eng.php
EBI/ChEBI	European Bioinformatics Institute/ Chemical Entities of Biological Interest	http://www.ebi.ac.uk/chebi/init.do
CHEC	Children's Health Environmental Coalition	http://checnet.org.master.com/texis/master/search/?q=&sufs=2&s=SS&prox=paragraph&xsubmit=Search
TERA	International Toxicity Estimates for Risk Database	http://www.tera.org/iter/ http://iter.ctcnet.net/publicurl/pub_search_list.cfm
PAN	Pesticide Action Network – Pesticides Database	http://www.pesticideinfo.org/
Cefas	Centre for Environment, Fisheries and Aquaculture Science	http://www.cefas.co.uk/products-and-services/environment/pollution--dta.aspx

Table 5.2 Chemical food additives, chemical carcinogens resources and pharmacology databases

Agency	Website Name	Link
US-FDA	EAFUS: A Food Additive Database	http://vm.cfsan.fda.gov/~dms/eafus.html
WHO-IARC	International Agency for Research on Cancer	http://monographs.iarc.fr/
	Pharmacokinetics information	http://www.diseasesdatabase.com/ddb30693.htm
EMBASE	EMBASE-Drug and Pharmacology (not free)	http://www.ovid.com/site/catalog/DataBase/66.jsp
FARAD	Food Animal Residue Avoidance Databank	http://www.farad.org/
Clark University Database	Human Interindividual Variability in Parameters Related to Susceptibility for Toxic Effects	http://www.clarku.edu/faculty/dhattis/
Phish-Pharm	Fish Drug Analysis – Phish-Pharm: A Searchable Database of Pharmacokinetics Data in Fish	http://www.fda.gov/cvm/addaquainfo.htm

2002a, 2005; WHO, 2004). Residues of chemicals potentially harmful to human health can be present both in fish from the wild and farm-raised fish. Over the last few decades, methods to detect chemicals have improved from detection levels near 1 mg/kg to near 1 ng/kg. Food regulatory agencies worldwide seek to protect consumers from ingesting harmful residues in any food (European Commission, 2004; USFDA, 1994). It is, however, both a scientific and a regulatory problem to define when a residue, which can now be measured in extremely minute levels, is considered harmful or safe. In order to accomplish this, the risk assessments mentioned previously are used to try to determine which concentration levels would be harmful and which levels would have no effect (NOEL) or no adverse effect (NOAEL) on consumer health.

In the case of veterinary drugs, a risk assessment is used when agencies designate concentrations of residues that may be considered acceptable or legally permitted in edible tissues. The Codex Alimentarius Committee on Residues of Veterinary Drugs in Foods (CCRVDF), a committee of WHO/FAO, seeks to establish internationally acceptable guidelines on such residue concentrations (FAO/WHO, 2006a,b). These values are called 'Maximum Limit of Residues of Veterinary Drugs' (MRLVD) by Codex, 'Maximum Residue Level' (MRL) by the European Medicines Agency (EMEA) and Canadian Food Inspection Agency (CFIA) or 'Tolerances' by the United States Food and Drug Administration (USFDA). Another numerical value established by regulatory agencies is the 'Acceptable Daily Intake' (ADI). ADIs are an estimate of the amount of residue considered to be without any toxicological hazard or appreciable health risk if ingested daily over a lifetime. ADIs and MRLs can vary between countries but there is an international effort to harmonize these values.

5.4 Withdrawal times versus drug half-lives

Developing MRLVDs or tolerance levels helps regulators establish appropriate withdrawal times, i.e. the time that must pass between when drug administration is stopped and when the animal is slaughtered for human consumption. This process usually begins when the drug sponsor conducts a 'total residue' study, usually using radioactive drugs, to track the fate of the drug in all the animal tissues. The sponsor is also responsible for developing an analytical method to detect the drug in edible tissues and for establishing a 'marker residue.' The marker residue is an identifiable residue present in edible tissues, usually the residue present over the longest period of time after dosing. Marker residues may be either the parent compound or a metabolite. If the marker residue is not found in a sample, one can be reasonably assured that there are no other violative residues present in the product. With information from depletion studies, agencies can derive withdrawal times.

At this point, it may be helpful to explain the difference between the *half-life* of a drug vs. the *withdrawal time*. Simply stated, the half-life of a drug in a

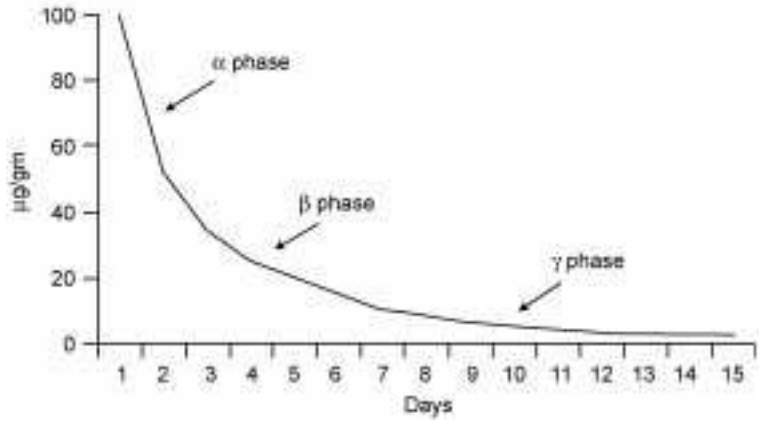

Fig. 5.1 Drug depletion.

particular tissue is the amount of time for the drug to drop from one concentration to half that concentration. There are, however, several different stages of metabolism and depuration which can affect the apparent half-life of a drug. For example, the drug may be rapidly removed from the bloodstream, but slowly be released from fatty stores. A half-life calculated during an early or redistribution phase (alpha) may be much faster than the elimination phase (beta) or the terminal phase (gamma) (Fig. 5.1). Drug residue data obtained by giving fish drugs and sampling at sequential times after dosing is used to calculate half-lives. This information is then used to develop a *withdrawal time*.

Half-life is only one of the factors used when determining a withdrawal time. A withdrawal time is the time that must pass after the last dose of drug and the time that the fish is slaughtered for human consumption. How much of the product is eaten, what parts of the animal are used, how toxic the drug is and the potential health risks to the consumer are all factors considered when developing a withdrawal time. For example, in the United States, catfish and trout have different withdrawal times for the sulfa drug 'Romet®'. Catfish have a three-day withdrawal period while trout have a 21-day withdrawal period. Both species have similar half-lives for this sulfa drug, but trout are sold with skin attached, catfish are sold without the skin. Since sulfa drugs have a longer half-life in skin than in muscle, any fish product sold with skin-on would need a longer depuration period. Fish marketed without skin can be granted a shorter withdrawal period, as the tissue being consumed does not contain the drug.

5.5 What is unique about using veterinary drugs in fish?

It is not the 'newness' of the drug that is stressed in this chapter, but the 'use in fish farming' that is the critical factor. As stated previously, most of the chemicals used in fish farming have a history of use in mammalian medicine or

agricultural application. It is the administration of these compounds to fish, and the resulting sequelae that are of concern.

5.5.1 Exposure methods for fish drugs and potential human hazards

There are three main routes by which fish are treated: oral, injectable, and waterborne.

1. *Oral* treatments are often the easiest method to treat fish, but frequently sick fish don't eat medicated feeds.
2. *Injectable* drugs ensure that the dose gets into the fish, but cause a significant amount of handling stress (for both the fish and commercial fish handlers).
3. *Waterborne* treatments consist of:
   a. Baths (and dips) where the drug is added to a static holding system;
   b. Flushes where the entire dose is added to flow-through systems in a short period (1–2 minutes), followed by unmedicated water which dilutes the dose;
   c. Constant flow where drug is added by continuously pumping a stock solution with a chemical dosimeter into a flow-through system.

The exposure route and associated factors, such as temperature, can change the amount of drug taken up by the animal. The chemical's physical properties often determine if it will be readily absorbed through skin or gills, or if absorption will not occur unless ingested or injected. Thus the exposure route affects potential residue levels within the animal that could enter the human food chain. Dosing method, however, also determines the amount of incidental exposure humans may have to the therapeutic compound.

Oral and injectable drugs are routinely used in mammalian medicine and human exposure (by contact) is generally minimal in these cases. Of concern with oral dosing are compounds that are sensitizing agents and especially dusty compounds that could irritate the skin and respiratory system. Allergic responses have occurred with several classes of antibiotic (Mooller *et al.*, 1984; Woodward, 2005b). Hazards associated with dusty medicated feeds can be reduced by requiring feed formulations that have minimal dusting potential. In addition, requiring personal protective equipment such as gloves and masks can reduce occupational exposures. Medicated feeds can also leave residues in sediment, either from uneaten food or unabsorbed chemical in the feces, resulting in other potential environmental effects (Bjorklund *et al.*, 1990, 1991; Capone *et al.*, 1996; Coyne *et al.*, 1994; Jacobsen and Berglin, 1988; Samuelsen, 1992).

Injectable compounds could pose occupational hazards following inadvertent self-injection. Injectable drugs present far less environmental risk, but there is the possibility of a single site of high residue being present in a small part of an edible filet if the injections are given intramuscularly (Reeves, 2007). These issues are the same for persons treating either terrestrial animal or aquatic animals, or the potential consumer.

Waterborne exposures more frequently used in aquatic animal medicine than terrestrial medicine, can be a much more significant source of human exposure. Contact with concentrated chemicals may result in adverse health effects. For example, organophosphates can be used to treat ectoparasites in fish (Bergh *et al.*, 2001) and thus present an occupational hazard. Although not exactly the same method of treatment, similar human health concerns arise in the case of ectoparasite dip treatments of organophosphate insecticides in mammals, e.g. sheep (Coggon, 2002; Woodward, 2005b). Such effects can be either acute or chronic and every effort should be taken to reduce human contact exposures.

Some waterborne medications are used in aquaculture to treat the water, not the fish per se. For example, copper may be used to reduce algal growth (Lewbart, 2005; Stoskopf, 1993) in a pond. Although not necessarily considered a 'fish drug' in the sense that the compound is treating something else in the water, with respect to human health, such treatments must be considered as potential chemical exposures either by contact or residue.

Waterborne treatments, can potentially release concentrated chemicals into the general environment resulting either in non-occupational contact exposures or drinking water contamination. In the USA, new aquaculture effluent guidelines have been recently published (USEPA, 2004). Many such guidelines and best management practices are being developed world wide to reduce pollution of receiving waters by aquaculture production systems (Boyd, 2003; FAO, 2004, 2006). Such guidelines consider drugs used to treat fish as potential human health hazards and seek to reduce discharge of such chemicals into the environment.

In addition to exposure by physical contact and drinking water contamination, drugs in aquaculture effluents could be absorbed by other non-target species (Coyne *et al.*, 1997; Samuelson *et al.*, 1992) including food plants (Blanchard and Diller, 1954; Delepee *et al.*, 2004; Klemmer *et al.*, 1955; Kumar, *et al.*, 2005; Migliore *et al.*, 2000, 2003). Many countries, especially in the developing world, use integrated aquaculture – horticulture systems (FAO, 2001; WWF, 2005). In these cases food plants could be directly exposed to any therapeutic agents used to treat fish. Drug accumulation in food plants, either cultured directly in effluents or down stream from an aquaculture farm, must be considered when regulating effluents from aquaculture systems and devising discharge permits.

Human and terrestrial animal medicines have also been reported in the aquatic environment (Batt *et al.*, 2006; Daughton and Ternes, 1999; DeLiguoro *et al.*, 2003; Gagne *et al.*, 2006; Kay *et al.*, 2005; Loraine and Pettigrove, 2006; Rooklidge, 2004; Thiele-Bruhn, 2003). Even though terrestrial medicines are not broadcast into water to treat the animals, those drugs enter the environment and potentially enter the human food chain or drinking supply (Bendz *et al.*, 2005; Kolpin *et al.*, 2002; Kummerer, 2001; Webb *et al.*, 2003). Therefore, human health impacts from pharmaceuticals entering into the environment need to be addressed in aquatic animal medicine, routine veterinary medicine and human medicine during the approval process and during post market surveillance. Risk

analysis will also be an important factor in this process (Cunningham *et al.*, 2006; Schwab *et al.*, 2005; US EPA, 2005; USFDA, 2004).

5.5.2 Other factors affecting drug residue levels in fish

As described in the previous section, exposure route and chemical properties can have an affect on drug absorption and excretion in fish. There are, however, many additional factors that affect these pharmacokinetic parameters. The fish species, the water temperature and salinity, and the dose and duration of treatment all affect how much drug ends up in the edible portion of the fish.

Species

Compared to mammals, there are many more species of fish, and aquaculture managers worldwide are exploring many different species and methods of farming. Some fish species are raised in ocean net pens, some in flowing freshwater, some in murky, nutrient-rich ponds and yet others are raised using indoor recirculation systems. Different species of fish can tolerate vastly different conditions, and may have specialized anatomical features which facilitate this adaptability. Those same features can affect residue levels in their bodies. One fish species with an anatomical variation which affects drug depletion is the toadfish, *Opsanus tau*. This fish has aglomerular kidneys. Drugs normally excreted by glomerular filtration have a much longer residence time in aglomerular species. The antibiotic gentamicin, eliminated primarily by glomerular filtration, is therapeutic in channel catfish at a dose of 3.5 mg/kg IM. When given to toadfish at this dosage, gentamicin is extremely nephrotoxic (Reimschuessel *et al.*, 1996). This is due, in part, to the fact that the half-life of gentamicin in toadfish is considerably longer than that of glomerular fish. In goldfish, plasma gentamicin concentrations drop from 6.4 (day one) to 2.3 μg/mL (day two), with a half-life of approximately two days. In toadfish, there is no appreciable difference between day one and day two values (7.9 and 7.8 μg/mL) and they only decline to 5.6 during the first week and 4.4 μg/mL by the second week after injection. The half-life of gentamicin in the toadfish is approximately 25 days (Jones *et al.*, 1997). Species specific factors must be considered when evaluating results of drug depletion studies in fish, especially if one is attempting to extrapolate data to other species.

Temperature

Temperature is another factor that can greatly affect pharmacokinetics and drug residue accumulation in fish (Borgan *et al.*, 1981; Rigos *et al.*, 2002; Sohlberg *et al.*, 1990, 2002). Generally, absorption increases as temperature increases, but excretion also accelerates with increasing temperatures. This can be seen in the study conducted by Bjorklund and Bylund (1990) (Table 5.3). Rainbow trout treated with a single dose of oral oxytetracycline (75 mg/kg) had a rapid rise in muscle drug concentrations during the first three to four days at the two higher temperatures (10 and 16 ºC), but after eight days the fish at 5 ºC had the highest

Table 5.3 Concentration in muscle (μg/kg) of oxytetracycline administered as a single oral dose of 75 mgt/kg body weight (data estimated from graph in Bjorklund and Bylund, 1990).

Temperature ºC	Day							$t_{1/2}$ hr
	3	4	6	8	14	21	36	
5	1	2	3	3	3	3	0.5	304
10	3.5	4	4	3	1	0.5	nd	182
16	3.5	3	1	0.5	0.3	0.1	nd	141

Note: OTC residues in fish held at 10 and 16 ºC rise to between 3 and 4 μg/kg by day 3, then decline. In fish held at 5 ºC residues take longer to reach their maximal concentration (day 6) and do not decline until after 21 days. nd = not detected

residue concentrations. Drug levels remained elevated in the 5 ºC fish throughout the 36 day depuration period. Thus, oral absorption was slower at 5 ºC, but once a steady state was achieved, the residue levels remained highest at the coldest temperature. The serum and liver concentrations had essentially the same pattern. As can be seen in the table, the tissue half-lives were much longer (304 hours) at 5 ºC than at 16 ºC (141 hours). In a similar study, Bjorklund *et al.* (1992) investigated the residue levels of oxolinic acid (single oral dose of 75 mg/kg) in rainbow trout at different temperatures. They also found that it took longer to reach the maximum concentration (C_{max}) at the lower temperature. C_{max} was reached within one day at 16 ºC, within four days at 10 ºC and within six days at 5 ºC. The tissue half-lives were much shorter at 16 ºC (48 hours) than at 5 ºC (161 hours).

Many compounds tested in fish seem to have a faster absorption and excretion at higher temperatures. For example, absorption studies show the anesthetics isoeugenol and tricaine methansulfonate reach higher muscle residues (over a period of 15–60 minutes) at warmer temperatures than at colder temperatures (5–7 vs. 15–17 ºC) (Houston and Woods, 1976; Meinertz *et al.*, 2006). Excretion studies of sulfadiazine and trimethoprim in carp (Nouws *et al.*, 1993), sulfadimidine in carp and rainbow trout (Van Ginneken *et al.*, 1991), and flumequine in rainbow trout (Sohlberg *et al.*, 1994) all show more rapid decline in muscle residues at higher temperatures. A few studies, however, have shown minimal difference in drug half-lives or muscle residue concentrations at different water temperatures (Chen *et al.*, 2004). Some studies have shown an actual increase in half-life with increasing temperature (Bowser and Babish, 1991; Bowser *et al.*, 1992). In the latter two studies, however, the experimental period was only 24 or 60 hours, and the temperature ranges were 10 and 15 ºC, not as low as 5 ºC where the major differences were noted in other studies. Coyne *et al.* (2005), however, also found that there was extensive fish to fish variation and that within a temperature range of 6–12 ºC there was minimal difference between plasma oxolinic acid concentrations.

Salinity

Water salinity may also affect drug absorption and depletion. Freshwater fish do not drink water and excrete copious urine. Saltwater fish constantly drink to maintain hydration, excreting salt in their intestinal tract while producing minimal urine. Thus, drugs in the gastrointestinal tract of saltwater fish may bind cations accelerating their excretion and reducing their absorption. For example, oxolinic acid (oral or injected) elimination is faster in rainbow trout held in saltwater than freshwater (Ishida, 1992). Oral difloxacin uptake by Atlantic salmon is ten-fold less in saltwater than freshwater (100 vs. 1000 ng/ml in plasma) (Elston *et al.*, 1994). Salinity shortens flumequine half-life when given orally but not when injected. Certain drugs, such as the tetracyclines bind cations, which can reduce their bioavailability even in freshwater fish, but more so in saltwater (Elema *et al.*, 1996). Thus, salinity, by affecting bioavailability, affects ultimate residue concentrations in fish.

Dose/duration

Residues present in the edible tissues of fish are generally dependant on dosage amount and duration. Multidose regimens usually achieve a steady state concentration with a higher C_{max} than that of a single dose. For the most part, higher dosages result in higher residues. This relationship is not necessarily a 1:1 effect, nor is it always the case. For example, isoeugenol in rainbow trout given at a lower dose for a longer duration (14 mg/L for 60 min) produces approximately double the residue than a higher dose of shorter duration (34 mg/L for 10 min) (Meinertz *et al.*, 2006).

5.6 Pharmacokinetics data and fish drug withdrawal times

Although there are a fair number of reports about specific drugs in specific fish, the general overview of drug residue data was not readily available. An extensive review of literature pertaining to fish pharmacokinetics (Phish-Pharm) has recently been published as a database, available to download from a free web-based journal (Reimschuessel *et al.*, 2005). This database allows one to search for information on drugs in different fish species dosed under varying conditions. An example of the type of information that can be obtained is in Table 5.4. In this search all the information available on enrofloxacin residues in muscles comes from studies that used oral dosing. As can be seen from the table, the drug half-life varies among the different species which were dosed at different temperatures.

Compared to mammalian residue studies, it is a more complex process to obtain adequate residue information to generate withdrawal times. Having said this, it is important to recognize that many generalities can be drawn by reviewing the literature and using tools like Phish-Pharm. In some cases fish are placed into groups for approval decisions (for example, salmonids are often considered as a group). Nevertheless, regulatory agencies must be careful to

Table 5.4 Enrofloxacin in muscle or skin + muscle of six fish species under varying conditions

Fish species	Depletion time	$t_{1/2}$ hr	Sample	Ave weight (gm)	Ave water temp (ºC)	Water	Dosage	Route	Comments	Reference
Atlantic salmon			Muscle		6	Salt	10 mg/kg 10 d	PO	Residue: 60d = 6, 80d = 0 ng/g	Steffenak *et al.* (1991)
Brook trout × Lake trout (Hybrid)	14 d	29	Muscle	2	9	Fresh	10 mg/kg 10 d	PO	No antimicrobial activity could be detected after 14 d post treatment.	Bowser and Babish (1991)
Rainbow trout	265 deg day (27 d at 10 ºC)	168	Muscle + skin	250	13	Fresh	10 mg/kg 5 d	PO	Residue 0d.5 = 0.56, 1d = 0.78, 2d = 0.68, 4d = 0.57, 7d = 0.18, 10d = 0.08 mg/kg. 20d, 30d, 40d, 48d, 60d all undetectable.	Lucchetti *et al.* (2004)
Rainbow trout	816 deg day for EU (82 d at 10 ºC)	168	Muscle + skin	250	13	Fresh	10 mg/kg 5 d	PO	Residue 0d.5 = 15.1, 1d = 14.2, 2d = 13.1, 4d = 8.4, 7d = 3.2, 10d = 1.5, 20d = 0.40, 30d = 0.24, 40d = 0.18, 48d = 0.14, 60d = 0.10 mg/kg	Lucchetti *et al.* (2004)
Sea bass		26	Muscle + skin	250	15	Salt	5 mg/kg single dose	PO	Residue: 10h = 1, 24h = 69, 36h = 0.26 μg/g	Intorre *et al.* (2000)
Seabream	> 120 h	72	Muscle + skin	150	26	Salt	10 mg/kg single dose	PO	Residue of Enro 1h = 0.2, 2h = 02, 4h = 0.4, 8h = 0.7, 12h = 0.6, 24h = 0.3, 48h = 0.07, 72h = 0.04, 96h = 0.03, 120h = 0.02 μg/g. No cipro detected.	della Rocca *et al.* (2004)
Tilapia	22 d		Muscle	200	27	Fresh	50 mg/kg 7 d	PO	Residue of Enro 0h = 3.6 d1 = 1, 4d = 0.3, 10d = 0.1, cipro 0h = 0.22, 1d = 0.15, 4d = 0.08, 10d = 0.1 μg/g (estimated from graph)	Xu *et al.* (2006)

have an adequate data package to ensure that consumers will not be exposed to harmful residues.

The basic information that needs to be evaluated, when considering drug residues in fish, is not very different from that used in mammals. An analytical method must be confirmed in the target species. The residue depletion pattern must be characterized in the target species. In fish, this may require additional studies which look at temperature range effects and perhaps at different salinities if the species is raised both in salt and fresh water. If MRLs have been developed, then depletion studies should show at what time point residue levels have dropped to acceptable levels. Again, those depletion times are used to develop withdrawal times, and in fish those withdrawal times may be linked to environmental factors such as temperature or salinity. For example, the EMEA uses 'degree days' when considering data for salmon. The time is linked to temperature, 5 days at 10 ºC equals 50 degree days, vs. 5 days at 20 ºC which equals 100 degree days. Temperature is also considered in regulatory decisions in the United States, i.e. USFDA specifies that oxytetracycline feed not be used in salmonids where the water temperature is below 9 ºC.

5.7 Regulations and exemptions

Veterinarians usually use regulated veterinary products in the species for which the drug is approved. There are, however, many 'minor' species for which there are no approved drugs. Of concern then, is the use of unapproved products under special conditions. In the United States and Canada this is called 'extra-label drug use' in the EU and Australia it is called 'off-label' (USFDA, 1997). There are times when using unapproved drugs is considered necessary by veterinarians treating minor species. For example, disease outbreaks in minor species may require drug treatment for humane reasons or to save endangered species.

Regulations regarding extra-label drug use in minor species vary greatly worldwide, and in some countries are completely absent. In a recent workshop (USFDA and NRSP7, 2004) it became rapidly apparent that many parameters have not been agreed upon by the worldwide community. For example, even what is considered a minor species is not universally accepted. In the USA, all fish are considered minor species, while in Japan and the EU, salmonidae are considered a major species. Likewise, there are different drugs approved for major species in different countries, so using a major species approved drug in a minor species means something different for each country (EMEA, 2004).

Also of concern for regulatory agencies is the use of drugs for which no ADIs have been established. This prompted the international community to try to develop guidelines for regulatory programs (FAO/WHO, 2004). The Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) made quite a number of recommendations to governments and international organizations concerning actions to develop information and harmonization in the areas of risk management and risk assessments. A few of these recommendations include:

a. CCRVDF should identify substances whose residues are highly toxic and develop a policy to ensure that they not be used in food animal production
b. CCRVDF should develop harmonization on parameters used for analytical methods to detect residues
c. CCRVDF should establish harmonized criteria/rules for evaluating food consignments containing such residues
d. CCRVDF should work with developing countries on drugs that are seen as important for those countries.
e. CCRVDF should facilitate completing MRLs within the next ten years for substances which have been evaluated by regulatory agencies and are legally used in many countries.
f. FAO and WHO should convene an expert workshop to consider the needs for veterinary drugs for aquaculture.

As can be seen even with this much abbreviated list, residues and use in aquaculture were considered areas that need concerted international effort.

Within the CCRVDF document are listed the plans that a number of countries have to draw up regulations for controlling veterinary drugs use (e.g., South America, Africa, Philippines, Thailand, Indonesia and Malaysia). Common to most of these evolving regulations is the prevention of violative residues in animal products for human consumption. Most of the developing programs include two main aspects:

1. a veterinary practice code with regulations to control veterinary drug use and
2. residue monitoring programs, using internationally recognized analytical protocols.

These programs have, in part, been instituted due to international economic pressures. Major international markets have, in the past, stopped all seafood imports from certain countries after identifying drug residues. For example, in 1991 and 1992 the EU banned imports of certain seafood products due to chloramphenicol contamination (UK Food Standard Agency, 2002b; Greenhalgh, 2004). Recently, in June 2007, the USA banned imports of Chinese seafood (http://www.shanghaidaily.com/sp/article/2007/200706/20070629/article_321490.htm). The ban was invoked because fish had tested positive for drugs banned or unapproved for use in US aquaculture. This ban followed the massive pet food recall due to the presence of melamine and other triazine compounds in Chinese products sold as 'wheat gluten' and 'rice protein concentrate'. These products were actually wheat flour with melamine or cyanurate added. The products ended up in pet food, swine and chicken feeds and also fish feeds (http://www.nytimes.com/2007/04/30/business/worldbusiness/30food.html?ex=1183608000&en=32161dee4c613ee1&ei=5070, Reimschuessel *et al.*, 2008). Such market restrictions motivated many countries to examine their regulation of veterinary drugs and to institute residue testing programs of their own.

5.8 Human health concerns

Many countries have banned a few specific drugs from use in aquaculture due to the potential for serious adverse effects in humans. These drugs include: chloramphenicol, nitrofurans and malachite green. The bans are due to data showing adverse effects from human drug use or from animal studies (WHO, 2004). Chloramphenicol can cause aplastic anemia when used as a drug in humans, while nitrofurans and malachite green are potential carcinogens (Best, 1963; Geary, 1974; NTP, 1989, 2004; Reimschuessel and Miller, 2006).

Residues of approved drugs in animals can also have a variety of potentially harmful effects on humans. Such effects may be direct effects of the drug causing acute or chronic toxicity, carcinogenicity, or allergic responses. There are also potential indirect effects. For example, antimicrobial residues in foods may change the bacterial flora of the human intestinal tract. Further, indirect effects on public health may occur due to residues entering into the environment, altering the antimicrobial susceptibility of environmental bacteria or even human pathogens.

5.8.1 Acute effects

There are few reports of acute toxic effects of veterinary drug residues in food. Fish or meat contaminated with heavy metals, polychlorinated or bromated biphenols have caused toxicity in consumers and these have been widely reported (Bernard *et al.*, 2002; Fries, 1985; Harada, 1995; Kay, 1977). Recently, dog and cat mortalities due to renal failure occurred following melamine and cyanurate being present in contaminated food. (http://www.fda.gov/oc/opacom/hottopics/petfood.html). Those triazines were also found in fish feeds and tissues, but at low concentrations (http://www.fda.gov/oc/po/firmrecalls/ziegler06_07.html). These compounds, however, were not veterinary drugs used in animals. They were the result of pollution in the environment, inadvertent feed contamination or, in the case of the triazines, addition to feed components to make them appear to have higher protein content. Toxicity due to veterinary drug residues in food have been seen with Clenbuterol (Brambilla *et al.*, 2000; Ramos *et al.*, 2004; Salleras *et al.*, 1995). Clenbuterol was used as a growth promoter in veal in 1990 in France and also in 1992 in Spain. The clinical signs in humans are primarily neurological, with relatively rapid onset of signs. This drug was banned for use as a growth promoter by the Commission of the European Community and is also illegal to use in food animals in the USA.

The main reports of human adverse responses to veterinary drugs are allergic reactions to antimicrobials in foods, especially to penicillin in milk or dairy products (Dewdney *et al.*, 1991; Schwartz and Shere, 1984; Vickers *et al.*, 1958; Wicher and Reisman, 1980). Streptomycin has also been implicated in an anaphylactic reaction (Tinkleman and Bock, 1984). The majority of responses consist of rashes and dermatitis. Quite a number of clinical reports of allergies, however, rely on circumstantial evidence, not ruling out other factors, such as

sensitivity to dairy products, which could have contributed to the apparent reaction (Dowling, 2006).

Another potential adverse response to antimicrobial residues in foods is the perturbation of gastrointestinal bacterial flora. Therapeutic concentrations of antibiotics can disturb the normal ecology of the human gut, resulting in diarrhea. Antimicrobial residues in food could conceivably kill off protective microflora, allowing access to pathogens. These effects are often considered indirect rather than direct effects. It is unclear whether this is a likely scenario, since the concentrations present as residues in food would probably be rather low.

Residual levels of antibiotic in food, even at low levels could, however, alter antimicrobial susceptibility of human pathogens (Phillips *et al.*, 2004; Yndestad, 1992). Changes in susceptibility and actual resistance to antimicrobials could then affect the ability of drugs to work during human disease outbreaks. In the United States, the USFDA developed Guidance Documents 152 and 159 (USFDA, 2003a,b, 2006) to help drug sponsors begin to assess these types of risks prior to approving new antimicrobials. Similar types of studies are being requested by other regulatory agencies throughout the world (Sarmah *et al.*, 2006).

5.8.2 Chronic effects

The affect that small quantities of residues in edible animal tissues pose for public health over a long period of time are very difficult to assess. It is for this reason that many drugs do not have established ADIs or MRLs. Most of the work that is being done to investigate potential chronic toxicity of drugs uses in vitro methods, or animals with short life stages (Anonymous, 1984; NTP, 2005; Pomati *et al.*, 2006). Another approach is to use epidemiological methods such as was done in the recent study evaluating mothers' beef consumption during pregnancy on semen quality in offspring. Of concern in that report is the use of anabolic steroids as growth promoters in the United States, with potential effects of those substances as minute residues on the developing fetus. These drugs were banned in Europe in 1988 (Swan *et al.*, 2007).

More frequently cited are residue levels and effects due to pesticides such as organophosphates, especially following agricultural use (Bradman *et al.*, 2005; Eskenazi *et al.*, 1999; Long *et al.*, 2006). Some effects have been noted as part of occupational exposures (Coggon, 2002), but others are recognized in persons living in agricultural communities (Rothlein *et al.*, 2006; Salvi *et al.*, 2003). Risks of pesticide exposures may, however, be more likely from terrestrial sources than from aquaculture use. Many fish species are quite sensitive to pesticides (Campagna *et al.*, 2006; Cox and Surgan, 2006; Feist *et al.*, 2005) especially those with estrogen receptor agonist activity (Ankley *et al.*, 2005). Since the water for most aquaculture facilities comes from the surrounding environment, there may be more risk of those facilities becoming contaminated from terrestrial pesticide use than vice versa.

However, since parasites are fish pathogens, pesticides are used in aquatic medicine. Regulations and the agencies that develop those regulations, vary from country to country. Some compounds fall under environmental regulatory jurisdiction, while others are under veterinary agency regulations (JSA, 2007). Nevertheless, most of the compounds used to treat fish parasites have been well studied both for human health effects and effects on non-target species (Ballent *et al.*, 2006; Davies *et al.*, 1998; Na-Bangchang *et al.*, 2006).

5.8.3 Indirect effects

As mentioned previously, there is concern about changes in antimicrobial susceptibility of both human and environmental bacteria following veterinary antibiotic use (CDC, 2006; Isaacson and Torrence, 2002; Puden *et al.*, 2006) A recent workshop held by FAO/OIE/WHO addressed this issue specifically in aquaculture (FAO/OIE/WHO, 2006). They concluded that antimicrobial resistance in human pathogens is largely due to drug use in human medicine but there could be components from veterinary use. The issues relative to resistance developing in farm or environmental bacteria and spread to human pathogens following aquaculture use are similar to those from terrestrial animal antimicrobial use (Alderman and Hastings, 1998; Cabello, 2006; Garrett *et al.*, 1997; Holmstrom *et al.*, 2003; Puden *et al.*, 2006; Teuber, 2001). The primary concern is the horizontal transfer of genes coding for resistant phenotypes to human flora or pathogens. These can occur either within the human host, altering the intestinal flora or in bacteria in the environment.

Intestinal bacteria in the target animal or in non-target animals can develop resistant phenotypes after the animal is treated with antimicrobials (Kerry *et al.*, 1997; Langford *et al.*, 2003; Shoemaker *et al.*, 1992). There has been some concern that human intestinal flora susceptibility patterns could be altered by residues in fish tissues, but there is no evidence that this occurs (Cerniglia and Kotarski, 2005).

The susceptibility of bacteria in the aquatic environment has also been shown to change following antimicrobial use in aquaculture settings (Guardabassi *et al.*, 2000; Kerry *et al.*, 1996; Miranda and Rojas, 2007; Sorum, 2006). In fact, the first florfenicol resistance plasmid was reported in a fish pathogen (Kim *et al.*, 1993). A number of antimicrobial resistance genes have been found to be present on transferable plasmids in fish pathogens and bacteria from the aquatic environment (Agerso *et al.*, 2007; Depaola *et al.*, 1988). Different use patterns worldwide also demonstrate that resistant phenotypes are present more commonly in fish pathogens where antibiotics are used. For example, in a survey of *A. salmonicida* susceptibility patterns, the minimum inhibitory concentration (MIC) was less than 0.12 μg/ml in 100% of the US isolates, but only 70% of the EU isolates (Miller and Reimschuessel, 2006). Oxolinic acid is not approved for use in US aquaculture but is approved in the EU.

Most countries are developing antimicrobial judicious (or prudent) use guidelines both for terrestrial and aquaculture use (AVMA, 2002; NAA, 2003;

Ungemach *et al.*, 2006; OIE, 2006). An important component of these guidelines is for regulatory agencies to develop programs to monitor antimicrobial use and antimicrobial susceptibility in bacteria from farm animals and the environment (Bertone *et al.*, 2003; Lunestad and Grave, 2005; Sarmah *et al.*, 2006; Woodward, 2005a). Such monitoring or pharmacovigilance programs provide an 'early warning' system to alert regulators of potential problems. In human medicine such programs have been used in hospitals to help physicians chose appropriate medications for patients. Such programs then recommend rotating drugs used in the hospital formulary to reduce nosocomial resistance 'hot beds' (Empey *et al.*, 2002; Gould and Jappy, 2000; Weinstein, 2001).

When conducting antimicrobial susceptibility monitoring programs it is important to harmonize methods across the international community. Recently, the Clinical and Laboratory Standards Institute published international guidelines for testing aquatic pathogens (CLSI, 2006a,b; Miller *et al.*, 2003, 2005). Since these organisms grow at lower temperatures (22, 28 ºC) than mammalian pathogens (35 ºC) it is important to use testing protocols designed for those bacteria. Such internationally validated standardized protocols allow interlaboratory comparisons and will help monitor bacteria globally for susceptibility changes.

In addition, many countries are developing 'Best Management Practices' (BMPs) with emphasis on husbandry and vaccination to reduce the need for antimicrobial therapy. Certain antimicrobials are also being listed as 'critically important for human medicine' (USFDA, 2003a,b; WHO, 2005). It is recommended that these antimicrobials be reserved for human use only and not be administered to farm/aquaculture animals.

The world community is attempting to foster aquaculture as a potential food source, while protecting public health. Thus, countries should establish or enforce existing regulations for veterinary drug use in aquaculture. These regulations should include the principals of pre-approval risk assessments, judicious use, and post-approval monitoring.

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6

Antibiotic resistance associated with veterinary drug use in fish farms

H. Sørum, Norwegian School of Veterinary Science, Norway

6.1 Introduction: veterinary drug use in aquaculture

The drugs used to control infections in aquaculture are normally the same or similar to those used in animal husbandry, and for treating household pets and humans. In fish farming, large numbers are concentrated in limited volumes of water. This high density makes individual fish more vulnerable to infections and the population in general more vulnerable to the rapid spread of disease. This is considered to result from several factors, two of the more important ones being physiological and social stress caused by intense production. Stressed individuals often have immune systems that show reduced functionality.[1,2]

When bacterial pathogens cause infections in fish, the causative agent is identified through autopsy of diseased individuals and laboratory diagnosis, and the appropriate antimicrobials (antibiotics and chemotherapeutics) are normally introduced to the fish through medicated feed. The optimal drug for feed medication is selected through susceptibility testing with the causative agent. Less commonly, antimicrobials are administrated as bath therapy, for instance for juvenile fish or crustaceans in hatcheries. Controlling bacterial diseases in fish farming always involves treatment of the entire population of individuals based on a diagnosis made from a few representatives. The population treated may consist of thousands of fish in a limited volume of water, resulting in treatment of completely healthy fish, together with those that are infected but not yet diseased and those already diseased. Early diagnosis is very important if antibiotic treatment is to be effective, and so fish farms should be connected to an up-to-date and well-run fish health service.

The large populations of fish in modern fish farms mean that there can be substantial economic losses if an infectious disease is not controlled quickly and effectively. There are risks when infected fish are treated with antibiotics without a proper diagnosis, or as a preventative measure in a high-risk situation. The use of antibiotic drugs has advantages and disadvantages. While the advantages include swift eradication of disease, the disadvantage is the risk of introducing drug resistance in the pathogen bacterium causing the infection. Consequently, fish farmers should take every opportunity to develop alternative methods to prevent infectious diseases. Optimal and up-to-date management of the fish population, including vaccination against potential disease agents in the area, should always be the first priority on a fish farm.

6.2 Current use of antibiotics in fish farming and the extent of antimicrobial resistance

6.2.1 Background

Antimicrobials have been used to control bacterial diseases in fish since the use of antibiotics in terrestrial animal husbandry started in the 1950s. The first reports of acquired resistance to antimicrobial drugs in fish pathogenic bacteria concerned sulphathiazol and tetracycline resistance in *Aeromonas salmonicida*, isolated from brook trout in the USA in 1959.[3,4] Several reports of resistance to antimicrobials used in Japanese fish farming appeared in the 1970s.[5] Freshwater and marine fish farms in Japan use antibacterial drugs to control infections in ayu (*Plecoglossus altivelis*), carp (*Cyprinus carpio*), eel (*Anguilla japonica*), tilapia (*Tilapia nilotica*), channel catfish (*Ictalurus punctatus*), large mouth bass (*Micropterus salmoides*), mullets (*Muogil cephalus*), sea bream (*Evynnis japonicus*), the salmonids amago (*Oncorhynchus rhodurus* f *macrostomus*) and yamame (*Oncorhynchus masou ishikawae*)[5,6] and yellowtail (*Seriola quinqueradiata*).[7]

Aquaculture production is largest in Asia, both in volume and in the number of species farmed.[8] Farming of Atlantic salmon (*Salmo salar* L) increased substantially in the 1980s and 1990s, and is still expanding in countries with cold marine coastlines, such as Norway, Scotland, Ireland, Iceland, Canada, USA, Chile and Tasmania.[9] Before effective vaccines against *Vibrio*- and *Aeromonas*-infections were developed, large amounts of antibiotics were used to control disease in Atlantic salmon farms.[10,11] Effective vaccines against piscirickettsiosis (*Piscirickettsia salmonis*) are still not available, and in Chile large amounts of quinolones are used to control this disease.[12]

More than 200 different aquatic animals and plants were farmed in the year 2000. Of these, only 29 species accounted for 78% of global aquaculture production.[8] In developing countries, aquaculture is often run as a small family business, while in developed countries multinational companies are becoming more important in fish farming, running fully integrated systems from fish egg to consumer fork.

6.2.2 Volume of current use of antimicrobials

It is not possible to get reliable estimates of the total use of antimicrobial drugs. Some European countries have systems for recording drug use accurately. In Norway, it has been mandatory to report all use of antibacterials in aquaculture to the National Institute of Public Health since 2002, a system which replaced a 20-year-old voluntary reporting system involving feed-mills and wholesale dealers. Similar reporting systems are now operating in other countries, including Sweden. The consumption figures for antibacterial drugs vary, from 2 grams per ton of fish produced in Norway and Sweden, 10–20 grams per ton in the UK, 40–100 grams per ton in Denmark, France and Greece, between 150 and 200 grams per ton in Canada and an estimated 700 grams per ton in Vietnam.[8]

In European and North American countries with strict regulations on the use of antimicrobials in fish farming, there are typically two to four antimicrobial agents licensed for use. These agents are normally quinolones, such as oxolinic acid and flumequine, florfenicol, oxytetracycline and a combination of sulphonamides and trimethoprim or ormethoprim. In some of these countries, the relatively high cost of licensing the agents for use in aquaculture results in reduced interest among the pharmaceutical companies who make them, due to the low sales volumes.

The opposite situation is common in countries where the use of antimicrobials in the aquaculture industry is not subject to such strict regulation. The number of available antibacterial drugs is normally higher and the amount of drugs used per ton of product is often high. However, exact consumption figures are normally not available, despite the fact that these countries provide the major part of total global aquaculture production. In many countries with limited regulation of drug use in the aquaculture industry, there is often a similarly low degree of regulation concerning the use of antimicrobials in human and veterinary medicine.[8] This scenario should be taken into account by regulatory authorities trying to reduce the use of antibacterial drugs in efforts to reduce antibiotic resistance in pathogenic bacteria across a society.

6.2.3 Antibiotic resistance related to drug consumption

Research related to antibiotic resistance in bacteria across various ecological niches, from human hospitals to fish farms, points to a strong link between use of antibacterials and development and spread of resistance against them. In fish, an example is the use of antibacterial drugs against cold water vibriosis (*Vibrio salmonicida*) in Norwegian salmonid farming in the 1980s and against furunculosis (*Aeromonas salmonicida* ss *salmonicida*) at the end of the 1980s and in the first half of the 1990s. Between 25% and 75% of all farmed salmonids produced in Norway in the 1980s received antimicrobial treatment, but this declined suddenly from 1993–94, resulting in minimal use of antimicrobials. Optimal vaccines and improved management lay behind this decline in antimicrobial use. In the 1980s, the majority of all *V. salmonicida* isolates were resistant towards the drugs used to treat cold water vibriosis. Similarly the

majority of *A. salmonicida* ss *salmonicida* isolates from the early 1990s were resistant to the drugs used, primarily quinolones.[13] Since the consumption of antimicrobials was almost eliminated, isolates from the few outbreaks that have occurred since have been found to be susceptible (data not shown).

6.2.4 Impact on the environment

The antibiotic drugs used to control infectious diseases need to reach the bacteria in the tissues of the infected and diseased individual. However, diseased fish often do not achieve the necessary levels of the drug in their body tissues due to loss of appetite, while healthy fish take in more of the drugs. In addition, the surrounding environment, i.e. water, sediments, wild fish and other biological systems, are directly exposed to the antimicrobial drug because it dissolves in the water or spreads on particles transported in the water.[14–16] This is dramatically different to the situation for land animals, because air is not a significant medium for spreading antimicrobial drugs into the environment.

Unintentional distribution of antibiotic drugs from an aquaculture system into the surrounding environment is unavoidable when the drugs are administered through medicated feed. There are many kinds of bacteria in the environment, not just pathogens but also many that decompose organic materials from the fish farms, and these will all be stimulated to develop antibiotic resistance. Drug-resistant bacteria in the environment can be a major source of antibiotic resistance genes, which can be transferred to pathogens of fish and other animals, including humans.[17]

6.3 Antimicrobial resistance in fish pathogens within the aquaculture industry

There are numerous examples of antibiotic resistance in human and veterinary medicine. Where pathogenic bacteria are drug resistant and there is an outbreak, the effect on the fish can be devastating and it is critical to find an effective replacement drug as quickly as possible. In aquaculture, drug resistance testing of the bacterial pathogen isolated from diseased individuals may indicate an effective and available alternative within a couple of days. However, increasingly often the drug-resistant pathogen turns out to be multi-resistant and there are no suitable antibiotics.

There are reports of antibiotic resistance in fish pathogens from all parts of the world, across the full range of climate zones, species farmed, water salinity and farming systems applied.[18]

6.4 Antibiotic resistance in *Vibrio* bacteria

The primary habitats of *Vibrio* bacteria are marine ecosystems and some of them cause disease in marine animals in general, and in farmed marine species in particular. *Vibrio anguillarum* is a global marine pathogen able to infect a wide

spectrum of farmed fish species at various salinities and temperatures, except in the coldest areas where water temperatures rarely reach above 10 ºC in summer.

In Japan, infections with multiple drug resistant *V. anguillarum* occurred in farmed ayu (*Plecoglossus altivelis*) from 1973.[19] Among 68 randomly selected *V. anguillarum* isolates from outbreaks of vibriosis in 1973, 65 carried transferable R plasmids encoding resistance to chloramphenicol, sulphonamides, streptomycin and tetracycline. Decreased susceptibility to furazolidone and nalidixic acid was found in more than half of the strains, but these characteristics were not transferable. It was assumed that use of antibacterial drugs in the farming of ayu had exerted a selective pressure, resulting in the occurrence of transferable R plasmids in isolates of *V. anguillarum* from infected ayu. Subsequently, 250 of 259 isolates of *V. anguillarum* taken from diseased ayu in farms in various districts of Japan during 1974–77 were found to be resistant to various combinations of six drugs. Transferable R plasmids were detected in 165 isolates (64%) and most R plasmids were found to carry resistance genes to chloramphenicol, sulphonamides and tetracycline.[20]

Similar studies were carried out in Japanese ayu farms during 1978–80, and 226 *V. anguillarum* isolates showed that almost all strains had developed non-transferable resistance to furazolidone and nalidixic acid. A change in the resistance patterns compared to the earlier studies was observed. Of the 112 isolates from 1978–79, 22 hosted a transferable R plasmid carrying resistance to only chloramphenicol. In 1980, the number of isolates with resistance to ampicillin, streptomycin, sulphonamides and trimethoprim was much higher. The R plasmids of these strains harboured genes encoding resistance to various combinations of ampicillin, chloramphenicol, streptomycin, sulphonamides and trimethoprim. A total of 75 of the 226 isolates of *V. anguillarum* harboured a transferable R plasmid.[21]

During 1981–83, 139 isolates of *V. anguillarum* with vibriosis from Japanese ayu farms were studied.[22] Transferable R plasmids were detected in 30 (22%) of the isolates and they always encoded resistance to ampicillin, chloramphenicol, sulphonamides, and trimethoprim, with some variation in the occurrence of resistance genes to streptomycin and tetracycline. The R plasmids showed extensive homology with minor variation in the drug resistance region. Two of the isolates were shown to be resistant to all 10 drugs used in the study.

The R plasmids of *V. anguillarum* isolated from ayu in Japan before 1980 belonged to two different groups, and both were different from the third group of R plasmids occurring from 1980. The first group of R plasmids (approx. 185 kb) was observed before 1978 and encoded resistance to chloramphenicol, sulphonamides and tetracycline. The second group occurred in the period 1978–79 and carried only chloramphenicol resistance, and the third group of R plasmids (approx. 200 kb) appeared in 1980 encoding resistance to ampicillin, chloramphenicol, sulphonamides, trimethoprim, and variable resistance to streptomycin and tetracycline.[23]

Finally, 114 isolates of *V. anguillarum* from Japanese ayu farms sampled in the period 1989–91 were analysed for drug resistance.[24] All except one isolate

were resistant to various combinations of drugs. Transferable R plasmids with resistance to 7 to 9 drugs were detected in 21 isolates. During this period, kanamycin was introduced as a resistance determinant in *V. anguillarum*. The R plasmids were shown to be very similar at this time, but they were generating a fourth group of R plasmids that was not similar to the R plasmids seen earlier in *V. anguillarum*.

From Galicia, Spain, *V. anguillarum* of serotypes not known to be pathogenic to farmed fish were sampled, mainly from diseased fish. A majority of the 46 isolates were resistant to streptomycin, and approximately 25% of the isolates were resistant to the combination of sulphamethoxazole and trimethoprim, while only four isolates were resistant to tetracyclines.[25]

In a study of 520 *V. anguillarum* strains isolated from fish and the environment primarily in Denmark but including the rest of Europe, minimal acquired resistance to antibacterial drugs was discovered.[26]

A total of 264 *Vibrio anguillarum* strains from Norway were examined for drug resistance. The strains were isolated mainly from nine different species of diseased or healthy farmed fish after routine autopsy. Resistance to commonly used antibacterial compounds was not demonstrated among the *V. anguillarum* isolates.[27]

A collection of 463 isolates of *V. salmonicida* isolated in the period 1980–95 and representing the majority of the outbreaks of cold-water vibriosis in Norway was screened for resistance to antibacterial drugs. Oxytetracycline, furazolidone, and sulphadiazine in combination with trimethoprim are the most important drugs in the treatment of salmon infected with *V. salmonicida*.[28] One third of the isolates were resistant to tetracycline, one third to trimethoprim, while 81% of the isolates were resistant to sulphonamides and 52% were resistant to furazolidone.[13,29]

Drug resistance in six *Vibrio harveyi* strains were studied. Four strains isolated from diseased penaeids and two reference strains were used. All three strains isolated in Taiwan exhibited resistance against nitrofurantoin, novobiocin and sulphonamide. The two reference strains and a strain from Indonesia were susceptible to these three antibiotics.[30]

From two outbreaks of disease on a Danish eel farm in 1996, 32 isolates of *Vibrio vulnificus* were screened for resistance to antibacterial drugs but no resistant strains were revealed.[31]

A Mg^{2+} dependent novel oxytetracycline resistance determinant, Tet 34, was cloned from chromosomal DNA of *Vibrio* sp. isolated from the intestinal contents of cultured yellowtail (*Seriola quinqueradiata*) in 1999.[32]

6.5 Antibiotic resistance in aeromonads

Aeromonas species thrive primarily in freshwater systems but they also cause disease in farmed fish in brackish and salt water. Furunculosis caused by *Aeromonas salmonicida* subspecies *salmonicida* is a major threat to salmonid

farming in temperate to cold water. Atypical *Aeromonas salmonicida* species cause disease in a wide range of species, including marine fish in the same areas.

Aeromonas hydrophila and related species of motile aeromonads cause infections in freshwater fish farms all over the world in warm and temperate water. It is also an important pathogen in ornamental and pet fish. *A. hydrophila* is also a potential enteric pathogen in humans and other animals (Table 6.1).[33]

6.5.1 Antibiotic resistance in *Aeromonas salmonicida*

In the 1950s, furunculosis in salmonid hatcheries in the USA was treated with sulphonamides, and an early report on acquired sulphonamide resistance in *Aeromonas salmonicida* comes from Leetown, West Virginia, with 36 out of 47 isolates from brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) found to be resistant in 1955.[3] In this report, the devastating effect of acquired sulphonamide resistance in the treatment of furunculosis was demonstrated by clinical trials. The resistant strain has since been shown to contain transferable resistance to both sulphonamides and tetracyclines.[5,34]

In France in 1971, 104 isolates of *A. salmonicida* were reported to be 100% resistant to sulphonamides while only 11.5% of the isolates were resistant to the antibiotics tetracycline, streptomycin and/or chloramphenicol.[35] Half of the drug-resistant Japanese *A. salmonicida* from salmonids in the 1960s had R plasmids hosting the drug resistance genes. Sulphonamides, tetracyclines, chloramphenicol and streptomycin were ineffective, although streptomycin was not used in the control of fish diseases.[5]

A study of drug resistance in *A. salmonicida* isolated from the intestinal tract of diseased pond-reared Amago (*O. rhodurus macrostomus*) and Yamame (*O. masou ishikawae*) was conducted in the early 1970s.[36] Fish ponds from the Nagano, Gifu, Shiga and Tokyo districts of Japan were studied, and all ponds (24) were supplied with fresh flowing water from springs or mountain streams. The drugs used for treating diseased fish in the ponds were chloramphenicol, sulphonamides, and to a minor extent tetracyclines. All the *A. salmonicida* isolates (20) found on media with chloramphenicol contained transferable R plasmids conferring resistance towards sulphonamides, streptomycin and chloramphenicol. A large portion of the *Aeromonas liquefaciens* (*hydrophila*) strains isolated from the pond-reared fish in this study also contained a transferable R plasmid conferring resistance to sulphonamides, streptomycin and chloramphenicol.

The transferable R plasmids with resistance towards sulphonamides, streptomycin and chloramphenicol isolated from both *A. salmonicida* and *A. liquefaciens* (*hydrophila*) were found to be of the same size and same incompatibility group.[37] No exchange of salmonid fish between different areas had occurred because this was restricted in an attempt to reduce transmittance of various diseases. In spite of that, the same R plasmid was detected in all fish farms in the four different districts in Japan, indicating that the plasmid occurred naturally in the environmental flora of the freshwater systems.

Table 6.1 R plasmids* in aeromonads

Bacterial species, host	Plasmid	Inc[a]	Size	Drug resistance region	Place/date	Refs
A. hydrophila, eel	RA3	U	29 MDa	*Int1*, *aadA2*, *orf513*, *catAII*, *sul1*	Japan, 1969	5
A. salmonicida, Biwamasu salmon	pAr-32	U	29 MDa	*Int1*, *aadA2*, *orf513*, *catAII*, *sul1*	Japan, 1970	38
A. salmonicida, salmonid	pJA8102-1	U	30 MDa	*Int1*, *aadA2*, *orf513 (2x)*, *catAII*, *sul1*	Japan, 1981	39
A. salmonicida, Atlantic salmon	pUG1001	U	40 MDa	Sm, Su, Tc, Tp[b]	Ireland, 1979–83	40
A. salmonicida, Atlantic salmon	pRAS1	U	25 MDa 45 kb	*Int1*, *dfr16*, *sul1* IS*6100*, Tn*1721′*, Tet A	W-Norway, 1989	41
A. salmonicida, Atlantic salmon	pASOT	U	47 kb	*Int1*, *aadA2/dfrIIc*, Tn*1721*, TetA, Su[b]	Scotland, 1982–93	41–43
A. salmonicida, Atlantic salmon	pASOT2	U	47 kb	*Int1*, *aadA2*, Su[b], Tet A	Scotland, 1988–93	41, 42
A. salmonicida, Atlantic salmon	pASOT3	U	39 kb	*Int1*, *aadA2*, Su[b], Tet A	Scotland, 1986–90	41, 42
E. coli, human pyelonephritis	pIE420	U	26 MDa 45 kb	Tet A, Su, Tm[b]	German Democratic Republic, 1976–79	43, 44
A. caviae, hospital effluent	pFBAOT6	U	85 kb	Tet A, Tn*1721* (cr376602, complete seq.)	England, 1997	43
A. hydrophila, eel/carp	pJA896	U	42 kb	Cm, Sm, Su[b]	Japan, 1970	45
A. salmonicida, atypical, fish	pU12652-97	U	58 kb	Tet A	Northeastern-USA	12
A. salmonicida, Atlantic salmon	pF1		50 kb	*Int1*, *dfrIIc*, TetA, Su[b]	Faroe Islands, 1991	46
A. hydrophila, eel/carp	RA1	C	86 MDa	Tc, Su[b]	Japan, 1970?	38
A. hydrophila, eel/carp	pJA5017	C	125 kb	*sul2*, Tc[b]	Japan, 1969	45
A. hydrophila, eel/carp	pTW64	C	125 kb	Tc, Su[b]	Taiwan, 1977	45
A. salmonicida, salmonid	R1491	C	100 MDa	Cm, Sm, Tc[b]	England	40
A. salmonicida, rainbow trout	p950704-2/2		150 kb	*Int1*, *dfr1, ant(3″)1a*, Tet A, Su[b]	Denmark, 1995	46
A. hydrophila, channel catfish	pES10		78 kb	*sul2*, Cm, Km, Sm, Tc[b]	Southern-USA, 1974	45, 47
A. salmonicida, Atlantic salmon	pRAS2		48 kb	Tet 31, *sul2*, *strA-strB*, Tn*5393c*	Norway, 1992	45, 47
A. salmonicida, salmonid	pJA8102-2		11.4 kb	Tet C	Japan, 1981	48
A. salmonicida, Atlantic salmon	pRAS3		11.8 kb	Tet C (ay043298-9, complete sequence)	Norway, 1980, 1991	49

* All plasmids in the table are conjugative, except pJA8102-2 and pRAS3 that has to be mobilized.
[a] Incompatibility group.
[b] Phenotypic resistance determinants; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Su, sulphonamides; Tc, tetracycline, Tp, trimethoprim.

A. salmonicida isolated during 1979–81 from fish farms in eight districts in Japan, including farms from three of the four districts studied by Aoki *et al.* in 1972,[36] were investigated for antibiotic resistance.[39] Of 129 isolates, only five were found to be susceptible to all antibiotic drugs tested. The other isolates were found to be resistant to up to six drugs. In particular, resistance was detected against quinolones and nitrofurans. Only two of the 124 resistant isolates studied were found to transfer drug resistance by R plasmids. These two isolates hosted an R plasmid (29 MDa) conferring resistance against sulphonamides, chloramphenicol and streptomycin. Variants of this *IncU* R plasmid have been found in *Aeromonas* bacteria all over the world.[18,41,50]

In 1989 in Atlantic salmon (*Salmo salar*) farms on the western coast of Norway, it was found that infections caused by atypical *Aeromonas salmonicida* could not be controlled with the quinolone drug oxolinic acid because of chromosomal quinolone resistance. In addition, these strains were found to carry transferable resistance towards sulphonamides, trimethoprim and tetracyclines. An R plasmid of 25 MDa (*IncU*) was found to be identical to an R plasmid isolated from salmon with furunculosis caused by *A. salmonicida* ss *salmonicida* in farms in the same area in 1991.[13]

In Scottish Atlantic salmon farms, *A. salmonicida* isolates from 229 outbreaks of furunculosis in 44 units at 34 locations during 1988–90 were investigated for antibiotic resistance. Among 304 isolates, 55% were found to be resistant to tetracyclines, 37% resistant to oxolinic acid, 31% resistant to sulphonamides and 10% resistant to a combination of sulphonamides and trimethoprim.[51] Among 40 oxytetracycline resistant isolates, 11 of the isolates transferred an R plasmid which encoded resistance to oxytetracycline and/or sulphonamides, trimethoprim and streptomycin to *E. coli.* This R plasmid was shown to be an *IncU* plasmid of the same type as found in Japan and Norway.[52]

In a study of 29 oxytetracycline resistant *A. salmonicida* isolates from Scotland, 19 transferred their tetracycline resistance to *E. coli*.[52] The Norwegian pRAS1 isolated from *A. salmonicida* were included in this study and restriction digests showed obvious similarity to the Scottish R plasmids, which were of similar size even if the featured resistance patterns were not identical. In another study, R plasmids featuring oxytetracycline resistance from mesophilic, motile *Aeromonas* spp. isolated from freshwater in a fish hatchery and from hospital sewage in England, were studied molecularly.[43] Six of 91 isolates from the fish hatchery water and 11 of 72 isolates from hospital sewage water were found to transfer oxytetracycline resistance. Seven of the 11 R plasmids from *Aeromonas* spp. from sewage were shown to belong to the *IncU* group.

In 23 drug-resistant *A. salmonicida* isolates from 13 different freshwater rainbow trout farms in Denmark, only three isolates transferred their R plasmid to *E. coli*. These three isolates transferred an R plasmid of 150 kb with resistance to oxytetracycline, sulphonamides, trimethoprim and streptomycin.[46] In addition, three oxytetracycline and sulphonamide resistant isolates of *A. salmonicida* from Canada and one oxytetracycline resistant isolate from USA, included as controls in the study, transferred R plasmids of 140–160 kb. Five *A. salmonicida*

isolates from the Faroe Islands resistant towards oxytetracycline, sulphonamides, trimethoprim and streptomycin were included in the study and they transferred a 50 kb (probably *IncU*) plasmid to *E. coli* encoding resistance to all their drug resistance features except streptomycin.

IncU R plasmids were detected in drug-resistant atypical *A. salmonicida* isolates from farmed and wild fish on the Northeastern coasts of USA and Canada.[53]

The occurrence of a transferable R plasmid belonging to the *IncU* group in *A. salmonicida* isolates from various parts of the world in both freshwater systems and in the marine environment is remarkable. This plasmid was found to be identical in all the sampled locations except for the drug resistance region. The *IncU* plasmid is probably particularly successful at establishing in *A. salmonicida*. However, the *IncU* plasmid is promiscuous and was found in *Aeromonas hydrophila* (see later), in atypical *A. salmonicida* on the coast of Norway, on the northern part of the east coast of North America and in human enterobacteria in Eastern Europe. Hedges *et al.*[40] suggested that the homogenic *IncU* plasmids are characteristic of aeromonads but do occasionally transfer to and establish in other genera. The plasmids may acquire some of the genetic factors linked to drug resistance in the gene pools of these bacteria and subsequently bring these back to aeromonads. However, one should not rule out the opposite possibility, that these *IncU* plasmids may be bringing antibiotic resistance factors from aquatic environments into our antibiotic consuming environments.

The use of quinolones, oxolinic acid and flumequine in the control of bacterial infections in farmed fish from the 1980s resulted in the occurrence of *A. salmonicida* with reduced susceptibility to quinolones.[54,55] Mutations in the gyrase A gene, *gyrA*, were found to be responsible for elevated MIC values against quinolones in clinical strains of *A. salmonicida*.[56] A study of 12 clinical *A. salmonicida* isolates from French marine fish farms in the period 1998 to 2000 showed that all quinolone resistant isolates carried a point mutation in *gyrA*.[57]

Quinolone resistant *A. salmonicida* isolates have been found to have changes in the outer membrane profile with loss of a 38.5 kDa protein and occurrence of a 37 kDa protein, probably linked to loss of porine function resulting in multiple low-level antibiotic resistance.[58,59]

In addition to amino acid substitutions in the gyrase A enzyme and porine changes in the outer membranes, it was indicated that an efflux mechanism might be involved in quinolone resistance in strains of *A. salmonicida* isolated from French marine fish farms.[57]

Quinolones, oxolinic acid and flumequine, were used extensively in fish farming to control infections caused by *A. salmonicida* from the late 1980s until effective vaccines containing oil adjuvants were introduced in the early 1990s. During that period, high MIC values for quinolones developed in isolates of *A. salmonicida* in all areas where furunculosis was endemic in salmon farming, resulting in repeated treatments of diseased fish during the summer months with

only weeks between each treatment. In Norway, the first resistance to quinolones were discovered in 1989, about one year after fish farmers started using oxolinic acid to control furunculosis.[55] It was found that 36% of the isolates were resistant to one or more of the drugs tested, with 30% resistant to quinolones as the most widespread feature. After many years of effective vaccination, there is minimal information on the occurrence of antimicrobial resistance in strains of *A. salmonicida* in general. It has been shown that *A. salmonicida* share similar genetic tools worldwide when drug resistance evolves.

6.5.2 Antibiotic resistance in *Aeromonas hydrophila*

Transferable antibiotic resistance in the fish pathogen *Aeromonas hydrophila* (*liquefaciens*) was seen in isolates from farmed fish in Japan in the early 1960s. Tetracycline and sulphonamide resistance determinants were most common.[38] In 1970, *A. hydrophila* was isolated from many Japanese freshwater fish farms, including carp (5 farms), eel (26 farms), ayu (17 farms) and salmonids (26 farms), and it was shown that they hosted transferable R plasmids with resistance genes against sulphonamides, tetracyclines, chloramphenicol, and streptomycin.[6]

A high proportion of drug-resistant bacteria from the intestines of farmed eel (*Anguilla japonica* and *Anguilla anguilla*) and from the water of Japanese farm ponds was found to harbour R plasmids. The resistance features transferred by the plasmids were predominantly the combined tetracycline and sulphonamide resistance, especially from *A. hydrophila* isolates.[60]

Representatives of R plasmids from isolates of *A. hydrophila* from Japanese salmonid ponds in 1969–71 were later studied in more detail.[45] All R plasmids conferring the combined resistance of tetracyclines and sulphonamides were found to belong to the *IncA-C* group. Strains from Taiwan and the USA were also included in the study. The *IncA-C* group plasmids mediating tetracycline and sulphonamide resistance were found to have a size of about 125 kb. This study of R plasmids included two other *IncC* plasmids harboured by *A. hydrophila* isolates from France,[61] and one *IncC* plasmid harboured by an *A. salmonicida* strain from the UK, which mediated different resistance features compared to the Japanese plasmids. All these *IncA-C* or *IncC* plasmids are probably the same plasmid with variations in the resistance factors at the drug resistance region. In a Danish study,[46] it was shown that transferable R plasmids of 150 kb harboured by *A. salmonicida* isolates from a Danish freshwater pond fish farm in 1995 mediated resistance towards oxytetracycline, sulphonamides, trimethoprim and streptomycin. In the same study, three Canadian *A. salmonicida* isolates from 1986 and 1990 were found to harbour a 160 kb transferable R plasmid mediating resistance towards oxytetracycline and sulphonamides. It is probably the same plasmid frame as the 130 to 160 kb *IncC* R plasmids of *A. hydrophila* and *A. salmonicida* from several regions around the world, as is the case with the *IncU* R plasmids already discussed.

In the Japanese study of R plasmids of *A. hydrophila* isolated from the salmonid ponds of various districts in 1969–71, two *IncU* R plasmids of 42 kb were also analyzed.[45] They mediated resistance against chloramphenicol, sulphonamides and streptomycin, and were identical to *IncU* plasmids isolated from *A. salmonicida*. *IncU* R plasmids of 34 to 40 MDa from three French isolates and two Irish isolates of *A. hydrophila* have also been studied.[40]

In a Danish study[62] of 313 isolates of motile *Aeromonas* spp. (*A. hydrophila* 35.3%, *Aeromonas bestiarum* 19% and *Aeromonas veronii* biovar *sobria* 15.3%) isolated from four freshwater fish farms, 69% of the isolates were resistant to oxytetracycline, 43% were resistant to sulphonamides/trimethoprim and 20% were quinolone resistant.[46] All the sulphonamides/trimethoprim resistant aeromonads carried a class 1 integron. Among 17 strains able to transfer an R plasmid, the integron and the Tet determinant were transferred together on the R plasmid in 15 cases.

Both *IncU* and *IncC* R plasmids are widespread within aeromonads and probably occur all around the world. Studies to date indicate that both these plasmids originated from local environmental water sources and adapted according to the drugs used in fish farms locally and regionally. Initially, variants of the two plasmids looked different due to their resistance features, but more detailed molecular study of the DNA sequence revealed that they are homologous.

A. hydrophila isolated from channel catfish (*Ictalurus punctatus*) farmed in ponds in the southern states of the USA have been typed for the determinants of tetracycline resistance. It was shown that Tet E was the dominating genetic determinant, occurring in 69% of the tetracycline resistant *A. hydrophila* isolates.[63,64]

Aeromonas hydrophila from skin lesions of cultured tilapia (*Tilapia mossambica*) from Malaysia were screened for antibiotic resistance and plasmid contents. Of 21 isolates studied, all were resistant to ampicillin, 12 were resistant to streptomycin, 10 were resistant to tetracycline and 9 were resistant to erythromycin. One isolate was resistant to six of the ten drugs included in the study.[65]

Rainbow trout (*Oncorhynchus mykiss*) in northern Portugal with clinical lesions caused by predominantly *A. hydrophila* were investigated in 2001. The pathogenic isolates and isolates from pond water were screened for resistance to β-lactams.[66] Among the 51 strains, resistance to amoxicillin, carbenicillin and ticarcillin was most common (between 76 and 88%). Of interest is that the level of resistance against β-lactam antibiotics among *A. hydrophila* isolates from rainbow trout was found to be lower than among isolates from human clinical infections.

Infection with *A. hydrophila* is an important cause of loss of ornamental fish in aquaria and use of antimicrobial drugs is common in the control of such infections. In a study of *A. hydrophila* isolated from the normal flora of commonly imported species of ornamental fish from southeast Asia in two pet shops in Georgia, USA, it was found that about half of the isolates were resistant

to one or more of the drugs tested and 25% of these isolates contained transferable R factors with resistance to primarily tetracyclines, sulphonamides and ampicillin.[67] One of the pet shops used tetracycline as a prophylactic in the aquarium water. Of additional interest in this study is that only the *A. hydrophila* isolates from the aquarium water of the pet shop using tetracycline prophylactically carried transferable R factors.

Future studies will most probably verify that both the *IncC* and *IncU* plasmids are common to the aeromonads with its global homogeneity.

6.6 Antibiotic resistance in *Photobacterium damselae* subspecies *piscicida*

P. damselae ss *piscicida* was given its name in the taxonomic system in 1995 after being named *Pseudomonas piscicida* and then *Pasteurella piscicida*.[68] Pasteurellosis or pseudotuberculosis, the clinical names of the disease, is a serious infection that has caused severe losses in marine yellowtail (*Seriola quinqueradiata*) farming in different areas of Japan since it was first observed in 1969.[69] It also affects cultured striped bass (*Morone saxatilis*) on the Gulf Coast of the USA.[70] In Mediterranean marine farming of mainly gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), pasteurellosis has been a serious problem with epizootics in the farms since the beginning of the 1990s.[71]

Among 60 isolates of *P. damselae* ss *piscicida* obtained from cultured yellowtail from various Japanese districts during 1975–80, transferable resistance against chloramphenicol, kanamycin, sulphonamides and tetracycline was found in five isolates from the same district. Non-transferable resistance to furazolidone was found in 21 isolates. This was the first report of multiple resistance in *P. damselae* ss *piscicida* and it was believed that the incidence could be explained by the abundant use of chemotherapeutics in yellowtail farming.[72]

Another collection of 281 isolates of *P. damselae* ss *piscicida* from the period 1981–83 were obtained from cultured yellowtail in mainly the same districts in Japan. After only a few years, as many as 262 isolates were resistant to various combinations of seven drugs and 168 isolates were able to transfer R plasmids to *E. coli*.[69] The most common profile of drug resistance encoded by the R plasmids was one containing resistance features to chloramphenicol, kanamycin, sulphonamides and tetracyclines.

Restriction and hybridization analysis of a random selection of 19 R plasmids from *P. damselae* ss *piscicida* isolates collected from 1980–83 demonstrated that all the plasmids were very similar, with only minor differences in the restriction profile.[69] These differences can be related to the variations in resistance gene profiles of the R plasmids.

Systematic collection of *P. damselae* ss *piscicida* isolates from diseased yellowtail in Japan and screening for drug resistance has been conducted for the periods 1984–85 (307 isolates),[73] 1986–88 (306 isolates),[74] and 1989–91 (175

isolates).[75] Identical resistance patterns were found throughout, from 1975 to 1991, and the majority of the isolates were found to be multi-resistant to a series of antibiotics. Almost all drug-resistant isolates harboured the same type of transferable R plasmid with variations only in the occurrence of the genes encoding the various resistance features in the drug resistance region of the plasmid.

A recent molecular study of the genes responsible for chloramphenicol resistance in the Japanese drug-resistant *P. damselae* ss *piscicida* isolates resulted in detection of three transferable R plasmids of 100, 50, and 40 kb, all encoding chloramphenicol resistance to the isolates.[76]

P. damselae ss *piscicida* isolated from outbreaks of disease in Mediterranean marine aquaculture also showed a high degree of genetic homology with Japanese and American strains.[71] In the European strains, resistance to erythromycin, kanamycin, streptomycin and sulphonamides was common. Plasmid profiling did not reveal a common R plasmid carrying the drug resistance genes in the *P. damselae* ss *piscicida* isolates. However, common DNA fragments of the plasmids were suggested to be carriers of drug resistance genes.

6.7 Antibiotic resistance in enterobacteria

Bacteria of the family Enterobacteriaceae are normally not an important part of the intestinal flora of fish. However, a few enterobacteria may cause serious outbreaks of infectious diseases in fish farming in particular *Edwardsiella tarda* and *Yersinia ruckeri*.

6.7.1 Antibiotic resistance in *Edwardsiella tarda*

E. tarda has been a significant pathogen in Japanese eel farms. The risk of bacterial infections is high in eel farming because the fish are kept at high densities in freshwater ponds enclosed in green houses, and high temperatures are maintained by artificial heating where necessary.[77]

Edwardsiellosis is also an important infection in other freshwater cultured fish, such as channel catfish (*I. punctatus*), Japanese flounder (*Paralichthys olivaceus*), largemouth bass (*Micropterus salmoides*), and chinook salmon (*Oncorhynchus tshawytscha*).[78] *E. tarda* has also been described as a potential human pathogen, and may cause disease in humans handling fish and fish products.[79]

A total of 168 isolates of *E. tarda* from eel (*A. japonica*), channel catfish (*I. punctatus*), and from the water of their ponds taken during 1972–79 in Japan were tested for susceptibility against 10 antibacterial drugs.[77] While 32 isolates were susceptible to all the tested drugs, the rest of the isolates were resistant to various combinations of eight different drugs. The most common combination of resistance features was against chloramphenicol, furazolidone, nalidixic acid, sulphonamides and tetracycline. Transferable R plasmids conferring resistance

to sulphonamides, tetracycline and chloramphenicol were detected in 38 of the isolates. The drug resistance patterns covered all the drugs used to control bacterial infections in the fish. The eel isolates of *E. tarda* were generally more resistant than the isolates from tilapia, consistent with a higher level of drug use in eel farming compared to tilapia farming.

The genetic relationship between the various conjugative R plasmids of the eel pathogenic isolates of *E. tarda* from Japan was studied and also compared to R plasmids of *E. tarda* from Taiwan.[80] This made it possible to identify two separate groups of transferable R plasmids in *E. tarda*. One of these groups contained R plasmids identical to the *IncC* R plasmids of *A. hydrophila*. The plasmids of this genetic group contain resistance genes against sulphonamides and tetracycline and sometimes chloramphenicol resistance as well. These two drug resistance profiles also occurred on plasmids in the other genetic group of R plasmids (approx. 120 kb) in *E. tarda*. They were identical except for a small variation in the drug resistance region. The results indicated that the two groups of R plasmids have common sequences in the drug resistance region and all information points to a class 1 integron as the common sequence between the two groups. The variations in drug resistance profile within and between the two plasmid groups of *E. tarda* were probably caused by the contents of drug resistance cassettes being different. Similar results were found in another study of 186 isolates of *E. tarda* isolated from diseased eel in culture ponds in various districts of Japan during 1983 and 1984.[81]

6.7.2 Antibiotic resistance in *Yersinia ruckeri*

Y. ruckeri is the primary cause of enteric redmouth disease in salmonid fish. Among 50 isolates of *Y. ruckeri* of various serotypes from North America and Europe tested for susceptibility to 23 various antibacterial drugs, two isolates were found to harbour a 36 MDa conjugative R plasmid encoding resistance to tetracyclines and sulphonamides.[82]

In Denmark, four freshwater rainbow trout farms situated along a stream system were screened for occurrence of bacteria with drug resistance in fish, water and sediment samples.[62] The 134 *Y. ruckeri* isolates from diseased rainbow trout were found to be largely susceptible to all five antimicrobial agents included in the screening.

In France, a study of resistance to chloramphenicol and fluorfenicol among fish pathogenic bacteria showed no reduced susceptibility to these drugs in *Y. ruckeri* despite wide use of florfenicol in French fish farming.[83]

Seven representative *Y. ruckeri* isolates from diseased fish in one Spanish rainbow trout farm with enzootic enteric redmouth disease taken during 1994–2002 were studied.[84] All isolates belonged to the same clone as demonstrated by pulsed-field gel electrophoresis restriction patterns. The *Y. ruckeri* isolates from the period 2001–02 had significantly lower MICs to all the quinolones tested compared to the isolates from 1994–98. Sequence analysis of the *gyrA* gene revealed a single amino acid substitution.

6.8 Transfer of antibiotic resistance between bacteria in fish farms

Various studies have characterized drug resistant bacteria from aquaculture in various parts of the world, and they reveal variable genetic backgrounds for the antibiotic resistance.[18] However, detailed molecular genetic studies of fish pathogenic bacteria are nowhere near as common as those of pathogenic bacteria in human medicine. Studies of drug resistant fish pathogens have been conducted in Japan since the early 1970s, and the results give an important indication of how bacterial pathogens in fish farming adapt to antibacterial drugs used for long periods. When adding the results from more recent studies in other fish farming countries, it is obvious that the spread and transfer of drug resistance genes occurs not only within the fish farm site, but also between fish farms and not least between bacteria in the fish farm and bacteria in the external environment.

The spread of resistance genes most probably occurs largely by selection of resistant bacteria during a period of drug use in the fish farm. The frequency of transfer of resistance genes to other members of the same species and transfer of resistance genes to other species of bacteria in the field scenario is not well known. However, extremely high conjugation frequencies of about 50% within one hour have been measured in the laboratory, with transfer of the common *IncU* plasmid pRAS1 of *Aeromonas salmonicida* to *Escherichia coli*, a mammalian enterobacterium.[41] This plasmid has been transferred by conjugation to *E. coli* under conditions mimicking natural habitats without reduction in transfer frequency.[85] This indicates that R plasmid transfer may occur with high frequency on fish farms. The most commonly used mechanism of drug resistance transfer is conjugation by R plasmids. A drug-resistant fish pathogen may replace resistance genes towards an antibiotic drug without an obvious reason, illustrating the flexibility and dynamic in the bacterial genetics behind the drug resistance features.

The tetracycline resistance determinant of R plasmids of *V. anguillarum* isolated from cultured ayu in Japan were analysed and the first group of R plasmids, occurring during 1973–77, were found to have the Tet B determinant. The second group, occurring during 1978–79, did not carry any tetracycline resistance and the third group of R plasmids, occurring from 1980–83, hosted a novel tetracycline resistance determinant.[86] This determinant was called Tet G.[87] However, the Tet G determinant was not found in the next group of R plasmids observed in *V. anguillarum* from ayu, in the period 1989–91.

Surprising and interesting is the fact that the Tet G determinant occurs in multidrug-resistant *Salmonella enterica* serovar Typhimurium DT104 strains on the genomic island, called *Salmonella* genomic island 1. This genomic island contains an antibiotic resistance gene cluster with both Tet G and the flu^R gene.[88] The flu^R gene was first detected in the fish pathogen *P. damselae* ss *piscicida*, also in Japan.[24,89,90]

The global occurrence of the same R plasmids in aeromonads only varying in

the number and types of drug resistance genes, demonstrates the enormous web of communication in the bacterial genetics of aquatic natural systems.

A study of motile aeromonads *A. hydrophila*, *Aeromonas veronii* and *Aeromonas caviae* from hospital effluents in England showed that the aeromonads from the hospital effluents harboured *IncU* R plasmids.[43] One of these *IncU* R plasmids, pFBAOT6, was sequenced.[50] An area of 30 kb of the 85 kb sequence of pFBAOT6 was very similar or identical to the *IncU* backbone of pRAS1.[41] The genes located in this part of pFBAOT6 were related to functions like replication and conjugal transfer, demonstrating that this region of 30 kb was the *IncU* backbone common to all these plasmids, while the other 50 kb part of the pFBAOT6 sequence constituted the variable drug resistance region of the plasmids.

The global stability of the plasmid backbones of *IncU* and *IncC* plasmids is contrasted by the plasticity of the drug resistance region, resulting in an adaptability that overcomes most genetic systems. The genetic systems responsible for gene mobility are quite well studied in aeromonads.

The 25 MDa plasmid (pRAS1) of *A. salmonicida* was found to be an *IncU* plasmid harbouring a class 1 integron with a *dfr16* cassette and the sulfonamide resistance gene *sul1*. Directly downstream of the integron, a deleted Tn*1721* with the TetA tetracycline resistance determinant was located. Comparison of restriction digests and other features, such as transfer frequencies of pRAS1, with published results from studies of other R plasmids led to the observation that the pAr-32[37] and pJA8102-1[91] from *A. salmonicida* isolated more than ten years apart in Shiga and Miyazaki prefectures in Japan, respectively, were similar. It was found that these three R plasmids had the same backbone structure, but that the drug resistance region differed; pRAS1 had an In*4* of Tn*1696* followed by a truncated Tn*1721* and pAr-32 had a complex class 1 integron structure of In*6* as seen in the plasmid Sa.[41]

In a Danish study of clinical *A. salmonicida* isolates common to all the conjugable R plasmids, the majority of the strains contained plasmids of 150 kb, and all strains with reduced susceptibility to sulphonamides contained class 1 integrons. The drug resistance cassette contents in *A. salmonicida* seemed to vary with the region from which they originated. Various trimethoprim resistance cassettes and the aminoglycoside resistance cassette *ant(3″)1a* were found in the integrons.[46]

A. salmonicida strains from Norway, including ten isolates of sulphonamide resistant atypical strains representing the various groups of atypical *A. salmonicida* isolated in Norway,[13] were screened for class 1 integrons and sulphonamide resistance genes.[47] *A. salmonicida* isolates from Switzerland, Finland, France, Japan, Scotland, and USA were also studied. Integrons were detected in the isolates of *A. salmonicida* from all the countries represented. In isolates from France and Switzerland, the *aadA1* cassette occurred in the integrons, while the *aadA2* cassette was found in the Scottish isolates and in the pAr-32 from Japan. The *dfr16* cassette was isolated from both atypical and typical *A. salmonicida* isolates from Norway, while the *dfr2c* cassette was detected in trimethoprim resistant isolates in Scottish isolates of *A. salmonicida*.

In South African drug resistant *Aeromonas* spp. from aquaculture systems, a similar situation with class 1 integrons and various combinations of drug resistance gene cassettes, such as *dfr1*, *ant(3″)Ia*, *aac(6′)Ia*, *oxa2a* and/or *pse1,* has been demonstrated.[92]

From molecular genetic research on drug resistant bacteria from fish farms available so far, it is obvious that fish pathogenic bacteria are not restricted to the fish farm when acquiring drug resistance features. It is important to remember that the environment surrounding the farm sites, which includes an invisible, partly unknown, web of bacterial genetics, is heavily involved.

6.9 Risk of transfer of antibiotic resistance from fish farm bacteria to human pathogens

A substantial number of studies demonstrate that antibiotic resistance genes and the structures responsible for their motility are shared between bacteria from very different ecological niches. For many years, research into the molecular mechanisms behind the development of antibiotic resistance in human pathogenic bacteria concentrated on clinical isolates from hospitals or similar health institutions. Results from studies in recent years indicate that the pathogens communicate genetically with harmless bacteria within the normal human or animal environment.[17,93]

There is little direct evidence that drug resistance genes from bacteria in aquaculture have been transferred to bacteria of importance to human health. Theoretically, such drug resistance factors could reach humans through their consumption of aquaculture products contaminated with drug resistant bacteria from the fish or its environment. Another source is environmental bacteria, primarily from aquatic systems that are influenced by aquaculture activity.

Again, the best indications of the potential impact of drug resistance genes from fish farms on human health can be found in detailed genetic analysis of the bacterial drug resistance factors in both ecological niches.

A collection of five plasmids isolated from human clinical infections and hospital sewage in Eastern Germany and Czechoslovakia during 1976–79 was found to belong to the *IncU* group.[44] Comparison of the restriction digests of these plasmids and those of pRAS1 showed a similarity between pRAS1 and all the *IncU* plasmids, including the *IncU* prototype plasmid RA3 isolated from *A. hydrophila* in Japan (identical to pAr-32).

The Tet G determinant seen in *V. anguillarum* from farmed ayu in Japan in the 1980s occurs in multidrug-resistant *Salmonella enterica* serovar Typhimurium DT104 strains on the genomic island, called *Salmonella* genomic island 1, which contains an antibiotic resistance gene cluster with both Tet G and the flo^R gene.[88] The flo^R gene was first detected in an R plasmid of the fish pathogen *P. damselae* ss *piscicida*, also in Japan.[89,94] This gene was located downstream of the *sul2* gene and has 47.4% identity with the chloramphenicol resistance gene *cmlA* of *Pseudomonas aeruginosa*. The identical flo^R gene of *P.*

damselae ss *piscicida* has also been detected in the chromosomal drug resistance region of the genomic island of multidrug resistant *Salmonella enterica* ss *enterica* Serovar Typhimurium DT104,[95] and in bovine isolates of *E. coli*, a florfenicol resistance gene with 98% homology is relatively widespread.[96]

Tetracycline resistant bacteria were isolated from fish collected from three different fish farms in the southern part of Japan.[97] Sequence analyses indicated a match between the tetracycline resistance genes of the fish farm bacteria and bacteria from humans. The homology was 99.3 to 99.9% for *tetB*, 98.2 to 100% for *tetC*, 99.7 to 100% for *tetD*, 92.0 to 96.2% for *tetG*, and 97.1 to 100% for *tetY*. The results suggest that tetracycline resistance genes from fish farm bacteria have the same origins as those from human pathogenic strains.

The majority of drug resistance genes studied and sequenced from fish pathogens are not related to or very distantly related to their analogs in drug resistant human pathogenic bacteria. For instance, the CAT gene of the R plasmids occurring in *V. anguillarum* isolated from farmed ayu in Japan in 1973 was found to be only 37–69% homologous with other CAT proteins of both Gram-negative and -positive bacteria when comparing the predicted amino acid sequences.[90]

Similarily, the tetracycline resistance determinant found in all resistant isolates of *V. salmonicida* was Tet E.[13,29] The Tet E determinant has primarily been found in aquatic environments in both Europe and America.[47,63,64,98,99]

6.10 Improving management and monitoring of antibiotic use in fish farms

Any use of antibiotics on fish farms impacts the bacterial flora of both diseased and healthy fish. In addition, the normal flora of the sediments and water of the aquaculture system will be disturbed. From this, it is obvious that all available measures should be taken to reduce the risk of a bacterial infection, including optimal management systems and procedures and vaccination strategies. When a serious bacterial infection is established in an aquaculture population, it will always be necessary to administer effective antibiotic drugs. Such drug administration should be based on proper diagnostic routines and resistance testing of the causative bacterium. Fish health services should be linked to the aquaculture site, and the use of antibacterial drugs should follow strict national or regional regulations, using only licensed drugs. Any use of antibacterial drugs should be reported to a central register for control and follow-up studies by authorities and researchers.

It is essential to develop and implement guidelines for prudent use of antimicrobials for veterinarians and other professionals prescribing antimicrobials, as well as for those working in the aquaculture industry. The WHO *Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food* provides a framework of recommendations to reduce the overuse and misuse of antimicrobials in food animals for the protection of human health. The

Global Principles were developed with the participation of FAO and OIE, as part of a comprehensive WHO Global Strategy for the Containment of Antimicrobial Resistance and can be found at: http://www.who.int/emc/diseases/zoo/who_global_principles/index.htm.[8]

6.10.1 Monitoring antibiotic resistance and preventing its spread

It is crucial that every outbreak of disease on a fish farm is monitored for the development of antibacterial drug resistance. Without this data, attempts to control subsequent infections may use ineffective drugs, resulting in failure of treatment and loss of large parts of the fish population before the most effective antibacterial drug is identified.

It is also important to keep track of drug resistance development in the various bacterial fish pathogens at district, regional and national levels. This type of surveillance needs to be organized as routine monitoring by the authorities and not left to occasional research projects. Monitoring antimicrobial drug usage and resistance serves several purposes, including: documentation of the situation; identification of trends; linkage of antimicrobial usage to antimicrobial resistance; basis for risk assessment; basis for interventions; evaluation of effectiveness of the measures implemented; a basis for focused and targeted research; and assessing compliance with regulations.[8]

When drug resistance has developed among the bacterial pathogens of a fish farm, it is difficult to control its spread to the environment if the farm is located in net pens in open water rather than land based. However, it is very important to follow traditional hygienic measures when handling diseased or dead fish, including organic material from the slaughter plants.

6.11 Future trends

The number of species under aquaculture will probably increase in the future. The systems involved will become more effective, with larger densities of animals and consequently increased risk of disease. Management routines will improve, but the risks of developing infectious diseases will remain relatively high, in particular within species that are new to aquaculture in a region. The focus on preventive strategies, including vaccination, will increase at all levels of aquaculture. However, the need for antibiotics will continue, but hopefully more as a preparedness measure in case preventive methods fail.

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7

Improving disease immunity to reduce antibiotic use in farmed fish

Ø. Bergh and A. H. Nerland, Institute of Marine Research, Norway

7.1 Introduction

There are several examples of epizootics with destructive socio-economic effects on the aquaculture industry. Introduction of exotic pathogens to new hosts and/or geographical areas may have particularly devastating effects. However, control with opportunistic pathogens is also a prerequisite for the modern aquaculture industry.

Aquaculture, by definition, implies rearing of aquatic animals in facilities at artificially high population densities in comparison to nature. High densities of hosts may provide improved opportunities for the proliferation of pathogenic agents, such as viruses, bacteria and parasites. Suboptimal rearing conditions, possibly compromising the immune defence of the cultured animals, may further benefit the pathogens. Thus prophylactic countermeasures towards important pathogens have been developed as an integrated part of modern industrialized aquaculture. Today, vaccination is by far the most important of the prophylactic countermeasures; however, nonspecific immunostimulants also play a significant role.

7.2 Immunity in fish

Today, the majority of knowledge of the immune system of vertebrates is based on studies of mammalians (mice and men). However, the information about other vertebrate species, including bony fish (teleostei), has increased rapidly during recent years, revealing that vertebrates to a large extent seem to share

similar organization of their immune system (Litman *et al.*, 2005; Robertsen, 2006; Magnadottir *et al.*, 2005). The immune system of vertebrates is quite complex, involving a variety of macromolecules (e.g. antibodies, cytokines and complement factors), a variety of cell populations (such as macrophages, granulocytes, T-cells and B-cells) and many specialized tissues/organs (thymus, spleen, etc.). When the immune system is stimulated, the following immune response is thus the outcome of complex interactions between macromolecules and cells, typically leading to cascade reactions involving activation of many different genes.

7.2.1 Innate and adaptive immunity

The vertebrate immune system may be divided into innate and adaptive immunity. Innate immunity means that specific patterns of pathogenic organisms (like LPS from bacteria or viral RNA) are recognized by parts of the immune system, leading to activation of effector systems like the complement system or phagocytic cells (macrophages, neutrophils) that will inactivate the pathogen. The patterns that are recognized are usually much conserved parts of the pathogenic organisms. This means that if a pathogenic organism manages to mutate in such a pattern, the innate immunity will not be able to confer protection. Innate immunity does not elucidate memory. However, innate immunity is usually the first line of defence against infections and is a very important part of the immune system of vertebrate organisms.

Adaptive immunity, on the other hand, is able to 'design' receptors that can recognize more or less any foreign components that enter the body. In addition, the adaptive immunity creates long-lived cell lines that confer memory and will thus be able to give a faster and stronger protective response if the foreign substance should happen to enter the body at a later stage.

There is no clear cut border between innate and adaptive immunity. Many components and cells can take part in both innate and adaptive immune responses. Often stimulation of innate immunity is the first step of the inflammation process which ends up with stimulation of adaptive immunity. Stimulation of adaptive immunity may, on the other hand, end up with an effector response which otherwise is a part of innate immunity (Kindt *et al.*, 2007; Murphy *et al.*, 2008).

7.2.2 Triggering adaptive immunity

The intention with vaccination is to stimulate the immune system in such a way that if an individual encounters a given pathogenic organism later in life, a strong protective response will rapidly be raised. Vaccines are therefore based on adaptive immunity and its inherent immunological memory. Central to adaptive immunity are the lymphocytes: B-cells and T-cells. B-cells are producing antibodies, which may be either membrane-bound (then often called B-cell receptors (BCR)), or released to the surroundings. T-cells express T-cell

receptors (TCR), which are always bound to the cell membrane. There are two subclasses of T-cells: T-helper (T_h) cells and T-cytotocxic (T_c) cells with distinctive functions in the immune system. Because antibodies are found in the blood plasma where the cells are removed, the B-cells system is often referred to as humoral immunity (humours – body fluids), whereas the T-cell system often is referred to as cellular immunity.

Circulating antibodies, if binding, may inactivate a pathogen either by generation of immobilizing complexes or by calling attention to phagocytic cells or the complement system. However, as antibodies are not able to cross the cell membrane, they will not be capable of inactivating a pathogen that has managed to hide inside cells. In such a case, protection will depend on T_c-cells which can lyse and kill target cells and thereby release the pathogens. One therefore can say that humoral immunity can protect against extracellular pathogens whereas cellular immunity is necessary for protection against intracellular pathogens. However, at least for a short period during infection, intracellular pathogens necessarily have to occur extracellularly, and can be reached by circulating antibodies.

In the body of vertebrates there is, by means of so-called somatic genetic recombination, a continuous generation of B- and T-cells expressing BCR and TCR with novel binding properties. However, to become effector or memory cells these native cells have to be activated. As an immune response directed against the body itself may be very destructive, this activation has through evolution become controlled by complex mechanisms (Fig. 7.1) If an antigen (foreign substance that is capable of stimulating the immune system) enters the body and binds to BCR, the native B-cell will not be activated unless it is at the same time stimulated by a nearby T_h-cell by released signal molecules (cytokines) as well as molecules bound to the membrane of the T_h-cell. To achieve such stimulation the T_h-cell must be stimulated itself by the same antigen. However, the antigen will not be able to stimulate the T_h-cell directly. First the antigen has to be engulfed by a so-called antigen-presenting-cell (APC) where it will be processed by digestion into fragments and these fragments have to be presented on the surface of the APC associated with special molecules called MHC (major histocompatibility complex) class II.

For the naïve T_c-cells the system is still more complex. For an antigen to be able to stimulate a naïve T_c-cell the antigen has to be synthesized inside a cell and then processed and the fragments presented on the surface associated with MHC class I. Simultaneously, the T_c-cell has to get additional stimuli by an adjacent T_h-cell that again has to have been stimulated as described above (Kindt *et al.*, 2007; Murphy *et al.*, 2008).

7.3 Developing vaccines

There is no standard way to develop a vaccine. Which effector molecules and/or effector cells have to be activated to get a proper protective immune response

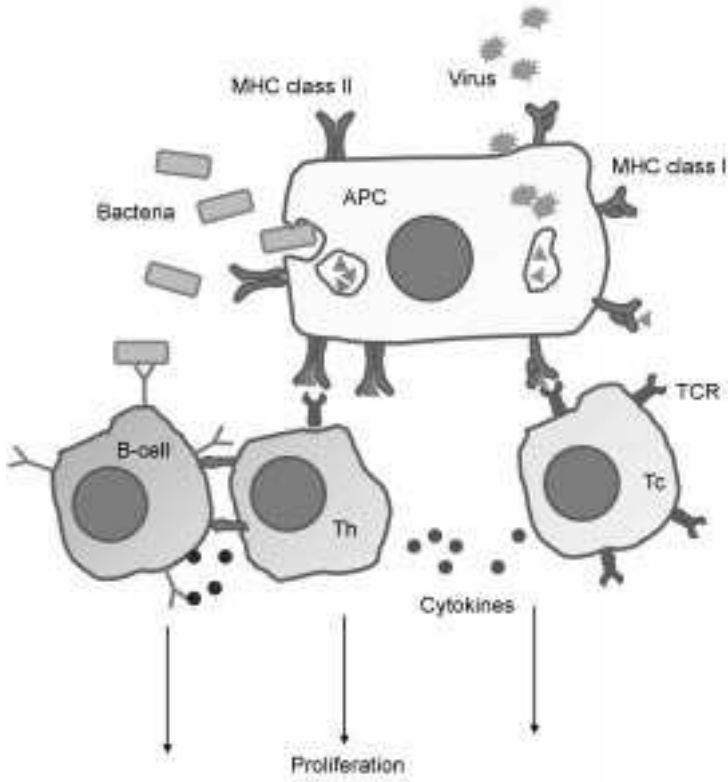

Fig. 7.1 A simplified picture of cells and events involved in the generation of immunological memory. Extracellular bacteria are taken up by an antigen-presenting cell (APC) where the bacteria are processed and the fragments are presented on the cell surface in association with MHC class II molecules. Viruses that replicate inside the cell are processed differently and presented on the surface together with MHC class I. T-helper cells recognize only antigens associated with MHC class II, whereas T-cytotoxic cells recognize only antigens associated with MHC class I. B-cells with membrane-bound antibodies can recognize unprocessed antigens. However, both B-cells and T-cytotoxic cells need additional stimuli (cytokines) for proceeding further to proliferation and generation of effector and memory cells.

depends on the biology and life cycle of the pathogen. As mentioned above, the path of the cascade of gene activations and signals between different cell populations depends of the nature of the antigen, and how the antigen is presented to the immune system. The only 'general' statement that can be said of vaccine development is that in order to obtain a protective immune response it is important that the 'right' antigen is presented to the immune system in the 'right' way, at the 'right' time, resulting in an appropriate cascade of signals ending with effector molecules and/or effector cells that are able to inactivate the pathogen in question. In general terms one can also say that humoral immunity may alone confer protection against extracellular pathogens while

cellular immunity is necessary to get protection against intracellular agents. For parasites (worms, flukes, and parasitic copepods) the knowledge about which parts of the immune system give protection is still limited.

The ideal vaccine for aquaculture should be of low cost for the farmer, easy to administer without any stress for the fish, without side-effects, and give a long-lasting protection covering the entire production cycle. In addition the vaccine should not be of any danger for the environment or for consumers. However, it has turned out to be difficult to fulfil all these criteria at the same time, and it is therefore necessary to compromise.

7.3.1 Administration of vaccines

In aquaculture, vaccines can be administered by immersion, injection or orally. Oral administration by mixing the vaccine components with feed would be the ideal method regarding labour effort and avoiding stress for the fish. However, it has turned out to be difficult to develop effective vaccines that can be given orally. One of the reasons is that it is difficult to avoid antigens being degraded in the gut. In addition, the vaccine dose per fish might be very variable. Immersion by adding the vaccine to the water, or by dipping the fish in a more concentrated suspension of vaccine, is moderately stressful and not much easier. The method has no limit for the size of the fish and a large number of individuals may be treated simultaneously. For some vaccines, administration by immersion gives satisfactory protection and the method is currently used in aquaculture. Injection (normally intraperitonally) is the most stressful and labour intensive method and it requires the fish to be over a certain size. In spite of that, injection is the most common method to apply vaccines in industrial aquaculture today. The reason is the use of multicomponent vaccines that confer protection against several pathogens. As proper protection against certain pathogens cannot so far be achieved without injection, all the vaccine components are combined and injected together. Advantages of injection are that a relatively small amount of vaccine is required and that the individual fish can be given the required dose.

The time of vaccination may be critical. From hatching it takes some time before the larvae is immune competent, which means that the immune system is developed to a stage where stimulation will induce a protective response. If the immune system is stimulated before this, it may induce tolerance instead of protection (Fig. 7.2). The time from hatching to competence may vary between the different fish species, and it depends on environmental parameters such as temperature (Pylkkö *et al.*, 2002). Salmonids hatch in fresh water which contains few microorganisms and the larvae are rather developed when leaving the eggs. Consequently, disease problems at the larval stage of salmonid fish have been a relatively few. The farming of marine species is different as the larvae often hatch at a far less developed stage and in seawater, which is more or less a soup of microorganisms. To prevent infections and diseases the vaccination has to take place as soon after hatching as possible. One therefore needs knowledge

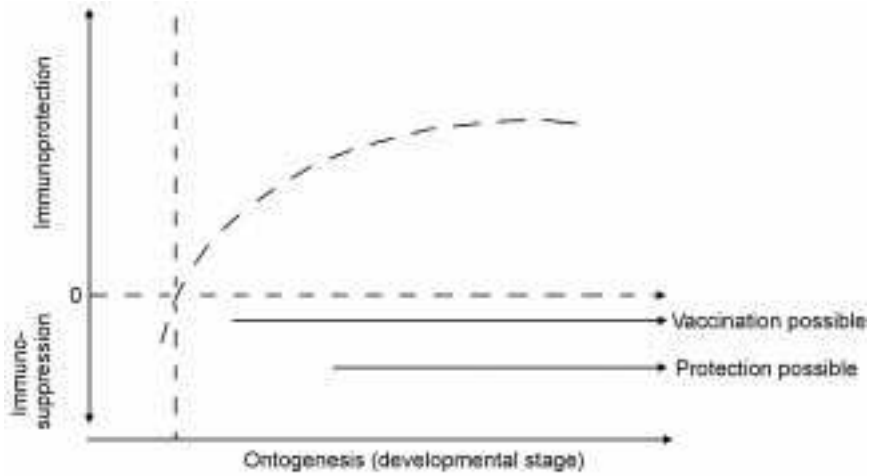

Fig. 7.2 Theoretical effect of vaccination as a function of ontogenetic stage at the time of vaccination.

about the ontogeny of the immune system of the fish species in question in order make vaccination regimes that induce protection and not tolerance.

7.3.2 Traditional vaccines

The traditional way to make vaccines in human and veterinary medicine has been to use either 'killed microorganisms' or 'live attenuated microorganisms'. The former is made by cultivating the pathogenic organism in large quantities and subsequently killing it, normally by adding formalin. Using such a suspension directly as a vaccine induces a protective immune response against some pathogens. For example, a vaccine consisting of formalin-killed *Vibrio salmonicida* will provide high protection of salmonids against vibriosis even when the vaccine is administered by immersion (Håstein *et al.*, 2005).

For other pathogens, like *Aeromonas salmonicida*, the protective effect of such a suspension is poor, even when injected. To get a satisfactory protection, the suspension of killed bacteria has to be added with adjuvants before injection. An adjuvant is some kind of materials that enhance the immune response. To get a proper protection against furunculosis, the killed bacteria are formulated as an oil-water emulsion. The adjuvant effect is probably a combination of induction of inflammation around the injected emulsion and a slow release of antigens (Håstein *et al.*, 2005).

For intracellular bacteria, like *Renibacterium salmoninarum*, the killed micro-organisms do not give any protection even if it is injected together with adjuvants (Kaattari and Piganelli, 1997). The explanation is probably that induction of a humoral immune response is not sufficient to get protection as the bacteria hide inside the cells.

Inactivated or killed viral particles used as vaccines might give reasonable

protection against viral infections. However, as the virus has to be propagated in cell cultures, the production cost of such vaccine is relatively high.

In human medicine several of the most successful vaccines are live attenuated microorganisms (e.g. vaccines against measles, mumps, polio). These vaccines are developed by manipulating the microorganism in such a way that it loses its pathogenic properties while the infectivity and the antigenicity remain. Today, the microorganisms are usually attenuated by using molecular biology. Such vaccines might be given orally even at a low dose, as the microorganisms will propagate in the body and induce both humoral as well as cellular immunity. However, there are concerns about using such vaccines in the aquatic environment, as there is a certain probability that the microorganism may mutate or recombine and in that way revert to a pathogenic form. In addition, as we are talking about live viruses and maybe genetic modifications, we have to take into account the opinion of consumers.

7.3.3 Recombinant subunit vaccines

Recombinant subunit vaccines are made by cloning genes encoding particular protein antigens from the pathogen in question and expressing the recombinant protein in large quantities in another host (bacteria, yeast, insect cells). The recombinant protein is then eventually added adjuvants and other components and formulated as a vaccine. The protection may depend on the host in which the antigen is expressed due to differences in post-translational modifications (i.e. glycocylation). Such vaccines have many of the same properties as vaccines based on killed inactivated microorganisms, but for virus vaccines the production cost will be far less. For a given pathogen it may only be a few key components that induce immunological protection. Other components may either induce little immune reaction or worse still induce an immune reaction that makes the fish more susceptible to the disease. To develop a recombinant subunit vaccine it is therefore critical to discover the protective protein antigens and their corresponding genes. As viruses have relatively few genes compared to bacteria, the selection of genes is normally far less laborious.

Several recombinant subunit vaccines have been developed and tested to give protection against viral diseases (nodavirus, VHS, IPN) (Sommerset *et al.*, 2005b; Lecocq-Xhonneux, 1994; Christie, 1997). For intracellular bacteria like *Renibacterium salmoninarum* and *Piscirickettsia salmonis* the recombinant approach has been less promising. However, the reason may be that the optimal antigen has not been used. So far only the vaccine against IPN is commercially available.

7.3.4 DNA vaccines

DNA vaccines are made by cloning gene encoding antigens into a plasmid vector behind a strong eukaryotic promoter. By injection of the plasmid into tissues, the antigen will be expressed in the host cell and processed in a similar

way as achieved by an infection and thereby inducing both cellular and humoral immunity (Utke *et al.*, 2008).

The first DNA vaccines that were tested on fish were against the rhabdoviral diseases VHS and IHN and were based on the viral glycoproteins that are normally located on the surface of the virus particles. The results were surprising. High protection was achieved only a few days after vaccination and it lasted for at least one year. Later studies have shown that the early protection is most probably due to stimulation of the innate immune system as the vaccines also give temporary short-term protection against unrelated viruses, whereas long-lasting adaptive immunity comes up after 3–4 weeks (Lorentzen *et al.*, 2001; Kurath, 2005; Lorenzen and LaPatra, 2005; Sommerset *et al.*, 2005b).

DNA vaccination against disease caused by intracellular bacteria has also been promising. A vaccine based on the gene encoding a fibronectin binding protein from *Mycobacterium marinum* protected fish in challenging experiments by inducing humoral as well as cellular immunity.

DNA vaccines are usually given as intra-muscular injections as muscle cells have turned out to be suited for expression of the antigen. A disadvantage is therefore that DNA vaccines are not compatible for administration together with the multivalent vaccines currently in use, which are given by intraperitonal injections. DNA vaccines will give a local, transient expression of foreign genes in the vaccinated individual. Whether a DNA vaccinated fish has to be regarded as a GMO (genetically modified organism), with all its implications, has been disputed, but so far not settled by the relevant bodies.

7.3.5 Live vector vaccines

Live vector vaccines are made by cloning the gene encoding antigens in such a way that they will be expressed by an apathogenic microorganism (preferentially on the surface), which can be applied to the fish orally, by immersion or injection. If this is a microorganism having an intracellular stage, both humoral and cellular immune response against the antigen will be induced. It could also be possible to use live vectors to deliver DNA into tissues and cells where the genes express antigens as described for DNA vaccines above. The live vector may be bacteria as well as viruses. However, there are similar concerns about this kind of vaccine as for the live attenuated virus vaccines.

An example of a bacterial live vector is *Aeromonas salmonicida* Aro^- mutants where gene encoding enzymes involved in the synthesis of aromatic amino acids have been deleted. Such bacteria can be grown in the laboratory as long as the medium is supplied with aromatic amino acids. However, if the bacterium is administered to fish, it will multiply a few times until it has run out of aromatic amino acids then it will vanish. Meanwhile it has triggered the immune system. As *A. salmonicida* is partially intracellular it will induce both humoral and cellular immunity (Vaughan *et al.*, 1993; Marsden *et al.*, 1996).

7.4 Developing feed-based immunostimulants

The innate defence includes both humoral and cellular defence mechanisms such as the complement system and the processes played by granulocytes and macrophages. Different substances such as β-glucans, bacterial products, and plant constituents may directly initiate activation of the innate defence mechanisms acting on receptors and triggering intracellular gene activation that may result in production of anti-microbial molecules. These immunostimulants are often obtained from bacterial sources, brown or red algae, and terrestrial fungi are also exploited as sources of novel potentiating substances (Bricknell and Dalmo, 2005).

The use of immunostimulants, as dietary supplements, can improve the innate defence of animals providing resistance to pathogens during periods of high stress, such as grading, reproduction, sea transfer and vaccination. The immunomodulation of larval fish has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals until its adaptive immune response is sufficiently developed to mount an effective response to the pathogen. To this end it has been proposed that the delivery of immunostimulants as a dietary supplement to larval fish could be of considerable benefit in boosting their innate defences with little detriment to the developing fish. Conversely, there is a school of thought that raises the concern of immunomodulating a neotenous fish before its immune system is fully formed as this may adversely affect the development of a normal immune response (Bricknell and Dalmo, 2005).

7.5 Impact of prophylaxis on the reduction of antibiotic use in fish

Effective vaccines for aquaculture have been developed during recent decades (Gudding *et al.*, 1999), and vaccination is one of the most important prophylactic measures against disease. The aquaculture industry in Norway is a good example of the great environmental impact vaccination may have. In 1987 almost 50 000 kg of antibiotics was used to produce 50 000 tons of salmon. The same year the vaccine against coldwater vibriosis was introduced, and later on programmes for vaccination against yersiniosis and furunculosis were implemented. The impact of these vaccination programmes was significant, and in 1997 only 746 kg of antibiotics was used to produce 316 000 tons of fish. Thus, whereas 1000 g of antibiotics was used per 1 ton produced in 1987, this was reduced to 2 g per 1 ton produced in 1997 (reviewed by Lillehaug *et al.*, 2003). Total consumption in 2006 was 1426 kg, measured as active substance.

Furunculosis is a 'classical' fish disease, caused by the Gram-negative bacterium *Aeromonas salmonicida* subspecies *salmonicida*. *Aeromonas salmonicida* has been recognized as a pathogen of fish for over 100 years. The first report of its isolation was during a disease outbreak at a Bavarian hatchery in 1884 (cf Hiney and Olivier, 1999). The original epizootic spread of

furunculosis in both Europe and America and particularly in the UK is strongly suggestive of an introduced pathogen (Bakke and Harris, 1998), although today the distribution is nearly global. Furunculosis was first found in Norway in 1964 following the importation of rainbow trout from Denmark. The disease was spread to several farms and to wild fish in the River Numedalslågen. In 1985 furunculosis was discovered in marine salmon farms to which infected salmon smolts from Scotland had been imported to Norway (Egidius, 1987a; Johnsen and Jensen, 1994). This resulted in a major epizootic that culminated in the years 1990–92 (Lillehaug *et al.*, 2003), after which the emergence of oil-based adjuvant vaccines has more or less eliminated the problem in farmed salmon (reviewed by Sommerset *et al.*, 2005a). In 1992 550 farms were infected, and the disease had spread from 22 natural watercourses in 1989 to 72 in 1992. Rapid spread of the disease was associated with several factors including escapes and natural movement of wild fish in the sea (Johnsen and Jensen, 1994). Ecological consequences are not known, but the effective elimination of the problem in aquaculture has probably resulted in a dramatically reduced infection pressure towards wild salmon. The disease is not considered a major problem to wild salmon in Norway today, but outbreaks in salmon rivers with low water flow are occasionally registered.

A similar example to furunculosis, although with a native pathogen, is *Vibrio anguillarum*, which has long been known as a causative agent of disease (classical vibriosis). It regularly causes epizootics in wild and cultured populations of several fish species (Egidius, 1987b, and articles cited therein). The oldest known descriptions of the disease date from 1817. Vibriosis is known in a wide range of fish species. It caused significant problems to salmonid farming before the advent of modern vaccines (reviewed by Lillehaug *et al.*, 2003; Sommerset *et al.*, 2005a). Contact between fish seems to be an important factor for the spread of the pathogen; however, the bacterium is widely distributed in wild fish as well as food organisms and is transmitted horizontally. Similarly, *Vibrio salmonicida*, although originally described from cultured fish (Egidius *et al.*, 1986), caused a major epizooitic of coldwater vibriosis, or so-called 'Hitra disease' during the late 1980s, is now completely controlled by vaccines (Lillehaug *et al.*, 2003; Sommerset *et al.*, 2005a). It is unlikely that the bacterium is not present in the wild.

In recent years, 'Winter ulcer' has been the most important bacterial disease in Norwegian salmon aquaculture from an economic and fish welfare viewpoint. *Moritella viscosa* (formerly *Vibrio viscosus*) is the primary cause of the disease, although other bacteria may also be involved. The disease causes major ulcerations, and there is little doubt that it is associated with animal suffering. The effect of vaccination has been considered variable, and antibacterial treatment may have little or no effect (Coyne *et al.*, 2004, 2006).

So far surprisingly few pathogens that cause disease in coldwater marine fish have been discovered. This is, however, most likely because aquaculture of coldwater fish is still in its infancy. As the industry develops, new pathogens will be discovered. So far it seems that the knowledge developed for salmonids

may to some extent also be used for coldwater fish. However, there are clear indications that vaccines should be designed according to the species, due to the differences in pathogens affecting the different species. The obvious positive effect of vaccination is reduced mortality, but for a sustainable biological production, the reduced need for medication is also significant.

Studies of vaccination of marine species are scarce, and little is published. Ingilæ *et al.* (2000) demonstrated protection in challenge experiments of halibut and spotted wolfish vaccinated intraperitoneally with oil-emulsified vaccines against atypical *Aeromonas salmonicida.* Several vaccines designed specifically for marine fish species are now available. Vaccines against viral diseases are presently at the experimental stage. Húsgarð *et al.* (2001) showed protection in turbot in challenge experiments with nodavirus, following vaccination with a recombinant vaccine. The increase in the Norwegian cod farming industry, however, has caused a significant increase in the consumption of antibiotics. In Norway in 2006, cod farming treatments constituted 647 kg active substance, of which 98% was quinolones. In comparison, the consumption for salmon was 347 kg, halibut 41 kg, and other species 165 kg.

For salmonids the change from a freshwater stage to a seawater stage creates some benefits for disease control. For marine fish that stay in seawater the whole lifecycle, it is therefore desirable to vaccinate at an early stage. However, the stress induced by vaccination at young stages may entail immunosuppression and increased susceptibility to pathogens, and it may also reduce the performance related to other aspects such as growth.

The problems connected with vaccination of young stages and the fact that the specific immune system is not fully developed at this stage may have a solution in the fact that egg/larvae 'inherit' specific immunity from the mother – maternal immunity. It has been demonstrated that it is possible to manipulate specific antibody composition and levels in eggs and larvae through immunization of the mother. Immunization of tilapia (*Oreochromis aureus*) broodstock with different proteins revealed a considerable increase in antibody activity. The maximum increase was 10–13 $\log_2$ units in embryos that hatched 15–35 days after immunization. Atlantic salmon broodstock vaccinated against yersiniosis revealed maternal transfer of specific antibodies to eggs and yolksac larvae, but at low levels insufficient to protect offspring against yersiniosis. Too little is known to fully evaluate the potential of stimulation of maternal immunity as a method in microbial management. However, it is reasonable to believe that at least for some diseases, vaccination or secondary stimulation of mothers with appropriate vaccines before the spawning season could protect the larvae against disease the first period after hatching.

7.6 Implications for farmed fish welfare, safety and quality

It is of major importance to keep the vaccines efficient while reducing their side effects. Although the majority of the farmed fish seem to possess side effects

within acceptable limits – given the prophylactic effects of vaccination – it is evident that some fish possess unacceptable levels of side effects. At present, adherations and skeletal deformations are probably the side effects with the most important implications to animal welfare in the salmon industry (Berg *et al.*, 2006a). Increased focus on vaccination protocols and formulations has greatly reduced the problems, but to some extent, vaccination still remains an ethical problem for salmon aquaculture.

There are several different effects of vaccination, such as:

- immune reactions and adherations
- melanin deposits
- effects on growth (short-term and long-term)
- skeletal deformations.

The rediscovery of oil-based adjuvants, and the use of such in furunculosis vaccines was the key to successful prophylaxis, and practical elimination of this disease problem from the Norwegian salmon industry (reviewed by Lillehaug *et al.*, 2003). Oil-based adjuvants ensured long-term protection and reduced mortality to very low numbers (Midtlyng, 1998). After 1993, furunculosis has been practically eliminated from Norwegian aquaculture (Lillehaug *et al.*, 2003). However, the discovery of gross and histopathological lesions in farmed salmon immunized by oil-adjuvanted vaccines has led to increased interest in vaccine side effects. One side effect of vaccination by injection is local reactions in the peritoneal cavity. The magnitude of such side effects is dependent on the formulation of the vaccine. Granulomatous inflammation and melanin accumulation is a side effect of the use of oil adjuvants (Koppang *et al.*, 2005). In severe cases, lesions consisting of granulomatous tissue adhere to and embed different visceral organs in such a way that normal function may become affected (Poppe and Breck, 1997).

Fish of other species than salmon may also develop adherations following vaccinations. Trout get less adherations than salmon following injection with oil-based vaccines. In addition water-based vaccines are frequently used with trout, cod and halibut. The use of water-based vaccines reduces the amount of vaccine-associated welfare problems considerably. Adheration is not considered a significant problem (Berg *et al.*, 2006a). It is difficult to determine to what extent this affects the welfare of the fish. However, it is known that intestinal adhesions give severe pains in humans.

Melanin deposits is a consequence of normal immune reactions. Melanomacrophages and other melanin-containing cells may deposit black pigment. Melanin on internal organs will normally be removed during processing of the fish, but in extreme cases may be visible in, or between viscera. Melanin deposits are generally not considered a welfare problem but may lead to declassification of the product, especially when hidden in fillet (Berg *et al.*, 2006a,b). Owing to use of water-based vaccines the problem is considerably less in trout, cod and halibut.

New vaccine concepts, such as recombinant vaccines based on genomics/

proteomics, may give better protection and considerably fewer side effects, if development and utilization is allowed to proceed. The same applies to DNA vaccination. Religious or political objections to new vaccine technologies must be weighed against concern for animal welfare, environmental and economic factors.

Reduced growth for a short period following vaccination has been well described. This is mainly a short-term effect, and is thought to be partly due to reduced appetite, stress, and the ongoing immune response following vaccination. However, there are reports demonstrating long-term reduced growth of vaccinated fish compared to unvaccinated fish, but this phenomenon is poorly understood. Time of vaccination, as well as the vaccine formulation may influence the effect on growth.

Deformed vertebrae may occur at different locations and at different life stages. Factors such as small smolt size, fast growth, early vaccination, low phosphorous content and bioavailability, contaminants and high temperatures may influence the deformities (Berg *et al.*, 2006b). These deformities have a different appearance in cod, and are mainly due to deformation of the notochord by pressure from the swim bladder, which may cause malformation of the vertebral column (Grotmol *et al.*, 2005).

7.7 The current situation and future trends

Currently commercial vaccines are available giving satisfactory protection against many of the bacterial diseases that previously caused great problems for industrial aquaculture. However, to get protection against these diseases, the fish are injected with multicomponent vaccines containing oil adjuvant causing unwanted side effects. Just a few of the vaccines can be administered by methods (oral, immersion) which involves minimal stress for the fish and which are labour-effective for the farmer.

There are several bacterial diseases where protective vaccines are still not available. Typically, the causing agents have a life cycle involving intracellular stages as *Renibacterium salmoninarum* and *Piscirickettsia salmonis*. The result is that unwanted large quantities of antibiotics are used in some countries, such as Chile.

For viruses, which are obligate intracellular pathogens, only few effective vaccines are available and there is a range of viral diseases that are causing serious obstacles for aquaculture. And so far no commercial vaccines are available against parasites such as sea lice which is one of the major problems in farming of salmonid fish. There is therefore a great need for development of new vaccines both for the fish species currently farmed and for new species coming up.

Worldwide, a great effort is taking place both from academic institutions and private industry to develop new and better vaccines. The hope is that improved vaccination formulations and principles will come up that can be applied for a

wide range of vaccines against pathogens where the protection so far has been unsatisfactory. However, this seems not to be straightforward. More knowledge and information are needed about the immune system of fish, making it possible to develop assays to monitor the different steps in the immune response of fish, which again will make it more feasible to design vaccines that trigger a proper immune response. In addition, detailed knowledge is necessary about the different pathogens, their infection mechanism and life cycle. That will make it possible to select antigens for the vaccines which will give an immune response that interferes and neutralizes the pathogen.

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8

Different methods to reduce antibiotic use in farmed fish

F. J. Gatesoupe, INRA – Ifremer, France

8.1 Introduction

The previous chapter highlighted the utmost importance of optimising innate and acquired immunity in fish with a view to limiting antibiotic use. The immune system plays a key role in disease control, but it can only operate fully when fish are reared in good conditions. While several vaccines are now available, they cannot protect fish against every potentially pathogenic bacterium. Efforts to avoid stopgap treatments by vaccination and immunostimulation must thus be placed within the context of a global prophylactic approach, in which other measures are necessary to deal with hygiene management or stress prevention, for instance. Such practices are – or should be – current in fish farms, and it is worthwhile describing them briefly in this chapter. Other methods can be aimed at the development of functional feeds and microbial management, but they still need further research and testing. These future trends deserve description in further detail, especially those relating to tailoring the diet for fish health, or to introducing or favouring beneficial bacteria in microbiota. Prevention is never foolproof, and in the case of bacterial disease, antibiotics are generally the last resort. However, alternative therapeutic treatments show promise of future application to fish culture, especially those inspired by traditional herbal medicine. There are also some new methods which offer great hope of combating pathogenic bacteria by disrupting their means of communication, introducing bacteriophages to lyse the infectious cells, or administering antimicrobial peptides produced by other living organisms as natural defences. These hopes rest, however, on the condition that such treatments prove less susceptible than antibiotics to give rise to bacterial resistance.

8.2 The prophylactic approach: hygiene, welfare and feeding

8.2.1 Improving hygiene

Good hygiene practices are the first requisites in limiting the need for antibiotics and the spread of antibio-resistance. The institutional authorities may publish rules and guidelines, but good practice of those rules lies in the hands of veterinarians and fish farmers. Some basic principles are outlined in this section, but for further detail of aquaculture biosecurity, see Scarfe *et al.*, 2005; Section 8.6.

Quarantine

When fish are introduced into a new area, they must be kept under quarantine until they can be declared disease-free. This also includes the search for the presence of asymptomatic carriers of pathogens in the origin area. The quarantine zone should be strictly delimited and dedicated equipment never taken out of the zone without being disinfected.

Sanitation

Whenever possible, dead fish and particulate matter should be removed from the rearing units, and then disposed of properly. It is essential to design facilities that allow the easy collection of solid waste, with special attention to water current, surface slopes and smoothness, gratings, filters and protein skimmers.

Disinfection

Disinfection is the principal key to hygiene management, and all incoming or outgoing materials should be treated using proper methods – including chemical baths and rinsing, autoclaving, etc. – while people should use footbaths and hand-washing devices. Water inlets and outlets should be treated constantly with ultraviolet light or ozonation. Water limpidity is essential for disinfection, reinforcing the need to remove suspended particles.

Fallowing

Last, but not least, each rearing season should end with a break to allow the system to dry out before the next run.

8.2.2 Improving fish welfare

The limitation of stress in husbandry is essential for fish health. This issue is discussed fully in Chapter 20, whilst the effect of stress on the immune system is described in Chapter 7. This section outlines two main issues of welfare preservation, which are also generally considered in health management.

Water quality

The oxygen supply should be monitored with automatic control devices. Respiratory demand fluctuates with planned events like feed distribution, but also with unexpected disturbances that may provoke reactions of fear. Gas

supersaturation should be controlled even though this risk can easily be forecast and prevented. The level of toxic compounds should be kept low, the most common threat being caused by ammonia and nitrite, with their toxicity linked to pH and temperature.

Rearing standards
Productivity may jeopardise fish welfare, as well as health and flesh quality. Compromise solutions must be found to assess the optimum conditions of stocking density, rearing temperature, feeding rate, etc. Glenn and Taylor (2006), for instance, recently demonstrated that it was possible to limit the incidence of furunculosis in rainbow trout by lowering the rearing density. Handling operations should be kept to a minimum since they can induce not only stress, but also physical damage, which is a common route for infection.

8.2.3 Improving feed

Feed quality is also important for fish health (Lim and Webster, 2001). In addition to the immunostimulants incorporated into the feed for this specific purpose, the dietary level of most nutrients may also affect the immune system (Waagbø, 1994). The main features are outlined here, but for further discussion, see Chapter 7.

Proteins
The dietary level of protein may affect the immune status of rainbow trout to some extent (Kiron *et al.*, 1995a), as also the partial replacement of fish meal by the fish protein hydrolysate in Japanese sea bass (Liang *et al.*, 2006). The replacement of fish meal by vegetable protein sources may be detrimental to the immune system when the rate of substitution is too large (Chapter 12). Soybean products may cause intestinal pathology in salmonids when they are incorporated at high dose (Burrells *et al.*, 1999; Krogdahl *et al.*, 2000). Conversely, some plant constituents are antibacterial (Section 8.4), or anti-oxidative (Sitjá Bobadilla *et al.*, 2005).

Antioxidants
Peroxidised lipids may endanger the immune status of fish (e.g., Obach and Baudin Laurencin, 1992). Lipid rancidity can thus increase the demand for antioxidants, mainly vitamin E (e.g., Obach *et al.*, 1993; Table 8.1). The supply of vitamin E is particularly important for health when the diet is rich in highly unsaturated fatty acids (Puangkaew *et al.*, 2005). However, an overdose of vitamin E may fail to stimulate immunity, probably due to imbalance with other antioxidants (Ortuño *et al.*, 2000). The interaction between vitamins E and C is complex, but anti-oxidative protective mechanisms may account at least partly for the immunostimulant effects of these vitamins (Waagbø, 1994; Table 8.2). Vitamin A also has antioxidant properties and stimulated the immune response of gilthead sea bream (Cuesta *et al.*, 2002b, 2003). Dietary carotenoids also

Table 8.1 Some examples of increased resistance to pathogens, and immunomodulatory effects due to vitamin E in fish, a non-exhaustive list

Species	Disease resistance	Cellular immunity	Humoral immunity	References
Dicentrarchus labrax	–	Phagocytosis	Lysozyme, complement	Obach *et al.* (1993)
Epinephelus malabricus	–	Respiratory burst	Lysozyme, complement	Lin and Shiau (2005a)
Ictalurus punctatus	*Edwardsiella ictaluri*	Phagocytosis	–	Wise *et al.* (1993)
Morone chrysops ♀ × *M. saxatilis* ♂	–	Respiratory burst	–	Sealey and Gatlin (2002)
Oncorhynchus mykiss	–	Lymphocyte proliferation, natural cytotoxicity	Antibody production, complement	Verlhac *et al.* (1993), Puangkaew *et al.* (2004)
Paralichthys olivaceus	*Edwardsiella tarda*	Respiratory burst	Lysozyme, complement	Wang *et al.* (2006)
Salmo salar	*Aeromonas salmonicida*	–	Complement	Hardie *et al.* (1990)
Sparus aurata	–	Respiratory burst, phagocytosis	Complement	Ortuño *et al.* (2000)

Table 8.2 Some examples of increased resistance to pathogens, and immunomodulatory effects due to vitamin C in fish, a non-exhaustive list

Species	Disease resistance	Immunomodulation	References
Cirrhinus mrigala	*Aeromonas hydrophila*	Phagocytosis	Sobhana *et al.* (2002)
Epinephelus malabricus	*Vibrio carchariae*	Respiratory burst Complement Lysozyme	Lin and Shiau (2005b)
Labeo rohita	–	Respiratory burst Phagocytosis Lymphokine production Lysozyme Serum bactericidal activity	Misra *et al.* (2007)
Oncorhynchus mykiss	*Vibrio anguillarum*	Antibody production Lymphocyte proliferation Wound healing	Navarre and Halver (1989) Hardie *et al.* (1993) Wahli *et al.* (2003)
Salmo salar	*Aeromonas salmonicida*	Antibody production Lysozyme Complement	Waagbø *et al.* (1993a)
Scophthalmus maximus	–	Lysozyme	Roberts *et al.* (1995)
Sparus aurata	–	Lysozyme Phagocytosis Complement Respiratory burst Natural cytotoxicity	Verlhac *et al.* (1993, 1998) Ortuño *et al.* (1999, 2001) Cuesta *et al.* (2002a)

Table 8.3 Some examples of increased resistance to pathogens, and immunomodulatory effects due to fatty acids in fish, a non-exhaustive list

Species	Experimental variable	Disease resistance	Immuno-modulation	References
Dicentrarchus labrax	Fish oil replacement	–	Respiratory burst Leucocyte proliferation	Mourente *et al.* (2005)
Epinephelus malabricus	Total lipid supply	–	Respiratory burst Complement Lysozyme	Lin and Shiau (2003)
Epinephelus malabricus	DHA/EPA ratio	–	Phagocytosis Respiratory burst Leucocyte proliferation	Wu *et al.* (2003)
Gadus morhua	Fish oil replacement	–	Respiratory burst	Bell *et al.* (2006)
Ictalurus punctatus	Lipid source	*Edwardsiella ictaluri*	–	Li *et al.* (1994) Fracalossi and Lovell (1994)
Oncorhynchus mykiss	Fatty acids	IHN virus	Antibody production Macrophage bactericidal activity	Kiron *et al.* (1995b)
Salmo salar	Lipid source	*Yersinia ruckeri* *Vibrio salmonicida*	Antibody production Macrophage bactericidal activity	Erdal *et al.* (1991) Waagbø *et al.* (1993b)
Salvelinus alpinus	Lipid source	*Aeromonas salmonicida*	–	Lødemel *et al.* (2001)
Sparus aurata	Fish oil replacement	–	Lymphocyte proliferation Respiratory burst Complement	Montero *et al.* (2004)

enhanced the immune status of rainbow trout, in connection with vitamins A, C, and E (Amar *et al.*, 2001).

Fatty acids

Dietary fatty acids not only have an indirect effect on fish health due to peroxidation, but they are also directly implicated in immunity as important cell membrane constituents, as well as being precursors of prostaglandins in immuno-competent cells (Rowley *et al.*, 1995; Chapter 11). The influence of essential fatty acids is further complicated by the variable requirements of fish species, according to bioconversion abilities that are quite different in salmonids and in marine fish, for instance. Lipids are a major source of energy and the optimum fatty acid supply is also dependent on the rearing conditions, which affect swimming and maintenance metabolism. Although literature on the effect of fatty acids on fish health is relatively abundant, a considerable research effort would prove necessary in this field, especially when faced with the need to replace fish oil by alternative lipid sources (Table 8.3).

Other nutrients

Many other nutrients may be involved in the immune defences of fish, such as vitamins acting as coenzymes (Waagbø, 1994). Dietary folic acid, for instance, increased the resistance of channel catfish to *Edwardsiella ictaluri*, in a dose-dependent manner, especially with a low supply of vitamin C (Duncan and Lovell, 1994). Li and Gatlin (2006) recently reviewed the importance of nucleotides for fish health. Trace elements may also play a role in immunity and organic forms seemed generally more efficient than minerals (Sealey *et al.*, 1997; Wang *et al.*, 1997; Gatta *et al.*, 2001; Apines-Amar *et al.*, 2004). Iron deficiency can cause anaemia and iron interferes with metabolism of vitamin C (Waagbø *et al.*, 1993a) and fatty acids (Rørvik *et al.*, 2003). Dietary-available iron should be adjusted carefully since bacterial pathogens may benefit from an excessive iron supply (Rodrigues and Pereira, 2004). Little is known about the immunomodulatory effects due to complex interactions between anti-oxidative vitamins and minerals. Hung *et al.* (2007) have recently investigated the effects of these combinations on macrophage proliferation and activity in hybrid tilapia.

8.3 Microbial management

A balanced microbiota is the first barrier against bacterial pathogens and the introduction of beneficial bacteria may reinforce this barrier. Depending on the target microbiota, one can distinguish several kinds of treatment. For instance, the following terminology has been suggested: ‘bioremediation’ to apply to microbial treatments attempting to improve water quality; ‘biocontrol’ to refer to the treatment of microbes on the external mucosa of animals or those intended to manage biofilms in tanks, filters or sediments; and ‘probiotics’ to describe those directed explicitly at the digestive tract (Gatesoupe, 1999). In practice,

however, the distinction may not be so simple, because of the complex relationship between the microbes, the aquatic environment and the fish.

8.3.1 Bioremediation

Microbes play a major role in preserving water quality in aquaculture ponds (Moriarty, 1997). However, few studies have considered the possibility of introducing beneficial bacteria to improve water quality. Dalmin *et al.* (2001) and Wang *et al.* (2005) showed the potential of *Bacillus* spp. in this regard in shrimp ponds. Experiments in fish ponds failed to evidence any effect on water quality, in spite of a significant increase in the survival and net production of channel catfish (Queiroz and Boyd, 1998), and a higher growth rate of common carp (Sharma and Bhukhar, 2000). More recently, a commercial preparation with four microbial strains improved water quality in a closed-circuit system for Japanese flounder, among other probiotic effects (Taoka *et al.*, 2006a). In this latter case, the water treatment seemed particularly beneficial since it increased fish growth and survival, unlike the same treatment administered via the feed, which had no effect on growth, but only on survival.

8.3.2 Biocontrol

Numerous strains of bacteria have been documented that exert antagonistic behaviour against fish pathogens, and the reader is referred to other reviews for further information (Section 8.6). The antagonistic strains are very diverse and they may inhibit the growth of pathogenic bacteria belonging to different orders, or even phyla (Table 8.4). The mechanisms for these antagonisms are not all known, but they seem variable – from the production of bacteriocin-like compounds (Gibson *et al.*, 1998) to the competition for iron (Smith and Davey, 1993). One important feature of antagonism is the inhibition of adhesion of the pathogens to fish mucus, possibly mediated by biosurfactants (Velraeds *et al.*, 1996). This adhesion has generally been tested with intestinal mucus, but skin and gill mucuses were also used by Chabrillón *et al.* (2005a,b, 2006a,b). Mucus was collected from various fishes, e.g. clownfish (Vine *et al.*, 2004), Senegalese sole (Chabrillón *et al.*, 2005a,b), gilthead seabream (Chabrillón *et al.*, 2006a,b) and rainbow trout (Balcázar *et al.*, 2007a).

Three kinds of tests have been defined to observe this inhibition of adhesion: (1) competitive exclusion, after prior binding of the probiotic; (2) direct competition, when mucus is exposed simultaneously both to probiotic and pathogenic bacteria; (3) displacement, when the probiotic is introduced after the pathogen. In displacement conditions, the reduction of adhesion seemed generally more significant than in the other conditions, especially direct competition (Chabrillón *et al.*, 2005a,b, 2006a,b). *Pseudoalteromonas* sp. AP5 (Vine *et al.*, 2004), candidate probiotic *Vibrionaceae* (Chabrillón *et al.*, 2005a,b, 2006a), and lactic acid bacteria (Chabrillón *et al.*, 2006b; Balcázar *et al.*, 2007a) have been shown to reduce the adhesion of several fish pathogens, mainly

Table 8.4 Some examples of references on bacteria antagonistic to fish pathogens, a non-exhaustive list

	Sensitive pathogens							
Probiotic	*A. hydrophila*	*A. salmonicida*	*V. anguillarum*	*V. salmonicida*	*V. vulnificus*	*E. tarda*	*Y. ruckeri*	*L. garviae*
A. media	Gibson *et al.* (1998)	Gibson *et al.* (1998)	Gibson *et al.* (1998)	–	Gibson *et al.* (1998)	Lategan *et al.* (2006)	Gibson *et al.* (1998)	Lategan *et al.* (2006)
Pseudo-alteromonas sp.	–	–	Longeon *et al.* (2004)	–	–	–	–	–
Pseudomonas sp.	Das *et al.* (2006)	Smith and Davey (1993)	Holmstrøm and Gram (2003)	–	–	–	–	–
V. alginolyticus	–	Austin *et al.* (1995)	Austin *et al.* (1995)	–	–	–	–	–
Roseobacter spp.	–	–	Hjelm *et al.* (2004)	–	–	–	–	–
Bacillus sp.	–	–	–	–	Sugita *et al.* (1998)	Chang and Liu (2002)	–	–
Carnobacterium sp.	Robertson *et al.* (2000)	Ringø and Holzapfel (2000)	Ringø and Holzapfel (2000)	Ringø and Holzapfel (2000)	–	–	Kim and Austin (2006)	–
Enterococcus faecium	–	–	–	–	–	Chang and Liu (2002)	–	–
Lactobacillus rhamnosus	–	Nikoskelainen *et al.* (2001a)	–	–	–	–	–	–
Lactococcus lactis	Balcázar *et al.* (2006c)	Balcázar *et al.* (2007a)	Balcázar *et al.* (2007a)	–	–	–	Balcázar *et al.* (2007a)	–
Weissella hellenica	Cai *et al.* (1998)	–	Cai *et al.* (1998)	–	–	Cai *et al.* (1998)	–	–

A.: *Aeromonas*; *E.*: *Edwardsiella; L.*: *Lactococcus*; *V.*: *Vibrio; Y.*: *Yersinia*

Vibrionaceae (Vine *et al.*, 2004; Chabrillón *et al.*, 2005a,b, 2006a,b), but also *Aeromonas hydrophila* (Vine *et al.*, 2004), *Aeromonas salmonicida*, *Yersinia ruckeri*, *Carnobacterium piscicola*, and *Lactococcus garvieae* (Balcázar *et al.*, 2007a).

Skjermo *et al.* (1997) developed a biofilter through which non-opportunistic microbiota were selected in the inlet water for rearing turbot larvae. Larval growth was thus increased, probably due to the limitation of opportunistic bacteria. The biofilms colonising the larval-rearing facilities seem favourable to antagonistic bacteria (e.g., *Roseobacter* spp. and *Vibrionaceae*; Hjelm *et al.*, 2004). The bacterial management of the inert surfaces may, therefore, be particularly important for larval rearing. In addition to bacterial antagonism, such treatments could act directly on the larvae. For instance, the pre-incubation of the eggs and hatching larvae of halibut with beneficial bacteria stimulated the proliferation of mucous cells and the non-specific defences of the early larvae (Ottesen and Olafsen, 2000). The potential of introducing beneficial bacteria into the rearing water is not limited to the larval stages. A prior bath treatment with some strains of *Pseudomonas* sp. limited the mortality of Atlantic salmon challenged with *Aeromonas salmonicida* (Smith and Davey, 1993), and in one of two experiments, the mortality of rainbow trout challenged with *Vibrio anguillarum* (Spanggaard *et al.*, 2001).

8.3.3 Dietary probiotics

Many probiotics have been introduced experimentally into fish feeds, with various effects including growth improvement or digestion efficiency, whilst the introduction of probiotics via live food organisms has been extensively tested for fish larval rearing (see reviews, Section 8.6). Among the various effects, those important for the reduction of antibiotic use are not only the ones based on the direct inhibition of pathogens, but also those that improve fish health, even though the mode of action is not clearly understood. For instance, the antibiotic florfenicol reduced the incidence of the vertebral column compression syndrome observed in rainbow trout, and this effect was reproduced by feeding the trout with a dietary probiotic, *Pediococcus acidilactici* (Aubin *et al.*, 2005a). Some challenges demonstrated the efficiency of probiotics to increase the resistance of fish to pathogenic bacteria, and the stimulation of the immune system has mainly been studied with lactic acid bacteria (Table 8.5; Gatesoupe, 2008).

One major interest of probiotics may lie in the combination of direct antagonistic effects with the reinforcement of the host's defences, leaving no chance for the pathogen to develop resistance. However, some yeast strains are among the best candidates for the reduction of antibiotic use, though they are not generally antagonistic to pathogenic bacteria (Gatesoupe, 2007). It is also important to consider whether the probiotics should be administered live, since the practical application of live probiotics in fish farming may be limited by safety constraints. In particular, the antibiotic resistance possibly acquired by some candidate probiotics may be of concern. In most cases, viability did not

Table 8.5 Some effects of dietary probiotics beneficial for fish health, a non-exhaustive list

Probiotic	Fish	Disease resistance	Immuno-stimulation	References
A. hydrophila	Rainbow trout	*A. salmonicida*	–	Irianto and Austin (2002a)
A. sobria	Rainbow trout	*S. iniae* *L. garviae*	Phagocytosis	Brunt and Austin (2005)
Bacillus subtilis	Gilthead seabream	–	Phagocytosis	Salinas *et al.* (2005)
Bacillus subtilis	*Labeo rohita*	*A. hydrophila*	Antibodies	Kumar *et al.* (2006)
Bacillus subtilis	Rainbow trout	–	Complement	Panigrahi *et al.* (2007)
Carnobacterium divergens	Rainbow trout	*A. salmonicida* *Y. ruckeri*	Respiratory burst, lysozyme	Kim and Austin (2006)
Carnobacterium maltaromaticum	Rainbow trout	*A. salmonicida* *Y. ruckeri*	Phagocytosis	Kim and Austin (2006)
Enterococcus faecium	European eel	*E. tarda*	–	Chang and Liu (2002)
Enterococcus faecium	Rainbow trout	–	Respiratory burst, complement, cytokine gene expression	Panigrahi *et al.* (2007)
Lactobacillus delbrueckii	Gilthead seabream	–	Phagocytosis	Salinas *et al.* (2005)
Lactobacillus rhamnosus	Nile tilapia	*E. tarda*	Complement	Pirarat *et al.* (2006)
Lactobacillus rhamnosus	Rainbow trout	*A. salmonicida*	Respiratory burst, complement, antibodies	Nikoskelainen *et al.* (2001b, 2003)
Lactobacillus rhamnosus	Rainbow trout	–	Phagocytosis, respiratory burst, complement, lysozyme	Panigrahi *et al.* (2004)
Lactococcus lactis	Rainbow trout	–	Phagocytosis	Balcázar *et al.* (2006b)
Lactococcus lactis	Brown trout	–	Complement, lysozyme	Balcázar *et al.* (2007b)

A.: *Aeromonas*; *E.*: *Edwardsiella*; *L.*: *Lactococcus*; *S.*: *Streptococcus; Y.*: *Yersinia*

Table 8.6 Some effects of the viability of probiotics on fish health, a non-exhaustive list

Probiotic	Fish	Disease resistance	Cellular immunity	Humoral immunity	References
A. hydrophila	Rainbow trout	*A. salmonicida* (V+, I+)	Phagocytosis (V+, I+)	Lysozyme (V-, I+)	Irianto and Austin (2002a, 2003)
Vibrio fluvialis	Rainbow trout	*A. salmonicida* (V±, I+)	Phagocytosis (V+, I+)	–	Irianto and Austin (2002a, 2003)
Vibrionaceae 51M6	Gilthead seabream	–	Phagocytosis, cytotoxicity (I+)	–	Díaz-Rosales *et al.* (2006)
Vibrionaceae Pdp11	Gilthead seabream	–	Phagocytosis (I+)	–	Díaz-Rosales *et al.* (2006)
Gram + coccus A1-6	Rainbow trout	*A. salmonicida* (V+, I+)	Phagocytosis (V+, I+)	Lysozyme (V+, I+)	Irianto and Austin (2002a, 2003)
Carnobacterium sp.	Rainbow trout	*A. salmonicida* (V+, I±)	Phagocytosis (V+, I+)	Lysozyme (V+, I+)	Irianto and Austin (2002a, 2003)
Lactobacillus rhamnosus	Rainbow trout	–	Phagocytosis (V+, I-)	–	Panigrahi *et al.* (2005)
L. lactis	European turbot	–	Nitric oxide production (V+, I+)	–	Villamil *et al.* (2002)
Leuconostoc mesenteroides	European turbot	–	Nitric oxide production (V+, I-)	–	Villamil *et al.* (2002)
[§]Alchem Poseidon®	Nile tilapia	*E. tarda* (V+, I-)	–	Lysozyme, bactericidal activity (V+, I+)	Taoka *et al.* (2006b)

Form of the probiotic: V, viable, or I, inactivated; +: stimulation; -: no effect, ±: variable effect; *A.*: *Aeromonas*; *E.*: *Edwardsiella*; *L.*: *Lactococcus*;
[§] Commercial preparation of *Bacillus subtilis*, *Lactobacillus acidophilus*, *Clostridium butyricum*, and *Saccharomyces cerevisiae*.

seem essential for the probiotics to stimulate the immune system of fish (Table 8.6), and formalin-killed cells of *Aeromonas hydrophila* even stimulated lysozyme activity in rainbow trout (Irianto and Austin, 2003), unlike the active form of the probiotic (Irianto and Austin, 2002a). However, Panigrahi *et al.* (2005) observed that heat-killed *Lactobacillus rhamnosus* lost the ability to stimulate phagocytic activity in rainbow trout. The heat-killing of *Leuconostoc mesenteroides* also inhibited the stimulation of nitric oxide production in turbot (Villamil *et al.*, 2002). In its viable commercial form, the preparation of four microbial strains used by Taoka *et al.* (2006b) increased the disease resistance of Nile tilapia to *Edwardsiella tarda*, although not after inactivation of the probiotics with formaldehyde.

In addition to the reduction of antibiotic use, probiotics seem able directly to improve eating quality. For instance, intramuscular lipid content and flesh pigmentation were increased in rainbow trout fed a diet supplemented with live *Saccharomyces cerevisiae* var. *boulardii* (Aubin *et al.*, 2005b).

8.3.4 Dietary prebiotics

A prebiotic is a 'nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson and Roberfroid, 1995). Application to fish intestinal microbiota is still very limited, though potentially beneficial microbes such as *Carnobacterium* sp. and *Bacillus* sp. can use inulin and Fructo-Oligo-Saccharide (FOS) respectively (Ringø and Holzapfel, 2000; Mahious *et al.*, 2006a). Gastrointestinal microbiota of fish have been documented for use in the fermentation of carbohydrates, resulting in the production of Short-Chain Fatty Acids (SCFA) (Clements, 1997). Intestinal microbiota from Siberian sturgeon produced SCFA from inulin and FOS in vitro (Mahious *et al.*, 2006b). Dietary lactosucrose increased the thickness of the intestinal wall in red seabream, probably due to the fermentation of the prebiotic (Kihara *et al.*, 1995). Intestinal microbes from common carp intensively fermented soybean oligosaccharides and raffinose (Kihara and Sakata, 2002). However, in Nile tilapia, α-starch seemed to be the preferential substrate for fermentation (Kihara and Sakata, 1997), whereas in rainbow trout, branched SCFA seemed to be produced by fermentation of nitrogenous compounds (Kihara and Sakata, 2001). A high dose of incorporation of inulin (15% of the dry diet) was clearly detrimental to gut integrity in Arctic charr reared in freshwater (Olsen *et al.*, 2001). The adherent intestinal microbiota, particularly gram-negative bacteria, were less numerous in fish fed this diet containing inulin than in fish fed a diet with 15% dextrin, and the dominant species of *Carnobacterium* was also different in both diets (Ringø *et al.*, 2006). In Atlantic salmon reared in seawater, the supply of inulin at 7.5% of the dry diet did not damage the intestine, but stimulated intestinal growth (Refstie *et al.*, 2006). Since dietary oxytetracycline did not affect this stimulation, it seemed to be due to the physical load of indigestible fibre in the intestinal tract, while prebiotic

effects were unlikely. The incorporation of 2% FOS in the diet for weaning turbot larvae improved growth, in comparison with a diet containing 2% cellulose, inulin or lactosucrose (Mahious *et al.*, 2006a). Among the vegetable sources of fructans that could be used as prebiotics, garlic could be of particular interest since it combines antibacterial and immunostimulant properties (Section 8.4; Sahu *et al.*, 2007), with the selective stimulation of certain strains of lactic acid bacteria in fermented fish fillet (Paludan-Müller *et al.*, 2002). Though prebiotics are selective by definition, their specificity may be unpredictable, especially in an aquatic environment rich in opportunists like *Vibrio* spp. and *Bacillus* spp. The pathogens are highly specialised and thus unlikely to benefit from the prebiotic, but some risk may arise from its continuous dietary supply, which could result in the emergence of pathogenic strains able to use the substrate. A good precaution would be to combine the treatment with a probiotic that can degrade the prebiotic.

8.4 The therapeutic approach: antimicrobial compounds

Whatever progress is expected in prophylaxis, fish will never be completely protected from bacterial infections. The drastic regulation of antibiotic use has made the search for alternative therapeutants urgent. Probiotics have sometimes been suggested for this purpose, but only bacteriophages seem able to propagate sufficiently rapidly, even benefiting from the proliferation of the pathogen. Most hopes, however, rest on alternative chemotherapies. These treatments can also be used in prophylaxis, but their routine preventive use seems inadvisable, due to the risk of selecting resistant pathogens.

8.4.1 Plant-based medicines and herbal derivatives

Herbs have been widely used in veterinary and human medicine, mainly in Asia. Their application to fish culture is growing, and significant limitation of antibiotics may be expected from this sustainable practice. The efficiency of many plant extracts against fish pathogens has been documented (Table 8.7; Direkbusarakom, 2004). Among many target pathogens, *Aeromonas hydrophila* is the most studied, probably due to the occurrence of strains pathogenic to humans (Stecchini *et al.*, 1993; Delamare *et al.*, 2007). Several active compounds have been characterised, such as allicin from garlic (Feldberg *et al.*, 1988), which inhibited a strain of *Mycobacterium marinum*, pathogenic to European sea bass (Colorni *et al.*, 1998). Other antibacterial substances identified in plants include many phenolic compounds (Beuchat and Golden, 1989). Thymol, carvacrol and cinnamaldehyde were found to be particularly efficient in controlling microbiota in carp fillets (Mahmoud *et al.*, 2004). The genus *Vibrio* is sensitive to plant polyphenols, in spite of variable susceptibility among strains (Taguri *et al.*, 2004). Vanillin and *o*-coumaric acid inhibited the growth of fish pathogens, while stimulating feeding activity in goldfish (Nakajima and Tsujiwaki, 2000). However, the purified extracts are not

Table 8.7 Some examples of references on plant extracts active against fish pathogens, a non-exhaustive list

Taxonomical position	Plant species	Sensitive bacteria	References
Campanulids; Apiaceae	*Cnidium officinale*	*V. anguillarum*	Ji *et al.* (2007)
Campanulids; Asteraceae	*Artemisia capillaris*	*V. anguillarum*	Ji *et al.* (2007)
Lamiids; Lamiaceae; Nepetoideae	*Ocimum sanctum*	*V. harveyi*	Sivaram *et al.* (2004)
Lamiids; Lamiaceae; Nepetoideae	*Rosmarinus officinalis*	*S. iniae*	Abutbul *et al.* (2004)
Lamiids; Lamiaceae; Scutellarioideae	*Scutellaria baicalensis*	*A. hydrophila*	Zhang and Yang (2005)
Lamiids; Solanaceae	*Whitania somnifera*	*V. harveyi*	Sivaram *et al.* (2004)
Caryophyllales; Chenopodiaceae	*Hammada scoparia*	*A. hydrophila, Ph. damselae, V. alginolyticus*	Abutbul *et al.* (2005)
Caryophyllales; Polygonaceae	*Polygonum sachalinense*	*Ph. damselae*	Kawai *et al.* (2004)
Caryophyllales; Caryophyllaceae	*Stellaria aquatica*	*A. salmonicida, E. ictaluri*	Tong *et al.* (1990)
Rosids; Cucurbitaceae	*Mormordica charantia*	*A. hydrophilia, V. harveyi*	Direkbusarakom *et al.* (1998)
Rosids; Rosaceae	*Crataegus* sp.	*V. anguillarum*	Ji *et al.* (2007)
Rosids; Resedaceae	*Ochrademus baccatus*	*S. iniae*	Abutbul *et al.* (2005)
Rosids; Sapindales; Anacardiceae	*Mangifera indica*	*A. hydrophila, E. tarda, Ps. fluorescens*	Mishra *et al.* (2002)
Rosids; Sapindales; Meliceae	*Azadirachta indica*	*A. hydrophila, Ps. fluorescens*	Das *et al.* (1999)
Rosids; Myrtaceae	*Psidium guajava*	*A. hydrophila, V. harveyi*	Direkbusarakom *et al.* (1998)

A.: *Aeromonas*; *E.*: *Edwardsiella*; *Ph.*: *Photobacterium*; *Ps.*: *Pseudomonas*; *S.*: *Streptococcus; V.*: *Vibrio*

necessarily the most convenient form to administer, since other compounds from plants can stimulate the immune system of fish, and thus decrease the risk of selecting resistant strains of pathogens.

Medicinal herbs have been tested as immunostimulants in fish, sometimes without considering the possible direct inhibition of pathogens. Such instances are listed by Ji *et al.* (2007), who simply recalled the fact that three of the four extracts investigated in their experiment inhibited *Vibrio anguillarum* (Ji *et al.*, unpublished data). These authors noted that the mixture of the four herbs was more efficient than the single preparations. Some examples of the effects on fish of plant extracts shown to inhibit fish pathogens are referenced in Table 8.8. Several of these treatments improved growth and feed conversion in fish, but they should be used carefully due to the risk of toxicity of some extracts, such as *Azadirachta indica* for Indian carps (Das *et al.*, 2002). There are also some complex commercial preparations containing plant products, in which it is difficult to evaluate the exact role of each component. Some of them could be of interest as an alternative to antibiotics, e.g. Biogen® – a mix of garlic, germanium-rich ginseng, organic selenium, *Bacillus subtilis* and hydrolytic enzymes – which reinforced the immune status and improved growth and nutrient utilisation in Nile tilapia (El-Gohary *et al.*, 2005; El-Haroun *et al.*, 2006).

8.4.2 Antibacterial compounds from algae

In addition to bactericidal activity, some plants are sources of glycoconjugates and saccharides that could be used to inhibit the adhesion of pathogenic bacteria to the tissues of the host (Ofek *et al.*, 2003). This new approach should be worth testing to control fish diseases.

Microalgae and seaweeds are good sources of antibacterial extracts (Table 8.9). The active compounds seemed diverse, e.g. fatty acids (Naviner *et al.*, 1999), lectins (Liao *et al.*, 2003), or volatile halogenated compounds (Bansemir *et al.*, 2006). Oligosaccharides prepared by hydrolysis of alginate are antibacterial, especially the hepta- and hexa-saccharides (Kitamikado *et al.*, 1993). Alginate can also be used as a prebiotic (Warrand, 2006), and it would be worth investigating whether some strains from fish intestinal microbiota could degrade alginate and produce the antibacterial oligosaccharides *in situ*. Austin *et al.* (1992) characterised the antibacterial substance from *Tetraselmis suecica* as a polysaccharide. The authors challenged Atlantic salmon with several pathogens and demonstrated the efficiency of the microalgal extract on fish survival. Interestingly, the treatment worked even when the extract was administered only after experimental infection, thus confirming its therapeutic value. Some antibacterial extracts from algae may be toxic to invertebrate larvae (Hellio *et al.*, 2001), but to my knowledge, the toxicity of these extracts has not yet been tested on fish.

8.4.3 Antibacterial compounds from fungi

The fungal kingdom is a source of classic antibiotics such as beta-lactams (penicillins and cephalosporins). New fungal metabolites with antibacterial

Table 8.8 Some effects of plant-based medicines beneficial for fish health, a non-exhaustive list

Medicinal plant	Fish	Rearing performances	Disease resistance	Immuno-stimulation	References
Cnidium officinale	Red sea bream	Growth; feed conversion	*V. anguillarum*	Lysozyme	Ji *et al.* (2007)
Artemisia capillaris	Red sea bream	Growth; feed conversion	*V. anguillarum*	–	Ji *et al.* (2007)
Ocimum sanctum	Greasy grouper	Growth; feed conversion	*V. harveyi*	Phagocytosis; bactericidal activity	Sivaram *et al.* (2004)
Rosmarinus officinalis	Tilapia	–	*S. iniae*	–	Abutbul *et al.* (2004)
Scutellaria baicalensis	Common carp	Growth; feed conversion	–	–	Liu *et al.* (2004)
Whitania somnifera	Greasy grouper	Growth; feed conversion	*V. harveyi*	Phagocytosis; bactericidal activity	Sivaram *et al.* (2004)
Crataegus sp.	Red sea bream	Growth	*V. anguillarum*	–	Ji *et al.* (2007)
Azadirachta indica	Common carp	–	*A. hydrophila*	Wound healing	Harikrishnan *et al.* (2003)

A.: *Aeromonas*; *S.*: *Streptococcus*; *V.*: *Vibrio*

Table 8.9 Some examples of references on algal extracts active against fish pathogens, a non-exhaustive list

Taxonomical position	Species	Sensitive bacteria	References
Bacillariophyta; Skeletonemataceae	*Skeletonema costatum*	*V. anguillarum*	Naviner *et al.* (1999)
Chlorophyta; Prasinophyceae; Chlorodendraceae	*Tetraselmis suecica*	*A. hydrophila, A. salmonicida, V. anguillarum, V. salmonicida, Y. ruckeri* (type I)	Austin *et al.* (1992)
Chlorophyta; Ulvophyceae; Ulvaceae	*Ulva conglobata*	*V. vulnificus*	Liao *et al.* (2003)
Phaeophyta; Sargassaceae	*Sargassum duplicatum*	*V. vulnificus*	Liao *et al.* (2003)
Rhodophyta; Bonnemaisoniales; Bonnemaisoniaceae	*Asparagopsis armata*	*A. hydrophila, A. salmonicida, P. anguilliseptica, V. anguillarum, Y. ruckeri*	Bansemir *et al.* (2006)
Rhodophyta; Ceramiales; Ceramiaceae	*Ceramium rubrum*	*P. anguilliseptica, V. anguillarum*	Bansemir *et al.* (2006)
Rhodophyta; Ceramiales; Delesseriaceae	*Drachiella minuta*	*P. anguilliseptica*	Bansemir *et al.* (2006)
Rhodophyta; Ceramiales; Delesseriaceae	*Hypoglossum hypoglossoides*	*V. anguillarum*	Bansemir *et al.* (2006)
Rhodophyta; Ceramiales; Rhodomelaceae	*Halopytis incurvus*	*A. hydrophila, A. salmonicida, P. anguilliseptica, V. anguillarum, Y. ruckeri*	Bansemir *et al.* (2006)
Rhodophyta; Ceramiales; Rhodomelaceae	*Laurencia chondriodes*	*P. anguilliseptica, V. anguillarum*	Bansemir *et al.* (2006)
Rhodophyta; Gigartinales; Solieriaceae	*Eucheuma serra*	*V. vulnificus*	Liao *et al.* (2003)
Rhodophyta; Gracilariales; Gracilariaceae	*Gracilaria cornea*	*P. anguilliseptica, V. anguillarum*	Bansemir *et al.* (2006)
Rhodophyta; Gracilariales; Gracilariaceae	*Gracilaria lemaneiformis*	*V. vulnificus*	Liao *et al.* (2003)
Rhodophyta; Nemaliales; Galaxauraceae	*Galaxaura marginata*	*V. vulnificus*	Liao *et al.* (2003)

A.: *Aeromonas*; *P.*: *Pseudomonas; V.*: *Vibrio* ; *Y.*: *Yersinia*

activity are frequently discovered, some of which could be useful in treating fish diseases. Marine fungi seem particularly promising in this regard. For instance, Christophersen *et al.* (1999) characterised several isolates active against *Vibrio parahaemolyticus* (*Aspergillus parasiticus*, *Penicillium phoeniceum*, and *Penicillium steckii*). More recently, Kwong *et al.* (2006) isolated from *Ampelomyces* sp. a novel antifouling and antibacterial compound, 3-chloro-2,5-dihydroxybenzyl alcohol, which is active against several pathogenic strains, including *Pseudoalteromonas piscicida* and *Vibrio harveyi*. The advantages and inconveniences of such new compounds remain to be evaluated, but they may be similar to those of classic antibiotics.

8.4.4 Antibacterial compounds from animals

Like plants and fungi, animals produce antimicrobial compounds that could be extracted and used as alternative drugs. Substances of this kind have been described in many animals (Table 8.10). Extracts from invertebrates such as sponges, corals and gorgonians have been tested against fish pathogens (*Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Edwardsiella tarda*, *Vibrio alginolyticus*; Choudhury *et al.*, 2002). Various chemical structures have been described for the active compounds, e.g. bromotyrosine derivatives (Matsunaga *et al.*, 2005), or pyridine alkaloids (Pelttari *et al.*, 2002).

Chitosan is used for the preservation of fish fillets (Tsai *et al.*, 2002). Its inhibitory activity is stronger against gram positive bacteria (No *et al.*, 2002), although chitosan can also interfere with the barrier function of the outer membrane in gram negative bacteria (Helander *et al.*, 2001). Chito-oligosaccharides are generally less efficient (No *et al.*, 2002), but a low-molecular-weight chitosan obtained by hydrolysis was found to be strongly active (Tsai *et al.*, 2004). Anas *et al.* (2005) proposed using chitosan to control pathogenic *Vibrio* in the larval rearing of the freshwater prawn, *Macrobrachium rosenbergii*. This could also be used in fish culture, possibly in combination with its immunomodulatory properties (Gopalakannan and Arul, 2006). Chitosan should be used carefully, however, since toxicity appeared when it was dissolved in the rearing water for common carp and rainbow trout (Bullock *et al.*, 2000; Dautremepuits *et al.*, 2004).

Antimicrobial peptides from marine animals seem particularly promising, such as the *Limulus* anti-LPS factor, which has been proposed as a potential therapeutic agent (Weiss *et al.*, 2000). This polypeptide of 102 amino acids was found to be bactericidal, but the active domain seemed to be limited to 27 amino acids. Short peptides from other animals are also active (e.g., Tincu *et al.*, 2003). These peptides seem to interact with lipids in bacterial membranes, which are thus permeabilised or disrupted (Matsuzaki, 1999), but the actual mode of action is flexible, depending on the environment (Patrzykat and Douglas, 2005). Many types of antimicrobial peptides have been described in fish (Noga and Silphaduang, 2003; Chang *et al.*, 2005; Zou *et al.*, 2007). Molecules of this

Table 8.10 Some examples of references on aquatic animal extracts with antibacterial activity, a non-exhaustive list

Taxonomical position	Species	Active compound	Senstive bacteria	References
Porifera; Ianthellidae	*Hexadella* sp.	Bromotyrosine derivatives	*A. hydrophila*	Matsunaga *et al.* (2005)
Bryozoa; Bugulidae	*Bugula pacifica*	n.k.	*E. coli, B. subtilis, S. aureus*	Shellenberger and Ross (1998)
Chelicerata; Limulidae	*Limulus polyphemus*	Polypeptide (102 AAs)	*P. aeruginosa*	Weiss *et al.* (2000)
Crustacea; Penaeidae	*Penaeus monodon*	Polypeptide (98 AAs)	*V.* spp., *E. coli* *B. megaterium, M. luteus*	Somboonwiwat *et al.* (2005)
Crustacea; Majidae	*Hyas araneus*	Polypeptide	*V. anguillarum, E. coli,* *C. glutamicum, S. aureus*	Haug *et al.* (2002)
Crustacea	Various	Chitosan	*A. hydrophila, V.* spp.	Taha and Swailam (2002), Anas *et al.* (2005)
Gastropoda; Haminoeidae	*Haminoea navicula*	Pyridine derivatives	*B. cereus, S. epidermidis*	Pelttari *et al.* (2002)
Bivalvia; Ostreidae	*Crassostrea gigas*	Polypeptide (65 AAs)	*M. lysodeikticus*	Gueguen *et al.* (2006)
Ascidiacea; Styelidae	*Styela plicata*	Peptide (8 AAs)	*P. aeruginosa, E. coli,* *S. aureus*	Tincu *et al.* (2003)
Teleostei; Moronidae	Hybrid striped bass	Peptide (22 AAs)	*A. hydrophila, E. coli,* *S. aureus*	Silphaduang and Noga (2001)

AAs: amino acids; n.k.: not known; *A.*: *Aeromonas*; *B.*: *Bacillus*; *C.*: *Corynebacterium*; *E.*: *Escherichia*; *M.*: *Micococcus*; *P.*: *Pseudomonas*; *S.*: *Staphylococcus*; *V.*: *Vibrio*

kind were tested in coho salmon experimentally infected with *Vibrio anguillarum*, and the peptides were only efficient in limiting mortality when they were delivered continuously (Jia *et al.*, 2000). Consequently, therapeutic treatments with antimicrobial peptides may be difficult to apply to fish, and Jia *et al.* (2000) considered that the best approach would be to select transgenic fish able to produce enough antimicrobial peptide. Risk assessment of using such transgenic fish remains an open question.

Terrestrial animals also have antimicrobial peptides, such as cecropins, of which synthetic derivatives have been produced. These compounds seemed efficient against fish pathogens (Kelly *et al.*, 1990; 1993; Kjuul *et al.*, 1999), while experimental production of transgenic fish harbouring cecropin genes was undertaken (Jia *et al.*, 2000; Dunham *et al.*, 2002; Sarmasik *et al.*, 2002; Sarmasik and Chen, 2003). There are also iron sequestrants of animal origin that could be used to limit the incidence of bacterial pathogens. For instance, the addition of bovine lactoferrin to the diet of Nile tilapia limited mortality due to an experimental infection with *Streptococcus iniae* (Welker *et al.*, 2007).

8.5 Future trends

Several other kinds of alternatives to antibiotics have been suggested, but their application to fish farming remains to be demonstrated.

8.5.1 Bacterial quorum sensing inhibitors

Quorum sensing has been defined as 'the regulation of gene expression in response to fluctuations in cell-population density' (Miller and Bassler, 2001). In gram negative bacteria, this regulation is mainly mediated by N-Acyl-Homoserine Lactones (AHL), while active peptides have been described in gram positive bacteria. The study of quorum sensing is in its infancy and the mechanisms of cell-to-cell communication need to be further explored. However, the disruption of quorum sensing has already been suggested as a promising alternative to antibiotic treatment in aquaculture (Defoirdt *et al.*, 2004). The first instance of an application of this kind seemed to be achieved by Manefield *et al.* (2000), who reduced the toxicity of culture exsudate from *Vibrio harveyi* for *Penaeus monodon*, due to the introduction of an AHL antagonist produced by the marine alga *Delisea pulchra* in the bacterial culture medium. Quorum sensing inhibition may thus be involved in the antagonistic effects of some probiotics and natural extracts, where it should be considered as a possible mode of action. Defoirdt *et al.* (2004), for instance, suggested that the strong inhibition of AHL activity by *Bacillus* spp. may partly explain the probiotic effects observed in aquaculture. Choo *et al.* (2006) showed that an extract from vanilla beans inhibited violacein production in *Chromobacterium violaceum*, which was induced by N-hexanoyl-homoserine lactone. The first attempted application to fish was made by Rasch *et al.* (2004), who used a

synthetic AHL inhibitor to treat rainbow trout challenged with *Vibrio anguillarum*. The treatment worked in fish exposed to the pathogen by cohabitation, but not by immersion. Moreover, the inhibitor was toxic to fish at high concentration. The search for less toxic compounds is crucial, as much higher doses seemed necessary to protect *Artemia* from pathogenic vibrios (Defoirdt *et al.*, 2006). In addition to toxicity, Defoirdt *et al.* (2004) considered that this approach has two main limitations: (1) there is a risk of favouring the emergence of resistant strains, even if the risk is assumed to be less likely than with antibiotics; (2) the lack of specificity could affect some beneficial quorum sensing mediated processes in microbiota.

8.5.2 Other biocidal products

Among chemical disinfectants and preservatives, some compounds could be used to treat live fish, on condition that toxic residues cannot contaminate the flesh. Following toxicity studies (e.g., Culp *et al.*, 1999), malachite green was thus banned from fish farming after decades of intensive use. New publications appear regularly showing the efficiency of biocides in aquaculture, but their application may pose safety problems, as in the case of formaldehyde (Sahul Hameed and Balasubramanian, 2000; Gatesoupe, 2002). However, further study of new ideas – such as the *in-situ* depolymerisation of poly-β-hydroxybutyrate, which inhibited *Vibrio campbellii* in *Artemia* (Defoirdt *et al.*, 2007) – would be worthwhile.

8.5.3 Bacteriophage therapy

Phages are probably the most abundant biological entities in the aquatic environment (Paul *et al.*, 2002). They are generally species-specific and play a major role in the maintenance of bacterial diversity by lysing the host cells. The lytic activity of phages specific to pathogens has been proposed for therapeutic applications, with a recent renewal of interest (e.g., Skurnik and Strauch, 2006). Summers (2001) considered phage therapy as particularly promising for fish and other animals who 'live in aqueous media and hence the therapeutic phage can have continuous and intimate physiological contact with the pathogens in a natural arrangement'. Nakai and Park (2002) observed high numbers of phages in fish infected with a pathogenic bacterium and they supposed that naturally occurring phages might contribute to fish survival in case of bacterial infection. These authors stressed the poor antibody response of fish against phages, whereas the strong immunogenicity of most phages has been put forward as a major concern for the efficacy of phage therapy in humans (Clark and March, 2006). Several phages specific to fish pathogens have already been described (Table 8.11; Ackermann and Krisch, 1997). It must be borne in mind that some phages may carry a virulence factor or toxin genes (Skurnik and Strauch, 2006) and they are thus unsuitable for therapeutic uses. For instance, Austin *et al.* (2003) demonstrated the implication of *Myovirus* VHML in the virulence of *Vibrio harveyi* to Atlantic salmon and *Artemia*. However, several promising

Table 8.11 Some examples of references on phage of fish pathogens, a non-exhaustive list

Host bacteria	Phage	Virus family	Biocontrol assay	Occurrence in diseased fish	References
L. garviae	PLgY	*Siphoviridae*	–	Yellowtail	Park *et al.* (1998)
L. garviae	PLgY-16	*Siphoviridae*	Yellowtail	–	Nakai *et al.* (1999)
A. hydrophila	AH1	n.k.	*Misgurnus anguillicaudatus*	–	Wu *et al.* (1981)
A. salmonicida	HER 110	n.k.	Brook trout	–	Imbeault *et al.* (2006)
E. tarda	Phi ET-1	n.k.	*Misgurnus anguillicaudatus*	–	Wu and Chao (1982)
Pi. Salmonis	–	n.k.	–	Coho salmon, rainbow trout	Yuksel *et al.* (2001)
Ps. plecoglossicida	PPpW-3	*Myoviridae*	Ayu	–	Park *et al.* (2000)
Ps. plecoglossicida	PPpW-4	*Podoviridae*	Ayu	–	Park *et al.* (2000)
Ps. plecoglossicida	PPpA	*Podoviridae*	–	Ayu	Park *et al.* (2000)
V. anguillarum	AS10	n.k.	Milkfish	–	Wu *et al.* (1986)
V. harveyi	VHML	*Myoviridae*	–	Atlantic salmon[§]	Austin *et al.* (2003)
V. harveyi	–	*Siphoviridae*	*Penaeus monodon*	–	Vinod *et al.* (2006)

n.k.: not known; [§]: experimental infection; *A.*: *Aeromonas*; *E.*: *Edwardsiella*; *L.*: *Lactococcus*; *Pi.*: *Piscirickettsia*; *Ps.*: *Pseudomonas*; *V.*: *Vibrio*

trials were carried out with phages that protected milkfish from vibriosis (Wu *et al.*, 1986), yellowtail from *Lactococcus garviae* (Nakai *et al.*, 1999), ayu from *Pseudomonas plecoglossicida* (Park and Nakai, 2003), and brook trout from furunculosis (Imbeault *et al.*, 2006). The risk of the emergence of phage-resistant bacterial strains remains to be evaluated.

8.6 Sources of further information and advice

8.6.1 Health management

International organisations have established rulings, such as the Office International des Epizooties (OIE, Aquatic Animal Health Code http://www.oie.int/eng/normes/fcode/en_sommaire.htm; Manual of Diagnostic Tests for Aquatic Animals 2006 http://www.oie.int/eng/normes/fmanual/A_summry.htm), or the European Union (http://europa.eu/scadplus/leg/en/lvb/f84008.htm; http://europa.eu/scadplus/leg/en/lvb/l12022.htm). There are many other sources of information and advice such as the University of Florida's Institute of Food and Agricultural Sciences (Extension Data Information Source, http://edis.ifas.ufl.edu/FA099). The Great Lakes Fishery Commission has published 'A guide to integrated fish health management', still fully available online (Meyer *et al.*, 1983, http://www.glfc.org/pubs/SpecialPubs/sp83_2/home.html).

With regard to the nutritional approach to health management, one issue of the magazine *Aqua Feeds: Formulation & Beyond* was entirely devoted to 'health additives' (Volume 2, Issue 3, 2005).

8.6.2 Microbial management

There have been many reviews dealing with live microbial additives for fish farming, notably: Abidi (2003); Balcázar *et al.* (2006a); Burr *et al.* (2005); Gatesoupe (1999; 2005; 2007; 2008); Gomez-Gil *et al.* (2000); Gram and Ringø (2005); Hansen (2000); Irianto and Austin (2002b); Maeda (2004); Ringø and Gatesoupe (1998); Ringø *et al.* (2005); Venkateswara Rao (2006, http://www.aquafeed.com/article.php?id=1618&sectionid=); Verschuere *et al.* (2000); Vine *et al.* (2006); Wang *et al.* (1998, http://alken-murray.hypermart.net/China98.htm).

8.6.3 Herbal medicines

General information about medicinal herbs can be found at HerbNet (http://www.herbnet.com/Herb%20Uses_AB.htm).

8.7 References

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9

Understanding factors affecting flesh quality in farmed fish

M. Espe, National Institute of Nutrition and Seafood Research (NIFES), Norway

9.1 Introduction

Generally fish muscle contains approximately 18% protein, about 80% water in lean species and from 74–80% in lipid rich species within lipids varying from a few percent (Atlantic cod) to more than 20% (Atlantic salmon). The remaining 2% constitute other nutrients (glycogen, minerals and vitamins). Some fish species show seasonal changes in muscle lipid content, and then the lipid content is exchanged with water, keeping the other nutrients more or less constant. Farmed fish are fed numerous variations in diet energy density, are subjected to changes in feeding rate, manipulations of the dietary compositions, and light regimes resulting in changed breeding season, all factors that may influence the nutritional and sensory quality of the fish flesh at harvest. Product tailoring is focussed on production of fish meat that is to contain a pre-defined nutritional composition when harvested. Lipid rich farmed fish like the salmonids are relatively easy to tailor when it comes to lipid soluble nutrients, but much harder when considering the water soluble nutrients. The fact that protein composition in fish is determined by the genetic code and the fact that free amino acids cannot be stored as such, means that the fillet protein cannot be tailored. A high flesh quality might be defined as a fish muscle containing nutrients that are health promoting for the consumer, and have the texture and taste required by consumers but concomitantly containing as little as possible of any harmful contaminants that may jeopardize human health.

Further, processing and slaughter methods might affect the final quality of the harvested fish. The current chapter will discuss factors affecting the quality of

the fish muscle, with emphasis on nutrient composition, harvesting methods and changes occurring during the *rigor mortis* process affecting flesh quality. Focus is laid on Atlantic salmon and cod representing a fat and a lean species. First a short summary of fillet structural organization and nutrient composition is given.

9.2 The structure and nutritional composition of fish muscle

Compared to terrestrial animals which contain many muscles (from 300 to 600, http://wikipedia.org/wiki/muscular-system), fish muscle is very simply constructed and consists of the large lateral muscle that runs on both sides of the body. These muscles are generally white to off-white in color, with the exception of red fleshed fish as salmonids, in which they are colored red due to the deposition of the carotenoids, mainly astaxanthin and/or cantaxanthin, or pelagic fish being brownish due to the high myoglobin content. The subcutaneous muscle contains higher amounts of the myoglobins as compared to the white muscle in all fish species and appears darker. Thus often this subcutaneous muscle is referred to as red muscle.

Fish muscle is divided by thin connective tissue membranes, the myocommata, into muscle segments of actin/myosin called the myotomes. Each of the myotomes consists of muscle fibers running parallel along the length axis of the fish. Surrounding each muscle fiber is a membrane called sarcolemma. The sarcolemma contains thin collagen fibrils that merge with the myocommata at the myotome-myocommata junction in contrast to terrestrial animals in which tendons are produced here (Dunajsky, 1980; Venugopal and Shahidi, 1996; Johnston, 2001). The muscle of fish consists of three major compartments, the contractile proteins, the lipids and the connective tissues. The contractile proteins are organized in myofibrils inside muscle fibers while the lipids are found as the major component in cell membranes (i.e., the phospholipids and cholesterol) as well as storage droplets in adiposities or lipid droplets in cytoplasm of the muscle fibers (Ackman, 1994; Nanton *et al.*, 2007) or in the myocommata (Zhou *et al.*, 1995). The third component in muscle constitutes the connective tissue that is mainly composed of collagen, forming the cytoskeleton of muscle cells. Fish muscle differs from the higher vertebrates in that the fiber types are present in two discrete layers, where the glycolytic (the anaerobic white muscle tissue) and aerobe (the red muscle tissue) constitute more than 90% of all muscle tissues present and constitute about 60% of the fish (Houlihan *et al.*, 1995). The anaerobe muscle is used by fish for the slow swimming motion, while the aerobe is used in rapidly energy bursts to escape from any danger. Most of the total muscle mass in fish is the white anaerobe muscle, while the red aerobe muscle is present along the lateral line and on the dorsal side of the fish. These two muscle types have different chemical composition and contain different enzymes. The aerobe muscle type contains more mitochondria and less sarcoplasmic reticulum as compared to the anaerobe muscle type. Aerobe muscle also contains larger concentration of lipids, the water

soluble B-vitamins, glycogen and nucleic acid than do the anaerobe muscle type. The aerobe muscle contains more of the glycogenolytic enzymes and enzymes of the Krebs cycles, the pentose phosphate shunt enzymes, those participating in the electron transport, glycogen synthesis enzymes and those of lipolysis as compared to the aerobe muscle. On the other hand, the anaerobic muscle contains less lipids, fewer mitochondria, fewer of the enzymes for energy turnover, but more ATPase activity, glycolytic acids and more water.

Further, fish as opposed to higher vertebrates, never cease to grow, thus have the capacity to both increase the size of the muscle cells as well as to form new muscle cells throughout the entire life span (Johnston, 2001). Like all muscle food items, fish muscle proteins are classified into sarcoplasmic, myofibrillar and stroma proteins. The sarcoplasmic proteins constitute 10–30% of the total muscle protein, myofibrillar protein accounts for 70–90% of the muscle protein, while the connective protein constitutes 3–10%. The corresponding values for cattle meat are 16–28, 39–68 and 16–28%, respectively (Dunajski, 1980; Huss, 1988; Bremner, 1992; Aidos *et al.*, 1999; Mommsen, 2001). The absence of tendons, the lesser content of connective tissue as compared to the higher vertebrates and the fact that the collagen forms less cross-binding in fish means it will need only a short cooking time before consumption (Dunajski, 1980; Sato *et al.*, 1986; Bremner, 1992).

The muscle fiber contains all the components of cells and the thread-like structures of myofibrils. Myofibrils can be segmented into sarcomers, showing alternate thin and thick filaments in electron microscopic pictures bordered by the Z-lines. The thick filament consists of myosin, while the thin are composed of a double treaded actin. These thin and thick filaments slide over one another during muscle contraction. Together with the thin filaments troponin and tropomyosin also exists. Generally the fish muscle consists of about 18% crude protein and very small fluctuations in crude protein exists between bony fishes (Table 9.1), while cartilaginous fish species contains more crude protein due to high content of both urea and trimethylamine oxide (TMAO).

Compared with protein, lipid content in fish varies widely from about 0.1 to more than 20 percent depending on species (Table 9.1). Fat fish store lipids in the muscular and intraperetoneal tissues, while the leaner fish species store the lipid almost solely in hepatic tissues (Bell *et al.*, 1998; Dias *et al.*, 1999; Torstensen *et al.*, 2000; Nanton *et al.*, 2003; 2007). Depending on their muscular lipid content the bony fish are divided into lean species (less than 2% lipids in muscle tissues), low lipid species (between 2 and 4% lipid in muscle tissues), medium fat species (having a muscle lipid content ranging from 4–8%) and high lipid containing species (containing muscle lipid in the range 8–20%).

Fish muscle, similar to meat from domestic animals, contains about the same amount of the essential and non-essential amino acids resulting in a ratio between essential to non-essential amino acids that is close to one, nicely fulfilling the human requirement (Table 9.2). The high content of free histidine present in the muscle tissue of mackerel (see Table 9.3) is typical for the migratory pelagic fish species such as mackerel, tuna and yellowtail. Also the clupeidae and the

Table 9.1 Crude chemical composition (g/160 g N) of some fish and shellfish species commonly eaten

Species	Dry matter	Crude protein	Crude lipid
Atlantic salmon (*Salmo salar*)	310	184	140–230
Atlantic cod (*Gadus morhua*)	196	181	3
Atlantic halibut (*Hippoglossus hippoglossus*)	279	162	104
Atlantic mackerel (*Scomber scombrus*)	220–500	140–180	30–300
Arctic charr (*Alpinus salvinus*)	269	161	71
Turbot (*Psetta maxima*)	209	159	24
Blue fin Tuna (*Thunnus thynnus*)	288	200	105
Eel (*Anguilla anguilla*)	541	173	325
Greenland halibut (*Reinhardtius hippoglossoides*)	285	176	132
Haddock (*Melanogrammus aeglefinus*)	192	166	2
Ling (*Molva molva*)	210	171	28
Plaice (*Pleuronectes platessa*)	177	134	14
Pollack (*Pollachius pollachius*)	184	160	2
Rainbow trout (*Oncorhynchus mykiss*)	302	172	102
Redfish (*Sebastes ssp.*)	210	171	28
Saithe, Coalfish (*Pollachius virens*)	200	165	3
Sprat (*Sprattus sprattus*)	491	124	176
Tusk (*Brosme brosme*)	181	161	2
Wolf fish (*Anarhichas ssp.*)	224	186	25

*historical data collected from NIFES

cyprinoidae contain high levels of the free histidine in their muscular tissues. The free histidine is functioning as a buffering agent in the muscle tissues of these species. Other fish species generally contain dipeptides of imidazol component acting as buffer agents within their muscular tissues. The salmonids, gadoids and elasmobranches generally contain the imidazole anserine as their buffering agent. Anserine is a dipeptide in which one of the amino acids is an imidazol component, β-alanyl-1-metylhistidine. The eels and sturgeon contains high amounts of carnosine (the dipeptide β-alanylhistidine) in the muscle tissue, being the buffer compounds in these species. The synthesis of these dipeptides has been found to occur in the myotomes and the white muscle having higher capacity for synthesis than do the red muscle tissue (van Waarde, 1988). The β-amino acid taurine is present in all fish muscle (Aksnes *et al.*, 2006; Gaylord *et al.*, 2007). Taurine occurs in substantial amounts in the migratory fish species, where it is the major nitrogenous substance after the imidazole components (Table 9.3). Physiologically the taurine participate in the osmoregulation in fish muscle, and is also conjugated with the bile acids in teleost as in higher vertebrates besides being crucial for the development of nerve tissues as the eye and brain (Sturman, 1993; Lima *et al.*, 2001). In the outgrowing sea water phase Atlantic salmon is capable of producing taurine through transsulfuration (Espe *et al.*, 2008), while other species including larvae and juveniles are found to require taurine in the diet (Kim *et al.*, 2005; Matsunari *et al.*, 2005; Gaylord *et al.*, 2006; Takagi *et al.*, 2006).

Table 9.2 Crude protein (g/16 g N) and its constituting amino acid composition (mg/g protein) in domestic (beef) and marine (fish) food items are almost equal and of which nicely fit requirement

Amino acids	Beef[a]	Fish[a]	Requirement (mg or g/kg BW/day)[c]	
			Child (10 year)[b]	Adult[c]
Protein	20–23	20–22	1.0	0.7
Met*	23	30	27[d]	16[d]
Lys*	85	89	60	19
Thr*	40	45	35	16
Val*	59	51	33	14
Ile *	52	49	30	18
Leu*	83	80	45	26
Trp*	11	12	4	4
Phe*	41	43	27[e]	20[e]
His(*)	32	34	?	?
Arg	67	63		
Tyr	33	36		
Gly	73	49		
Ser	40	41		
Pro	56	36		
Ala	60[b]	64		
Asp	88	97		
Glu	149	152		
ΣAA	991	971		

[a] From historical data NIFES.
[b] From FAO (1985).
[c] From Millward (1999), crude protein requirement is g/kg BW/day while IAAs are mg/kg BW/day
[d] Includes Met+Cys.
[e] Includes Phe+Tyr
AAs followed by * are regarded as essential for humans, histidin is still disputed

Marine lipids consists of triacylglycerols (TAG), phospholipids (PL), sterols, wax esters as well as some more unusual lipids like the glyceryl esters, glycolipids and sulfolipids (Ackman, 1994). Muscle lipids generally are stored as TAG in fatty fish, while lean fish store lipids mainly as TAG in the hepatic tissues, thus lipids present in the lean muscle mainly consists of phospholipids making up the cell membranes. The distribution of lipids within the fish muscle is not uniformly distributed, as the head region generally contains more lipids than the tail regions and even more is present in belly flaps (Bell *et al.*, 1998; Nanton *et al.*, 2007). Total lipid content in fish flesh varies between species from values below 0.5% in Atlantic cod to more than 20% in farmed Atlantic salmon (Table 9.1). Some wild species as for example herring and mackerel shows great seasonal variation in total lipid content (Huss, 1988) while farmed fish, i.e. Atlantic salmon and cod, show less annual variations in lipid storage (Hemre *et al.*, 2004; Espe *et al.*, 2004; Roth *et al.*, 2005).

Additionally to the variable lipid content due to anatomical position and

Table 9.3 The free amino acids (mg $100\,g^{-1}$) present in some commonly eaten fish and shellfish species. As can be read from the table, the amount of free amino acids present in different fish species varies widely

Amino acids	Atlantic salmon	Atlantic cod	Flatfish	Mackerel
Met*	2	1	1	2
Lys*	7	27	17	93
Thr*	10	6	4	11
Ile*	3	1	1	7
Leu*	6	2	1	14
Val*	6	2	1	16
His(*)	11	23	1	676
Phe*	4	2	1	4
Trp*	–	–	–	–
Arg	2	7	3	11
Tyr	5	2	1	7
Gly	41	36	5	7
Ser	13	9	3	6
Pro	4	11	1	26
Ala	27	33	13	26
Asp	2	2	–	–
Glu	53	10	6	18
Tau	42	–	171	84
ΣAA	236	167	227	997

Based on historical data from NIFES

season, increased energy intake in the form of energy-dense diets and feeding regimes results in increased lipid deposition in the medium to fat species (Sheehan *et al.*, 1996; Hemre and Sandnes, 1999). In the lean species on the other hand increased dietary lipid is reflected in the size of the liver tissues thus might have great impact on the condition factor of the fish (Lie *et al.*, 1988; Nanton *et al.*, 2003; Rosenlund *et al.*, 2004). Modern fish farming using energy-dense feeds and intensive feeding regimes has resulted in production of Atlantic salmon in which muscle may contain as much as 23% lipids. The variation in total lipid content of fish muscle is due to variations in TAG as all fish have a basic cellular lipid that averages 0.6 to 0.7% of the wet wt of the muscle and which mostly is phospholipids (Ackman, 1994; Serot *et al.*, 1998). Fish PL is dominated of phosphatidylcholine (60%, PC) and phosphatidylethanolamine (20%, PE) while the minor PL being phosphatidylserine (PS) and phosphatidylinositol (PI). PL differs from TAG in having less 20:1 and 22:1, and more of the 16:0 and 22:6n-3. The differences in fatty acids within PL and TAG thus results in a much higher ratio of n-3 to n-6 in PL as compared to TAG (Lie and Lambertsen, 1991; Ackman, 1994; Nanton *et al.*, 2007). Although the amount of PL is the same in different species the fatty acids present in PLs reflect the dietary fatty acids (Hemre *et al.*, 2004; Nanton *et al.*, 2007).

The fatty acids present in fish meat differ from mammalian fatty acids in that they consist of long chain fatty acids which are highly unsaturated. The chain

Table 9.4 Total lipid content (%) and fatty acids (g 100 g^{-1} lipid) in fish meat of some fish species

Fatty acid	Atlantic cod	Atlantic haddock	Atlantic halibut	Atlantic salmon
Total lipid	*0.3*	*0.2*	*10.4*	*14–23*
14:0	2.0	1.1	4.5	4.0–7.0
16:0	16.3	20.1	9.0	12.0–14.0
16:1*	2.5	1.6	8.7	4.0–7.0
18:0	2.9	4.0	1.9	2.0–3.0
18:1*	9.0	11.0	24.4	15.0–18.0
18:2n-6	1.2	0.9	1.3	2.0–16.0
18:3n-3	0.3	0.2	0.5	0.5–2.5
18:4n-3	1.1	0.3	0.7	0.5–2.5
20:1*	4.7	1.6	18.5	5.0–14.0
20:4n-6	1.8	4.6	0.4	0.2–1.0
20:4n-3	0.4	0.4	0.4	1.0–2.0
20:5n-3	14.5	16.1	2.7	4.0–10.0
22:1*	1.3	0.7	19.6	4.0–15.0
22:5n-3	1.2	2.0	0.6	1.5–5.0
22:6n-3	36.8	31.4	2.9	7.0–15.0

Based on historical data from NIFES, * sum of isomers

length of the fatty acids present in fish muscle range from C14 to C24 (Ackman, 1994). Mammalian fat rarely contains more than two double bonds per fatty acid, while the depot fat in fish contains many fatty acids with five or six double bonds. The fatty acids present in fish meat are the saturated (20–35% of fatty acids), the monoenic (15–40%) and the polyeneic fatty acids (38–51%). The marine fish species generally have a higher content of polyeneic fatty acids as compared to freshwater fish, and the majority of the polyenic fatty acids (PUFA) are the n-3 fatty acids. Marine lipids generally are high in the nutritionally important n-3 fatty acids; EPA (20:5n-3), and DHA (22:6n-3). The fatty acid composition in fish meat of some species is listed in Table 9.4. The fatty acid composition of PL and even more the stored lipids as TAG is highly dependent on dietary fatty acid composition (Bell *et al.*, 2001; Torstensen *et al.*, 2005; Mørkøre *et al.*, 2007) as well as affected by the rearing temperature (Corraze and Kaushik, 1999). Cholesterol is an integrated part of the cell wall in fish as in other species. Most fish species have cholesterol content in the muscle tissue of about 50 mg 100 g^{-1} wet weight (Ackman, 1994).

9.3 The biological basis for growth and development in fish

Generally the white muscle constitutes the main edible part (Houlihan *et al.*, 1995) with the exception of fish which swim more or less constantly, i.e. the herring in which the red muscle tissue may constitute as much as half of the

muscle (Love, 1980). When fish develop from embryo until harvest size several phases of growth occur which are regulated by diet and hormones (Johnston, 2001; Mommsen, 2001). Fish always grow faster the smaller they are, but opposite to terrestrial animal fish never cease to grow (Mommsen, 2001); have the ability of both hyperthropic and hyperplastic growth during their whole life span (Johnston, 2001). The recruitment of fibers and the hyperthropic growth differ by both season and growth rate (Johnston, 2001; Bjørnevik *et al.*, 2004; Johnston *et al.*, 2007). Kiessling *et al.* (1991) reported that rapid growth increase in fish favored hypertrophy, while fish with slower growth increase favoured fiber recruitment. The fiber size has been linked to texture as the smaller fibres results in a muscle with firmer texture as compared to fish having bigger fibres (Mørkøre *et al.*, 2001; Sigurgisladottir, 2001). The phenomenon of gaping, i.e. the fish muscle falling apart (Lavety *et al.*, 1988), generally occurs concomitantly with textural changes, that is the softer the muscle the higher the gaping score (Bremner, 1992; Einen *et al.*, 1999), and as muscle softens during ice storage, so does the frequency of gaping (Einen *et al.*, 1999; Mørkøre and Rørvik, 2001; Espe *et al.*, 2004). Gaping probably occurs concomitantly with the textural changes, as both show seasonal variation and both of them show interactions with the pre-harvest feed intake and end pH (Love, 1980; Mørkøre and Rørvik, 2001; Espe *et al.*, 2004; Bjørnevik *et al.*, 2004).

Farmed fish do not have the possibility to catch their own feed and thus rely totally upon the feed offered by the fish farmer. The feed has to provide all the nutrients necessary for growth and development at all life stages to secure a healthy fish with good growth performance, still having a muscle composition and quality acceptable for the consumer. That is being of high nutritional quality, having a normal texture, colour and taste.

The ready and constant availability of energy-dense feed is one of the reasons that the farmed fish grow fatter or produce bigger livers than their wild counterparts, thus changing the stages of growth, storing more lipids in both muscle and in intraperetoneal cavity (Sheehan *et al.*, 1996; Mørkøre *et al.*, 2001; Rosenlund *et al.*, 2004; Nanton *et al.*, 2007). This, of course, will affect the yield and thus the economy of fish farming as well as the textural properties of the farmed fish (Sheehan *et al.*, 1996; Mørkøre *et al.*, 2001).

Lipids are needed to provide energy, but also the diets should provide essential/non-essential fatty acids to provide a healthy fish. Fish to a large extent deposit the fatty acids as they are fed, thus the muscle and liver lipid depots reflect the fatty acid pattern of the diet (Waagbø *et al.*, 1993; Rørå, 2003; Nanton *et al.*, 2007). The probability for lipid oxidation increases exponentially with the number of double bonds in the fatty acid. Thus marine lipids containing high levels of these long chained fatty acids are more prone to oxidize than are domestic meat. Lipid oxidation is an autocatalytic cyclic process, where the product of one cycle of oxidation initiates the next cycle. The process is usually divided into an initiation, propagation and termination phase. Oxidation occurs *in vivo*, but only at a very low rates due to the organisms many mechanisms of protection against this process. *Post mortem* the organism does not produce

endogenous antioxidants, the antioxidant enzymes lose their activities and natural antioxidants deposited pre slaughtering become depleted. Lipid oxidation post harvest thus is dependent on the antioxidant status of the fish at death, possible addition of antioxidants during processing and conditions during processing and storage of the fish product.

Fish fillet can be protected against lipid oxidation, firstly by preventing its initiation. The fish should be well bled to prevent large amounts of blood, and thereby hemoglobin with iron, being present in the fillet during processing and storage. When fish is salted, the salt should be selected to contain a minimal amount of transition metals. Excluding air by airtight packaging will reduce the amount of reactive oxygen species that can initiate lipid oxidation and the amount of oxygen that can be consumed during propagation. Contact with air will increase oxidation and whole fish is more protected than fillet, which is more protected than minced fish (Unneland, 2001). Protection against light will reduce initiation by photo-oxidation. As with other deterioration processes the lipid oxidation process is also slowed down by low temperature, but may be accelerated during the freezing and thawing process. Therefore it is important that the freezing process is as fast as possible and thawing occurs at low temperatures. Storage at −30 ºC results in slower oxidation than storage at −20 ºC, but thawing and refreezing will increase lipid oxidation and mostly so at higher storage temperatures (Nordtvedt *et al.*, 2007). It is possible to increase shelf life by increasing the levels of natural antioxidants as tocopherols or ascorbic acid prior to harvesting through increased dietary supply protection against lipid oxidation *post mortem* (Gatlin *et al.*, 1992; Hamre *et al.*, 1998; Scaife *et al.*, 2000; Tocher *et al.*, 2002; Ruff *et al.*, 2002).

In contrast to lipids or lipid soluble vitamins, amino acids cannot be stored as such, which means if available in cell compartments in imbalanced or too high concentrations for what is actually needed for protein synthesis, amino acids are oxidized and utilized for energy purposes. This protein oxidation may increase nitrogen waste and additionally contributes to a fattier fish as the dietary lipids will be stored until being needed for energy purposes. This is illustrated in Fig. 9.1 where a diet which was balanced in all amino acids except the essential lysine was fed to Atlantic salmon in the fast growing seawater phase, showing that at equal nitrogen intakes, protein accretion was low, while lipid accretion was high until dietary lysine balanced the need for protein deposition. Nitrogen intake exceeding what is actually being deposited in the body will also increase the nitrogen excretion due to increased oxidation of the ingested amino acids and, of course, increase the cost of production. This increased nitrogen excretion may affect pollution of nitrogen in the water near the net pens and may results in pollution problems and farming conditions not favoring the growth and quality of the farmed fish. Therefore balancing the dietary amino acids is of importance for partitioning of growth (i.e. lipid and protein deposition) in muscle tissues in the fattier fish and shape of the leaner fish. The easiest way of balancing the dietary amino acids is by accepting the ideal protein concept as introduced for domestic animals in the eighties (Wang and Fuller, 1987, 1989). According to

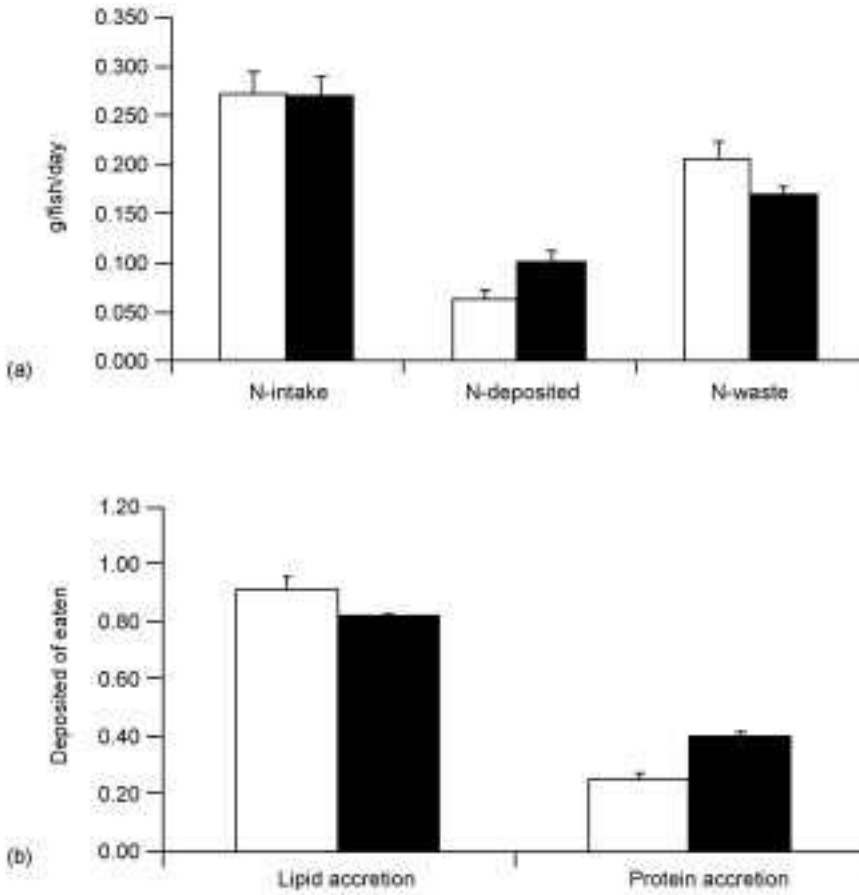

Fig. 9.1 The N waste (intake – deposited) is higher when inadequate lysine is fed to Atlantic salmon (a). Additionally lipid accretion (deposited of eaten) is higher and protein lower when lysine intake is inadequate (□) as compared to adequate (■) (calculated from Espe *et al.*, 2007).

Wang and Fuller the ideal protein in pig should contain relative to lysine: 18% tryptophane, 75% isoleucine, 63% total sulphur amino acids, 72% threonine, 75% valine and 120% total aromatic amino acids. Fish also require arginine and histidine, which mammalian species do not. As the constituent amino acids are relatively similar in fish species (Table 9.2), so should the ideal protein be also. But differences in lysine requirement are reported (Table 9.5) resulting in variable ideal protein levels (Table 9.6). Harvest quality by accepting the ideal protein thus might have impact on the ratio of lipids to protein in muscle tissues in fat fish species, and might also affect the shape of the lean fish by increasing the condition factor having impact on processing quality. However, whether the fish grow fast or slow will not affect the nutritional quality of the fish protein for

Table 9.5 Lysine requirement in different fish species and life stages

Fish species	Lysine requirement	Life stage	Reference
Atlantic salmon (*Salmo salar*)	3.98	F	Anderson *et al.*, 1993
Jundia (*Rhamdia quelen*)	5.10	F	Montes-Girao and Fracalossi, 2006
Indian major carp (*Cirrhinius mrigala*, Hamilton)	5.75	F	Ahmed and Khan, 2004
Sea bass (*Dicentrarchus labrax*)	4.82	F	Tibaldi and Lanari, 1991
Gilthead seabream (*Sparus aurata*)	5.04	J	Marcouli *et al.*, 2006
Nile tilapia (*Oreochromis niloticus*)	5.23	J	Furyama *et al.*, 2006
Grass carp (*Ctenopharyngodon idella*)	5.44	J	Wang *et al.*, 2005
Japanese seabass (*Lateolabrax japonicus*)	5.80–6.05	J	Mai *et al.*, 2006
Grouper (*Epinephelus coioides*)	5.56	J	Luo *et al.*, 2006
Yellowtail flounder (*Pleuronectes ferrugineus*)	4.5–4.7	J	Kim and Lall, 2003
Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*)	4.03	J	Keembiyehetty and Gatlin, 1992
Rainbow trout (*Oncorhynchus mykiss*)	7.91	G	Rodehutscord *et al.*, 1997
Atlantic salmon (*Salmo salar*)	5.04	G	Espe *et al.*, 2007

F, J and G means fry, juveniles and grow out phase, respectively.

the consumer, but might affect other quality aspects such as lipid to protein ratio, texture and gaping.

As well as dietary lipid and protein levels the balance between macro and micro nutrients might also affect the quality. In the red fleshed fish such as the salmonids, the color of the muscle is very important for the eating quality. The

Table 9.6 The ideal protein in fish as compared to ideal protein accepted for fattening pigs. Essential amino acids (EAAs) relative to lysine in fish meal are listed for comparison

EAAs	Juveniles[a]	Fattening pigs[b]	Fish meal
Arg	75–125	–	77
His	29–40	–	35
Ile	44–76	Nd	53
Leu	58–100	Nd	98
Lys	100	100	100
Thr	45–80	72	55
Trp	10–21	18	15
Val	55–76	75	63
TSAA	45–80	63	49
TAA	75–131	120	90

[a] Calculated from NRC (1993).
[b] From Wang and Fuller (1989).
TSAA includes Met+Cys, TAA includes Phe+Tyr.

main carotenoid in Atlantic salmon is astaxanthin and the color of the harvested fish muscle depends on both the level of dietary carotenoid used and on the strategy chosen for feeding the carotenoids (Schiedt *et al.*, 1981; Torrissen *et al.*, 1989; Torrissen, 1995; Torrissen and Christiansen, 1995; Bjerkeng, 1997). Interactions between lipid level and absorption of carotenoids exist (Shahidi *et al.*, 1998) and low dietary lipid levels results in weaker coloring of the fish flesh (Torrissen *et al.*, 1990; Sheehan *et al.*, 1996; Einen and Roem, 1997). However, production of a very lipid-rich muscle may result in a post-harvest muscle appearing whiter to the human eye in spite of containing the same concentration of carotenoids. Additionally, as growth is not constant throughout the life cycle (Johnston, 1982), seasonal variation in deposition and thus visible coloring is to be expected (Nickell, 1997; Torrissen *et al.*, 1995; Einen and Thomassen, 1998). As opposed to the red colored fish muscle, white fish muscle as, for example, the Atlantic cod, should be shiny white to bluish *post mortem*, which is secured by low stress and good bleeding upon harvesting (Olsen *et al.*, 2006).

The word product tailoring has been used to describe production of fish meat that is to contain a defined chemical composition. For example, one might want to produce fish meat that contains more of the fatty acids EPA and DHA. Farmed Atlantic salmon is found to have great potential to be tailored for the lipid soluble nutrients (Waagbø *et al.*, 1993; Sigurgisladottir *et al.*, 1994; Hamre and Lie, 1997; Grahl-Madsen and Lie, 1997; Horvli *et al.*, 1998; Rørå, 2003; Nanton *et al.*, 2007), but much harder for those that are not lipid soluble. On the other hand, lipid levels in the lean cod fillet are generally below 1%, but the fatty acids in the phospholipids will reflect the dietary fatty acid profile (Hemre *et al.*, 2004). When it comes to the minerals, some may be increased or decreased depending on the source fed to the fish (Lorentzen *et al.*, 1994), while others do not seem possible to tailor (Maage and Julshamn, 1993).

9.4 Understanding the effects of environmental parameters on flesh quality

In addition to the fact that feed composition and feed intakes have an impact on the quality of the fish muscle *post mortem*, the process and methods of slaughtering have an impact on the final flesh quality. When death occurs, the tissues will not receive any more oxygen for aerobe metabolism, thus lactic acid will be produced and the energy of the muscle (i.e., glycogen, ATPs and creatin) will be depleted. As energy is needed for disrupting the actin myosine binding occurring in muscle contractions, energy depletion results in a stiff muscle – *rigor mortis*. The time before fish enters *rigor mortis* depends of the energy status in muscle tissues at the time of death, and will determine the ultimate pH of the muscle (Huss, 1988). A muscle containing high energy status, not being stressed or depleted of glycogen will result in longer periods of time before entering *rigor* as well as having lower ultimate pH as compared to a energy depleted muscle at the time of death (Huss, 1988). Thus the reduction of stress

during slaughter has been paid a lot of attention in farmed fish production as this has direct impact on quality as well as being ethically correct. Reduced stress results in longer periods of time until the fish enters *rigor mortis*, affecting the end pH and thus the texture of the fillet (Robb and Kestin, 2002; Van de Vis *et al.*, 2001; Pottinger, 2001; Skjervold *et al.*, 2001b; Morzel *et al.*, 2003; Kiessling *et al.*, 2004).

The low ultimate pH produced by low stress harvesting, also produces a fish meat post rigor that can be stored for longer periods of time due to a longer *in rigor* period (Huss, 1988; Roth *et al.*, 2006). During the *rigor mortis* process, the muscle is regarded as sterile, but *post rigor* fish will immediately start to deteriorate due to endogenous enzymes present in the flesh meat, mostly the lysosomal cathepsins (Haard, 1994). At the beginning of the storage process, enzymatic activity will contribute to the taste of fish but if stored for too long periods of time, nutrients produced may contribute to bacterial contamination. In addition, the deterioration *post rigor* affects both gaping as well as the texture of the fish meat, becoming softer and showing higher gaping scores (Sigurgisladottir, 2001; Espe *et al.*, 2004).

After harvest, TMAO present in fish muscle is converted to trimethyl amine (TMA) contributing to the smell of fish meat. In gadoid species TMAO might also be broken down to dimetylamine (DMA) and formaldehyde (FA) by the enzyme aldolase and the FA might result in cross bridges between the FA and protein, affecting the texture upon successive frozen storage (Badii and Howell, 2002; Nielsen and Jorgensen, 2004), this, however, is not a problem in the fresh muscle.

After death, the nucleotides will immediately start to break down to less energy-containing compounds and in the end to uric acid through the following pathway:

$$\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP} \rightarrow \text{I} \rightarrow \text{HxR} \rightarrow \text{Hx} \rightarrow \text{X} \rightarrow \text{U}$$

where ADP and AMP are adensosine di- and mono phosphate, IMP and I are inosine mono phosphate and inosine and finally HxR, Hx, X and U is hypoxanthine ribose, hypoxanthine, xanthine and urea, respectively. The degradation of nucleotides has been used as quality index for fish muscle, due to the fact that inosine is regarded as having a good taste while the hypoxanthine has a bad taste and therefore is used as a spoilage indicator during storage of fish and fish products (see Huss, 1988). The rate at which the nucleotides are broken down to hypoxanthine has been reported to correlate to stress upon harvesting, storage time and temperature, but not to feeding prior to slaughter (Erikson, 2001).

The amides (asparagines and glutamine) are present in all animal tissues. Amides are synthesized by the enzyme amide synthetase from ammonia and the corresponding amino acid (aspartic acid and glutamic acid) and are broken down by amidases. In the living tissue, amides are involved in the control of the ammonia level within tissues as well as its excretion rates. Post harvest the amide-N might contribute to the total volatile nitrogen (TVN). Upon prolonged

storage N arriving from bacterial degradation of amino acids will also contribute to volatile N as increasing amounts of ammonia are produced, leaving the fish meat unacceptable for consumption after approximately 8–10 days of ice storage (Conell, 1995). Feeding and feeding rate prior to harvesting will have no impact on TVN development, as this solely is a storage phenomenon.

External environmental factors such as light and water temperature have an impact on the quality of the fish flesh post harvesting, which are observed as seasonal variations in wild caught fish. The global increase in temperature might reduce the feed intake and consequently affect the quality of the farmed fish. Fish not being fed for several weeks prior to harvesting or fed reduced feed rations show changed protein as well as lipid content in the flesh (Einen *et al.*, 1998; 1999). Annual variations in colour and lipid depositions, texture as well as the phenomenon of gaping, changes with season and feeding behavior in both wild and farmed fish. Generally gaping is higher in the well-fed farmed fish as compared to the wild counterparts and the intense feeding regime results in lower end pH (Lavety *et al.*, 1998; Mørkøre and Rørvik, 2001; Sigurgisladottir, 2001; Espe *et al.*, 2004; Kristoffersen *et al.*, 2006). Neither texture nor gaping has been linked directly on the fast growth period prior to harvesting (Johnston *et al.*, 2007). Upon storage post harvest, both gaping and softness will increase, rendering annual variations less (Espe *et al.*, 2004). Fish that are filleted *pre rigor*, however, will result in a fillet showing less gaping, having a firmer texture and more intense color due to the shortening of the fillet (Skjervold *et al.*, 2001a; Kristoffersen *et al.*, 2006). Pigmentation may also show seasonal variations, although variations and lipid depletion are to the strongest degree influenced by sexual maturation (see Nickell and Springate, 2001) and less by diet.

When fish is not consumed after being harvested, both temperature and period of storage will of course have a huge impact on the quality of the fish meat for the consumers. Fish that is stored on ice generally shows acceptable quality until about 10 days after slaughter (Conell, 1995). Some exceptions exist as the Atlantic halibut can be stored refrigerated for 21 days post harvesting and still show acceptable eating quality (Guillerm-Regost *et al.*, 2006). Texture is changing during storage and due to endogenous and exogenous enzyme activity and bacterial deterioration pH will increase, thus rendering the fish unacceptable for human consumption after a certain period of time (Huss, 1988; Conell, 1995). Fish containing high amounts of the free imidazole, histidin, will also increase the production of the corresponding amine, histamine, which may result in scromboid poisoning if consumed. Therefore fish that is not to be consumed during a short period post harvesting should preferably be stored frozen or processed by, for example, salting or cold smoking to increase the storage time to maintain a high quality product (Røra, 2003; Birkeland, 2004). The processing of farmed fish will not be discussed here.

Of course pollutants as well as possibly contamination will have impact on the flesh quality as will microbiological aspects but these aspects are discussed elsewhere in this book.

9.5 Understanding the effects of genetics on flesh quality

Farmed fish such as Atlantic salmon has been bred for fast growth and not for the quality of the produced fish. As growth mainly consists of the gains in the major components of the muscle, protein and fat, and the fact that the protein content is rather constant, salmon have been bred to maximize lipid stores thus contributing to farmed salmon containing more fat as compared to the ocean ranched and wild salmon (Espe *et al.*, 2001; Mørkøre *et al.*, 2001; Blanchett *et al.*, 2005; Johnston *et al.*, 2006). As the farming of salmon uses energy-dense diets and intensive feeding this also contributes to maximizing growth, concomitantly increasing the lipid deposition. However, as the fat in salmonids is stored in the belly flaps, in the liver and visceral tissues, breeding programs to produce fish containing different storage patterns of the lipids to make fillets containing different amounts of fat is possible and has been done (Quillet *et al.*, 2005; 2007). Less lipid deposition in the flesh will interfere with the textural properties of the meat, as the fillet becomes softer as the fat content increases (Mørkøre *et al.*, 2001). Breeding programs might be useful for improving color intensity of the flesh post harvest as well as postponing sexual maturation (Alderson, 2001). To gain maximal growth and accretion without excessive lipid stores, the breeding programs for improving quality should preferably be run in close coordination with nutrition and feeding regimes. Lean strains and less lipid deposition in fillets exist, but these fish store more lipids in the intestinal tissue, thus this might change the shape of the fish and thus affect technological quality. The breeding for farmed fish quality is discussed elsewhere in this book.

9.6 Future trends

Fish oils have long been regarded to have beneficial health effects on human health as they may counteract the 'lifestyle diseases' commonly found in most industrialized and semi-industrialized countries. The positive effects of the marine n-3 PUFAs to counteract artherosclerosis, stroke, inflammatory diseases, and hypertension are discussed elsewhere in this book. As farmed fish may be tailored to contain specific fatty acids or levels of fat, concomitantly being low in possibly harmful substances, farmed fish have at least as beneficial effects preventing life style diseases as consumption of the wild catches (Cahu *et al.*, 2004; Seierstad *et al.*, 2005).

In recent years, fish protein has also been found to have cardio-protective effects as consumption of the lean fish meat has been found to improve consumer health. Cholesterol excretion has been found to be higher in poultry (Iritani *et al.*, 1985) and rat (Wergedahl *et al.*, 2004; Gudbrandsen *et al.*, 2005) fed soy and fish protein as compared to those fed the milk protein casein. This was partly due to the amino acids present in the different protein sources, but also the soy protein contribute to health improvements due to their isoflavone content (Gudbrandsen *et al.*, 2006). Further, all marine fish meat, as meat from domestic animals, contains the amino acid taurine, which is known to enhance

cholesterol degradation and thus reduce hepatic cholesterol concentration due to increased excretion in the bile (Yokogoshi *et al.*, 1999; Gudbrandsen *et al.*, 2005). In the future it will probably be shown that eating both lean and fat fish reduce the probability for development of life style diseases as well as cardiovascular diseases. Farming fish for health counteracting life style diseases will probably be intensified in the future.

In addition to understanding the health-promoting effects and the possibility of producing fish containing high contents of components promoting such effects by product tailoring, the production of niche products containing different amounts of preferred quality post harvest might occur also. The changed composition of the fish as occurring when the marine ingredients are exchanged with the plant ingredients must be focused in more detail as having impact on both the health of the farmed fish and the health of the consumer.

9.7 Sources of further information and advice

Farmed fish is a healthy food item, and should preferably be consumed at least twice a week. As the farming of fish allows for controlling the feed ingredients used and the fact that more and more of the marine lipids are substituted with plant oils, the farmed fish will be low in pollutants as long as the feed components used are clean. Farmed fish may be tailored to contain health-promoting substances such as, for example, omega-3 fatty acids. However, as fish easily deteriorate post harvest, it is of utmost importance that the fish are harvested with low stress, under high hygienic conditions and when not eaten soon after harvest, are stored cold to reduce deterioration and prolong shelf life. However, the storage period prior to consumption as well as the rearing conditions may affect the quality of the fish. If harvested or stored at suboptimal conditions, the intrinsic quality might be destroyed completely in very short periods of time.

More information regarding the impact on quality of farmed fish as affected by harvesting and storage conditions as well as the changes in quality can be found in the following books:

BREMNER AH (ed.) (2002) *Safety and Quality Issues in Fish Processing*, 1st edition, Woodhead Publishing Limited, 507pp, ISBN 0-8493-1540-9.

KESTIN SC and WARRISS PD (eds) (2001) *Farmed Fish Quality*, 1st edition, Fishing News Books, 430pp, ISBN 0-85238-260-X.

CONNELL JJ (ed.) (1995) *Control of Fish Quality*, 4th edition, Fishing News Books, 245pp, ISBN 0-85238-226-X.

SHAHIDI F and BOTTA JR (eds) (1994) *Seafoods: Chemistry, Processing Technology and Quality*, Chapman and Hall, 342pp, ISBN 0-7514-0218-4.

HUSS HH, JAKOBSEN M and LISTON J (eds) (1992) *Quality Assurance in the Fish Industry*, Elsevier, 588pp, ISBN 0-444-89077-7.

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10

Improving farmed fish quality by selective breeding

T. Gjedrem, Institute of Aquaculture Research, Norway

10.1 Introduction

In aquaculture production it is possible to influence product quality in a number of different ways, whereas in capture fisheries the product quality is determined by what is caught at any given time. Since cultured animals are raised under controlled conditions from fertilisation until slaughter, it is possible to regulate the environment and nutrition throughout the whole life cycle, in addition to the control of breeding. Through detailed studies of the effects that different factors have on quality traits, it is possible to make changes during the farming process in order to meet particular consumer preferences.

In order to make changes or to improve quality traits through selective breeding, information on multiple parameters or characteristics for each trait is required. In addition to calculating trait averages and standard deviations, the heritability for each trait, and phenotypic and genetic correlations between the traits in question must be known. Heritability (h^2) describes the amount of variation in a trait that is caused by genetic factors; for example a h^2 value of 0.25 means that 25% of the total phenotypic variation is genetic. The phenotypic correlation is the correlation between two traits that are recorded on a group of animals. The genetic correlation is estimated from the genetic covariance between two traits, and can be estimated from the average of two traits measured on many animals from a large number of families. A genetic correlation is usually caused by genes that affect both traits in question, a situation known as pleiotropy.

To obtain genetic improvement by selective breeding, some conditions must be met:

- The trait must show variation.
- Some of the variation must be heritable since it is only the genetic variation which is transferred to the next generation.
- A candidate trait should be of economic importance.
- It must be possible to measure the trait accurately and at a reasonable cost.
- It must be possible to control the whole life cycle of an animal because selection can only be performed once there is sufficient information available on the candidate parents.
- Each animal must be identifiable.

10.2 Quality traits to be improved

A candidate trait for selection must be precisely defined and the trait measurement should show a high degree of repeatability. Traits of highest economic importance should be selected since increasing the number of traits included in the breeding goal is expected to result in a reduced rate of gain for each individual trait. It has been shown repeatedly that when there are several traits in the breeding goal, they should be selected for simultaneously (Hazel and Lush, 1942). This can be achieved through the use of an index or total score to estimate breeding values. The index should include available records for all traits together with pedigree information. In the index, each trait is weighted according to its genetic variation, genetic correlation with other traits, and economic importance.

There are several quality traits that can be changed permanently by selective breeding. All the quality traits discussed below are considered to be quantitative traits.

10.2.1 Fillet yield

Fillet yield is the ratio between fillet weight and body weight and is a measure of the edible part of the body. This trait is of economic importance in all species and is considered to be the most important quality trait in many, including Atlantic salmon, rainbow trout, river catfish, Atlantic cod and Nile tilapia. The trait is expensive to record and can presently only be measured accurately after slaughtering.

10.2.2 Fat content

Fillet fat per cent is an important economic trait for salmonids and other species. However, since the consumers' preferences vary from one market to another and also within countries, it is difficult to precisely define the optimum fat per cent in carcasses. The general impression is, however, that values above 16–18% are too high for this trait. For species with a relatively low fat per cent such as cod and halibut, fat content will not be a focus for selection.

Chemical analysis provides the most accurate measurement of fillet fat per cent, but it is expensive and time consuming to record and most recording methods require that animals be slaughtered. Computerised tomography is an alternative method to predict fat per cent with high accuracy (Rye, 1991; Gjerde, 1987). The method is non-destructive and is easy to use on either fresh or frozen carcasses; however, the equipment used to take the measurements is large, expensive and immobile, making it impossible to use in the field. An alternative method for measuring fat per cent is the Torry fat meter (Kent, 1990), which is non-destructive and measures the fat from outside the fish by touching the skin. This instrument is inexpensive and portable, but does not have the same accuracy as computerised tomography. AKVAFORSK has recently developed image analysis technology that shows a high degree of accuracy for rainbow trout filets, with an R^2 of 0.83 between image and chemical analyses of fat in fillets (Rørvik and Austreng, personal communication).

10.2.3 Fat distribution

Several species store fat in various fat depots around the body. For example, salmonids have fat depots in the belly and around the fins and intestine, Atlantic halibut have fat depots around the fins and cod have fat depots in the liver. These fat depots reduce the product quality and increase the quantity of waste in processing, and should therefore be reduced as much as possible. Fat depots can be observed and the size of the fat depot can be measured by means of computerised tomography in both salmonids and Atlantic halibut (Rye *et al.*, 1994; Kolstad *et al.*, 2002).

10.2.4 Colour of flesh

Flesh colour is of economic importance in several fish species. In salmonids, the red colour comes from carotenoids supplied in the feeds. Added carotenoids are expensive and in fact represent around 15% of the feed cost. However, retention rates of dietary carotenoids in salmon are low at around 10% (Bjerkeng *et al.*, 2007). Flesh colour can be measured by chemical analyses, however, this strategy is expensive and time consuming. Visually scoring the fillet colour has been used as a substitute, but shows low repeatability due to different interpretations of those doing the scoring. Colour meter readings (Minolta Chroma Meter CR-300) have been found to correlate well ($r = 0.80$) with chemical analyses of astaxanthin levels in salmon flesh (Rye *et al.*, 1994). The image analysis technology developed by AKVAFORSK provides an even higher degree of accuracy in rainbow trout filets, with an R^2 of 0.86 between the values obtained by image and chemical analyses of astaxanthin (Rørvik and Austreng, personal communication). The two last methods are currently used by breeding companies to calculate breeding values for rainbow trout and Atlantic salmon.

For species with generally white flesh, consumer preference is for a purer white colour, as opposed to being grey or reddish. The whiteness may be scored

subjectively by visual interpretation but accuracy would most likely be increased through the use of instrumental analysis.

In rainbow trout, a bright skin colour is of importance in some markets, particularly in Japan. At present, this trait is evaluated subjectively.

10.2.5 Texture

Texture is a quality trait of importance both in fresh as well as in refined products. Instruments applying mechanical pressure can be used to measure texture. Mørkøre and Einen (2002) found that sensory hardness correlated highly ($r = 0.70$) with a Warner-Bratzler blade of 12.5 mm in diameter in raw salmon. Despite its importance, texture is not frequently included in breeding goals for fish species. In Atlantic cod there is a relatively higher level of phenotypic variation in texture than in salmon (Mørkøre, personal communication).

10.2.6 Condition factor

Condition factor is commonly used to measure the conformation of the fish. It is the ratio of body weight and body length cubed. This trait is not considered to be an important economic trait and is rarely included in breeding goals.

10.2.7 Dressing percentage

Dressing percentage is a measure of the proportion of the body that is classified as waste. Such waste includes blood, intestine, fat in and around the intestine, and the head (in some cases). Ulla and Gjedrem (1985) found that the intestine including fat represented 75% of the loss at gutting. Including dressing per cent in a breeding program may be risky as it could lead to reduction in size of the intestine, an organ of vital importance to metabolism and feed digestion. The internal fat may, however, be reduced without much risk for animals under farming conditions.

10.2.8 Other traits

Taste and smell are two important quality traits. At present, these traits are only measured by test panels, which is very laborious and expensive. As a result, it is very difficult to include them in a breeding programme as selection criteria. Gaping is another trait of importance. Like taste and smell, there are currently no instruments available for measuring this trait, therefore it must be judged by visual scoring. Some fish species like carp, silver barb and sea bream have intramuscular bones. These small bones are a problem since they are difficult to remove and undesirable to the consumer.

Future challenges include the development of indirect methods for measuring quality traits on animals before slaughtering. This would allow individual selection, and therefore an increased rate of genetic gain could be achieved.

10.2.9 Quality traits of most importance in fish species

In fish species with high fat content such as salmonids, the most important quality traits to be included in breeding goals are:

- fillet yield
- fat content
- fat distribution
- flesh and skin colour.

In fish species with low fat content such as tilapia, Atlantic cod, Atlantic halibut, river catfish and carp, the most important product quality traits to be included in breeding goals are:

- fillet yield
- intramuscular bone quantity (carp and sea bream)
- flesh colour – whiteness.

10.3 Genetic parameters

10.3.1 Genetic variation – heritability

The magnitude of genetic variation is critical for the rate of improvement which can be obtained in a breeding program. Genetic variation should be estimated from a large data set with hundreds of genetic groups (half- and full-sib families). Heritability (h^2) is estimated by

$$h^2 = \sigma_A^2/\sigma_T^2$$

where σ_A^2 is the additive genetic variance and σ_T^2 is total phenotypic variance. Heritability is not a constant figure, but relates to a specific population. To obtain reliable results, heritability estimates must be based on a large quantity of data from many genetic groups reared under ordinary farming conditions. Table 10.1 shows that heritability estimates of quality traits vary from 0.23 to 0.47 with the exception of colour score for rainbow trout and Atlantic salmon and fat per cent for Arctic char. This tells us that quality traits measured with relatively high repeatability have quite high heritability. Taking into account that the coefficient of variation ($(\sigma_T^2/X) \cdot 100$) is quite high for most traits, it can be concluded that the genetic variation for quality traits in salmonid species is generally large. So far, there is limited information on the genetic parameters of quality traits for marine species. Knowledge of such parameters is mandatory when planning breeding programmes.

10.3.2 Phenotypic and genetic correlations between quality traits

Two traits that are genetically correlated do not vary independently. This is primarily caused by genes that affect both traits. Consequently, a change in one trait will cause some change in the correlated trait. When planning a breeding

Table 10.1 Average heritability estimates for quality traits for different species. Reproduced from Gjedrem (2005, Table 5.7) by permission of Springer

Species	Trait	Average	Coefficient of variation	Heritability	No. of estimates
Rainbow trout	Fillet yield[a]	63.2	–	0.33	1
	Fat percentage	13.1	17	0.37	3
	Colour score	3.6	19	0.17	2
Atlantic salmon	Fillet yield[e]	68.2	2	0.23	2
	Fat percentage	18.8	17	0.38	2
	Fat distribution	8.3	21	0.37	2
	Colour score	3.5	17	0.05	2
	Colour image	7.7	18	0.47	1
	Texture[b]	9.7	4	0.26	1
River catfish	Fillet yield[c]	35.6	20	0.03	1
Channel catfish	Fat percentage	–	4	0.23	3
Nile tilapia	Fillet yield[d]	37.3	16	0.12	1
Arctic char	Fat percentage	20.0	12	0.06	1
	Colour score	–	46	0.28	1

[a] Kause *et al.* (2002); [b] Refstie *et al.* (1999); [c] Sang (2007) personal communication; [d] Rutten *et al.* (2005); [e] Thorland (2007) personal communication.

programme, the magnitude of genetic correlations between traits should be known. Response to selection will be reduced if there are negative genetic correlations between some of the traits included in the breeding goal.

In Table 10.2, phenotypic and genetic correlations are shown for various quality traits. The correlations are averages of reported estimates. Unfortunately, relatively few estimates are available in the literature and the majority of these are from Atlantic salmon and rainbow trout. Since body weight is an important trait in all breeding programmes, it is also included in the table.

Table 10.2 Average estimates of phenotypic (below diagonal) and genetic correlations (above diagonal) between quality traits measured in Atlantic salmon and rainbow trout. Reproduced from Gjedrem (2005, Table 5.5) by permission of Springer, correlations between texture and other traits are from Refstie *et al.* (1999)

Trait	Body weight	Fat percent	Fat distribution	Flesh colour	Texture
Body weight		0.13^{4}	0.17^{1}	17^{4}	0.15^{1}
Fat per cent	0.47^{5}		0.71^{1}	-0.23^{3}	0.19^{1}
Fat distribution	0.53^{1}	0.86^{1}		–	0.10^{1}
Flesh colour	0.14^{4}	-0.03^{3}	–		0.14^{1}

[1–5] Number of estimates

Generally, there are positive genetic correlations between body weight and product quality traits, indicating that selection for increased body weight will result in an increase in the quality traits. For fat per cent and fat distribution, this is not desirable because selecting for faster growth will tend to increase both fat percentage and amount of fat depots. However, when selecting for faster growth rate, the fish will reach market size at younger physiological ages. A general law in physiology is that young animals use most energy for growth of muscles, while older animals deposit more fat. Therefore, this positive genetic correlation between body weight and fat per cent and area of fat depots will not necessarily cause long-term problems. Furthermore, in a selection program, all traits in the breeding goal should be included in the selection index and each trait should be weighted according to genetic correlations and economic weights. When the genetic correlation is small to medium, which is typically the case as seen by the estimates in Table 10.2, it should be possible to achieve selection response for body weight as well as for fat per cent and fat distribution.

Genetic correlations between growth rate and flesh colour are favourable and will result in a correlated response when selecting for growth rate only and a higher response when selection for both traits is performed simultaneously.

Fat per cent and flesh colour show a negative genetic correlation, which is favourable when selecting for reduced fat per cent and increased flesh colour. Texture is positively correlated with body weight and flesh colour, while selecting for reduced fat per cent and fat distribution will tend to reduced texture or firmness in fillets.

In a breeding programme that does not include condition factor and dressing per cent, a correlated response may occur. Selection for increased growth rate tends to increase dressing per cent with little influence on condition factor, and selection for reduced fat per cent will tend to reduce condition factor as well as dressing per cent (Gjedrem, 2005).

10.4 Possibilities for genetic improvement in quality traits

10.4.1 Introduction

With present technology, it is extremely difficult to record the quality traits (at least with any degree of accuracy) listed in Table 10.1 in live fish. As a result, the quality traits must be measured (generally destructively) on relatives of potential broodstock. Due to the very high fecundity of most fish species, testing of full- and half-sib families is the best alternative. Relatively high family breeding value estimation accuracy can be achieved for quality traits in the breeding goal through slaughter and quality trait measurement of 10 to 15 family members. Fish used for carcass evaluation should be slaughtered at normal market size. An efficient breeding programme for the improvement of quality traits must test a large number of families, since the genetic gain will increase with an increasing number of families tested per generation.

The traditional strategy in breeding programmes is to select for all economic

traits simultaneously in the form of a selection index. This has been shown to be more efficient than selecting for each trait independently or using independent culling levels. The advantage of this strategy is that it results in a continuous genetic improvement for all economic traits. The genetic gain per trait will be reduced, however, as the number of traits included in the selection index increases.

In order to meet different requirements in the market, it is possible to develop multiple genetic lines for quality traits. This could be of particular interest for fat percentage since some factories and markets prefer fattier while others prefer leaner carcasses. Crosses between lines could be performed in order to meet even more particular market requirements. A potential disadvantage of such a strategy could be that the quality lines may have a reduced rate of improvement in other economic traits such as growth rate. The development of genetic lines is of particular interest when genotype by environment interaction is significant, but so far this has not been shown to be of importance for quality traits. If interaction is important, special lines should be developed for each environment.

10.4.2 Estimation of genetic improvement

It is possible to make theoretical estimates of genetic improvement based on knowledge of phenotypic and genetic parameters for the traits in question. The following example is given to show how the magnitude of improvement for family selection for fillet yield in tilapia can be estimated using figures given in Table 10.1. Family selection must be applied since fillet yield can only be measured after slaughter. The formula for estimating response to family selection is according to Falconer and Mackay (1996):

$$\Delta G = i\,h^2\,\sigma_P(1 + (n-1)r)/\sqrt{n(1 + (n-1)t)}$$

where ΔG is the genetic gain per generation; i is the selection intensity (in this case broodstock will be selected from 15 families out of total 200 tested, therefore $i = 1.9$); $h^2 = 0.12$; $\sigma_P = 6$ per cent; n is number of progeny per family (set to $n = 15$ in this case); r is the relationship between family members (for full-sib members $r = 0.50$) and t is the correlation between phenotypic values of members of the families (set to $t = 0.20$ in this case).

The expected genetic gain resulting from selecting for fillet yield in one generation will be:

$$\Delta G = 1.9 \cdot 0.12 \cdot 6 \cdot [1 + (15-1) \cdot 0.50]/\sqrt{[1 + (15-1)0.20]} = 1.45$$

The selection response was a fillet yield increase of 1.45 per cent per generation which is very high. Since Nile tilapia has a generation interval of only one year, the improvement in fillet yield will be very rapid, and since tilapia has a relatively low fillet yield, the scope for long-term genetic improvement is large.

10.4.3 Quality traits included in breeding programmes

In 2006 a survey was carried out to establish which quality traits were frequently included in breeding goals. Body weight at harvest was recorded in all programmes and usually included from the beginning. The first family breeding programme in aquaculture, started by AKVAFORSK in 1975 and now run by Aqua Gen AS, did not include quality traits before 1990. The first quality trait included was flesh colour, followed by fillet fat per cent, skeletal deformities and fillet yield. As can be seen from Table 10.3, some programmes include several quality traits in their breeding goal while others only include one or two traits. It is expected that quality traits will be more frequently selected for in the future.

As mentioned previously, some important quality traits are difficult to measure accurately, and some of the methods used are expensive and time consuming. Therefore, there is an increasing need to develop cheap, non-destructive methods for measuring quality traits.

Table 10.3 Number of breeding programmes selecting for quality traits based on a survey of breeding companies in 2006, V. Gjerde (2007) personal communication

Traits recorded and selected for	Atlantic salmon	Rainbow trout	Chinook salmon	Coho salmon	Sea bass, sea bream	Nile tilapia	Atlantic cod
Harvest body weight	9	9	1	2	2	8	2
Dressing percentage	2						
Fillet yield	4	1				4	2
Fat content	5	1	1	1			
Fillet colour	7	3	2	2			
Gaping	1						
Liver index							1
Skin colour		6					
Skeletal deformities	3	2	2		2		1
Condition factor	1	3			2		

10.5 Conclusions

- The demand for a higher quality of farmed fish is increasing and therefore quality traits should be included in breeding goals, particularly in large-scale breeding programmes.
- Each quality trait must be defined precisely.
- Family selection is a necessity until traits can be measured non-destructively on breeding candidates.
- There is a need to develop non-destructive methods for measuring quality traits, particularly on live animals, which will allow individual selection in addition to family selection.
- In general there is a large amount of genetic variation in quality traits and the possibility for genetic improvement is therefore very good.

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11

Alternative lipid sources for fish feed and farmed fish quality

J. G. Bell, University of Stirling, Scotland

11.1 Introduction: why do we require alternative lipid sources for fish feeds?

Aquaculture production globally is estimated to be increasing by around 10.5% per annum, over the last 10 years (Tacon, 2003). This has meant that demand for extruded feeds has increased in line with production. Culture of fish has traditionally used diet formulations based on fish meal (FM) and fish oil (FO). However, while this has been successful, and is scientifically and nutritionally sound, fish oil production worldwide is static, or in decline, and there is no likelihood that production can be increased if fisheries are to be managed in a sustainable fashion (Pike, 2005). Current estimates suggest that > 85% of global fish oil production will be used in aquafeeds by 2010 (Tacon, 2005). The distribution pattern of FO and FM usage in 2002, compared to estimated values for 2012, are shown in Fig. 11.1. Therefore, continued expansion of aquaculture production will require the use of alternative oil sources, with terrestrial plant oils the most likely candidates, to provide the large quantities of oil required, using sustainable methods of crop production. In addition, the rapid rise in prices of marine-derived raw materials, in the past few years, has meant that FO is now more expensive than a number of the major oilseed crops. A further reason to include more plant products, especially vegetable oils, is because levels of persistent organic pollutants, principally dioxins/furans and polychlorinated biphenyls (PCBs), in some FO from the northern hemisphere, may breach new EU limits and prevent their inclusion in aquafeed formulations (Easton *et al.*, 2002; Foran *et al.*, 2005; EC, 2006a,b). In this chapter I will discuss the effects of aquafeeds containing reduced levels of marine raw materials on fish growth

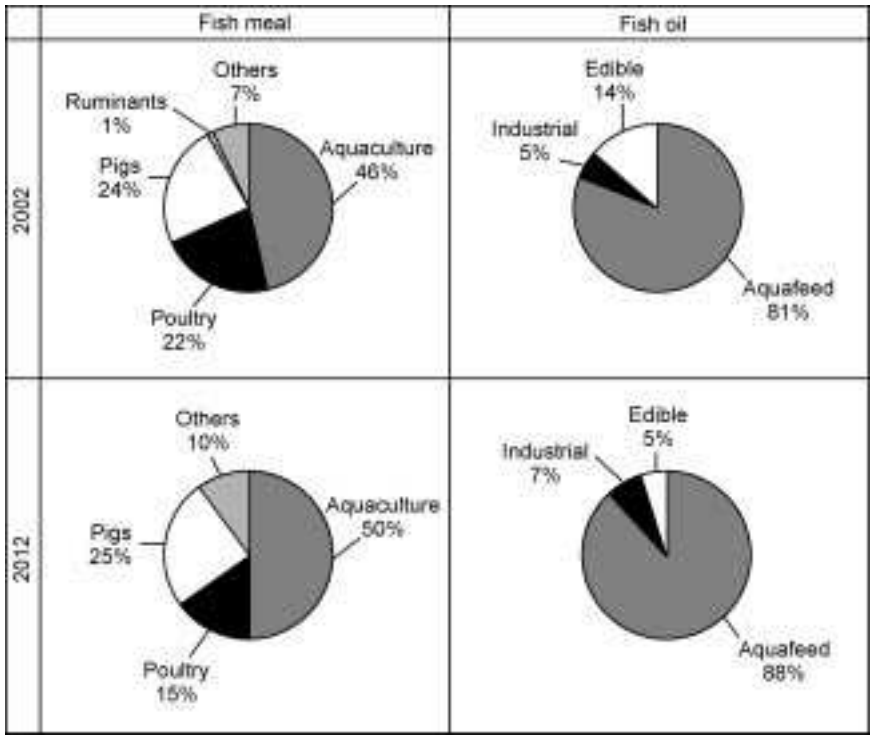

Fig. 11.1 Estimated use of fish meal and fish oil in animal and aquaculture feeds in 2002 and 2012 (source: Pike, 2005).

and health. In addition, the effects of including plant oils on the nutritional quality of farmed fish, in terms of n-3 HUFA content, and their nutritional benefits for human nutrition will be discussed.

11.2 Role of dietary lipids in fish growth performance

The lipid component in aquafeeds must meet both energy and essential fatty acid (EFA) requirements to allow the rapid growth and development required in modern aquaculture production (Sargent *et al.*, 2002). Literature evidence suggests that energy production by the β-oxidation pathway in salmonids shows a substrate preference for 16:0, 16:1, 18:1n-9 and 22:1n-11 as well as 18:2n-6 (Henderson and Sargent, 1985; Henderson, 1996). However, more recent studies with salmon and rainbow trout suggest that linolenic acid (18:3n-3; ALA) as well as eicosapentaenoic (20:5n-3; EPA) and docosahexaenoic acids (22:6n-3; DHA) may also be utilised for energy production when present at higher concentrations (Bell *et al.*, 2001a,b; Stubhaug *et al.*, 2005a,b,c). The high latitude FO, that have been used extensively in salmonid culture, are very rich in 20:1n-9 and 22:1n-11 while VO are rich in different proportions of 16:0, 18:1n-9,

18:2n-6 and 18:3n-3. Several studies have shown that salmonids can be cultured for up to 30 weeks using diets with up to 100% replacement of added FO by VO (Torstensen *et al.*, 2000; Bell *et al.*, 2002; Caballero *et al.*, 2002). Similar studies in marine fish have also confirmed that moderate VO inclusion can be accepted by a range of species including gilthead seabream (*Sparus aurata)*, European sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maxima*) (Caballero *et al.*, 2003; Parpoura and Alexis, 2001; Regost *et al.*, 2003). While salmonids have a limited ability to convert C_{18} polyunsaturated fatty acids (PUFA) to their long chain essential HUFA products, marine fish have a higher requirement for EPA and DHA, as they are unable to perform these bioconversions (Tocher *et al.*, 2002; Sargent *et al.*, 2002). However, even in salmonids the increased flux through the fatty acid desaturation and elongation pathways does not compensate fully for reduced dietary EPA, DHA, and possibly arachidonic acid (20:4n-6; ARA), and sufficient dietary supply of these HUFA will be needed in freshwater, anadromous and marine species (Bell *et al.*, 2001b; Sargent *et al.*, 2002).

Possible criticisms of trials conducted on FO replacements suggest that conducting short-term studies, that represent only a small part of the growth cycle for that species, does not allow a thorough investigation of possible detrimental effects and may be of limited relevance to producing market-sized fish. In the last three or four years a number of long-term feeding studies have been conducted that have gone some way to answering such criticism. In salmon post-smolts, replacement of FO with 25–100% of linseed oil (LO), rapeseed oil (RO) and olive oil (OO) had no effect on growth or survival (Torstensen *et al.*, 2004a; Bell *et al.*, 2004) and confirms results of similar studies using both low (Hardy *et al.*, 1987; Bell *et al.*, 1991; Waagbø *et al.*, 1991, 1993a) or high energy diets in Atlantic salmon (Bell *et al.*, 2001a, 2002; Rosenlund *et al.*, 2001; Torstensen *et al.*, 2000, 2004b). These studies show similar growth responses to other studies that have used RO (Bell *et al.*, 2001a, 2003a,b; Rosenlund *et al.*, 2001) or LO (Bell *et al.*, 1997, 2003a; Tocher *et al.*, 2000) as full, or partial, replacement of FO and show that salmon energy requirements can be satisfied by VO with variable fatty acid compositions. In addition, the lack of any negative growth response suggests that the levels of EPA and DHA, derived from dietary fishmeal, are adequate to fulfil EFA requirements of salmon up to 100% substitution of FO with VO.

A full production cycle trial was conducted in salmon that lasted for 24 months from first feeding larvae to 3 kg market-sized salmon (Torstensen *et al.*, 2005). The dietary fatty acid composition was formulated to provide similar levels of the saturated, monounsaturated and polyunsaturated fatty acid classes to capelin oil, by blending three VOs, namely RO, LO and palm oil (PO) in a ratio of 3.7:2:1 w/w/w. By adopting this approach it was hoped the dietary fatty acid balance might be better physiologically for fish health and welfare than using a single VO. Thus, when salmon were fed either 75 or 100% of the VO blend for the whole production cycle, growth rates were good in all treatments. Interestingly, for the 100% VO treatment, significantly higher final mean weight was found compared to the FO group (Torstensen *et al.*, 2005). The higher mean

weight after 22 months post-first feeding (PFF) correlated with higher protein sparing in the 100% VO group compared to the FO group suggesting that, during the late autumn and winter period of the sea water growth phase, the fatty acid composition of the 100% VO diet favoured protein growth and spared dietary protein from energy production (Torstensen *et al.*, 2005). Previously, studies have shown that dietary lipid content, but not dietary oil source (Torstensen *et al.*, 2000; Bendiksen *et al.*, 2003), affected protein utilisation, growth rate, muscle lipid level and feed conversion (Watanabe, 1977; Arzel *et al.*, 1993, 1994).

In rainbow trout fed 100% of LO, RO or OO for 12 weeks, or the 75% and 100% VO blend for 62 weeks, there were no significant effects of diet on final weight, SGR, TGC or FCR (Kaushik and Corraze, 2004). Several studies with rainbow trout and other salmonids showed similar results where reduction in growth was observed with different VOs, including soybean oil (SO), RO, OO, PO, LO and lard (Dosanjh *et al.*, 1988; Greene and Selivonchick, 1990; Guillou *et al.*, 1995; Caballero *et al.*, 2002; Figueiredo-Silva *et al.*, 2005; Fonseca-Madrigal *et al.*, 2005). However, unlike salmon, no increase in final weight of rainbow trout fed the 100% VO blend was observed. In salmon, the increased growth observed during the winter in Norway may have been due to improved fatty acid digestibility, and thereby increased protein sparing, which lead to better growth at low water temperatures of less than 5 ºC (Torstensen *et al.*, 2005). A similar effect of protein sparing and improved SGR and TGC was seen in a recent study where salmon were fed diets containing 30 and 60% of RO, compared to 100% FO, and grown at low water temperatures (Karalazos *et al.*, 2007). By contrast, the rainbow trout trial was conducted at a constant 17 ºC such that any seasonal differences in digestibility would not be apparent (Kaushik and Corraze, 2004).

In sea bass and sea bream, replacement of up to 60% of FO with VO had no detrimental effects on growth or feed conversion (Izquierdo *et al.*, 2003, 2005; Mourente *et al.*, 2005, 2007). However, replacement with 80% linseed oil or 100% of a VO blend in sea bream did reduce growth rates (Izquierdo *et al.*, 2003, 2005) although, with the VO blend, growth reduction was not observed in fish over 250 g suggesting that EFA requirements in larger fish were lower than in smaller fish. This data is in agreement with a 12-week study using sea bream of start weight 10 g (Izquierdo *et al.*, 2003) while growth reduction was also seen in sea bream fed 80% SO for seven months (Menoyo *et al.*, 2004). However, this result of Izquierdo *et al.* (2005) is contrary to earlier data where growth depression was observed in sea bream fed diets containing 50% VO (Alexis, 1997; El-Kerdawy and Salama, 1997). This can probably be explained by the higher dietary lipid level in the more recent studies (Izquierdo *et al.*, 2003, 2005) (20–25%) compared to the latter (10–12%) (Alexis, 1997; El-Kerdawy and Salama, 1997) as the diets in the more recent studies contained higher n-3 HUFA levels that could maintain optimal growth rates. This is also supported by the data of Ibeas *et al.* (1994) where smaller bream had a high requirement for n-3 HUFA and appeared more susceptible to low HUFA diets in short term growth trials than larger fish.

11.3 Role of dietary lipids in fish health and welfare

In the 21st century, ensuring good standards of animal health and welfare is a very high priority for producers and consumers (Appleby, 1999). Although a precise definition of welfare is contentious, it can be described as changes in culture techniques that induce changes in behaviour, leading to the development of physiological indicators of stress that impact on the health of the animal (FSBI, 2002). Included among factors that may induce physiological stress are provision of sub-optimal nutrition. Formulation of fish diets should always ensure that minimal nutritional requirements are met but changes in the degree of unsaturation of fatty acids, as well as altering the ratios and chain lengths of the EFA, could potentially induce physiological stress in fish. These changes in EFA might subsequently lead to increased disease susceptibility, due to impairment of innate and inducible immune function, and the development of pathology. Development of pathologies may proceed initially without compromise to growth performance but may develop quickly and lead to welfare problems, and ultimately, mortalities. Therefore, early identification of immune problems is vital in maintaining healthy fish stocks and delivering a high quality product.

The nutritional status of an animal, including fish, can influence immune functions and the overall resistance of an organism to disease is thus dependent on their nutritional status (Blazer, 1992). The first review paper implicating fatty acids as important factors in immune status was by Meade and Mertin (1978), while more recent publications have confirmed the importance of the PUFA of both the n-6 and n-3 series, as modulators of immune function in mammals (Calder, 2001; Yaqoob, 2004). Dietary fatty acids are incorporated into the plasma membrane such that the fatty acid composition of cellular membranes closely reflects the composition of the diet (Clamp *et al.*, 1997; Sargent *et al.*, 2002). In fish, changes in the dietary fatty acids, especially PUFA and HUFA, as well as the n-3/n-6 ratio, can influence the composition of immune cells, including circulating blood leukocytes (Thompson *et al.*, 1995; Bell *et al.*, 1996; Mourente *et al.*, 2007).

11.3.1 The role of fatty acids in immune function

Fatty acids have a wide range of functional roles in all cells. In addition to being an important source of energy, they also serve as structural components of cell membranes as well as being precursors for a number of molecules involved in cell signalling. In mammals, dietary fatty acids may be able to modulate the immune system through several mechanisms including reduction of lymphocyte proliferation, phagocytic activity and altered cytokine synthesis and also by modification of natural killer cell activity (De Pablo and De Cienfuegos, 2000). The major factor in the modulation of immune function may be associated with changes in the cell membrane due to dietary fatty acid manipulation. It is probable that modulation of the immune system arises due to alterations in

membrane fluidity, lipid peroxidation, eicosanoid production or regulation of gene expression (Calder, 2006).

Alteration of dietary fatty acid compositions has been shown to affect both innate (Sheldon and Blazer, 1991; Obach *et al.*, 1993; Waagbø *et al.*, 1993b, 1995) and adaptive immunity (Thompson *et al.*, 1995; Waagbø *et al.*, 1993b,c; Erdal *et al.*, 1991; Fracolossi and Lovell, 1994), as well as susceptibility to pathogens (Thompson *et al.*, 1995; Erdal *et al.*, 1991; Waagbø *et al.*, 1993c; Fracolossi and Lovell, 1994; Salte *et al.*, 1988). However, the role and mechanisms by which n-3 and n-6 fatty acids exert their effects on fish immune functions is still unclear and literature evidence is not conclusive and studies are often contradictory.

The modulation of immune activity is likely to occur at different cellular levels, and involve different cell systems, with the most likely being changes in cell membrane phospholipid fatty acid composition, thereby affecting the activity of membrane-bound enzymes, receptors and ion channels (Theis *et al.*, 2001). Furthermore, eicosanoids, a series of bioactive molecules derived from ARA, EPA and dihomo-γ-linolenic acid (20:3*n*-6), and include prostaglandins (PG), thromboxanes, leukotrienes and lipoxins, are involved in regulation of the immune response (Hwang, 1989; Yaqoob, 2004). Additional immune regulatory processes, involving fatty acids, include control of intracellular signalling pathways (Khan *et al.*, 1995) and direct interactions between fatty acids and nuclear transcription factors in immune cells, such as the peroxisome proliferator activated receptors (PPARs), that act to regulate cellular functions (Calder, 2006).

11.3.2 The effect of dietary oils on immune function

Fish tissues and cell membranes, including phagocytic cells (macrophages, neutrophils), contain high concentrations of n-3 HUFA, and their compositions can be altered by changes in dietary lipid (Bell *et al.*, 1996). The importance of EPA, and especially DHA, in immune cell function can be evidenced by the relatively high retention of these HUFA in immune cells, compared to other tissues, when grown on diets with low HUFA for an extended period. In sea bass fed diets containing 60% VO for 64 weeks, DHA in peripheral blood leucocytes was only reduced by 30% while in liver values were reduced by over 50% (Mourente *et al.*, 2007). The fatty acid compositions of monocytes, macrophages, lymphocytes and polymorphonuclear cells (PMN) reflect the dietary fatty acid composition, in mammals (Johnston and Marshall, 1984). In cod, sea bass and sea bream, dietary fatty acid compositions were closely related to the fatty acid profile of macrophages and other immune cells (Waagbø *et al.*, 1995; Farndale *et al.*, 1999; Montero *et al.*, 2003). Montero *et al.* (2003) observed selective incorporation of specific fatty acids, especially DHA, into head kidney macrophages of seabream. Generally, fish that were fed a high FO diet showed the highest n-3 HUFA content in their immune cells (Waagbø *et al.*, 1995; Farndale *et al.*, 1999; Montero *et al.*, 2003; Mourente *et al.*, 2007). Literature

evidence suggests that altering the fatty acid composition of immune cells can influence immune function by changing the physico-chemical properties of the cell membrane but also by influencing the production of regulatory prostaglandins and leukotrienes (Calder, 2006). The synthesis of eicosanoids is influenced, in part, by the availability of C_{20} fatty acids and, especially, the EPA/ARA ratio. In a study with Atlantic salmon fed single replacement VOs, a three-fold difference in the EPA/ARA ratio of immune cells was recorded (Bell *et al.*, 1996) while in a more recent study in sea bass, using blends of VOs, the difference in the EPA/ARA ratio, between the three dietary treatments, was only 13% (Mourente *et al.*, 2007). The relatively minor changes to the EPA/ARA ratio in the latter study might explain the absence of effects on innate immune functions observed in that study.

Reduction in macrophage respiratory burst activity in head kidney has been recorded in sea bass and sea bream fed single replacement VOs (Montero *et al.*, 2003; Mourente *et al.*, 2005). Sea bass fed 60% RO, LO or olive oil showed significantly reduced phagocytic capacity of head kidney macrophage to engulf yeast particles (Mourente *et al.*, 2005), while Montero *et al.* (2003) found reduced macrophage activity in sea bream fed 60% RO. In addition, Sheldon and Blazer (1991) found that macrophage killing activity in channel catfish was positively influenced by increased dietary n-3 PUFA content. They observed that phagocytosis of live *Edwardsiella ictaluri* by catfish head kidney macrophages was not significantly affected by feeding soybean oil compared to fish fed menhaden oil or beef tallow. However, dietary soybean oil significantly reduced the macrophage killing activity of engulfed bacteria, compared to macrophages from fish fed menhaden oil. Macrophages from the menhaden group also had a significantly better killing index than macrophages from fish fed soybean oil (Sheldon and Blazer, 1991). Atlantic salmon fed diets with high n-3 PUFA significantly reduced the bacterial killing ability of macrophages at 12 ºC, but not at 18 ºC, suggesting that temperature, possibly related to membrane fluidity had influenced the macrophage activity (Waagbø *et al.*, 1993b). In contrast, Thompson *et al.* (1995) found no effects on phagocytosis or bactericidal activities of head kidney macrophages in Atlantic salmon fed diets enriched in either n-3 or n-6 PUFA.

There is considerable evidence that dietary fatty acids impact on immune function and, thereby, fish health and welfare although there is considerable variation between different studies both within and between species. Clearly, when using 'alternative' oil sources to replace FO, care must be taken when developing feed formulations to reduce any potential impact on fish health as much as possible. Therefore, current evidence suggests that, at high levels of FO replacement (>60%), a balanced fatty acid composition, similar to that of FOs or natural prey species should be less stressful physiologically compared to the more extreme fatty acid compositions that result when replacing FO with a single VO.

11.4 Role of dietary lipids in eating quality

11.4.1 Effects on lipid content and fatty acid compositions

Fish is rightly regarded as a healthy food choice and increased fish consumption is generally encouraged. The efficacy of EPA and DHA in preventing or attenuating inflammatory disease in humans was first reported around 1970 when epidemiological studies found a reduced incidence of cardiovascular disease in Inuit populations in Greenland and that coastal populations had different disease patterns compared to inland dwellers (Bang and Dyerberg, 1972; Dewailly *et al.*, 2001a,b). The explanation for the differences in disease patterns was due to higher fish and n-3 HUFA consumption in coastal populations.

Aquaculture diets based largely on marine raw materials are of high nutritional quality being rich in EPA and DHA and with an n-3/n-6 ratio of ~4:1 (Kalogeropoulos *et al.*, 1992; Bell *et al.*, 1998, 2004). By contrast, fish grown on diets where >50% of added FO has been replaced with VO show significant reductions in flesh EPA and DHA concentrations (Bell *et al.*, 2003a; Caballero *et al.*, 2002; Montero *et al.*, 2003; Parpoura and Alexis, 2001). Clearly, from a human health perspective, changes to flesh n-3 HUFA content should be minimised to preserve endogenous n-3 HUFA levels as much as possible. But how much fish and n-3 HUFA do we need to consume to provide realistic health benefits? Over the last 20 years public health advisers have sought to recommend beneficial intake levels for n-3 HUFA. One of the first was established by the Committee on Medical Aspects of Food Policy (COMA) that recommended an intake of n-3 HUFA of 200 mg/day compared to an estimated daily intake, in the UK in 1994, of 100 mg/day (DH, 1994). More recently, the recommendation of the Scientific Advisory Committee on Nutrition and the Committee on Toxicity recommended increasing the intake to 450 mg/day (SACN/COT, 2004), against current UK intake values of 282 mg/day of which 244 mg were from EPA + DHA (Givens and Gibbs, 2006). At trans-national level, the International Society for the Study of Fatty Acids and Lipids (ISSFAL) suggested that an intake of 500 mg/day or 3.5 g/week of EPA + DHA should provide optimal cardiac health in humans (www.issfal.org.uk).

In terms of European aquaculture produce, how much fish requires to be consumed to meet these recommended intake values and what is the relative contribution from fish cultured using predominantly FO compared to those with increased inclusion of VO? Consuming around 200 g/week of Atlantic salmon, grown on FO containing diets (capelin oil), can provide the ISSFAL weekly recommended intake of 3.5 g EPA + DHA while around 460 g/week of a salmon grown on 75% VO or 750 g/week of salmon grown on 100% VO would also provide the ISSFAL weekly EPA + DHA intake (Table 11.1; Torstensen *et al.*, 2005). By comparison, salmon grown on a high n-3 HUFA diet of sardine oil can provide up to 5.8 g of EPA and DHA from a 200 g portion (Table 11.1). These values in salmon, grown on capelin oil, were slightly higher than those reported for wild salmon, that would generally have a lower lipid content, but lower than those reported for farmed salmon in recent published reports (SACN/

Table 11.1 Quantity of EPA + DHA (g) provided by a 200 g portion of fish flesh

Species/dietary oil	EPA + DHA g/200 g portion
[a]Atlantic salmon, 100% capelin oil	3.2
[b]Atlantic salmon, 100% sardine oil	5.8
[a]Atlantic salmon, 75% VO/25% capelin oil	1.5
[a]Atlantic salmon, 100% VO	1.0
[c]Rainbow trout, 100% capelin oil	2.0
[c]Rainbow trout, 100% VO	1.2
[d]Gilthead sea bream, 100% anchovy oil	1.2
[d]Gilthead sea bream, 60% VO	0.7
[d]Gilthead sea bream, 100% VO	0.3
[e]European Sea bass, 100% anchovy oil	1.0
[e]European Sea bass, 60% VO	0.7
[f]Atlantic cod, 100% FO	0.6
[f]Atlantic cod, 100% VO	0.3

[a]Torstensen *et al.*, 2005; [b]Bell, unpublished; [c]Kaushik and Corraze, 2004; [d]Izquierdo *et al.*, 2005; [e]Mourente and Bell, 2006; [f]Bell *et al.*, 2006.

COT, 2004; EFSA, 2005). The fish grown on capelin oil, which contains relatively low levels of EPA + DHA, give lower values than in salmon grown using oils with higher n-3 HUFA levels such as sardine oil. By comparison, 200 g of rainbow trout, grown on FO diets, can provide almost 60% of the ISSFAL weekly recommended intake of EPA + DHA and ~340 g/week would be required to fulfill the ISSFAL intake recommendation. Six hundred and fifty grams of trout grown on 100% VO would meet the ISSFAL weekly intake value for EPA + DHA. The values of ~1.0 g EPA + DHA/100g wet flesh reported for trout are similar to reported literature values (SACN/COT, 2004; EFSA, 2005).

In the marine species, 200 g of sea bream, fed FO diets, can provide 35% of the ISSFAL weekly recommended intake (3.5 g) of EPA + DHA while around 580 g/week of bream would be required to fully meet the ISSFAL recommendation. By comparison, consuming more than 1 kg of bream grown on 60% VO, or 2.3 kg of sea bream grown on 100% VO, would meet the ISSFAL weekly intake value for EPA + DHA. Sea bass present broadly similar values to bream such that 200 g of flesh, from fish fed FO, can provide 29% of the ISSFAL weekly recommended intake (3.5 g) of EPA + DHA and around 700 g/week would be needed to fully meet the ISSFAL recommendation. Similar to bream, around 1 kg of sea bass grown on 60% VO would satisfy the ISSFAL weekly intake value for EPA + DHA. In Atlantic cod, 200 g of flesh, from fish fed FO, would provide 17% of the weekly ISSFAL value whereas 200 g of flesh from cod fed 100% VO would provide half that value.

These data show that the delivery of EPA + DHA to the consumer is related to both species and diet and that the species effect is related to the oil content range for that species. Thus, fish with oily flesh, such as salmonids, mackerel, herring or sardines, can provide a higher dietary intake of EPA and DHA, compared with leaner fish, although the contribution of n-3 HUFA from the

leaner species is still significant. Even in very lean white fish, including cod and plaice, whose flesh lipid content is only ~1% of wet weight, the EPA + DHA content of ~0.25 g/100 g can still make an important contribution to the human diet (SACN/COT, 2004; EFSA, 2005). It is also important that the HUFA in lean fish are concentrated in the phospholipid fraction which is regarded as being more digestible and more readily deposited in cell membranes than HUFA supplied as triacylglycerols. Therefore, the current recommendation of the UK Food Standards Agency that we should consume two portions of fish per week, of which one should be lean and the other oily, is sound nutritional advice (www.food.gov.uk).

11.4.2 Effects of dietary oils on organoleptic properties

Although numerous studies have been conducted on replacement of FO in different species there is relatively little data in the literature on organoleptic qualities. In a recent study where salmon were fed either 100% FO, 75% VO or 100% VO the objective sensory taste panel found only minor, although statistically significant, differences between fish fed FO or 100% VO but not between FO and 75% VO (Torstensen *et al.*, 2005). Principal components analysis (PCA) indicated that flesh from salmon fed FO grouped separately to that from salmon fed 100% VO. Specifically, intensity of odour, marine oil odour, colour tone, marine oil flavour and rancid oil flavour all gave significantly higher scores in fresh cooked flesh of salmon fed FO compared to those fed 100% VO. Conversely, vegetable oil flavour scored significantly higher in salmon fed 100% VO compared to those fed FO. However, after feeding a FO-finishing diet for five months, to both FO and 100% VO groups, there were no differences detected in sensory parameters, either by PCA or by the sensory parameter scores. When panellists were asked to rank product preference from 0 (extreme dislike) to 10 (extreme like) no major differences were discerned although a ranking test generally showed a preference for the 100% VO group over the 75% VO or 100% FO group (Torstensen *et al.*, 2005).

Although differences between the FO and VO fish were minor some significant effects were observed in fresh cooked salmon. There was a general preference for the 100% VO salmon which may have been due to reduced rancid and marine flavours and rancid odour detected in this group. However, after feeding a FO finishing diet for five months no differences between fish previously fed FO or VO were detected. In the study described above salmon were fed a blend of RO, PO and LO. In earlier trials, where fish were fed single VOs, at lower levels than the 75% VO used here, organoleptic panels detected differences in salmon taste and odour, taste intensity, fattiness and juiciness and fish aroma (Waagbø *et al.*, 1993a; Thomassen and Røsjø, 1989; Skonberg *et al.*, 1993). In contrast, several studies observed no differences in organoleptic qualities of salmonid flesh when dietary FO were replaced by VOs (Hardy *et al.*, 1987; Koshio *et al.*, 1994; Rasmussen, 2001) although these trials generally used diets with much lower levels of dietary lipid than in the trial of Torstensen *et al.* (2005).

In non-salmonid species, including gilthead seabream and European sea bass, slight changes in organoleptic profiles were observed when FO was partially replaced by sunflower, rapeseed or linseed oils, or a blend of all three. However, all flesh samples produced using any of the FO replacements were well received by the trained sensory panel (Izquiredo *et al.*, 2003). Similarly, when FO was replaced with either soybean or linseed oils in the diets of turbot (*Psetta maxima*) changes in sensory characteristics were perceived, although, as with salmon, the use of a FO-finishing diet eliminated these differences (Regost *et al.*, 2003).

11.4.3 Effects on lipid soluble micronutrients

Fish are rightly regarded as a healthy and nutritious food and this is not only due to their content of n-3 HUFA, as discussed above, but also on the balanced content of micronutrients that they contain. A number of vitamins and minerals, present in high concentrations in seafood, are of particular value due to their roles in prevention of common life style disorders. The FO component of aquafeeds can deliver valuable sources of fat soluble nutrients in particular vitamins A and D that have been implicated with preventative roles in a number of diseases and conditions including osteoporosis (vitamin D), vision disorders (vitamin A), anti-viral activity (vitamin A) and developmental disorders (vitamin A).

Fish provide one of the few natural sources of vitamins A and D where they originate in the lower trophic levels of the marine food chain. The oily fish species with high flesh oil, including salmonids, mackerel, herring, sardines and anchovies among others, contain significant concentrations of vitamin D in their flesh (Ostermeyer and Schmidt, 2006) but lower amounts of vitamin A. By contrast, fish species with low flesh oil levels normally store higher concentrations of fat soluble vitamins in their livers which are not usually consumed directly as foodstuffs but may be taken as vitamin A and D supplements (e.g., cod liver oil). FO-based aquafeeds usually provide sufficient amounts of the lipid soluble vitamins A and D to support fish growth and maintain fish product quality (Graff *et al.*, 2002; Ørnsrud *et al.*, 2002). FO, as well as the oil component of fish meals, contain considerable amounts of vitamins A and D, so provided fish meal and FO are not reduced to zero levels in aquafeeds there should be no risks for vitamin deficiencies when using moderate vegetable-based meal and oil substitutes. However, the benefits of the current vitamin rich aquaculture products, when fish are fed fish meal and FO, would be reduced to some degree, if replacement of these marine raw materials was extensive. Partial restoration of these levels could probably be achieved using dietary supplementation but this may require changes in current legislation, for some supplements, regarding supplementation levels of micronutrients in animal feeds.

11.5 What are the benefits and risks of using different lipid sources?

As discussed earlier in this chapter, the main benefits of reducing dependence on dietary FO would be reduced pressure on a finite resource derived from feed grade fisheries. There is good evidence that salmonids can be grown on diets where 100% of the FO is replaced by VO, and that marine fish can be fed up to 60% VO with no detrimental effects on growth. The principal disadvantage in using high levels of VO is that reductions of DHA and EPA, of between 50 and 65%, have been observed (Bell *et al.*, 2004; Torstensen *et al.*, 2005; Izquierdo *et al.*, 2005; Mourente *et al.*, 2005). A potential benefit of replacing FO with VO would be reductions in persistent organic pollutants (POPs) as the main source of these contaminants in fish flesh is from the FO component of aquafeeds. The sources of environmental contaminants and the risks and benefits of fish consumption are discussed in more detail in Chapters 1 and 2 of this book so only a quick overview of the benefits of FO replacement, with respect to POPs levels is presented here.

The principal organic contaminants of interest are the polychlorinated dibenzodioxins and polychlorinated dibenzofurans, collectively known as dioxins, the dioxin-like polychlorinated biphenyls (DL-PCBs) and the polybrominated diphenyl ethers (PBDEs). Seventeen dioxins and 12 DL-PCBs have been shown to be toxic although individual congeners have different levels of toxicity. For this reason the World Health Organisation (WHO) have established toxic equivalency factors (TEFs), according to their relative toxicity, enabling the calculation of toxic equivalents based on the concentrations of the 29 congeners present in a feed or food (TEQs; Van den Berg *et al.*, 1998). The PBDE flame retardants currently have no toxic values assigned. Replacing FO with VO has potential as a method of reducing POPs in fish flesh as VO generally contain lower levels of these pollutants than most marine fish oils (SACN/COT, 2004). The EU has recently revised dioxin limits and assigned new limits for the 12 DL-PCBs, such that combined values for dioxins and DL-PCBs of 24 ng TEQ/kg for FO, 1.5 ng TEQ/kg for VO, 7 ng TEQ/kg for fish feeds and 8 ng TEQ/kg for fish products have now been implemented (EC, 2006a,b).

The concentrations of dioxins in aquafeed raw materials can vary widely depending on fish species and geographical location of capture. In general, levels in European FO average 4.8 ng TEQ/kg (range 0.7–20) while in FO from the Pacific the average is much lower at 0.6 ng TEQ/kg (range 0.16–0.61) while VO have an average value of 0.2 ng TEQ/kg (SCAN, 2000). Clearly, using FO from the Pacific Ocean could have immediate benefits in reducing the dioxin and DL-PCB levels in feeds and fish (Lundebye *et al.*, 2004), with no reduction in n-3 HUFA levels, although this would only be a short-term solution, as comprehensive switching to Pacific FO would not be sustainable in the longer term. In salmon fed 100% FO, 75% VO or 100% VO over a whole production cycle, the flesh concentrations of dioxins + DL-PCBs, when harvested at 2.5–3 kg, were 1.9, 0.63 and 0.30 ng TEQ/kg, respectively (Berntssen *et al.*, 2005; Bell *et al.*,

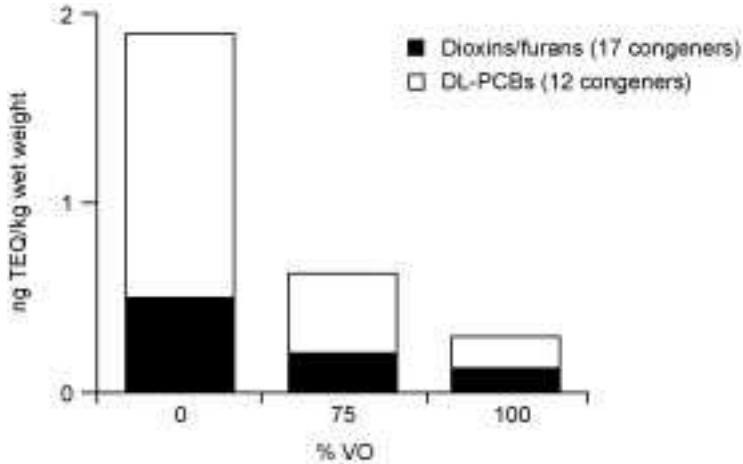

Fig. 11.2 Concentrations and dioxins/furans (■) and dioxin-like PCBs (□) in flesh of Atlantic salmon fed either fish oil (0), 75% vegetable oil (VO) or 100% VO for the whole production cycle. (Data from Berntssen *et al.*, 2005 and Bell *et al.*, unpublished.)

unpublished; Fig. 11.2). This represents a 67% reduction in fish fed 75% VO and an 84% reduction in fish fed 100% VO, compared to fish fed 100% FO. To summarise, these values confirm that replacement of marine FO with VO, in aquafeed formulations, can significantly reduce dioxin and DL-PCB concentrations in farmed salmon flesh.

11.5.1 Using finishing diets to restore n-3 HUFA levels in flesh

The potential health benefits of fish consumption, due to the n-3 HUFA content, are currently well recognised (Connor, 2000; SACN/COT, 2004) and it is vital that cultured seafood maintains a healthy profile by producing a product that is as good, if not better than that produced from capture fisheries. However, the evidence from trials where fish are cultured on diets containing VO, particularly where >50% of FO is replaced, results in significant reductions in flesh EPA and DHA (Bell *et al.*, 2004; Torstensen *et al.*, 2004a,b; Menoyo *et al.*, 2004; Mourente *et al.*, 2005). To restore n-3 HUFA levels, fish can be fed a FO-containing finishing diet in the weeks leading up to harvest in an attempt to restore flesh HUFA concentrations. Generally, the restoration of EPA and DHA was more readily achieved than the removal or dilution of the 18:2n-6 and 18:3n-3 present in the VO (Bell *et al.*, 2004; Torstensen *et al.*, 2004b, 2005; Mourente *et al.*, 2005; Izquierdo *et al.*, 2005). In salmon, the DHA and EPA were restored, to at least 80% of the values in fish fed FO, in fish previously fed VO, compared to fish fed FO throughout the production cycle, with only small increases observed after a further eight weeks on FO (Bell *et al.*, 2004). In comparison, sea bass and sea bream showed almost complete restoration of flesh EPA and DHA concentrations after 14 weeks on a FO-finishing diet (Mourente *et al.*, 2005; Izquierdo *et al.*, 2005).

Thus, the use of FO finishing diets, at the end of the production cycle, can largely restore n-3 HUFA levels in fish prior to harvest. However, it is likely that, by using more FO at the end of the production cycle to increase n-3 HUFA, dioxin and DL-PCB concentrations could also be increased. Bell *et al.* (2005) successfully used a 24-week finishing diet period to restore flesh DHA and EPA concentrations to >80% of the value seen in fish fed only FO. In addition, the flesh dioxin and DL-PCB concentrations, in the fish previously cultured on 100% VO diets, following the 24-week finishing diet period, were still 60 and 47% lower, respectively, than in fish cultured on FO for the whole production cycle (Bell *et al.*, 2005). This indicates that FO finishing diets can be used to successfully restore n-3 HUFA levels, at the end of the production cycle, with only slight increases in flesh contaminant concentrations.

11.5.2 What are the best oils to use as fish oil substitutes?

The lipid component of aquafeeds must meet both energy and essential fatty acid requirements of the fish to allow the rapid growth and development required in modern aquaculture production (Sargent *et al.*, 2002). Evidence suggests that β-oxidation activity in salmonid fish has a substrate preference for 16:0, 16:1, 18:1n-9 and 22:1n-11 as well as 18:2n-6 (Henderson and Sargent, 1985; Henderson, 1996) although more recent studies with salmon and rainbow trout suggest that 18:3n-3, EPA and also DHA, may be utilised for energy production when present at higher concentrations (Bell *et al.*, 2001b, 2003a; Stubhaug *et al.*, 2005a,b,c). The high latitude FO, that are currently favoured in salmonid production, are very rich in 20:1n-9 and 22:1n-11 while the different VO are rich in some or all of 16:0, 18:1n-9, 18:2n-6 and 18:3n-3. Ideally, the 'optimal oil' should contain largely saturated and monounsaturated fatty acids that are readily utilised for energy production allowing maximum retention of existing HUFA for essential functions in cell membranes and/or enhancing production of HUFA from moderate amounts of C_{18} precursor fatty acids. Generally, reduction of DHA and EPA appeared to be less in fish fed VO with a low PUFA content e.g. olive oil, (Bell *et al.*, 2004; Torstensen *et al.*, 2004b), palm oil (Bell *et al.*, 2002; Ng *et al.*, 2004, 2007), a blend of FO, palm and rapeseed oils (Ng *et al.*, 2003) or a blend of rapeseed, palm and linseed oils (Torstensen *et al.*, 2005; Izquierdo *et al.*, 2005; Mourente *et al.*, 2007) than in fish fed oils high in PUFA.

The selective deposition and retention of specific fatty acids, rather than mobilisation and metabolism for energy, may reflect the structural importance of these fatty acids in membrane phospholipids, where 16:0 and 18:1n-9 are often located in the *sn*-1 position of phospholipids, especially in phosphatidylcholine (PC) and phosphatidylethanolamine (PE), with PUFA and HUFA being favoured in the *sn*-2 position (Bell and Dick, 1991; Sargent *et al.*, 2002). This specific incorporation of saturates and monoenes in the *sn*-1 position, and PUFA and HUFA in the *sn*-2 position, may be important in the relative retention efficiency of EPA and DHA when fish are fed different oils as described above.

When a VO low in PUFA is used in an aquafeed, there will be less competition for the *sn*-2 position and retention of EPA and DHA will be enhanced compared to feeding a VO with high PUFA levels that will tend to compete at the *sn*-2 position and displace EPA and DHA, thereby, reducing tissue HUFA levels. For that reason the best oils for FO substitution will be oils that are high in monounsaturates and saturates, providing digestibility of the latter is not compromised by low culture temperature, and have only low or moderate PUFA levels, especially 18:2n-6 which is not especially favoured for β-oxidation. These oil compositions can be fulfilled by olive, palm and high oleic sunflower and rapeseed oil varieties, or by using oil blends while oils with high PUFA levels such as soybean, linseed, sunflower, etc., are better avoided, or used only sparingly.

However, there is evidence that tissue HUFA content is a heritable trait in poultry (Mennicken *et al.*, 2005), and it is likely that flesh n-3 HUFA content will similarly be a heritable trait in fish. Thus, in addition to dietary oil selection, the development of selective breeding strategies that select fish strains that have a high HUFA retention will be of great benefit to the future development of commercial aquaculture, particularly if FO prices continue to rise and are replaced by more sustainable VO that are devoid of HUFA.

11.6 Future trends

It has been established that, with judicious care, much, if not all, of the FO currently used in the production of freshwater, anadromous and marine fish can be replaced with single VOs, or blends of VOs, without comprising growth performance. Changes in fatty acid composition of flesh arising from VO inclusion can be readily restored using a FO finishing diet. Thus, much of the FO used in aquafeeds is currently wasted in that it can easily be replaced with more readily available and sustainable VOs. This is logical since most of the oil used in fish feeds is catabolised to provide energy for growth. A suitably blended VO can be catabolised and provide as much energy for growth as FO, provided the EFA requirements of the fish are met. The EFA requirements can be met either from the FO component of fish meal or by addition of small amounts of FO or other marine raw materials rich in EPA and DHA.

In the short term, the currently available high production volume VOs are likely to meet most of the requirements as FO replacements. These will include rapeseed and palm oils with smaller additions or blends including soybean oil and linseed oil. The new high oleic acid variants, developed either by selective breeding or genetic modification, including high oleic sunflower oil (sunseed), rapeseed and soybean oils may also have practical application in fish feeds, if the price is competitive with the conventional oilseeds described above. In the longer term, other VOs, that are not currently high production oils, may have compositions that would make them suitable for inclusion in fish feeds. These include *Camelina sativa* a plant that is known by many names in many regions

(e.g., German Sesame and False Flax) due to its long history as an oilseed crop. The principal fatty acids are 18:3n-3 (~38%), 18:2n-6 (~18%) and interestingly 20:1n-9 (~16%), which is more commonly found in high latitude fish oils and is a rich energy source (Marquard and Kuhlmann, 1986; Sargent *et al.*, 2002). Another potentially interesting plant oil is from *Limnanthes alba* (Meadowfoam seed oil^{TM}). This plant oil, which has been likened to sperm whale oil, in terms of its functional characteristics, is composed of four principal fatty acids namely, 20:1n-15 (~60%), 22:1n-17 (5%), 22:1n-9 (~12%) and 22:2n-9 (18%) (Pollard and Stumpf, 1980). As Meadowfoam is rich in monounsaturates this oil should be a good source of dietary energy. An additional plant oil that may have potential for use in aquafeeds is from the genus *Cuphea*. *Cuphea* species are characterised by high concentrations of medium chain saturated fatty acids, especially 8:0, 10:0, 12:0 and 14:0. For example, *Cuphea koehneana* has 95% of fatty acids as 10:0, *Cuphea carthagenesis* has 5% 8:0, 81% 10:0, 5% 12:0 and 9% 14:0 and *Cluphea epilobiifolia* has 20% 10:0, 68% 12:0 and 12% 14:0 (Hirsinger, 1985). Since these medium chain fatty acids should not be stored as triacylglycerols in vertebrate tissue they can provide an easily metabolised energy source in fish feeds thereby sparing other more essential fatty acids, such as n-3 HUFA, from oxidation.

In recent years significant advances have been made in the production technology of single cell oils. Photo-bioreactors have been used to generate enriched EPA oils from algal species such as *Phaeodactylum and Porphyridium* although the EPA tends not to be in triacylglycerol form but associated with galactolipids (Yongmanitchai and Ward, 1989; Cohen, 1990). However, the heterotrophic algae, such as *Crypthecodinium* species, and the marine protists including *Schizochytrium* and *Thraustochytrium* species, store large amounts of DHA and other HUFA as triacylglycerols and phospholipids (Ward and Singh, 2005). While single cell oils can provide a high quality product rich in HUFA, that is essentially contaminant-free, the current production costs for these oils, which are mostly used for human supplements, would only allow small amounts to be used in fish feeds. Therefore, presently they would not be regarded as cost-effective options but could conceivably be used in the future as HUFA concentrates to provide EFA requirements in diets with low or no marine-derived raw materials. In recent years, advances in genetic modification technology have resulted in a number of transgenic oilseed variants, including soya, canola and the mustard-type species, *Brassica juncea* and *Arabidopsis thaliana,* being developed that can produce EPA and DHA (Napier *et al.*, 1999; Lopez and Maroto, 2000; Wu *et al.*, 2005). While production of variants with moderate amounts (10–15%) of EPA has been possible only minor production of DHA (<1%) has been possible. Thus, at the present time GM oilseeds are unlikely to fill the role of FO in aquafeeds but may become more important in the future as the technology is developed.

A further alternative to FO may lie in the use of marine oils from lower trophic levels, such as krill and calanoid copepods. Both *Euphasia suberba* from the Antarctic and krill from northern latitudes (*Meganyctiphanes norvegica* and

Thysanoessa inermis) are rich in HUFA, especially EPA and DHA, which means they can provide the essential fatty acids required for both freshwater and marine fish (Ackman *et al.*, 1970; Ellingsen, 1982; Falk-Petersen *et al.*, 1982). Krill oil is also a rich source of the carotenoid astaxanthin with values ranging from 727–1080 mg/kg (Yamaguchi *et al.*, 1983; Fujita *et al.*, 1983). Recently, oils have been produced from *Calanus finmarchicus*, which is the most abundant herbivore in the Nordic seas (Melle *et al.*, 2004). However, while these oils are rich in n-3 HUFA, as well as energy-rich long chain monoenes, this is mainly found as wax esters rather than triacylglycerols (Sargent and Henderson, 1986). Wax esters can be digested by fish but generally at a slower rate than triacylglycerols (Mankura *et al.*, 1984; Olsen and Ringø, 1997). Studies on the application of copepod oils in aquafeeds are currently being conducted with a recent study in Atlantic salmon showing a high acceptability and growth compared to fish fed FO (Olsen *et al.*, 2004). The main problems associated with the use of krill and copepod oils are likely to be the high energetic costs in capturing the animals, the processing of the material that can degrade rapidly and the possible concerns of NGOs regarding sustainability and potential damage to the marine food web.

At the present time an increasing amount of FO available in the northern hemisphere is unsuitable for use in aquafeeds due to high levels of POPs that are above current EU permitted levels (EC, 2006a,b). Recently a number of pilot plant facilities have been developed in Europe to remove dioxins and PCBs from FO largely by the use of activated carbon stripping (Maes *et al.*, 2005). Further development of this process using a combination of activated carbon, coupled with low pressure and low temperature stripping, could effectively remove around 90% of all dioxins and PCBs from FO (www.desmet.com). Although these processes will increase the cost of FO slightly, the benefits in terms of reduced POP transfer to aquaculture products and economy advantages with scaled up production plants mean that decontaminated FO are likely to be widely used in the near future.

11.7 References

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12

Plant proteins as alternative sources for fish feed and farmed fish quality

S. J. Kaushik, INRA, France and G.-I. Hemre, National Institute of Nutrition and Seafood Research (NIFES), Norway

12.1 Introduction

On a worldwide scale, fisheries production remains constant at about 90 million tons of fish whereas aquaculture already supplies about 50 million tons and is on a constant increase, growing at a rate of over 8% per annum, well above the average growth rate in terrestrial animal production. Almost half of the fish products eaten by man thus come from aquaculture. This growth of aquaculture, especially of finfish and prawns, has resulted in an increased need for specialised compound foodstuffs, estimated at 20 to 25 million tons. Although this is only a small portion of the global animal feed production of around 620 million tons, the very specific nature of the aquafeeds implies more attention as regards implementation of principles of sustainability than for terrestrial animal feeds. Most of the intensively farmed fish species are recognised as having high dietary protein requirements. Consequently, the feeds used in aquaculture generally have higher total protein (>35% of dry matter) and fat (>10%) contents than those used for conventional animal feeds. In intensive farming, the major portion of proteins and lipids are supplied in the form of fish meals and oils.

12.2 Global fish meal resources and availability

Worldwide annual production of fish meal (about 6 million tons) and fish oil (< 1 million tons) has remained fairly stable for the last 20 years. Fish meal and fish oil are produced from dedicated pelagic fisheries, mainly from South America and northern Europe. Efforts are constantly underway to ensure that the marine

fisheries on which fish meal and fish oil depend remain sustainable and are not over-exploited. Fish meal and oil are also produced from trimmings, offal and/or by-catch, although to a limited extent. But, within the European Union, it was estimated that in 2002 about 33% of the fish meal produced was manufactured from trimmings from food fish processing (Huntington *et al.*, 2004). No comparable data is available at the global level (FAO, 2005).

The high dependence of aquaculture on these ingredients, themselves derived from dedicated feed-grade fisheries, prompts questions as to the sustainability of such practices (FAO, 2002). Given the predictable increase in requirements for aquaculture, the risk of deficits in these ingredients is real. From a global perspective, it is recognised that the pressure on natural marine resources should be diminished. For the preservation and optimal use of natural fish stocks and for the sustainable development of aquaculture, research on alternative protein and oil sources is essential (FAO, 2003) and has gained momentum over the last few years, the main objective being to meet the protein and amino acid requirements of fish and shrimp without relying far too heavily on fish meal and fish oil. We shall focus here mainly on plant protein ingredients as alternatives to fish meal, other separate chapters in this book dealing with other protein sources and replacement of fish oil.

12.3 Meeting protein and amino acid requirements in fish

For fish to thrive and grow well, it is most important that we provide, more than dietary proteins *per se*, adequate amounts of the indispensable amino acids (IAA) and in the right proportions. For fish and shrimp, the ten IAA are: arginine, histidine, isoleucine, leucine, lycine, methionine, phenylalanine, threonine, tryptophan and valine (Halver *et al.*, 1957; Cowey and Forster, 1971). Thus, the quality of a protein depends upon its amino acid composition and the availability of the constituent amino acids to the animal that consumes it (Friedman, 1996). An amino acid is usually classified according to whether or not protein synthesis and growth can proceed in the absence of a dietary supply. In addition to being component parts of proteins, several of the amino acids are precursors for the synthesis of other biologically active compounds (Table 12.1): for instance, histidine is decarboxylated to form histamine, tyrosine is iodinated to form thyroid hormones, and is also used in the synthesis of the catecholamines; tryptophan is the presursor of serotonin and melatonin.

From a quantitative point of view, although hundreds of species of finfish and a score of crustaceans are being farmed, precise data on requirements for all the ten IAA are not available for all species and there is much variability in the literature on amino acid requirements of fish. Data on average values on IAA requirements of fish and of shrimp are reported in Table 12.2. Cowey (1994) observed that much of this variability can be attributed to methodological issues: experimental conditions, levels and sources of amino acids, dietary energy density, fish size, growth rates, etc. Some recent reviews have updated the IAA

Table 12.1 Examples of non protein nitrogenous compounds derived from amino acids

Amino acid	Product(s) formed
Arginine	Polyamines, urea, nitric oxide, creatine
Histidine	Polyamine (histamine)
Lysine	Carnitine
Methionine/cystine	Taurine, choline, creatine, putrescine, glutathion
Phenylalanine	Catecholamines
Tyrosine	Thyroid hormones
Tryptophan	Serotonine, melatonin
Alanine	Dopamine
Aspartic acid	Pyrimidines
Glutamine	Purines, pyrimidines
Glycine	Creatine, purines, porphyrines
Serine	Ethanolamin

needs of major species of interest (Lall and Anderson, 2005; Tibaldi and Kaushik, 2005).

A critical analysis and re-evaluation of lysine requirement data from all species was undertaken by Hauler and Carter (2001). Their major conclusion was that despite the variance in available data on lysine requirement, attributable to differences in experimental designs, conditions, species, methods of interpretation, etc., the amount of lysine required per unit body mass gain would be relatively constant. They proposed a value of 18.5 g lysine required for 1 kg of whole body weight gain. Since almost all of these data on IAA requirements are based on studies with young fish of small size over relatively short periods, the question arises as to whether these data reflect the requirements for fast growing fish over the full cycle and the best criterion to use. Recent data from the studies of Espe *et al.* (2007) with big Atlantic salmon

Table 12.2 Average data on indispensable amino acid requirements (expressed as g/16 g N) of several teleosts compared to that of shrimp

	Different fish		Shrimp
IAA	Avg	std	
Arg	4.1	0.72	5.3–5.8
His	1.62	0.22	2.2
Ile	2.3	0.28	4.6
Leu	3.21	0.36	6.8
Lys	4.62	0.23	6.4
Met + Cys	2.74	0.56	2.6
Phe + Tyr	4.83	0.49	5.2
Thr	2.5	0.78	4.6
Trp	0.59	0.19	1.2
Val	2.85	0.32	4.8

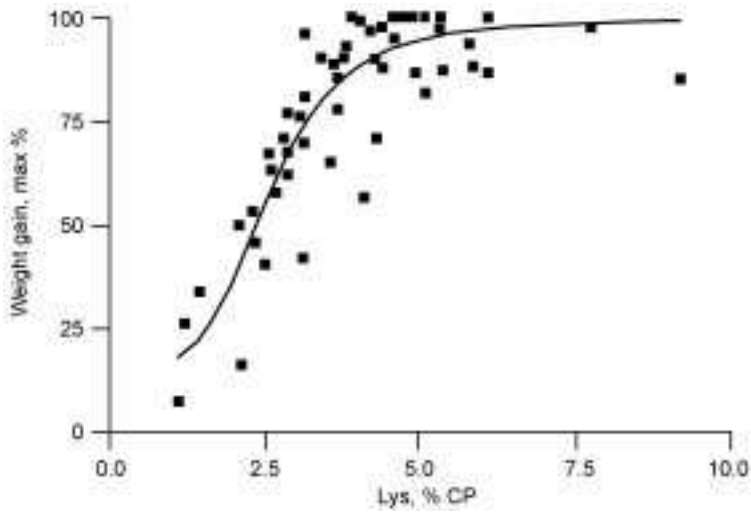

Fig. 12.1 Relation between dietary lysine (g/16 g N) and maximum weight gain in salmonids. Data for Atlantic salmon are from Anderson *et al.* (1993), Berge *et al.* (1998) and Espe *et al.* (2007) and those for rainbow trout are from Ketola (1983), Walton *et al.* (1984), Kim *et al.* (1992) and Encarnacao *et al.* (2004).

(>300 g initial weight) suggest that for maximum protein accretion, a dietary lysine supply should be 5.04 g/16 g N, a value higher than earlier data for the same species (Anderson *et al.*, 1993). A meta-analysis of available data on lysine requirement for maximum weight gain of salmonids indeed shows that the requirement would be around 5 g/16 g N (Fig. 12.1). More than whole body weight gain, it is necessary that we use body protein accretion as a criterion. Green and Hardy (2002) undertook a study with rainbow trout and showed that the IAA pattern associated with IAA requirements as published by NRC (1993) was found to result in the best protein utilisation and so was considered the best estimate of optimum IAA pattern.

Mambrini and Kaushik (1995) compared the indispensable amino acid composition of a number of tissues and species of fish and found the IAA composition of a given tissue to be highly conserved among species, irrespective of biotic or abiotic factors and that the whole body amino acid composition best reflected the ideal pattern for a reference protein. Akiyama *et al.* (1997) used the A/E ratios to compare the amino acid profiles of fish to the IAA requirement profiles and found that differences between species in their whole body amino acid profiles were in fact lower than those between reported data on requirements, again suggesting that the observed differences in requirement data are more apparent than real. Applying the same approach to data on the whole body amino acid composition from a greater number of species and size groups, a dissimilarity diagram of whole body IAA profiles of different teleosts, prawn and pig was drawn and it was shown that there is indeed very good homogeneity between species that are phylogenetically very distinct (Kaushik and Dias,

2001), which is not surprising given the relatively great constancy of the different protein fractions in most animals. Rollin *et al.* (2003) attempted to determine the optimum IAA balance for Atlantic salmon fry by calculating the balance from the reduction in N gain after replacing about 45% of a single IAA by a mixture of dispensable AA in isonitrogenous diets. They found that the mixture of AA simulating the AA pattern of cod-meal protein and gelatine was used with the same efficiency as cod-meal protein and gelatine. Expressed relative to lysine (100), the optimal balance for different amino acids was derived. This AA composition was found to be close to that of the Atlantic salmon whole-body, with some restrictions as regards some amino acids.

Given that muscle protein growth is the ultimate objective, studies have been undertaken with rainbow trout and gilthead seabream (de Celis *et al.*, 2004) to check whether diets with IAA profiles reflect either that of the muscle or that of the whole body. Growth rates were not significantly different among groups, showing that whole body IAA profile is well suited. As regards ideal ratio between indispensable to dispensable amino acids (IAA:DAA ratios), Green *et al.* (2002) fed rainbow trout six levels of dietary IAA:DAA ratios ranging from 23:77 to 66:34 and found that maximum N retention could be achieved with an IAA:DAA ratio of 46:54; they also concluded that there are close similarities between different animal species including mammals, birds, and fish regarding the optimum range of IAA/DAA ratio for maximum protein utilisation.

12.3.1 Protein and amino acid metabolism: general pathways of protein utilisation

One major explanation for the high protein requirements in fish is that they utilise a significant portion of the dietary protein to meet their energy needs (Cowey and Luquet, 1983). But, despite their high protein/amino acid requirements, fish are recognised as being more efficient converters of dietary digestible protein to edible protein available for man than other farmed animals. Although the major pathways of intermediary metabolism are common to fish and terrestrial animals, the ammonitelic nature confers fish distinct advantages in terms of metabolisable energy available per unit protein intake. Nevertheless, as fish diets contain high amounts of protein, a large amount of dietary energy is derived from nitrogenous compounds, leading to increased production of ammonia. Enhancing nitrogen retention through optimisation of digestible protein to digestible energy ratios contributes to protein-sparing and to a reduction of ammonia production, a phenomenon demonstrated in almost all finfish and crustaceans. Besides, the quality of dietary protein and amino acid balance is known to affect nitrogen excretion. Thus, in the context of fish meal replacement, the ideal amino acid balance deserves attention.

12.3.2 Protein synthesis and degradation

Rates of protein synthesis, both in the whole-body and in individual tissues, correlate well with whole-body growth in fish (Fauconneau, 1985; Houlihan *et*

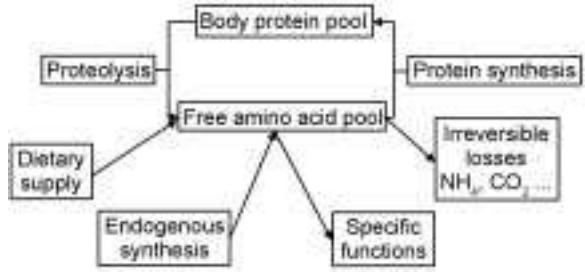

Fig. 12.2 Schematic diagram of protein/amino acid utilisation.

al., 1995; Bolliet *et al.*, 2000). However, overall protein growth is a resultant of differences between protein synthesis and degradation (turnover) and is constantly in a dynamic state (Fig. 12.2). Increased protein growth can occur either due to an increased protein turnover or via a reduction in protein degradation at a given rate of protein synthesis. Fractional rates of protein synthesis measured in several fish show that the rates are low in white muscle in comparison to those in other active tissues such as the liver or the digestive tract; but given the relatively high proportion of muscle tissue, muscle protein synthesis contributes to most of whole body protein synthesis and growth. From a comparative point of view, although the protein synthesis rate is low in trout, the efficiency of deposition of synthesised protein in terms of muscular growth is very high, the efficiency being above 75% (Fauconneau, 1985; Houlihan *et al.*, 1995).

An increase in the supply of some of the dietary IAA is known to increase protein synthesis and/or deposition in terrestrial animals. No such increase in protein accretion has so far been demonstrated in fish receiving indispensable amino acid supplements above the requirement levels. A dietary amino acid imbalance, on the other hand, increases protein synthesis as well as degradation in fish (Langar *et al.*, 1993). Similarly, in the European eel, de la Higuera *et al.* (1999) observed that muscle protein synthesis rates were modified by dietary protein sources and that supplementation with limiting amino acids increased protein synthesis rates.

It has been shown that in all teleosts, protein breakdown contributes to a great extent to the provision of energy and the values are much higher in fish than in terrestrial vertebrates. But we do not yet have an easy way of measuring protein degradation *in vivo*. We also do not have a clear picture as regards the relative importance of the different pathways involved in muscle protein degradation: ubiquitine-proteasome system, lysosomal system involving cathepsins or Ca-activated system involving calpains and calpastatins. Mommsen (2004) suggested that in migrating salmon, lysosomal cathepsins, especially cathepsin D and sometimes cathepsin L, are responsible for the degradation of muscle protein during fish migration, maturation and starvation. This is in contrast to what is generally known in mammals, where the ubiquitin proteasome, in conjunction with ancillary systems, constitutes the major pathway for muscle

protein degradation. Martin *et al.* (2001) similarly found that expression and the amount of cathepsin D, a lysosomal endopeptidase involved in protein degradation, were increased in rainbow trout under starvation conditions. Amino acids are known to be regulators of protein degradation (Kadowaki and Kanazawa, 2003), but precise knowledge on the impact of dietary amino acids on muscle protein degradation in aquatic organisms, generally confronted with high dietary amino acid intake, is yet to be obtained.

Recent data show that muscle protein accretion is closely linked to muscle myosin heavy chain expression in fish (Hevrøy *et al.*, 2006). As regards the impact of dietary protein sources on changes in muscle protein metabolism, very few studies have dealt with potential effects on muscle protein degradation (Langar *et al.*, 1993) or muscle protein fractions (von der Decken and Lied, 1993). The latter found that decreasing dietary fish meal level led to significant reduction in the amount of sarcoplasmic protein in the muscle of cod.

12.3.3 Plant protein sources as alternatives to fish meal in the diets of fish and shrimp

Compared with terrestrial animal and plant protein sources, fishmeal is unique in that it is not only an excellent source of high quality animal protein and essential amino acids, but is also a good source of digestible energy, essential minerals and vitamins, and lipids (Hertrampf and Piedad-Pascual, 2000). At present, most formulated feeds used in aquaculture of fish and shrimp already contain variable levels of protein-rich plant derived ingredients. The limitations as regards their incorporation in the diets for fish and shrimp as in any other farmed animal are related to the amino acid profiles of these feedstuffs and the presence and levels of potential anti-nutritional factors (ANF). For instance, plant protein sources that are particularly low in lysine are corn and wheat and most oil-seeds have low levels of methionine. Table 12.3 gives a small overview of amino acid profiles in some of the most abundant plant protein ingredients used as alternatives to fish meal.

12.3.4 Protein-rich plant feedstuffs

A few feedstuffs of plant origin contain relatively high crude protein levels (>65%), similar or close to the levels found in fish meals. These include soy or rape protein concentrates or isolates, corn gluten meal, wheat gluten, rice protein concentrate, etc. Given that extraction procedures vary, the biological values of such ingredients are often found to vary but they hold much promise in terms of supplying high levels of protein compared to conventional oilseed meals or pulses.

Soy protein concentrate (SPC) was found to hold potential even as total fish meal substitute in the diets for rainbow trout (Kaushik *et al.*, 1995). They found that total replacement of fish meal with alcohol-water extracted SPC sufficiently supplemented with methionine did not have any adverse effect in terms of

Table 12.3 Amino acid profile in commonly used plant protein resources, expected to be important for fish feed (data expressed as g/16 g N, are of indicative nature only, since large batch variations can occur)

	Soy prot conc	Wheat gluten	Corn gluten meal	Soybean meal	Rapeseed meal	Sunflower meal	Lupin	Peas	Fish meals
ARG	7.7	2.5	3.2	7.4	6.0	8.2	10.9	8.6	5.8
HIS	2.4	1.5	2.1	2.6	2.6	2.4	2.2	2.5	2.4
ILE	4.8	2.9	4.1	4.6	4.0	4.1	4.6	4.2	4.3
LEU	8.1	5.3	15.9	7.4	6.7	6.1	7.3	7.1	7.2
LYS	6.5	1.2	1.8	6.1	5.3	3.6	4.9	7.3	7.5
MET	1.2	1.4	2.4	1.4	2.0	2.3	0.8	1.0	2.8
CYS	1.2	1.5	1.8	1.5	2.4	1.7	1.6	1.3	0.8
MET+CYS	2.4	2.9	4.2	2.9	4.5	4.0	2.4	2.3	3.7
PHE	4.9	4.0	6.2	5.0	3.9	4.4	3.9	4.7	3.9
TYR	3.7	2.5	5.0	3.3	2.9	2.3	4.7	3.1	3.0
PHE+TYR	8.6	6.5	11.2	8.4	6.8	6.7	8.6	7.8	7.0
THR	4.2	2.1	3.3	3.9	4.3	3.6	3.7	3.8	4.2
TRP	1.1	0.6	0.5	1.3	1.2	1.2	0.7	0.9	1.0
VAL	4.5	3.2	4.6	4.8	5.0	4.9	4.3	4.7	5.1
ALA	4.2	2.0	8.7	4.4	4.4	4.4	3.4	4.4	6.3
ASP	11.7	2.6	6.0	11.3	7.1	8.8	10.6	11.6	9.2
GLU	18.0	26.5	20.7	17.8	16.9	19.0	20.8	16.3	12.9
GLY	4.5	2.6	2.7	4.2	5.0	5.7	3.9	4.4	5.7
SER	5.0	3.6	5.1	5.0	4.4	4.3	5.3	4.7	3.9
PRO	5.0	7.0	8.8	4.9	6.1	4.4	4.2	4.2	4.1

growth, nitrogen utilisation or flesh quality. Yet, Mambrini *et al.* (1999) showed that with another source of SPC the possibility of replacing 50% of fish meal in high fat extruded diets. Similar success has not been reported in other salmonids such as the Atlantic or Pacific salmon, for high levels of substitution of fish meal, even by protein-rich soybean products (see Kaushik, 2007). Fagbenro and Davies (2004) found that 75% of fish meal protein can be replaced with SPC in the diets for African catfish; they also showed that catfish can effectively utilize supplemental methionine, thereby allowing up to 100% of the dietary protein from fish meal to be replaced by SPC. In marine fish such as European seabass, gilthead seabream, turbot, red sea bream, yellow tail or Atlantic halibut, SPC can contribute between 20 and 60% of protein in replacement of fish meal with equal performance (Peres and Lim, 2007).

As regards rapeseed protein concentrate (RPC), even early studies by Teskeredzic *et al.* (1995) pointed out that RPC can supply more than 60% of dietary protein (fish meal only 11%) for rainbow trout without adversely affecting performance or body composition. A reduction of anti-nutrients such as phytic acid or glucosinolates can highly improve their use. Data of Thiessen *et al.* (2004) confirm that properly dephytinised RPC can replace substantial amounts of fish meal in the diets of rainbow trout. Kissil *et al.* (2000) also found that both SPC and RPC can be used as partial fish meal substitutes in the diets of gilthead sea bream. Other protein concentrates such as potato protein concentrate (PPC) have also been tested with some species with varying degrees of success. While Xie and Jokumsen (1997) found a decrease in growth with increasing levels of PPC, incorporation of PPC with low levels of glycoalkaloids appears to be well suited as a partial substitute for fish meal (Refstie and Tiekstra, 2003). Similarly, some studies with rainbow trout show that rice protein concentrates can replace up to 20% of fish meal (Palmegiano *et al.*, 2006). Lupin protein concentrates also appear to be extremely potent substitutes for fish meal in diets for rainbow trout (Glencross *et al.*, 2006).

Wheat gluten is an excellent protein source, having very high protein digestibility and amino acid availability to salmonids (Storebakken *et al.*, 2000) as well as marine fish (Robaina *et al.*, 1999). Rainbow trout grow equally well with a diet consisting of 100% wheat gluten as a sole protein source duly supplemented with amino acids as with a fish meal based diet (Rodehutscord *et al.*, 1995). Similarly, corn gluten meal (CGM) is rich in protein and has a high protein digestibility and amino acid availability in fish. Inclusion of up to 20% in the diets did not have any adverse effects even in turbot (Regost *et al.*, 1999). Pereira and Oliva-Teles (2003) showed that CGM can replace up to 60% fish meal protein in diets for gilthead sea bream juveniles. With salmonids, however, one of the limits for the use of corn gluten meal is also the presence of xanthophyll pigments, which can interfere with flesh pigmentation (Hatlen *et al.*, 1992), recently also confirmed in rainbow trout (unpublished personal data).

12.4 Oilseed meals

12.4.1 Soybean meals

The world-wide availability of soybean products in great quantities definitely makes them a reliable and viable alternative to fish meal in fish and shrimp diets. Although SBM having crude protein levels ranging from 45 to 50% cannot be used as a total substitute for fish meal, low to moderate levels of soybean of various qualities may be included in diets for almost all fish and crustaceans (Amaya *et al.*, 2007; Hansen *et al.*, 2007; Kaushik, 2007; Peres and Lim, 2007). Salmonids appear to be sensitive to several of the anti-nutritional factors present in soybean products. There are inter-species differences, but the level of available information is not of equal value in all species to undertake reliable comparisons. High levels of dietary soy products have been related to a number of adverse effects: poor feed intake; decreased nitrogen, phosphorus, or trace element availability; digestive and immune function disorders; antigenic or estrogenic response; and poor nutrient utilisation affecting overall performance. At the metabolic level, soybean products appear to induce changes in lipid metabolism, where further insight on the roles of specific anti-nutritional factors (ANFs) is needed. While the essential amino acid profile can be reasonably well adjusted through adequate supplementation or through judicious mixture of other ingredients, the major problem often encountered is related to one or more anti-nutritional factors.

12.4.2 Rapeseed/canola meal

In contrast to RPC or isolates which are well utilised by fish, the incorporation of rapeseed/canola meal in the diet of most fish or crustaceans still remains a difficult issue, despite the reduction of ANFs such as glucosinolates through genetic selection or of fibre and tannins by technical processes such as dehulling. A detailed literature review was recently made by Burel and Kaushik (2008): for all practical purposes, it is suggested to use heat-treated meals at levels not exceeding 20–30% of the diet depending upon the species. Besides palatability problems, fibre, phytic acid and tannins, involvement of glucosinolates in thyroid metabolism has been incriminated, possibly alleviated by a supplementation with iodine.

Other oilseed meals such as peanut meal, sunflower meal or cotton seed meal have been found to be more or less well utilised by warm water fish such as carp, tilapia or catfish when included at levels generally below 10% of the diet. It is worth recalling that a mixture of oil cake meals along with some cereal (wheat, rice) bran is a very common mixture used for pond culture of cyprinids in Asia where they constitute the major farmed finfish group. High levels of inclusion of these oilseed meals have not met with success in intensively cultivated finfish or shrimp. Sunflower meal, although recognised as having high protein digestibility, cannot be included in high levels due to the high fibre content, generally encountered in untreated meals. Efforts are underway to decrease the fibre content and make better use of this potentially useful protein source.

12.4.3 Pulses

Pulses are grain legumes, having about 25–40% of crude protein, generally used directly for human consumption. Although there is a large variety of pulses, the most commonly used in the animal feed industry are lupins, peas and some beans.

Three major species of lupins, *L. angustifolius* (narrow-leafed sweet lupin), *L. albus* (white lupin) and *L. luteus* (yellow lupin) are of interest. Although lupin is of Mediterranean origin, more than 75% of the world production of the >1.3 million tons occurs in Australia, mainly of *L. angustifolius*. Lupin seeds have protein contents ranging from 32 to 38% and about 6 to 9% of fat. Lupin kernel meals have higher protein (39–52%) and fat (9–11%) contents. In all aquaculture species for which a nutritional assessment has been made on the value of lupins, they have been shown to be a well-accepted and nutritionally useful ingredient (see Glencross, 2001). The digestibility of lupin proteins has been reported to be high (Gomes and Kaushik, 1989) and improved by extrusion (Burel *et al.*, 2000), affected by the levels of non-starch polysaccharides (NSP) present (Glencross *et al.*, 2003). Up to one third of fish meal can be replaced by lupins in the diets of salmonids as well as marine fish (Burel *et al.*, 1998, 2000; Carter and Hauler, 2000).

Pea proteins are interesting in terms of their IAA composition: rich in lysine and in arginine, but limiting in sulphur amino acids, when compared to fish meal protein. Early studies by Kaushik *et al.* (1993) and Gomes *et al.* (1995) demonstrated that dehulling, extrusion and milling improved apparent digestibility of pea starch from almost nil to 96%. Such extruded peas can replace a portion of fish meal in almost all finfish such as trout, Atlantic salmon, sea bass, sea bream or turbot as well as shrimp diets (Thiessen *et al.*, 2003; Bautista-Teruel *et al.*, 2003; Young *et al.*, 2006). Pea starch also has interesting binding properties. Recent developments in high protein pea products are again very promising in terms of their further use in aquaculture. Other pulses such as faba beans have been tried in different species including salmonids or marine fish (Gouveia *et al.*, 1993; Gomes *et al.*, 1995; Fontanhas-Fernandes *et al.*, 2000; Booth *et al.*, 2001).

12.4.4 Other sources

Malt protein combined with soybean protein was proved to support growth in diets for fingerling rainbow trout (Akiyama *et al.*, 1995; Toshia *et al.*, 1995), combining soybean meal with brewers yeast and tomato meal resulted in acceptable growth in African catfish (Hoffman *et al.*, 1997). Similarly, brewery draff could be used to a limited extent in the diets for tilapia (Pouomogne *et al.*, 1992). In the context of the rapid growth in ethanol/biofuel production, use of distillers dried grains with solubles (DDGS) from such plants, which can contain high levels of protein, has been attempted in a number of species. Currently, in intensive culture conditions and in salmonids or marine fish, their levels of incorporation do not exceed more than 5–8%. Extrusion technologies, addition

of phytase (Cheng and Hardy, 2004a) or proper supplementation with amino acids (Cheng and Hardy, 2004b) make these byproducts of potential value in feeds for aquaculture. Yet another source of plant origin is that of aquatic plant kingdom, macro or micro algae. Their use in the culture of marine fish and crustacean larvae is well established. Sporadic attempts have been made, mostly under pond farming conditions, to make better use of plants such as azolla or of algae (spirulina, chlorella to cite but a few) or sometimes under experimental conditions (Zaki and El-Ebiary, 1997). Their potential as essential fatty acid source (Harel *et al.*, 2002) and as an amino acid source, which is currently not yet fully exploited. Spirulina can replace up to 40% of the fish meal protein in diets for tilapia (Olvera-Novoa *et al.*, 1998) or for sturgeon (Palmegiano *et al.*, 2005).

12.5 Reducing anti-nutrients and other undesirable compounds in alternative protein ingredients

When using plant protein sources, a major concern is the level of anti-nutritional factors (ANFs) normally present in these ingredients. Plant ingredients contain varying levels of ANFs and fibres, such as protease inhibitors, lectins, antigenic proteins, phenolic compounds, oligosaccharides and phytates (Kaushik, 1990; Francis *et al.*, 2001). These ANFs can intervene at different levels such as protein digestion and amino acid availability, mineral and trace element availability or as anti vitamins; there are also a number of possible methods of reduction or alleviation (Table 12.4).

As fish are generally more sensitive to these anti-nutritional factors than land animals, and as aquaculture feeds are rich in proteins, the destruction of these factors is essential when incorporating them into aquafeeds. Presence of ANFs from soybean causes an inflammatory response in the distal intestine of salmonids (Bakke-McKellep *et al.*, 2000), the severity of which is dose dependent (Krogdahl *et al.*, 2003). The inflammation appears to cause a decrease in brush border and cytosolic enzyme activities, and decreased nutrient transporter activities or numbers of the distal intestinal epithelia cells (Nordrum *et al.*, 2000). These may be contributing factors to lowered feed digestibility and efficiency in soybean meal fed carnivorous fish. A deleterious effect of the induced enteritis to disease resistance has also been suggested (Krogdahl *et al.*, 2000).

There is as yet no clear information as regards which particular ANF specifically exerts its effects on a particular physiological function, affecting overall growth performance and nutrient utilisation. In fact, it is often found that specific purified ANFs do not have the same degree of adverse effects as seen with soy products containing the same (Bureau *et al.*, 1998), showing that a combination of factors induces such effects. This, combined with poorly defined quality criteria for choice of raw materials, often makes it difficult to make progress towards the use of soybean products in a larger scale than is practised today. Given the urgent need for research on alternatives to fish meals,

Table 12.4 Classification of some anti-nutritional substances commonly encountered in different feedstuffs

Major factor	Commonly found in	Means of alleviation
Interaction with protein nutrition		
Protease inhibitors	(Trypsine) soybean	Heat, autoclaving
Heamagglutinins	(lectins) soybean	Heat, autoclaving
Saponins	Peas, alfalfa,	
Polyphenols	Tannins, sorghum,	
Chlorogenic compounds		Supplementary methionine or choline
Interaction with mineral availability		
Phytic acid	Soybean	Supplementation
Oxalic acid	leaf proteins	Heat treatment
Glucosinolates	Rapeseed	New varieties
Gossypol	Cottonseed	New varieties
Interaction with vitamin availability		
Vit A (lipoxygenase)	Soybean	Heat treatment
Vit D	Soybean	or autoclaving
Vit E (oxidase)	Kidney beans	Autoclaving, addition of vit E
Thiaminase	Raw fish flesh	Heat, additional B1
Anti-nicotinic acid	(niacinogen) corn	
Anti-pyridoxine	Linseed meal	Water extraction, heating
Anti-vitamin B12	Raw soybean	Heat treatment
Cyanogens	Cassava, sorghum	Heat treatment

especially for the salmonids, standardised information based on soybean products, including the technological treatments involved, is warranted. Thus, full advantage may be taken of the availability of soybean products in the global market.

In parallel with the development of plant genotypes with low levels of anti-nutritional factors (e.g., Primor 000 or canola-type rapeseeds), the appropriate technological processing (dehulling, extrusion-cooking, co-extrusion, micronisation, etc.) can lead to products with low levels of anti-nutritional factors. Heat-processing of ingredients as well as feed extrusion technologies thus enable us to reduce the anti-trypsic factors and improve amino acid availability (Cheng and Hardy, 2003).

Plant protein ingredients also contain relatively high amounts of non-starch polysaccharides (cellulose, hemicellulose, β-glucans, pectins and gums) and belong to the natural diet of herbivorous and omnivorous fish, but not carnivorous species. (Krogdahl *et al.*, 2005). In the herbivore *Labeo rohita* feeding on macrophytes, carbohydrate digestibilities vary between species of macrophytes (Ray and Das, 1994) supposedly due to variation in carbohydrate composition. A comparative study between an herbivorous and an omnivorous species fed diets with algae showed comparable and low digestibilities for algae fibre and algae cell walls (Galetto and Bellwood, 1994). Efforts to evaluate nutritive value

of NSP in the omnivorous pinfish (*Lagodon rhomboides*) indicate some ability to utilise soluble low molecular carbohydrates from seagrass, but little or no utilisation of the complex carbohydrates (Lindsay and Harris, 1980). So, rather than supplying nutrients, non-starch polysaccharides may reduce utilisation of other nutrients and thereby act as anti-nutrients. This appears to be true for both carnivore and herbivore fish. Information on anti-nutritional effects of NSP in fish is incomplete. However, some NSP seem to increase the transit rate through the gut (Montgomery and Targett, 1992) which is possibly related to the decreased nutrient concentration in the intestine. There is also experimental evidence that increased inclusion of ingredients high in NSP reduces utilisation of digestible carbohydrates, proteins and lipids *in vivo* and the *in vitro* protein digestibility (Ryu *et al.*, 1992). An *in vitro* study of effects of pectin, gum karaya, alginate and cellulose showed that all these NSP may inhibit protein hydrolysis. Including alginate and guar gum in diets for rainbow trout reduced protein and lipid digestibility to a great extent (Storebakken, 1985).

12.6 Development of non-fish meal or low fish meal diets

Some studies by Watanabe and his co-workers in Japan showed that use of non-fish meal diets in yellowtail led to some hepatic malfunctioning. Watanabe *et al.* (1998) demonstrated that rainbow trout could be grown over long periods with properly balanced non-fish meal diets even without any supplementary IAA. Later, they also showed that such non-fish meal diets based on soy protein concentrate, defatted soybean meal, corn gluten meal, meat meal, and krill meal can be used with success even with yellowtail, provided proper attention is also paid to the feed processing techniques (Watanabe *et al.*, 2001).

There is accumulating evidence to confirm that it is possible to considerably reduce the use of fish meal by using plant protein sources with certain species, such as the rainbow trout (Kaushik *et al.*, 1995), European sea bass (Kaushik *et al.*, 2004), gilthead sea bream (Sitja-Bodadilla *et al.*, 2005), Atlantic salmon (Espe *et al.*, 2006) or peneid shrimp (Amaya *et al.*, 2007). There is thus much latitude for the reduction of fish meal levels in the feeds for almost all intensively farmed finfish and shrimp, thus reducing the pressure on marine resources. Specific methods have also been proposed to evaluate the biological value of individual protein sources (van der Meer and Verdegem, 1996; Booth and Allan, 2003).

In red sea bream, non-fish meal diets based on a mixture of other protein sources such as soy protein concentrate, soybean meal, corn gluten meal and meat meal led to similar growth and flesh quality to that of fish fed a commercial diet containing 64% fish meal (Watanabe *et al.*, 2001). Consistent and promising results have been reported when including a small portion of fish meal (as low as 5% inclusion) in diets for European sea bass (Kaushik *et al.*, 2004), and Atlantic salmon (Espe *et al.*, 2006). A general conclusion from the two latter studies is that fish can grow and utilise plant protein diets well, as long as these contain

small proportions of marine protein stimulating appetite and the dietary amino acid composition mimics the amino acid composition of fish meal. Palatability was in particular stimulated by additions of stick-water or squid hydrolysate, both good sources of peptides, in the studies with Atlantic salmon (Espe *et al.*, 2006).

Adelizi *et al.* (1998) evaluated several diets with rainbow trout by using different combinations of soybean meal, soy flour, SPC, peanut meal and also blood meal, and their results showed that there is quite some latitude of action for developing fish meal-free diets without leading to any adverse effects in terms of digestive or hepatic functions (Sitjà-Bobadilla, 2005).

A point worth taking into account is that more than protein and amino acid supply, replacement of fish meal by plant by-products also requires attention as regards mineral and trace element availabilities. Indeed, depending on the ingredient, availability of minerals (Ca, P, Na) and of trace elements (Cu, Fe, Mg, Mn, Sr or Zn) can vary (Yamamoto *et al.*, 1997; Suguira *et al.*, 1998).

12.7 Supplementation of fish meal with limiting amino acids

Some controversy exists as regards the efficacy of utilisation of supplementary amino acids, arising from the fact that absorption of dietary free amino acids might be faster than protein-bound amino acids. The difference in those results might be the quality and mixture of plant proteins, and how the amino acids were incorporated into the diet. Cowey and Walton (1988, 1989) indeed found that protein-bound amino acids were incorporated into body protein to a greater extent than free amino acids. However, if proper precautions are taken (pH adjustment, coating), crystalline amino acids are as well utilised as protein-bound amino acids in fish as well as shrimp. Rainbow trout successfully grow as well on a diet consisting of 100% plant protein with added crystalline amino acids as on a pure fish meal diet (Rodehutscord *et al.*, 1995). Now it is becoming more and more clear that crystalline amino acids are well utilised when included in purified or practical diets in widely different fish and shrimp (Fournier *et al.*, 2002; Fagbenro and Davies, 2004; Alam *et al.*, 2005; Espe *et al.*, 2006).

12.7.1 Issues related to voluntary feed intake

It should be recognised that independent of ingredients, there are large batch differences in quality of both plant and marine ingredients, which will also result in differences in how well these stimulate appetite and growth in fish. Incorporation of high levels of plant ingredients often leads to reduced feed intake and consequently affects growth. This can at least partially be alleviated by addition of appropriate feed attractants: amino acids, peptides, fish protein hydrolysates, beatine, inosine all appear to exert such phagostimulatory effects to varying degrees in different species (Carr *et al.*, 1996).

12.7.2 Issues related to the use of reference proteins

Even with regard to fish meal, it is established that a reduction in nutrient bioavailability might result from high temperature processing of, e.g. fish meal, if the quality of the batch is low, formation of enzyme-resistant cross-linkages between protein chains might occur, oxidation of amino acids, such as methionine and tryptophan, or a reaction of lipid oxidation products with amino acids (Camire *et al.*, 1990; Macrae *et al.*, 1993). Reduced nutrient availability may, in turn, have a number of adverse effects in terms of nitrogen utilisation in fish fed on diets containing fish meals produced using harsh processing conditions (Pike *et al.*, 1990; Aksnes and Mundheim, 1997). Thus there may be differences in growth performance between fish fed diets manufactured from high quality fish meals (e.g., Norse-LT 94) and those fed diets containing lower quality meals (e.g., NorSeaMink): high quality meals have been reported to give increased growth and feed efficiency in a range of species. When trying to reduce the fish meal inclusion to a minimum, it is therefore of great importance to test against high quality fish meal.

12.7.3 Issues related to the use of genetically modified (GM) plant feed ingredients

Changing from fish meal to plant meal results in substantially lowered levels of organic pollutants such as dioxins and dioxin like-PCBs, but unfortunately new unwanted substances (pesticides or herbicides) might also influence the safe utilisation of various plant ingredients. Little information is available on the biological consequences, but further detailed information can be found in Chapters 2 and 3. One question which often arises concerns the use of feed ingredients derived from genetically modified (GM) plants, especially with regard to soybeans and maize, which are the largest grown GM plants of interest to the feed industry today, constituting 60% and 24% of global GM cultivated area, respectively (James, 2005).

Since 2004, GM food and feed applications have been regulated in the European Community, which requires labelling of products containing more than 0.9% of approved GM ingredients, and $\leq$0.5% for non-approved GM ingredients. The European Food Safety Authority (EFSA) is responsible for the science-based assessment of GM food and GM feed in Europe. The term 'Substantial equivalence' was formulated by the Organisation for Economic Cooperation and Development as a guiding tool for safety assessment of genetically modified foods, and is based on the idea that an existing plant with known feed value and with a history of safe use, can serve as comparator when assessing the safety and nutritional value of a genetically modified plant (OECD, 1993). Substantial equivalence is the starting point for safety assessment of a GM variety, with comparisons of the GM variety with its closest traditional counterpart, and can be described as a comparative approach to the assessment of safety (EFSA, 2004). Compositional equivalence is based on compositional analysis, which is a part of the safety assessment, and in studies

with fish, is defined as experimental diets being equal in macro- and micronutrients, and as equal as possible in ANFs. However, the latter is difficult to obtain as the modification of plants very often leads to alterations in ANF amount and profile between the GM variety and its near-isogenic parental line (Flachowsky *et al.*, 2005). Safety assessments include toxicity testing of the newly expressed proteins, potential occurrence of secondary effects, potential for horizontal gene transfer to other species, the potential allergenic effects of newly inserted traits, and the role of the new food in the diet (Kuiper *et al.*, 2001; Herrero *et al.*, 2007).

Random integration of DNA into the plant genome can result in DNA rearrangements of the transgenic construct and of the target site, and cause alteration or disruption of DNA (Cellini *et al.*, 2004). The presence of rearrangements is often associated with instability in transgene expression (Fladung, 1999; Kumar and Fladung, 2000). Complex integration patterns have been seen with *Agrobacterium*-mediated integration, such as directed and inverted repeats (Krizkova and Hrouda, 1998) and integration of vector 'backbone' sequences from outside the left and right borders of the transgenic DNA (De Buck *et al.*, 2000). Evidence exists that integration of transgenic DNA causes mutations due to loss of gene function. Gene-rich regions are known to be *hot spots* for recombination of transgenic DNA (Gill *et al.*, 1996; Cellini *et al.*, 2004), which increases the risk of altering the gene expression profiles of plants in the process of transgenic DNA insertion. The potential risk of unintended effects are one of the concerns in genetic engineering (Kuiper *et al.*, 2001; Cellini *et al.*, 2004). Unintended effects might be silencing or activation of present genes or origin of new genes, which might lead to the formation of new proteins or altered levels of the existing ones (Kuiper *et al.*, 2001), such as new anti-nutritional factors. Another concern is the risk of horizontal gene transfer (HGT) of novel DNA to prokaryotic and eukaryotic organisms. The latter often deals with possible transfer of antibiotic-resistant genes, which are constituents of the transgenic DNA, inserted to be able to sort out the GM organisms successfully, and which eventually might result in an increase in antibiotic-resistant pathogens as a secondary effect (Gay and Gillespie, 2005).

Concerns arise as regards possible toxicity or secondary effects of intended or unintended transgenic proteins expressed in the GM organism. Force-feeding and intraperitoneal injection of Atlantic salmon with pellets spiked with round-up ready soybean (RRS) DNA-sequences of various sizes from 100 to 1000 base-pairs, showed that all sizes were absorbed and entered blood, kidney and liver tissue, with maximums found 6 to 8 hrs after feeding (Nielsen *et al.*, 2005). By means of *in-situ* hybridisation techniques, DNA from RRS soya, were identified in the epithelial cells in salmon intestine (Sanden *et al.*, 2006). The process of transgenic DNA insertion and possible secondary effects of diet exposure of GM maize and GM soybean to Atlantic salmon (which would apply to fish in general) are illustrated in Fig. 12.3 (Sagstad, 2006).

Whether or not the inclusion of GM soybean or GM maize affects the fish in any possible manner has been investigated in several studies with fish, and with

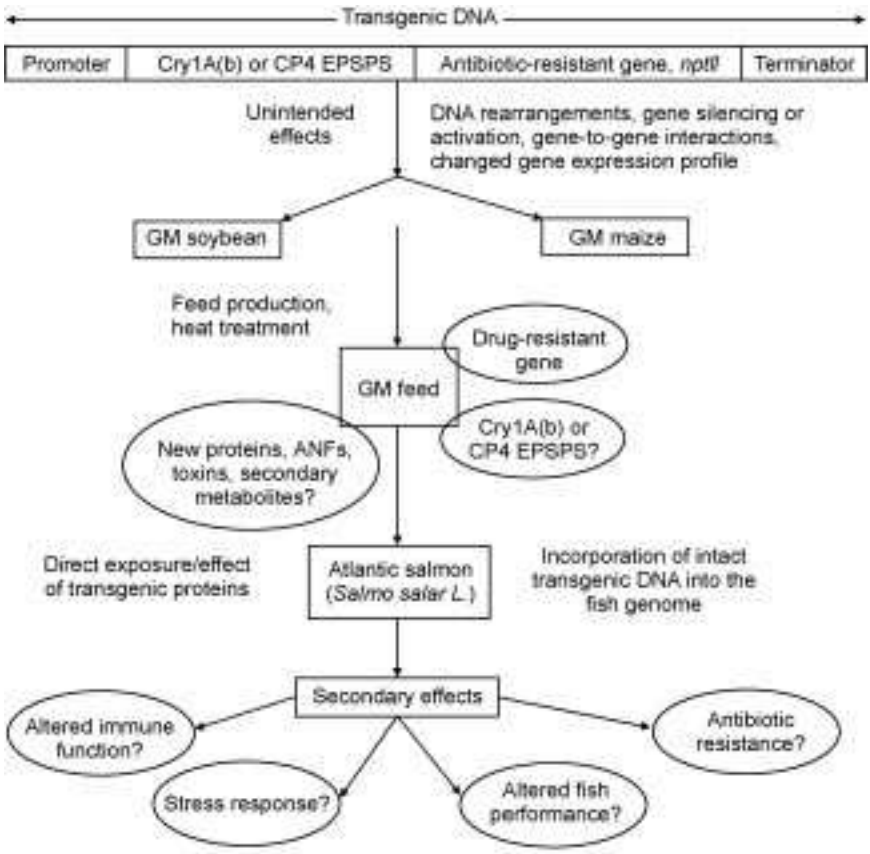

Fig. 12.3 Illustration of the transgenic DNA inserted into GM plants, and possible secondary effects resulting from feeding GM feed to Atlantic salmon (*Salmo salar* L.). (Sagstad, 2006).

various results (Hammond *et al.*, 1996; Sanden, 2004; Sagstad, 2006). The major conclusions are that growth, digestibilities, feed utilisation and general health parameters are more influenced by the plant material as such, than the plants being genetically modified. Chainark *et al.* (2006) investigated the use of GM soybean meal in the diets of rainbow trout. A GM defatted soybean meal was compared to a regular soybean meal fed at two levels (15 and 30%) in a 42% protein diet fed to juvenile trout (48.3 g) for 12 weeks. The results showed no significant differences in growth or feed performance between the two soybean meal sources. Promoter fragments were detected in muscle of fish fed the GM soybean meal, initially, but were not detected after five days on a non-GM meal-based diet. Some indications of alterations of the immune system were found by Sagstad (2006). Further studies on how metabolic pathways are influenced are under way, in order to identify which pathways are altered (Sanden, 2007, unpublished; Séralini *et al.*, 2007). In Atlantic salmon, no major differences in

organ sizes, except for distal intestine, are reported due to feed containing GM; organs investigated were liver, heart, brain, kidney, head-kidney, spleen, and the different sections of the gastrointestinal tract. Whole body or liver proximate compositions were not altered due to GM, and only minor effects were identified on the stress-response system; parameters used in the evaluations were heat-shock protein 70, catalase, superoxide dismutase, and leakage of organ specific enzymes to the plasma compartment (Sanden, 2004; Sagstad, 2006). The transport mechanism for glucose in the anterior gut was however found to be altered by the plant being GM (Hemre *et al.*, 2007).

12.7.4 Consequences for flesh quality

When comparing diet × genotype interactions, Smith *et al.* (1988) observed that while differences in morphometric parameters could be attributed to genotype, none were attributed to dietary protein source. Kaushik *et al.* (1995) did not find any major difference in flesh quality attributes between trout fed a fish meal based diet or those fed a SPC-based diet. Feeding diets with high levels of plant protein ingredients over more than six months to red sea bream did not induce any major change in the physical flesh quality characteristics or distribution patter of chemical constituents such as free amino acids or fatty acids (Aoki *et al.*, 1996); although there were some differences in sensory evaluation parameters, the general conclusion is the absence of distinct differences in flesh quality caused by dietary protein sources. Adelizi *et al.* (1998) found some differences in fillet flavour in trout fed different fish meal-free diets, but these differences were mostly attributable to dietary oil sources rather than the protein sources. Similar observations have also been made in Atlantic salmon (Bjerkeng *et al.*, 1997). Feeding rainbow trout or the gilthead sea bream over long periods with diets containing very high levels of plant protein ingredients led only to minor changes in flesh quality parameters (de Francesco *et al.*, 2004).

12.8 Future trends

In the context of the need to reduce the use of fish meal to the minimum, efforts should focus on developing high quality fish meal for aquaculture uses, and to use reference fish meal for studies on substitution. Data need to be obtained over the full life cycle rather than on short stages of specific physiological growth, evaluating all possible physiological and environmental consequences. Since fish meal is a complex and complete ingredient in terms of different essential nutrients that they supply, studies should encompass not only the IAA balance or ANFs, but also look into the supply and bioavailability of all essential nutrients. The bottom line is the maintenance of the physiological well being of farmed fish and shrimp. Complementary to whole animal studies, novel approaches in integrative biology (transcriptomics, proteomics) can possibly provide new insights on the physiological consequences often overlooked in conventional studies (Panserat *et al.*, 2005).

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13

Alternative marine sources of fish feed and farmed fish quality

R. W. Hardy, University of Idaho, USA

13.1 Introduction

Feeds and feed ingredients from marine sources have long been used in feeds for farmed fish. However, in recent years, efforts to identify and utilize marine products have accelerated due to pressure associated with the growth of aquaculture, higher production of aquafeeds, and the realization that traditional marine products used in aquafeeds, e.g. fish meal and oil, are finite resources that cannot support continued growth of the aquaculture sector. In some regions, marine products are harvested, or processing by-products are collected and used as supplemental feeds for pond culture of shrimp and fish. Similarly, pelagic fish are harvested and used directly as feed or mixed with other ingredients to produce 'on-farm' feeds. This is mainly practised in developing countries in Asia. However, effective efforts to utilize marine materials as components of aquafeeds must involve some form of processing to convert the materials into stable forms suitable for storing, shipping and use in pelleted feed manufacturing

The potential for higher recovery and utilization of marine materials in aquafeeds has always existed, but this potential has not been realized because the economics of production have been unfavourable. This situation is changing as market prices for marine protein (fish meal) and oil used in aquafeeds have increased. In addition, there is a growing demand for grains and oilseeds as feedstock for ethanol and biofuel production. This has doubled the value of corn from its 30-year average price, thereby raising prices of other grains and oilseeds as well as prices of value-added feed ingredients produced from them. As a result, there are now economic incentives to recover and utilize marine materials

for use in aquafeeds. In the near future, the bulk of protein in aquafeeds for many farmed species of fish will be supplied by plant protein concentrates, and plant oils will increasingly replace portions of fish oil. As this shift occurs, opportunities will arise to produce specialty products from marine sources designed to overcome shortcomings of plant proteins and oils, to cultivate marine organisms for use in specialty feeds in aquaculture, such as larval or maturation feeds, and to develop supplements that will be essential to ensure high-quality consumer products from farmed fish.

13.2 Fisheries by-catch and seafood processing by-products

13.2.1 Fisheries by-catch

Marine fisheries operate by locating schools of targeted species and using gear that not only captures the target species, but also is designed to exclude small fish that cannot be processed by mechanical equipment. However, these efforts are not 100% successful, leading to landings of undersized fish of the targeted species, and to landings of non-targeted species. These landings are collectively called by-catch. In some cases, by-catch contains fish species having economic value but, in most cases, by-catch has little value as seafood and is discarded at sea.

The volume of by-catch is difficult to estimate with any degree of accuracy because most by-catch is thrown overboard and not weighed. FAO (1999) estimates annual discards from the fishing industry to be approximately 20 million metric tons (mmt). This estimate is a combination of by-catch and processing waste. Although precise values for global by-catch are not known, accurate measurements have been made for some fisheries. In the UK, for example, by-catch is estimated to be about 50% of the weight (range 40–60%) of landings from demersal fisheries, whereas for pelagic and shellfish fisheries, the average is 13%, with a range of 5–20% (Archer, 2003). In Alaska, by-catch is highly regulated by limiting total landings of non-targeted species, e.g. halibut by-catch associated with the arrowtooth flounder fishery. When the allowable by-catch of halibut is reached, the arrowtooth flounder fishery is closed. In many parts of the world, by-catch is unregulated and unreported. In the USA, data from shrimp fisheries in the Gulf of Mexico are the most accurate with regards to by-catch. By-catch of non-targeted species that are discarded at sea is estimated to range from five times the landed weight of shrimp to 15 times, resulting in an estimate of by-catch of as much as 15 mmt worldwide from shrimp fisheries (McGinn, 1998).

13.2.2 Fish processing by-product recovery and utilization

The volume of fish processing by-products is much better known than the volume of by-catch because the volumes of landed fish are recorded and yields of edible products from various species are known (Table 13.1). Yields vary

Table 13.1 Average processing yields (% of whole fish) from species of marine fish (adapted from Crapo *et al.*, 1993)

Common name	Species	H & G[a]	Skinless fillets
Arrowtooth flounder	*Atheresthes stomias*	74	34
Atlantic salmon	*Salmo salar*	88	68
Black rockfish	*Sebastes melanops*	57	27
Chinook salmon	*Oncorhynchus tshawytscha*	72	46
Dover sole	*Microstomus pacificus*	65	29
Pacific cod	*Gadus macrocephalis*	63	39
Pacific halibut	*Hippoglossus stenolipis*	72	41
Pacific Ocean perch	*Sebastes alutus*	62	30
Walleye pollock	*Theragra chalcogramma*	62	34
Shrimp	*Pandalas* sp.	53 (headless)	36 (peeled)

[a] H & G refers to fish with head, gills and viscera removed.

greatly with species and processing methods (Crapo *et al.*, 1993). For wild salmon, yields range from 70–83% of the whole weight of the dressed and head-off fish and approximately 46–56% for skinless fillet production. For pollock, the largest fishery in Alaska, the yield of skinless, boneless fillets ranges from 24–36%. The remaining material (64–76%) is fish processing by-product. The yield for surimi production from pollock can be as low as 15–20%. In Alaska alone, the volume of seafood processing by-products exceeds one million metric tons (Crapo and Bechtel, 2003). Globally, processing by-products and by-catch together are estimated to be 25–30 (mmt), equivalent to the average volume of landings used to produce fish meal and oil (Barlow, 2003). Thus, if all fisheries by-product and by-catch were used to produce fish meal and oil, annual global production of each would double. In 2002, an estimated 5.6 mmt of processing byproducts, e.g. trimmings and rejects from food fish, were converted into fish meal and oil (Tacon *et al.*, 2006). However, for processing by-processing waste and by-catch to be recovered and utilized, they must be available in sufficient quantities and over a sufficient period of time to justify the construction and operation of processing factories to convert them to meal and oil, at least if the processing factories employed conventional wet reduction technology. Except for certain fishing ports, e.g. western Dutch Harbour and Sitka Alaska, this is not the case. At ports where landings are relatively small or occur over a short fishing season, other economical processing methods, such as ensiling or hydrolysate production (see next section), must be used to convert the material into intermediate stable products that can be collected for final processing without spoiling or losing nutritional value.

Fish are either processed at sea on large processing vessels or on shore in factories. Many at-sea processing vessels contain small fish meal processing plants and utilize a large proportion of the by-products of fish processing. However, most at-sea processing vessels do not recover and concentrate the soluble protein (or the oil fraction) produced during wet reduction due to space

limitations. The liquid removed from presscake, containing water, oil and the soluble protein fraction, is discarded at sea and the resulting meal, made from insoluble, structural proteins, is called presscake meal. In contrast, shore-based seafood processing plants produce whole fish meal from processing by-products that contain both soluble and insoluble protein fractions, plus they recover fish oil from the liquid fraction.

Fish meals produced from processing by-products differ in composition from fish meals produced from whole fish because a large proportion of the structural protein, e.g. muscle, is removed to enter the human food stream (Hardy and Masumoto, 1990). This alters the composition of the meal by decreasing the protein content and increasing the ash (bone) content compared to conventional fish meal (Table 13.2). High ash content is a concern in fish meal because the ash fraction is mainly composed of fish bones from the skeletal system of the fish, and is rich in calcium and phosphorus. High levels of calcium and phosphorus in aquafeeds can cause antagonistic interactions in the gastro-intestinal tract of farmed fish, resulting in the formation of insoluble calcium-phosphates when digested food leaves the acid environment of the stomach and enters the neutral or slightly alkaline environment of the intestine. Calcium and phosphorus are soluble in acid environments, but precipitate when pH rises. Complex salts that bind divalent cations, especially zinc, can form, lowering bioavailability and inducing zinc deficiency in farmed fish (Shearer and Hardy, 1987). This problem is exacerbated in the presence of phytic acid, the storage form of phosphorus in seeds, which is present in meals and concentrates made from grains and oilseeds (Richardson *et al.*, 1985). Zinc deficiency affects

Table 13.2 Proximate and mineral composition of fish meals produced from seafood processing by-product (adapted from Babbitt *et al.*, 1994)

Proximate category	Category of meal		
	Regular	Screened[a]	Deboned[a]
Protein (%)	62–64	69.5 (73.7)	73.9 (81.7)
Fat (%)	6–8	7.0 (7.4)	9.6 (10.6)
Moisture (%)	6–8	5.4	10.0
Ash (%)	23–25	17.8 (18.9)	7.0 (7.7)
Minerals (expressed on dry weight basis)			
Calcium (%) 6.74 2.74			
Phosphorus (%) 3.54 1.67			
Sodium (%)		0.68	0.81
Magnesium (%)		0.23	0.20
Potassium (%)		0.56	0.62
Iron (ppm)		73.0	133.0
Copper (ppm)		3.0	4.0
Zinc (ppm)		82.0	96.0
Manganese (ppm)		10.0	8.0

[a] Dry matter basis values given in parentheses.

growth and bone development, but more importantly, can cause cataracts in salmonids and other species (Hardy, 2001). The ash level in meals made from processing by-products can be reduced by mechanical screening of material as it is discharged by the dryer before final grinding to reduce particle size (Babbitt *et al.*, 1994), or by air-classification. There is a mechanical limit to how much bone can be removed by screening or air-classification, plus bone contains protein as well as minerals, so aggressive bone removal alters the composition of fish meal and lowers yields. Any process that fractionates marine material must upgrade the product to one with a higher value, but also produce a secondary product, i.e., the material being removed, that has some economic value and market to justify the expense of removing it.

The nutritional value of fish processing by-product meals depends on the conditions of manufacture, e.g. bone removal (Rathbone *et al.*, 2001) and on the components of the processing waste stream used to make the meals (Bechtel and Johnson, 2003). Meals made in Alaska from the entire processing waste stream have been shown to be equivalent in nutritional value to whole fish meals when used in aquafeeds (Forster *et al.*, 2004). This is not surprising, given the fact that the by-products used to make Alaskan fish meals are human food grade in terms of degree of freshness. This is not the case, however, with meals produced from shrimp or crab processing by-products. Approximately 50% of the landed weight of shrimp is processing by-product, e.g. heads, and a large percentage is shell, primarily chitin. Chitin is a polymer of glucosamine, and is indigestible to most species of fish. Shrimp by-catch and processing by-product meals have been evaluated as replacements for fish meal in aquafeeds for red drum and rainbow trout. Meals made from whole fish by-catch are suitable replacements as they are essentially whole fish meals, but meals made from shrimp processing waste are inferior in nutritional quality to conventional fish meals (Li *et al.*, 2004; Hardy *et al.*, 2005).

Researchers have evaluated the fish processing waste stream by collecting various components as they are removed from whole fish, analyzing their composition, and making meals from each component for chemical and nutritional evaluation (Bechtel and Johnson, 2003). As fish are processed to remove edible muscle (fillets), the heads, viscera, frame after filleting, and finally the skin are removed. In some fish processing schemes, such as pollock roe production, livers and testes can be collected separately from the viscera component. Not surprisingly, the proximate, mineral, and amino acid composition of the component meals vary considerably (Table 13.3). Lipid class profiles and fatty acid content of liver also vary considerable among species of marine fish and throughout the fishing season (Bechtel and Oliveira, 2006).

Of the important quality attributes of farmed fish affecting the consumer, the use of meals produced from seafood processing by-products in aquafeeds is most likely to influence fatty acid composition. Marine meals produced using fish viscera, liver, or heads contain relatively high quantities of the omega-3 fatty acids, specifically docosahexanoic acid (C22:6, DHA) and eicosapentanoic acid (C20:5, EPA). Other proximate or nutritional constituents of seafood processing

Table 13.3 Composition of meals (%) made from components of the Alaska pollock seafood processing by-product waste stream. Values are adjusted to 10% moisture for comparative purposes (adapted from Bechtel, 2003)

Meal	Crude protein	Crude lipid	Ash
Whole fish	67.4	14.8	10.7
Head	68.1	0.5	20.6
Viscera	37.5	46.8	3.9
Frame	76.8	4.2	16.0

by-product meals do not influence product quality, but, as noted above, may affect fish health or growth rate. These effects are well characterized, and modern aquafeeds are formulated to avoid them. Flavour of farmed fish products may be influenced by the presence of residual lipids in meals produced from seafood processing by-products, but, as mentioned, the meals are often made from food-grade material, making it unlikely that any off-flavours associated with lipid spoilage or rancidity might affect flavour.

13.2.3 Fish silage and hydrolysate meals

Fish silage and fish hydrolysates are both liquid products resulting from enzymatic hydrolysis of processing by-product or by-catch. Silage production depends on endogenous enzymes in the by-product material to hydrolyze protein, whereas hydrolysates are produced by adding exogenous enzymes after thermal treatment of by-product material to denature endogenous enzymes. Silage and hydrolysates can be used as liquids, or they can be dried to produce meals. Spray-drying, fluidized bed drying and co-drying with fish meal or plant proteins are several methods by which fish hydrolysates are converted to dry meals for shipping, storing and use in pelleted feeds. If liquefied fish silage or hydrolysates have been acidified, neutralization prior to drying improves product quality (Hardy *et al.*, 1983).

Fish silage is prepared by combining ground material with inorganic acids or *Lactobacillus* sp. plus a suitable carbon source to lower the pH of the material to 4.0. At this pH, bacterial growth is inhibited and spoilage is prevented. Formic acid lowers pH and also inhibits mold growth, whereas other acids, such as phosphoric, only lower pH. With such acids, a mold inhibitor, such as propionic acid, must be added. After acidification, proteins are hydrolyzed by endogenous enzymes until substrates are exhausted or the material is heated to 65 ºC or above to stop enzymatic activity. The fish by-products can also be heated before acidification to denature endogenous enzymes, then enzymes can be added to produce hydrolysates with specific peptide lengths, to minimize the production of free amino acids or to achieve some other mixture of products in the final hydrolysate. In some systems, enzymes are added and the material is held for a period at 40–50 ºC without acid addition to hydrolyze proteins within a short period before spoilage occurs. When the desired degree of hydrolysis is

achieved, the mixture is heated further to denature added enzymes and sometimes to pasteurize the material, then either acidified to control microbial spoilage or dried at once. The pH of the mixture, the enzymes present (or added), the temperature of the mixture, and the length of time enzymes are allowed to remain active to hydrolyze proteins dictate the proportion of protein fragments, peptides, free amino acids and ammonia in the final product (Stone and Hardy, 1986). Fish processing waste made from filleting material tends to be high in bone content, but after proteins are hydrolyzed, bones can be easily removed by screening the liquefied material.

In general, fish hydrolysates are inferior to fish meal in nutritional value because they are pre-digested, meaning that intact proteins have been converted to peptides or free amino acids that are absorbed and metabolized more quickly than intact proteins. This can result in an unbalanced mixture of amino acids arriving at cells for protein synthesis (Stone and Hardy, 1989). Amino acids that are present in excess of cellular needs for protein synthesis are deaminated and used for metabolic energy, resulting in a reduction in protein retention in farmed fish when hydrolysates comprise a high proportion of dietary protein. For this reason, hydrolysates are not used to supply a large proportion of protein in aquafeeds. On the positive side, however, the production of fish silage and fish hydrolysates is relatively simple, and the equipment needed is much less expensive than that used to produce conventional fish meal. Hence, production of fish silage or hydrolysates is well suited for locations where the volume or seasonal availability of fish processing by-products is insufficient to justify operation of a conventional fish meal plant. Fish silage and hydrolysates contain 65% or more water, making it uneconomical to ship any distance without the material being concentrated or dried.

Fish hydrolysates are added to aquafeeds to improve feed palatability and feed attraction, and also used in feeds for fish larvae during the period of early life when the digestive system of the larvae is not fully developed. Their use has no impact on farmed fish quality because they are added in small proportions to aquafeeds, or added only to feeds for fish larvae or fry.

13.2.4 Trash fish

Fish farmers in some regions use fresh-caught or frozen whole fish to raise farmed fish. Generally, these are low-value fish that are not commonly used as human food; hence the name ‘trash’ fish, but, in some situations where high-value species, such as bluefin tuna, are farmed, edible pelagic species such as sardines or pilchards are fed. Whole fish are sometimes used as the sole food, as used to be the case with yellowtail farming in southern Japan before the introduction of pelleted aquafeeds, or the trash fish is combined with a dry meal mix (broken rice, seaweed, oilseed cake) to make a fresh, moist feed that is made at the farm and fed fresh. Farming of basa in Vietnam has followed this model, although pelleted aquafeeds are replacing farm-made feeds in Vietnam. According to Tacon *et al.* (2006), over 60% of the total fish catch from the

South China Sea and the Gulf of Thailand, 30–80% of the catch from Vietnam (includes freshwater landings in the Mekong River Delta) and 50% of the catch in Malaysia is trash fish and used in on-farm aquafeeds. In addition to basa, bluefin tuna and yellowtail, trash fish feeds are used in the production of marine species in China, lobsters and crabs, and snakehead, sand goby and *Pangasius* catfish in Southeast Asia (Tacon *et al.*, 2006). The quantity of trash fish used directly as feed or to produce on-farm feeds is estimated to range from five to six million metric tons (Allan, 2004; Tacon, 2004). As information about the nutritional requirements of these species expands, the use of on-farm feeds will diminish and be replaced with pelleted aquafeeds.

Using on-farm feeds containing unprocessed (raw) trash fish or feeding trash fish as the sole food presents risks associated with the introduction of pathogens to the farmed fish via the feed. In addition, using trash fish feeds lowers water quality, further increasing the risk of disease or fish loss. From the perspective of quality of farmed fish to the consumer, the use of trash fish has little impact, aside from the risk of loss due to pathogens in culture. Wild fish consume trash fish as their normal prey, albeit fresh, live fish.

13.3 Crustacean and shellfish by-products

13.3.1 Shrimp by-products

As mentioned in Section 13.2.1, shrimp meal is a product made from shrimp heads and viscera. Compared to menhaden fish meal, it is relatively low in crude protein (52–55%) and high in ash (21–23%) (Li *et al.*, 2004). It also contains lower levels of the essential amino acids lysine and methionine than fish meal. These amino acids are typically limiting in aquafeeds containing high proportions of plant protein concentrates. The apparent digestibility coefficient for protein, measured in both red drum and rainbow trout, was significantly lower for shrimp meal than for fish meal (Li *et al.*, 2004; Hardy *et al.*, 2005). Not surprisingly, growth performances of red drum and rainbow trout were significantly lower when shrimp by-product meal replaced 50% of menhaden fish meal in aquafeeds for these species.

Inclusion of shrimp by-product meals in aquafeeds at appropriate levels, e.g. <10%, is unlikely to result in any change in farmed fish quality relevant to the consumer. Higher levels of inclusion lower farmed fish growth performance and are generally avoided in formulation and production of aquafeeds.

13.3.2 Squid by-products

Squid processing by-products possess several attributes that make them highly desirable marine materials from which to produce ingredients for aquafeeds (Chamberlain and Hunter, 2001). Squid oil, squid liver meal and squid liver paste, produced from viscera and trimmings, are used in aquafeeds to add essential nutrients and to increase feed intake, presumably by increasing feed

palatability. Squid oil contains omega-3 fatty acids and sterols including cholesterol, an essential nutrient for shrimp. Squid meal is a high-protein ingredient that averages 73% crude protein (range 60–80%) and is added to aquafeeds for shrimp and selected fish at 2–8% of the formulation. Squid liver paste, in contrast, is a high-moisture product, high-lipid product (30% and 27%, respectively), and is used only in specialty feeds. Squid products are relatively expensive and therefore used sparingly for specific purposes in aquafeeds.

13.3.3 Shellfish by-products

Crab meal is produced from crab processing waste and consists of the crab shell, gills, viscera, and muscle not removed during processing. The ash content of crab meal exceeds 40%, and consequently crab meal is used sparingly as a feed attractant and as a carotenoid pigment source, primarily in shrimp feeds.

13.4 Krill

Krill are small, pelagic crustaceans found in abundance in temperate and subarctic waters, such as the North Atlantic (*Megayctiphanes norvegica*), North Pacific (*Euphausia pacific*) and the Antarctic Ocean (*Euphausia superba*). Krill are abundant; the biomass of *Euphausia superba* in the Antarctic Ocean is estimated to be between 125 and 750 mmt, the largest biomass of any species on earth. Annual production of krill in the Antarctic Ocean ranges from 13 mmt to 2000 mmt per year. Krill are the keystone species of the Antarctic Ocean, feeding on plankton and themselves being the main food source for whales, seals, squid and many species of birds; annual consumption of krill by these predators ranges from 152 to 313 mmt per year. *Euphausia pacific* are a primary food source for wild Pacific salmon, supplying the carotenoid pigment, astaxanthin, that imparts colour to the muscle tissue of salmon.

Annual krill landings were reported to be 117 120 tons in 2003, mainly *Euphausia pacific*, down from 300 000 to 500 000 tons in the 1970s and 1980s (FAO, 2005). Despite their small size (2 g maximum), *Euphausia superba* are fished to produce tail meat which is frozen or pickled and in high demand in Japan, Korea, Russia and former Soviet Union countries. Krill processing by-product and krill unsuited for producing tail meat are used to make krill meal, krill hydrolysate and krill oil. Krill products are expensive, approximately twice the cost of fish meal. As a result, they are used as supplements in aquafeeds to supply astaxanthin to feeds for salmon and marine fish, and to increase feed palatability. Typically, no more than 10% krill meal or hydrolysate is added to aquafeeds. Krill product supplementation is limited to feeds used for larval or first-feeding stages or when aquafeeds for marine species contain high amounts of oilseed proteins that lower feed intake, e.g. soy protein concentrate or rapeseed protein concentrate. Between 10 000 and 15 000 tonnes of krill products are used annually in aquafeeds (Tacon *et al.*, 2006).

13.5 Other alternative marine sources of fish feed

13.5.1 Marine algae

Marine algae and plants have been used to make products for a range of uses for many years, and products for use in aquafeed have been used off and on for decades. Most commonly, alginates have been used as binding agents to increase water stability of moist pellets. Kelp meal was used in aquafeeds in the past as a source of iodine and other minerals. This practice became obsolete when other sources of iodine and trace minerals became common additives to aquafeeds, and also with the transition from moist and semi-moist feeds to pelleted dry feeds made by cooking-extrusion. However, interest in meals produced from marine algae and plants has increased in recent years, this time as additives to increase shrimp pellet water stability (Cruz-Suarez *et al.*, 2007). Shrimp feed slowly, making it critically important that pelleted feeds remain water stable for hours until shrimp locate and consume them.

13.5.2 Cultivated marine organisms

Cultivating marine organisms to feed to farmed fish or shrimp is a challenging enterprise. The cost of cultivating marine organisms is high and the inevitable metabolic inefficiency of converting food into growth makes it unlikely that cultivated marine organisms will ever be a major component of aquafeeds. However, some cultivated marine organisms have unique characteristics, some known and others as yet unknown, that are essential and therefore valuable to fish farmers and therefore justify their high prices. At present, many marine organisms are cultivated for use in aquafeeds, including rotifers, *Artemia*, the ragworm (*Nereis virens*), the marine algae *Haematococcus pluvialis* and the marine microalgae *Crypthecodinium cohnii*.

Rotifers and *Artemia* sp. are small zooplanktonic crustaceans that are prey for many species of farmed fish, especially those having a larval stage at first feeding. Fish larvae are too small to consume pellets or feed particles, making it necessary to supply microparticulate feeds to fish larvae being cultivated. However, many fish larvae do not recognize microparticulate feeds as prey items, making it necessary to supply live prey, e.g. rotifers and *Artemia* at the first feeding stages. Larvae are weaned from live prey as they grow and switched to microparticulate and pelleted feeds. Rotifer and *Artemia* culture are well developed, although in the case of *Artemia*, many growers utilize wild-caught *Artemia* nauplii hatched from cysts rather then grow *Artemia*. Using wild zooplankton as live feed for fish larvae contains the risk of introducing disease organisms into the larval culture system, whereas producing live prey in the hatchery allows some level of biosecurity to be employed. Since rotifers and *Artemia* are only used at the first feeding stage, their use has little impact on the quality of farmed fish for consumers, other than in terms of ensuring the supply of farmed fish in the market.

The ragworm, also called the sandworm, is a marine polychaete that grows in intertidal areas in temperate zones of the Atlantic Ocean. Ragworms can grow to

over one metre in length and are prey for many marine fish. Cultivated ragworms are used in hatcheries as a component of maturation feeds for a growing number of farmed marine fish, e.g. sole, European sea bass and gilthead sea bream, and are also used as a feed for maturing shrimp. Ragworms are grown under controlled conditions and flash-frozen. Ragworm products are also used in starter feeds for marine fish larvae to stimulate feeding and to supply essential nutrients. These stages of life are critical in the context of aquaculture production, thereby justifying the cost of ragworms as aquafeeds and/or supplements. Ragworms are excellent sources of omega-3 fatty acids, but they evidently contain other compounds that are essential nutrients and feeding stimulants. They are not used in grower feeds for any species and therefore have no connection to farmed fish quality for the consumer, other than in the context of enhancing maturation and larval survival, thereby increasing productivity of marine and shrimp aquaculture to make farmed products more available.

The marine algae *Haematococcus pluvialis* produces astaxanthin when grown under stressful conditions, and this property has been exploited to produce dried *H. pluvialis* as a supplement for aquafeeds (Guerin *et al.*, 2003). Currently, *H. pluvialis* is produced commercially by several companies, but the cost of dried *H. pluvialis* as an astaxanthin supplement exceeds that of astaxanthin produced by industrial synthesis. This price disparity limits its use in aquafeeds to situations where a natural astaxanthin supplement is desired by the consumer. The efficiency of pigmenting salmonids by astaxanthin from *H. pluvialis* is similar to that of synthetic astaxanthin, but the proportion of stereoisomers in astaxanthin produced by *H. pluvialis* differs from that of synthetic astaxanthin. The main stereoisomer in astaxanthin produced by *H. pluvialis* is 3S,3′S, the same form found in wild salmon that obtain astaxanthin via the food chain. The synthetic form of astaxanthin is an equal mixture of 3S,3′S and 3R,3′S forms. The metabolic significance of this difference is not known, but both forms are deposited in salmon tissues more-or-less equally. There are no reported differences in health of salmon fed feeds supplemented with synthetic astaxanthin or astaxanthin produced by *H. pluvialis*. In fact, research demonstrating that astaxanthin is an essential nutrient for Atlantic salmon was conducted using synthetic astaxanthin supplementation (Christiansen *et al.*, 1995).

Consumers wishing to avoid farmed fish, mainly salmon, that have consumed feeds supplemented with synthetic astaxanthin have an alternative, namely farmed salmon fed feeds supplemented with astaxanthin from *H. pluvialis*. Such products are available in the market.

The marine microalgae *Crypthecodinium cohnii* can be grown under controlled, continuous fermentation to produce a high-lipid product that is rich in omega-3 fatty acids, mainly DHA and EPA, essential nutrients for farmed fish and shrimp. The traditional source of DHA and EPA in aquafeeds has been fish oil produced along with fish meal from marine fish. The trade name for this one product produced from *Crypthecodinium cohnii* is AquaGrow®. Given the finite annual production of fish oil (average 1.24 mmt, worldwide) and the fact that nearly 80% of annual fish oil production is now used in aquafeeds, the prospects

for AquaGrow® or similar products as ingredients in aquafeeds are good. The advantages of cultivated algae products as sources of omega-3 fatty acids include sustainability, traceability, and consistent product quality. The disadvantage at present is cost, but cost could be reduced if production reaches an industrial scale.

Farmed fish require dietary sources of omega-3 fatty acids, particularly long-chain fatty acids of marine origin, such as DHA and EPA, for normal growth and health (NRC, 1993). However, dietary requirements are lower than the dietary amount required to produce fast-growing, healthy fish. Thus, edible products, e.g. fillets, from farmed fish fed feeds containing levels of omega-3 fatty acids necessary for optimum growth and health do not necessarily contain omega-3 fatty acids at levels typical of wild fish. In fact, numerous studies have demonstrated that salmonids and other species can grow normally when fed diets containing any number of alternative lipids from rendering of slaughter by-products or plant oils, providing feeds contain 1–2% long-chain, omega-3 fatty acids (Hardy *et al.*, 1987; Higgs and Dong, 2000). However, one of the desirable attributes of fish consumption is their high omega-3 fatty acid content compared to other food items. The fatty acid profiles of fish fed high levels of alternative lipids reflect dietary fatty acid profile. Farmed fish producers are acutely aware of this and employ a range of feed strategies to ensure that consumer expectations concerning the omega-3 fatty acid content of their edible products are met. High omega-3 fatty acid sources are increasingly needed as components of finishing feeds for farmed fish to boost omega-3 fatty acids in fillets. This is necessary to achieve omega-3 fatty acid levels that impart healthful benefits to consumers, especially in light of the growing trend to add plant oils as partial replacements for fish oil in the grow-out stage of farmed fish production.

13.6 Future trends

Marine products, primarily fish meal and oil, have traditionally constituted a high percentage of aquafeeds. Annual global production of fish meal and oil has been relatively constant for 20 years, but the rapid growth of aquaculture with a concurrent growth in aquafeed production has increased the proportion of fish meal and oil used in aquafeeds to 50% and 80%, respectively, raising concerns about the sustainability of aquaculture production growth. Prices for fish meal and oil increased substantially in 2006, making alternative plant protein concentrates economical alternatives. Plant protein concentrates are inferior in nutritional value to fish meal, but nutritional value can be improved by adding relatively small amounts of marine proteins to aquafeeds based upon plant protein concentrates. For these reasons, the role of marine proteins and oil will shift from that of primary aquafeed ingredients to that of supplements to supply essential nutrients, increase palatability, to supply as-yet unidentified metabolically active components to plant-based aquafeeds, and to ensure healthful levels of omega-3 fatty acids in farmed fish for the consumer. Recovery and utilization

of components of the seafood processing by-product waste stream will be driven by the need to add highly refined marine materials in plant protein-based aquafeeds. The impacts of these changes on the quality of farmed fish will be negligible, but changes in lipid source from marine oils to plant-derived oils will have a large impact on fish quality, changing both fatty acid profiles and also flavour. Marine-derived, lipid-rich ingredients will therefore be in increasing demand to maintain farmed fish quality for the consumer.

13.7 Sources of further information and advice

Maximizing the value of marine by-products (2006) F. Shahidi (Ed.) Woodhead Publishing Ltd, Cambridge.

Information on global trends in aquaculture production can be obtained from the following website: FAO http://www.fao.org/fi/website/FIRetrieveAction.do?dom=topic&fid=16073

Commercial websites containing information on cultured marine products include:

- Advanced BioNutrition, source of the *marine microalgae Crypthecodinium cohni* used to produce AlgaeGrow®: www.advancedbionutrition.com.
- Cyanotech Corporation, source of astaxanthin produced by the marine algae *Haematococcus pluvialis*: www.cyanotech.com.
- Igene, source of astaxanthin produced by *Phaffia* yeast: www.tateandlyle.com
- Seabait, source of ragworm (*Nereis virens*) products: www.seabait.com

Information can also be obtained by searching the world wide web using the search terms *Artemia*, rotifers, kelp products, fish hydrolysate, krill and other such terms for specific products.

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14

Tailor-made functional seafood for consumers: dietary modulation of selenium and taurine in farmed fish

J. Luten, Nofima Marine, Norway, E. Schram, Wageningen IMARES, The Netherlands and E. Elvevoll, University of Tromsø, Norway

14.1 Introduction

The primary role of a diet is to provide sufficient nutrients to meet the nutritional requirements of an individual. There is now an increasing amount of scientific evidence to support the hypothesis that some foods and food components have beneficial health effects beyond the provision of basic nutrients.

Scientific and technological developments in the field of food have led to a marked shift in how consumers deal with food and health. There is a growing awareness among consumers that the dietary source and form of food may affect their overall health. The role of food as an agent for improving health has initiated the development of new classes of tailor made food – functional foods.

The term functional food has been linked with several definitions that vary from simple statements to rather more complex scientific ones. In 1999, a European Community Concerted Action on Functional Foods Science in Europe (FUFOSE) tightened the definition of functional foods. The Concerted Action came to the conclusion that food can be regarded as functional if it is satisfactorily demonstrated to affect one or more target functions in the body beyond adequate nutritional effects, in a way that is relevant either to an improved state of health and well-being and/or the reduction of risk of disease (Diplock *et al.*, 1999). Functional foods must remain food and must demonstrate their effects from amounts that can normally be consumed in the diet.

The concept of functional foods was born in Japan 25 years ago. Consumer

demands and scientific results have contributed to the increase in functional foods and functional food ingredients in USA and Europe (Lewis, 2006). Suppliers now offer a large variety of these foods and ingredients.

In essence a functional food can be produced from a food by natural enhancement of the health beneficial components through growing conditions. Addition of the component to the food in order to provide the benefits is another option. Also the removal of component(s) in order to reduce any reverse health effect can be regarded as a process for functional foods. Chemical modification of one or several components in the food or changing the bioavailability of its healthy components to improve absorption is another tool to develop functional foods. Natural enhancement of health-beneficial components via the feed during farming is an ideal opportunity to develop 'new' functional foods. Also this natural enhancement may be better accepted by consumers (Frewer *et al.*, 2003) than addition of the health-beneficial component to the final product.

It is well known that fish is nutritious and healthy. It is, in particular, well documented that the polyunsaturated fatty acids (PUFA) in fish have health-beneficial effects. There is strong evidence that eating fish reduces the risk for coronary vascular diseases, even at low consumption in comparison with no consumption (He *et al.*, 2004). Risk factors affected by fish consumption (1–2 times per week) in a positive way are the triglyceride levels (decreasing) and the good HDL cholesterol level (increasing).

The PUFA content in fish depends on several factors and in the case of fish farming the PUFA content can be tailor-made by the selection of the type and amount of oil (e.g., fish oil versus vegetable oil) in the composition of the feed.

Selenium (Se) and taurine are other important micronutrients for human beings. These micronutrients are present in seafood and contribute significantly to the daily intake from food by consumers. The possibilities of tailor-making farmed fish with optimal levels of Se and taurine will be discussed in this chapter within the context of the health-beneficial effects of selenium and taurine for human beings.

14.2 Nutritional and health-promoting properties of selenium

14.2.1 Selenium intake and selenium status

Selenium is an essential element and it forms a part of at least eleven seleno-proteins in two groups of seleno-enzymes in the human body: glutathione peroxidases (GPx) and iodothyronine deiodinases. Selenium is beneficial at lower concentrations, whereas at higher concentrations it becomes toxic. The range between deficiency, essentiality and toxicity, however, is rather narrow. The European Commission Scientific Committee on Food (SCF) (2000) recommended that the intake should not exceed 300 μg Se per day. The Dietary Reference Intake[1] established by the National Academy of Sciences (2000) in the USA is

1. Also called Recommended Daily Allowance or Recommended Dietary Intake.

Table 14.1 Se intake in European countries (European Commission Scientific Commission on Food, 2000)

Country	Se intake (μg/day)
Belgium	28–61
Denmark	41–47
Finland	100–110
France	29–43
UK	63
The Netherlands	40–54
Norway	28–89
Spain	79
Sweden	24–35

55 μg Se/day for adult men and women. A so-called Population Reference Intake (PRI) of 55 μg Se/day was established by the SCF (1993). However in some European countries different PRI values are recommended varying from 30–150 μg Se/day. The Se intake based upon different studies in Europe, reviewed by SCF (2000), is shown in Table 14.1. Rayman (2005) argued that the current plasma and serum Se concentrations do not allow maximal expression of plasma GPx, the criterion adopted by most authorities when setting their dietary reference values for Se. The situation for some countries in Europe is of concern due to falls in Se intake (Rayman, 2005). This reduction is suggested to be a result of the reduced import of North American wheat, rich in Se for bread-making. Cereals are an important source for Se intake besides meat and fish.

14.2.2 Selenium in fish

Although fish consumption is lower than meat consumption, it is clear that fish is an important source for the intake of Se because of the rich Se content of several fish species and shellfish. An overview of the selenium content in fish species and shellfish (Barclay *et al.*, 1995; Oehlenschläger, 1997, Souci *et al.*, 2000; Plessi *et al.*, 2001) is presented in Table 14.2. The results show that the Se content in the edible part of fish and shellfish varies between 0.1 and 0.8 mg/kg with a mean value of 0.4 mg/kg.

However, fish as a whole or the edible part might undergo a number of processing steps (e.g. storage in ice after catch, salting, smoking, marinating, canning, etc.) and household preparations (baking, boiling, frying) before it is consumed. In two recent papers (Mierke-Klemeyer *et al.*, 2008; Careche *et al.*, 2008) it was reported that hardly any losses of Se occur during household preparation (baking, boiling or deep frying) in the case of African catfish, flounder and tuna.

14.2.3 Speciation of selenium in fish

From a nutritional and health-beneficial point of view it is even more important to know the chemical form in which Se is present in the edible part of the fish.

Table 14.2 Se content in edible part of fish and shellfish

Fish species	Average Se content in edible part (mg/kg)	Reference
Crab	0.84	Barclay *et al.* (1995)
Lobster	0.54	Barclay *et al.* (1995)
Regular prawns	0.19	Barclay *et al.* (1995)
Tiger prawns	0.26	Barclay *et al.* (1995)
Saithe, cod, haddock and whiting	0.30	Oehlenschläger (1997)
Hake, mackerel and horse mackerel	0.40	Oehlenschläger (1997)
Sardines, plaice and herring	0.50	Oehlenschläger (1997)
Lemon sole	0.60	Oehlenschläger (1997)
Salmon	0.25	Souci *et al.* (2001)
Rainbow trout	0.29	Souci *et al.* (2001)
Tuna	0.82	Souci *et al.* (2001)
Herring	0.43	Souci *et al.* (2001)
Mackerel	0.39	Souci *et al.* (2001)
Pilchard	0.60	Souci *et al.* (2001)
Halibut	0.13	Plessi *et al.* (2001)
Tuna	0.73	Plessi *et al.* (2001)
Spiny lobster	0.07	Plessi *et al.* (2001)
Mussels	0.61	Plessi *et al.* (2001)

The chemical form determines the degree of bioavailability and bioactivity after absorption. Although the methodologies for speciation trace element analysis have improved over the last ten years, the speciation of selenium in fish is still not optimal. Poor recovery of the extraction of Se from the raw material is one of the obstacles in that respect.

Early efforts to determine Se speciation in fish (plaice, cod, mackerel, herring) were performed by Åkesson and Skrikumar (1994), Crews (1998), Shen *et al.* (1997) and Önning and Bergdahl (1999) using various forms of size-exclusion chromatography with off- or on-line detection of Se. These studies demonstrated that the extraction of Se from the edible part was poor (less than 30% for some species). The soluble fraction (mainly water soluble proteins and low molecular weight (LMW) compounds) from plaice and mackerel contained a high proportion of LMW Se compounds while high molecular weight Se compounds were present in cod.

More recently selenomethionine and selenocystine were identified in enzymatic hydrolysates from Antarctic krill (Siwek *et al.*, 2005). Several other studies (Quijano *et al.*, 2000; Moreno *et al.*, 2004; Cabanero *et al.*, 2005) identified selenomethionine as the main Se component in the soluble fractions of sardine, swordfish, tuna, trout, krill, oyster and mussel. Also trimethyl Se^{+} and inorganic Se was found in some species.

A complete review of selenium speciation from food source to metabolites has recently been published by Dumont *et al.* (2006). This review shows that selenomethionine and selenocystine are the main Se components in Brazil nuts. Selenomethionine is the major component in cereals. In garlic and broccoli γ-

glutamyl-Se-methylselenocysteine and Se-methylselenocysteine are predominantly present. Se-yeast supplements contain mainly selenomethionine. The structure of the main identified organic selenium compounds is presented in Table 14.3.

14.2.4 Bioavailability of selenium from fish

The bioavailability and metabolic fate of Se from dietary fish in humans have not been studied extensively. A cross-sectional study (Hagmar *et al.*, 1998) with 68 men (24–79 years) from the coast and inland Latvia was carried out in order to investigate the relationships between fish intake, different markers of Se status and thyroid hormone function. The number of fish meals per month was correlated with plasma Se, selenoprotein P and GPx. The mean plasma Se level in those with high fish intake was 81% higher than in those with the lowest intake.

An intervention study (Fox *et al.*, 2004) was carried out to measure the bioavailability of Se from trout, yeast and selenate. The study was a parallel, randomized, reference substance controlled design carried out with 35 volunteers in the Netherlands and United Kingdom. Apparent absorption of Se from cooked or salted enzymatic ripened trout (88 $\pm$ 5% respectively 90 $\pm$ 3%) was similar to selenate in human volunteers (93 $\pm$ 4%). The study also showed that there was no difference between the two fish processing methods used. Apparent absorption of yeast Se (54 $\pm$ 7%) was significantly lower than from Se in trout and selenate. However, Se retention from trout (86%) was significantly higher than selenate (60%). The retention of Se from yeast (59%) was lower.

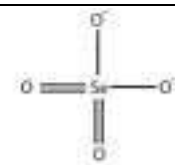
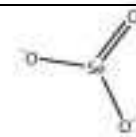
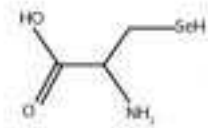
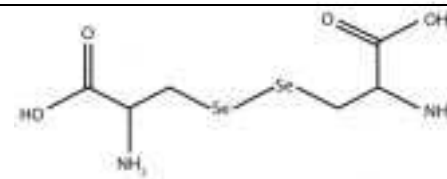
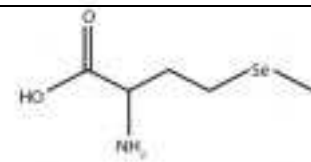
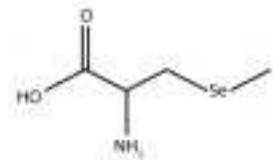
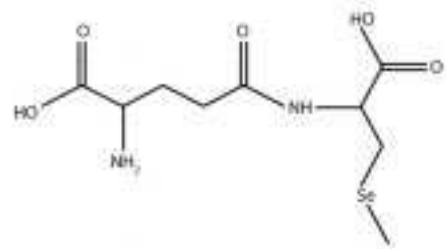
In another study by Fox *et al.* (2005) wheat, garlic and cod intrinsically labelled with Se-77 or Se-82 stable isotopes were consumed in random order by 14 adults, with a minimum washout period of six weeks between each test meal. Se absorption was significantly higher from wheat (81 $\pm$ 3%) and garlic (78 $\pm$ 14%) than from cod (56 $\pm$ 4%). The form of Se and food constituents appear to be key determinants of post-absorptive metabolism.

14.2.5 Health-beneficial effects of selenium

Several review papers have reported the health-beneficial effects of Se in relation to cardiovascular disease, other oxidative stress conditions, immune function, viral infections, reproduction, thyroid function, mood and cancer (Rayman, 2002; Whanger, 2004; Rayman 2005; Combs, 2005). It is clear that in some cases, where the Se-intake has been above those levels required for repletion of the seleno-enzymes, these may give health benefits. This also seems to be valid for immune system stimulation and reductions of cancer incidence and cancer mortality.

In rat studies, supplementation of a Se-garlic diet at different levels (1–3 mg Se/kg) consistently resulted in a lower tissue selenium accumulation when compared to Se-yeast (Clement *et al.*, 2000). On the other hand, Se-garlic was

Table 14.3 Name and structural formulae for some important Se components

Name	Structural formulae
Selenate	
Selenite	
Selenocysteine	
Selenocystine	
Selenomethionine	
Se-methylselenocysteine	
γ-glutamyl-Se-methylselenocysteine	

significantly more effective in suppressing the development of tissue abnormalities related to cancer. In another study (Finley *et al.*, 2000) diets with various amounts (up to 2.0 mg Se/kg diet) and forms of Se (selenate and selenized broccoli) were given to rats. Supranutritional amounts of Se supplied as broccoli significantly decreased the incidence of the precursors for colon cancer.

Prospective studies of Se and cancer in human beings, reviewed by Knekt (2002), showed that in approximately 50 out of 72 studies a lower cancer risk was associated with higher Se intake. The strongest evidence for a beneficial effect of Se appears to be related to lung cancer, oesophageal and gastric-cardia cancers and most notably, prostate cancer. The strongest evidence of the efficacy of Se as a cancer preventative, particularly for prostate cancer, is provided by the Nutritional Prevention of Cancer trial. Subjects with a history on non melanoma skin cancer were treated for 4.5 years with yeast-Se (200 μg Se/day) with a follow-up time was 6.5 years. Fifty percent lower total cancer mortality and 37% lower total cancer incidence was found (Clark *et al.*, 1996).

Further randomized clinical trials using defined Se-compounds are needed for confirmation of the above mentioned clinical results. One such study is currently in progress in the USA, the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Planned as a 12 years trial involving 400 sites and an enrolment of 32 400 men, SELECT will test the hypothesis that selenomethionine (200 μg/day) and/or vitamin E supplementation can reduce the risk of prostate cancer (Klein *et al.*, 2000).

In order to test the possible role of selenium in cancer prevention, a group of researchers from Denmark, Sweden and from the UK have designed the Prevention of Cancer by Intervention with Selenium (PRECISE) study. Over a period of 8 years a planned total of 42 000 Europeans will receive a supplement of yeast-selenium at 100, 200 and 300 μg/day or placebo in a randomized order.

A number of mechanisms have been suggested to explain the anti-cancer effects of Se. Although it is fairly well accepted that methylselenol (CH_3SeH) is involved in the anti-cancer effects of Se at supra-nutritional doses, evidence is growing that the seleno-enzymes do play a role, particularly at nutritional levels of intake. CH_3SeH can be formed by methylation of H_2Se as part of the Se excretory pathway or formed from a storage form of Se in plants (broccoli and garlic), i.e. γ-glutamyl-Se-methylselenocysteine. Metabolism removes the γ-glutamyl group to give Se-methylselenocysteine which is converted to CH_3SeH (Fig. 14.1).

14.3 Nutritional and health-promoting properties of taurine

14.3.1 Taurine intake and taurine status

Humans have a limited ability for biosynthesis of taurine and it may be regarded as conditionally essential, as its physiological concentration can be partly regulated endogenously (Roe and Weston, 1965; Gaull, 1986; Stapleton *et al.*, 1997; Niittynen *et al.*, 1999; Schuller-Levis and Park, 2003; van de Poll *et al.*,

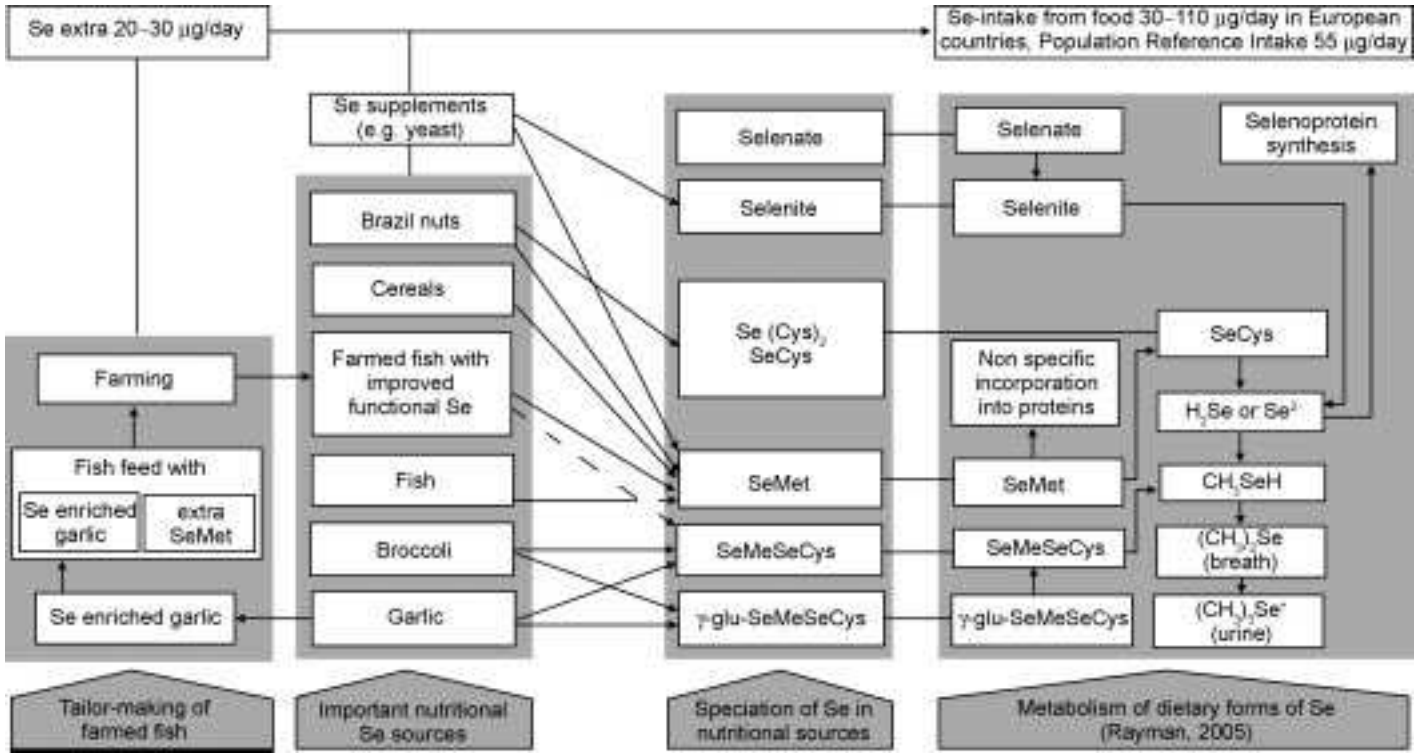


Fig. 14.1 Schematic overview of dietary modulation of farmed fish with Se, nutritional Se sources, speciation Se and metabolism of Se. $Se(Cys)_2$ = selenocystine, Se(Cys) = selenocysteine, SeMet = selenomethionine, SeMeSeCys = Se-methylselenocysteine and γ-glu-SeMeSeCys = γ-glutamyl-Se-methylselenocysteine.

2006). Newborns and infants have even no or lower ability to synthesize taurine, and therefore taurine is added to infant formulas (Gaull, 1986; Schuller-Levis and Park, 2003).

Taurine is synthesized from cysteine via the sequential actions of cysteine dioxygenase (CDO), which gives rise to cysteinesulfinate and cysteinesulfinate decarboxylase (CSD), which decarboxylates cysteinesulfinate to hypotaurine. Hypotaurine is further oxidized to taurine. The capacity for taurine biosynthesis varies between species. Compared with the rat, for example, man, primates, the cat and even fish (Park *et al.*, 2002) exhibit very low activities in vitro of CSD which is believed to be the rate-limiting enzyme responsible for the formation of taurine from cysteine.

As a result, dietary taurine uptake accounts for the majority of the taurine supply. The taurine body content is derived from direct taurine intake from the diet, taurine synthesis by the liver and kidney reabsorption (Bouckenooghe *et al.*, 2006). As a consequence, vegans and parenterally fed individuals not supplemented with taurine have a low plasma taurine content and may need to be supplemented.

Taurine is an end product of sulphur amino acid metabolism and thus considerations of the need of supplementation or dietary changes have to include consumption data on methionine and cysteine. Methionine is the only essential sulfur amino acid (SAA) and can provide sulfur for cysteine and taurine synthesis. Animal protein is generally considered to be a better source of SAA than vegetable protein. There is apparent consensus concerning normal SAA requirements. The classical experiments of Rose (1976) have been reproduced by the World Health Organisation (FAO/WHO/UNU Joint Expert Consultation, 1985) and several other authors (van de Poll *et al.*, 2006) and an SAA intake of 13 mg/kg per 24 h is still recommended for healthy adults.

Due to the lack of inclusion of taurine, while not generally regarded as essential, in standard tables of food composition, it has been difficult to calculate the dietary taurine intake. There are thus less data, or fewer studies than expected, assessing the actual dietary intake of taurine. The daily intake of taurine in western Europe and the safety of drinks enriched with taurine, were reviewed by The European Scientific Committee on Food (EFSA Scientific Committee on Food, 1999). The daily intake reported from different studies, varied between 40 and 400 mg with a mean of 58 mg (Rana and Sanders, 1986), although it was generally less than 200 mg (Laidlaw *et al.*, 1990).

The mean daily intake of taurine in nine different Japanese districts was 194 mg/day (males 226 mg/day and females 163 mg/day). Many subjects were recorded with intakes between 300 and 600 mg/day and highest intakes noted were over 1 g/day. There was a significant correlation between fish and shellfish intake and taurine intake, while no correlation between meat intake and taurine intake could be established (Kibayashi *et al.*, 2000). The taurine content in Chinese food, including seafood, fresh water fish, meats and some plants, was examined by Zhao *et al.* (1998). The daily taurine intake, based on food intake and analyzed taurine content in food, ranged from 34 to 80 mg/day. Another

study assessed both the dietary intake and the serum taurine level of women from a fishing district and a city area of Korea. The dietary intake ranged from 8.4 to 767 mg/day (mean 164 mg/day) with a significant difference between the city area (115 mg/day) and the fishing village (216 mg/day).

14.3.2 Taurine in fish

Differences in muscle osmolality, e.g. between marine and non-marine animals, are mainly due to nitrogenous solutes such as certain free amino acids, among which is taurine (Abe, 2000). Seafood, especially invertebrates such as molluscs and crustaceans (300–800 mg per 100 g edible portion), are high in taurine (Roe and Weston, 1965; Laidlaw *et al.*, 1990; Zhao *et al.*, 1998; Spitze *et al.*, 2003; Gormley *et al.*, 2007; Dragnes *et al.*, 2008) and by-products from seafood may thus serve as raw materials for ingredients in functional food (Elvevoll, 2005). The taurine concentration in a variety of fish species recently reported (Gormley *et al.*, 2007; Dragnes *et al.*, 2008) is presented in Table 14.4. Variation in taurine concentration among species and between individuals of the same species may be due to a variety of factors. Marine invertebrates generally have a higher cellular osmolarity than fish and since taurine has an osmoregulatory role, this may explain the higher concentrations. Other factors influencing the taurine concentration *in vivo* of the species are seasonal variations, temperature, salinity and diet (Jones, 1954). The concentration of taurine will reflect the contents of sulphur containing amino acids in the feed (Kim *et al.*, 2005).

14.3.3 Bioavailability of taurine from fish

As mentioned previously the body content of taurine in humans is derived mainly from direct taurine intake from the diet and kidney reabsorption and to a lesser extent, taurine synthesis by the liver (Bouckenooghe *et al.*, 2006).

Table 14.4 Taurine content in edible part of fish and shellfish

Fish species	Average taurine content in edible part (mg/100 g)	Reference
Haddock (*Melanogrammus aeglefinus*)	27	Gormley *et al.* (2007)
Atlantic salmon (*Salmo salar*)	60	Gormley *et al.* (2007)
Plaice (*Pleuronectus platessa*)	146	Gormley *et al.* (2007)
Cod (*Gadus morhua L.*)	108	Gormley *et al.* (2007)
Mackerel (*Scomber scombrus*)	78	Gormley *et al.* (2007)
Atlantic salmon (*Salmo salar*)	94	Dragnes *et al.* (2008)
Cod (*Gadus morhua L.*)	120	Dragnes *et al.* (2008)
Saithe (*Pollachius virens*)	162	Dragnes *et al.* (2008)
Haddock (*Melanogrammus aeglefinus*)	57	Dragnes *et al.* (2008)
Shrimps (*Pandalus borealis*)	220	Dragnes *et al.* (2008)
Mussels (*Mytilus edulis*)	510	Dragnes *et al.* (2008)

Studies in rat models have shown that ^{14}C-taurine is rapidly absorbed from the gastro-intestinal tract, distributed widely to tissues, and undergoes elimination unchanged in the urine. The studies indicated more extensive elimination during the first six hours after supplementing with higher doses (Sved *et al.*, 2007).

Fish muscle consumption is reported to result in increased concentrations of serum taurine when compared with beef and chicken muscle (Uhe *et al.*, 1992) and humans on a diet high in seafood are reported to have high serum taurine levels (Zhao *et al.*., 1998; Kibayashi *et al.*., 2000; Kim *et al.*., 2003) whereas humans on diets relatively low in taurine are reported to have low serum taurine levels (Brøns *et al.*, 2004). Human urinary excretion of taurine is also known as a marker for seafood consumption (Biosca *et al.*, 1990; Kim *et al.*, 2003).

14.3.4 Health-beneficial effects of taurine

Studies have suggested that taurine might be a candidate for use as a nutritional supplement to protect against oxidative stress, neurodegenerative diseases and atherosclerosis (Bouckenooghe *et al.*, 2006; Yamori *et al.*, 2006) and is regarded as essential for neuronal (retinal) development in childhood (van de Poll *et al.*, 2006). Taurine appears to have multiple functions and plays an important role in many physiological processes, such as osmoregulation, immunomodulation and bile salt formation.

Beneficial effects of dietary taurine have been observed in animal and human studies (Militante and Lombardini, 2004; Yamori *et al.*, 2004; Bouckenooghe *et al.*, 2006) and suggestions of a reduced cardiovascular risk through taurine alone or in combination with n-3 polyunsaturated fatty in seafood acids have been put forward (Mizushima *et al.*, 1997; Yamori *et al.*, 2001, 2006) and several positive effects on the cardiovascular system has been reviewed by Niittynen *et al.* (1999). First, taurine has antioxidant activity. This may reduce the production of pro-inflammatory products. Secondly, taurine has been shown to lower blood pressure in borderline hypertensive patients. It has also been reported that taurine can improve cardiac performance, reduce blood cholesterol values and suppress platelet aggregation.

14.4 Dietary modulation of selenium and taurine in farmed fish

There has been recently an explosion of consumer interest in the health-enhancing role of specific food, containing bio-active components in addition to nutrients, so-called functional or designer foods.

Based upon the difference between the PRI (55 μg/day) for Se and the measured intake of Se from food (including fish) in Europe (see Section 14.2.1) it is assumed that an increase of 20–30 μg Se/day might be appropriate to create a more optimal Se status in human beings. This suggested increase is well within

safe limits because losses (although minimal for total Se), less than 100% absorption and retention are not taken into account in the assumption. In general this increase in Se intake could be reached by consuming more fish products. However, there is a decline observed in seafood consumption in some European countries and the consumption of seafood among young consumers is low. Therefore the enhancement of Se in farmed fish by dietary modulation is an excellent opportunity to contribute to an improved health status of consumers. This particular is valid when bio-active, health-promoting Se forms are incorporated in the edible part. Assuming that a portion of 150 g of fish is consumed, the increase in the Se concentration should be approximately 0.2 mg/kg fillet. Using ingredients in the fish feed with bio-active Se components (γ-glutamyl-Se-methylselenocysteine and/or Se-methylselenocysteine) from natural resources (garlic, broccoli, etc.) or selenomethionine, would make it possible to create tailor-designed farmed fish. A similar strategy could be followed for taurine with the aim of developing new products with an increased taurine level.

In Fig. 14.1 a schematic concept of the development of farmed fish as a functional food by dietary modulation is presented with focus on Se in various forms including the health-beneficial Se compounds γ-glutamyl-Se-methylselenocysteine and Se-methylselenocysteine present in garlic, broccoli, etc.

14.4.1 Dietary modulation of selenium in farmed fish

The dietary Se requirements and effects of the Se concentration in feed on the final quality of farmed fish have been studied for several species. Most of the studies were not intended for tailor-making the selenium content in the edible part. However, in the context of this chapter these studies are also described here.

The dietary Se (added as Na_2SeO_3) requirement of fingerling channel catfish (*Ictalurus punctatus*) was studied by Gatlin and Wilson (1984). A minimum requirement of 0.25 mg Se/kg dry feed was established. Se concentrations in edible muscle tissue increased almost linearly from 0.07 mg Se/kg when no supplemental Se in the feed to 3.5 mg Se/kg in the edible part at the highest level of Se in the feed (15 mg/kg). At this highest level intoxication effects (reduced growth) in the channel catfish were observed. The minimum Se requirement of fingerling channel catfish was 0.25 mg/kg which will result in a Se content in the edible part of approximately 0.2 mg/kg.

Lorentzen *et al.* (1994) investigated the effects of Se supplementation in feed for Atlantic salmon (*Salmo salar*). The basal diet for the salmon contained 1.2 mg Se/kg. To this diet 1 or 2 mg Se/kg diet was added in the form of selenite or as selenomethionine. There were large differences in Se accumulation between groups fed the two chemical forms of Se. The Se concentration in the muscle tissue of salmon fed with the basal diet was 0.48 mg/kg at the end of the feeding period of 8 weeks. Similar Se concentrations were measured in the fillets (0.43 mg/kg and 0.57 mg/kg) of salmon fed with a supplementation of selenite at the two levels. However, in case of supplemental selenomethionine in

the feed the Se concentrations were much higher in the muscle tissue (1.57 mg/kg and 2.57 mg/kg, respectively). The metabolism of the added selenite and selenomethionine was different. Selenite was metabolized following the regular Se pathway which included storage in the liver. Selenomethionine could also follow the pathway of methionine which leads to incorporation in proteins. This study showed for the first time that increasing the Se level in the edible part of salmon is possible using selenomethionine as the source.

The bioavailability of selenite, selenomethionine and selenoyeast were measured by feeding fingerling of channel catfish (*Ictalurus punctatus*) for nine weeks (Wang and Lovell, 1997). The supplement dietary Se content in the feed varied between 0 and 0.40 mg Se/kg. The results demonstrated that up to a concentration of 0.1 mg/kg organic Se in the feed resulted in greater bioavailability compared to selenite resulting in higher Se content in the muscle tissue within the same period of feeding. A dietary Se content of 0.1 mg Se/kg deriving from selenomethionine or selenoyeast increased the level of Se in the muscle tissue from 0.1 mg/kg to 0.5 mg/kg at the end of the feeding period. A content higher than 0.1 mg/kg in the feed did not increase the Se level any further in the muscle tissue. The better bioavailability is suggested to be due to improved absorption of the organic forms in the fish.

Dietary Se requirements of juvenile grouper (*Epinephelus malabaricus*) were determined by Lin and Shiau (2005). Selenomethionine was added to a basal diet up to a concentration of 4 mg/kg. The adequate dietary requirement was estimated at 0.7 mg Se/kg feed. The Se content in the muscle tissue was increased from 0.64 mg/kg in the group fed on the basal diet up to 2.19 mg/kg in the group feed with a supplemental dose of 4.0 mg Se/kg feed.

A few studies with African catfish (*Clarias gariepinus*) given a Se-enriched diet were carried out by Luten and Schram (2006) and Schram *et al.* (2008). African catfish were fed during a period of six weeks with feed containing selenomethionine or organoselenium (γ-glutamyl-selenomethyl-selenocysteine and selenomethyl-selenocysteine) enriched in garlic. The Se dose in the feed varied between the normal doses of approximately 1 mg Se/kg and 8 mg Se/kg feed. It was shown that African catfish growth was not affected by the dietary Se level. The Se content in the edible part of the African catfish increased linearly with increasing dietary Se levels. The increase was approx. three times higher using selenomethionine than with the organoselenium compounds from Se enriched garlic supplemented to the feed.

Further optimization of the selenium enrichment of African catfish included experimental research on the required length of the enrichment period to reach a target concentration and the effects of depuration on selenium retention in the fillet. Preliminary results indicate that only approximately 10 enrichment days are required to reach a target concentration of 0.6 mg Se/kg fillet upon harvest for a dietary level of 11.7 mg Se/kg. In addition, preliminary results indicate that depuration, a necessary procedure to eliminate off-flavours, has little or no effect on total selenium level in the fillet. At the same time, these experiments indicate that the results previously obtained in relatively small fish (400 g) are

reproducible for market sized African catfish, where the average weight was approximately 1 kg.

Sensory evaluation revealed that fillets from catfish fed with feed containing garlic have a different taste when compared to the fillets from the control treatments. However, it was also found that this difference in sensory properties disappears as a result of depuration.

14.4.2 Dietary modulation of taurine in farmed fish

Dietary taurine requirements and effects of the taurine concentration in feeds on the final quality of farmed fish have been studied for several species. The aim of these experiments has mainly been to study growth and feed efficiency and not to increase the content of the edible flesh (functional foods). The effect of taurine, added as synthetic taurine (Kim *et al.*., 2005), inclusion of organic material high in taurine (Matsunari *et al.*, 2005) and diets enriched in precursors of taurine, methionine and cysteine and taurine (Yokoyama and Nakazoe, 1992), up to high levels in dry feed (20 mg/g dry weight diet) have been studied. When the levels are comparable to the levels present in the diets of wild fish enrichment in the flesh/fillets has been achieved. The use of fish hydrolysates (4.3 g taurine/kg protein) was shown to be less promising to achieve an enrichment of taurine in the flesh/fillets of Rainbow trout (*Oncorhynchus mykiss*) (Aksnes *et al.*, 2006).

It was concluded that taurine is essential for normal growth and development in 0.3 and 4 g Japanese flounder (Kim *et al.*, 2005, Park *et al.*, 2002). Suggestions for the dietary taurine requirement of 0.2 g Japanese flounder are 15–20 g/kg diet. In other studies no effect was observed on growth of chum salmon (Sakaguchi *et al.*, 1988) or of rainbow trout (Yokoyama and Nakazoe, 1992), by taurine supplementation, but taurine supplementation led to elevated levels of taurine in fish muscle.

An experiment in which African catfish (*Clarias gariepinus*) were given a taurine-enriched diet was carried out recently by Schram (personal communication). African catfish were fed during a period of approximately six weeks with a fish meal based diet, enriched with synthetic taurine. The taurine dose in the feed varied between approximately 1 and 18 mg taurine per g wet feed. The taurine content in the edible part of the African catfish increased in a non-linear manner with increasing dietary taurine concentrations leveling off when inclusion exceeded 8 mg taurine per g wet feed. A twofold increase of taurine in the muscle was reached by inclusion of 18–20 g/kg diet.

14.5 Future trends of dietary modulation of farmed fish

It might be expected that consumer demands for functional foods will increase in particular in Europe. The recent EC Regulation No. 1924/2006 on nutrition and health claims made for food regulates consumers' protection against misleading

health claims and creates room for the industry to develop functional foods with certain specific nutritional profiles and/or with health claims.

Marine fish is also a rich source for iodine, and iodine deficiency is still a major problem in some parts of Europe. The commonest strategy to ensure an improved iodine supply is the iodisation of salt, but on a voluntary basis. Schmid *et al.* (2003) showed that the iodine content in freshwater fish chars (*Salvelinus sp*.) could be increased by using feed supplemented with brown algae (*Laminaria digitata*) from 143 μg I/kg wet weight to almost 540 μg I/kg wet weight in nine months. Julshamn *et al.* (2006) performed a study to assess to what degree supplemental dietary iodine was retained in the fillet of adult Atlantic salmon (*Salmo salar*) reared in sea water. Atlantic salmon were fed with moist pellets (based on minced saithe and herring) supplemented with 0–80 mg I/kg on a dry weight basis for 150 days. This experiment showed that fillet iodine levels in adult Atlantic salmon can be increased to 1.4 mg I/kg wet weight by dietary iodine at 80 times the minimum requirement for salmonids, without impacting health and performance.

Above all, it is evident that all functional foods should have good sensorial properties for consumers. For both wild and farmed fish the taste, odour and texture are very important sensorial properties for consumers' (not) liking of seafood (Olsson *et al.*, 2007). Designing farmed seafood with a certain sensory functionality via dietary modulation is another challenging option. In particular, taste and odour in the final product may be affected by the ingredients of the feed.

The increase in knowledge by the application of genomics and proteomics might enhance the possibilities for designing farmed seafood with improved, nutritional functional and sensory properties.

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15

Reducing production-related diseases in farmed fish

R. Waagbø, National Institute of Nutrition and Seafood Research (NIFES), Norway

15.1 Introduction

Sustainable fish farming includes the capability to successfully handle all stages of fish production, from brood fish, early offspring, and a healthy rearing of juveniles and adults. All biological production, however, includes risks for infectious diseases and non-infectious disorders, affecting animals at different stages of the life cycle and in correlation with production intensity. Although aquaculture disease surveillance programs normally cover infectious diseases and how to meet these challenges by adequate biosecurity measures, the term *disease surveillance* has lately been defined more broadly, to include animal health and welfare problems related to toxicity and inherently new diseases, as well as human food safety (Scudamore, 2003). This is in line with national and international feed legislation, which aims to make sure that feed and feed compounds are safe for fish health, result in safe produce for human consumption, and make no risks to the environment.

The present chapter deals with production or husbandry-related disorders of nutritional origin or disorders that in any way may be related to feed and feeding, mainly with examples from salmonid production. It includes symptoms and disorders that often develop relative to periods of intensive growth, exposure to unfavorable environmental conditions or related to borderline nutritional deficiencies and toxicologies. This covers normally non-infectious diseases, although nutritionally induced immunodeficiencies may increase the susceptibility of farmed fish to normally occurring infectious agents (Sealy and Gatlin, 2001; Waagbø, 2006). The main focus is on developmental disorders such as bone deformities, cataracts, skin disorders, heart disorders and digestion

disorders. The role of nutrition as determinant for resistance to stress and infectious diseases was recently reviewed by Waagbø (2006), which included nutritional and toxicological immunomodulation. Very often production disorders can also partly be attributed to genetic relations such as breeding or inbreeding (Ferguson and Danzmann, 1998).

15.2 Fish nutrition as a major determinant for fish health

The modern intensive aquaculture industry is characterized by increased effectiveness in all parts of the production cycle. This means larger production units, higher biomasses, more exposed sea farms, use of light for improved growth and reduced early sexual maturation, water quality improvements such as oxygenation and pH buffering, mass vaccination and medical treatment, etc. Fish feeds and feeding strategies have also developed dramatically over recent decades, such as the introduction of high energy diets for salmonids (>30% lipid, Fig. 15.1), more flexible use of feed ingredients, and optimization of feeding regimes; all for maximal nutrient retention (i.e., seafood produce). Aquaculture represents, however, an industry that is dependent on a series of supporting industries and disciplines, all with their own research and development. Diverging developmental interests and lack of communication between disciplines may end in unfavorable practical and technical solutions or situations that cause predisposition for infectious diseases or result in development of production disorders.

For fish, we normally experience the most sensitive stages at hatching and during juvenile stages where organ development and growth rates are at their highest. This holds especially for the marine species in aquaculture, since the offspring are smaller and less developed than the juveniles (Cahu *et al.*, 2003).

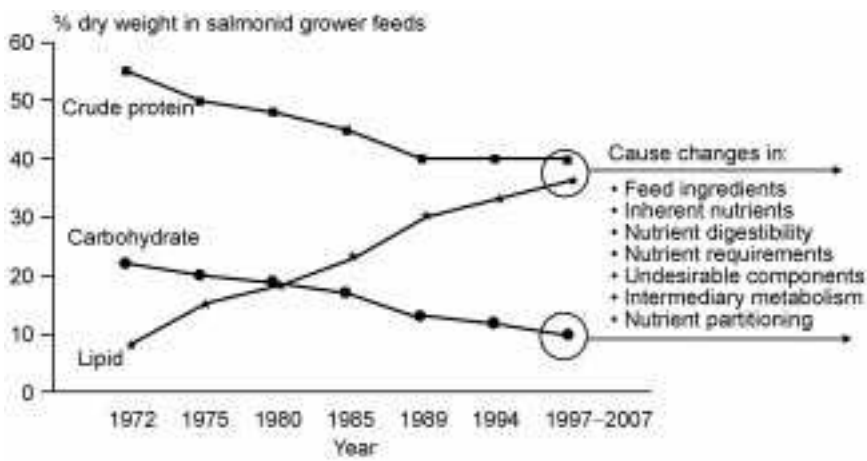

Fig. 15.1 The development of commercial salmon grower diets with time, with marked changes in macro nutrients and protein energy ratio.

Several disorders also occur around smoltification in salmonids, a sensitive period when the salmon prepare for and adapt to sea water, or at adult stages as a consequence of intensive and economically driven farming strategies, including feed types and feeding. Feed constituted as much as 50–55% of the production costs per kg of sea farmed Atlantic salmon (*Salmo salar*) in Norway during the last decade (Fisheries, 2007) and consequently the economy depends on the price of globally and locally available bulk feed ingredients. Inappropriate choices of feed compounds, mainly based on economic considerations may affect digestibility, may contain inadequate levels of nutrients or include toxicants that lower the margins of biological systems and leave the fish at the edge of tolerance. Additional fluctuating or challenging farming environmental conditions may easily move the fish beyond the tolerable limits, resulting in gross visual disease symptoms. Large-scale production also implies that favorable conditions are sought and applied for the majority of the fish population, while a minority may live under suboptimal conditions, which can be stressful and end in development of different disorders for this part of the population. One has also to take into account that artificial juvenile fish production in aquaculture includes raising of less fit individuals that normally would disappear in the wild.

Single nutrient deficiencies cause more or less specific disease symptoms (Halver and Hardy, 2002; Tacon, 1993). While most nutrient requirement studies are performed on juveniles, less information is available on nutrient deficiencies or suboptimal nutrition in adult stages, despite the fact that these represent the main economic investment and seafood produce (Hardy, 2001). The fish farmers are, however, often faced with similar gross symptoms in adults, suggesting that feeding and nutrition may constitute important factors in diseases of multi-factorial origin.

15.2.1 Present status of production-related diseases in farmed fish

Global aquaculture production steadily increases both in biomass (54.8 million tons in 2003) and species (246 reported species in 2003) (Tacon *et al.*, 2006). From the literature on fish farming disorders, it is not surprising that new farmed species experience more difficulties than established ones, despite the fact that experiences and knowledge can be drawn comparatively across species. However, from time to time severe outbreaks of non-infectious disorders, such as bone deformities, cataracts, heart disorders, skin and fin diseases also occur in well-run aquaculture industries. It is difficult to estimate losses from non-infectious production disorders since only severe cases or outbreaks affect growth and result in mass mortalities. Fish with visible signs of such disorders are, however, less marketable, and in the slaughterhouses these end in downgrading and indirect economic losses. An example from Norwegian salmon industry statistics shows that 645 000 metric ton of salmonids were produced for sale in 2005 (Fisheries, 2007). The estimated yearly losses the last 10 years were between 6 and 12% of which a major part (60–90%) was not

specified in detail but reported as non-specific mortalities (Fisheries, 2007). Other statistics from Norwegian salmon aquaculture operates with relatively constant yearly losses at approximately 20% during the last decade (Lillehaug and Skrudland, 2007), while this latter figure includes mortality, escapees, predation, culled fish at slaughter, and undefined losses. Larger aquaculture companies have their own health surveillance programs, which include more specified data recording production, losses and downgrading, but such information is not available to the public. For the individual farmer, however, losses may be critically high during outbreaks. Experience also shows that some sites, for unknown reasons, may be more and repeatedly exposed to disorders than others, illustrating a complex etiology.

15.2.2 Nutrition as one factor in disorders of multi-factorial origin

Modern fish farming relies on a well-qualified feed industry that normally takes care of a sound feed production according to established nutrient requirements and given national and international regulations, and suggests feeding practices for actual fish species. Even though classical nutritional deficiencies mostly belong to yesterday's aquaculture, they are likely to also occur in modern fish farming due to changes in requirements under intensive production conditions and for new species, relative changes in energy-giving nutrients, use of novel feed ingredients, but also inadequacies during feed production, handling and storage (Hardy, 2001). So, despite a modern industrial setup, the fish farmer may experience disorders that can be suspected to have an etiology related to feed or nutrition. Many historical incidences of disorders concern the pressure towards quantitative replacement of traditional high quality marine resources by more or less sustainable alternatives. Today major fish feed ingredients are recruited among products of plant origin but also increased utilization of existing marine resources, like marine by-products from fishery related production and by-catch from the fisheries; alternative marine resources such as krill, algae and cuttlefish; by-products from land animal production; and industrially produced (single-cell protein) or modified feed stuffs (Tacon *et al.*, 2006; Sørensen *et al.*, 2007; Waagbø *et al.*, 2001). Such alternatives may introduce health implications that need to be addressed and acted upon before these can be commonly used (see Table 15.1). National and international feed regulations on upper limits for undesirables may also prohibit selected feed stuffs from being used in fish feed for safety reasons. In general, adverse components can normally be removed from feed stuffs by refinement (Sørensen *et al.*, 2007). Since this generally means higher feed costs, a balance between a safe inclusion level and grade of refinement are often considered in diet formulation.

Plant feed resources

Worldwide aquaculture of carnivorous fish species has hitherto relied on the supply of marine feed ingredients as main feed sources with high protein quantity and quality, and lipids with highly unsaturated n-3 fatty acids (n-3

Table 15.1 Examples of possible fish health implications by use of plant ingredients as fish meal and fish oil replacers in aqua feeds

Plant component (key references)	Suspected disorders or symptoms	Suggested cause-relationship
Proteins		
(Li and Robinson, 2006)	Growth reduction, body composition	Amino acid imbalance/deficiency; disturbed protein digestion by phytate
Antinutrients		
(Storebakken *et al.*, 1998)	Reduced mineral absorption with risk for bone deformity	Mineral deficiency due to phytate binding
(Francis *et al.*, 2001)	Growth reduction	Disturbance of enzymes, digestion and absorption by antinutrients
(Baeverfjord and Krogdahl, 1996; Knudsen, 2007)	Distal intestinal changes (enteritis)	Lectins; antinutrients; saponins
(Krogdahl *et al.*, 2000)	Immunity and disease resistance	Local inflammation by antinutrients
(Yildirim *et al.*, 2003; Garcia-Abiado *et al.*, 2004; Li and Robinson, 2006; Tripathi and Mishra, 2007)	Toxicity	Plant inborn toxic compounds, like gossypol and glucosinolates
Lipids		
(Waagbø *et al.*, 2004; Bjerkås *et al.*, 2006)	Cataracts	Lens fatty acid composition; peroxidation; membrane permeability
(Balfry and Higgs, 2001; Waagbø, 2006)	Reduced immune and stress resistance	Membrane composition; imbalanced C20 fatty acids and derived eicosanoids; peroxidation
(Bell *et al.*, 1991; Seierstad, 2008)	Cardiac pathology	Imbalanced C20 fatty acids and derived eicosanoids; peroxidation
(Olsen *et al.*, 2000)	Intestinal mucosa	Phospholipid deficiency
Gene modified (GM) ingredients		
(Hemre *et al.*, 2007; Sagstad *et al.*, 2007, 2008)	Immuno-organ enlargement; stress or immune response (?)	Metabolic changes; phytotoxic effects (transgenic DNA or proteins); antinutrients (lignin); inflammation
(Hemre *et al.*, 2007)	Gastrointestinal tract physiology	Effect of plant glycosides (flavonoids) on intestinal glucose transport
Possible contaminants		
(Metcalfe, 1998)	Toxiopathic lesions in organs	Oxidation; membranes related cellular events; endocrine disruption of pesticides
(Hendricks, 1994; Manning, 2001)	Hepatocellular carcinomas/adenomas	Aflatoxin B_1 contamination of feed/plant ingredients with *Aspergillus* spp.

HUFA). With limited global resources from marine fisheries and increasing competition for these among farmed animal production, and higher proportions in direct use for human consumption, suitable alternatives need to be provided in the near future. Along with the intensive search for and use of fish meal and fish oil replacers in the farming industry, dietary challenges occur, such as changes in feed technical and nutritional quality, nutrient bioavailability, and in the concentration of antinutrients, and more or less known undesirable substances (Table 15.1). The price of the marine ingredient replacers not only depends on availability on the market but also increases upon refinement procedures as discussed above. The final quality and price are important issues in the evaluation of feedstuffs and in the decision-making processes of the feed producers. Several protein and vegetable lipid sources or their mixtures have been evaluated successfully for growth and overall performance in fish, mostly at inclusion levels <30% (Hertrampf and Piedad-Pascual, 2000). This depends on ingredient suitability (technical quality), digestibility (nutritional quality), palatability (feed intake) and nutrient utilization (growth), possible metabolic interferences (energy), and not least the impact on the final seafood quality. These topics were recently reviewed by Glencross *et al.* (2007) in relation to suggested evaluation strategies for feed ingredients in aquaculture feeds. With elevated proportions of feed lipids as in modern salmonid diets (30–40%) it is extremely important to select suitable and safe alternatives.

Possible fish health implications introduced by use of plant ingredients are also listed in Table 15.1. The main challenge for plant protein ingredients is the more or less imbalance in amino acid composition for optimal protein deposition in fish, mainly the indispensable amino acids methionine, lysine, cysteine, threonine and tryptophan (Hertrampf and Piedad-Pascual, 2000). These can, however, easily be completed by mixing protein ingredients or by supplementation of approved products of crystalline amino acid. Espe *et al.* (2006) showed, for example, that Atlantic salmon could be fed on diets devoid of fish meal as long as the amino acid composition was balanced with up to 10% crystalline amino acids.

For fish oil replacers, problems arise related to fatty acid composition, pigmentation and technical quality of the produce for human consumption (Bell and Waagbo, 2008). However, lately coronary heart disorders, cataracts, bone deformities and reduced immune competence have occurred in farmed fish that have been suspected to be attributed feed composition, and the use of plant lipid sources (Table 15.1). Relative to the above, a major question is whether these conditions rely on the technical, nutritional or toxicological conditions of the oils, such as oxidation status, unfavorable fatty acid composition or traces of lipid soluble contaminants like pesticides, respectively.

Genetically modified plant ingredients

Genetically modified (GM) plants represent an opportunity not only to improve plant resistance to infectious diseases and harsh environmental conditions, but also to improve the nutrient composition like the amino acid (Glencross *et al.*,

2003) or fatty acid compositions (Torstensen *et al.*, 2000) relative to the requirements for the farmed animal and the produce. In later generations of GM plant development one may also obtain reduced levels of normally occurring undesirable substances, like selected antinutrients. Since the introduction of GM plants for human consumption in 1996, several feeding studies have been conducted to support risk evaluation of using GM plants in food and feeds (Flachowsky *et al.*, 2005). Most studies conclude that GM products are as safe and nutritious as conventional non-GM products. There are, however, still safety concerns based on the earlier reported effect in the gastric mucosa in rats fed GM potatoes (Ewen and Pusztai, 1999). Examples of potential risks from GM feed ingredients in feed for Atlantic salmon are given in Table 15.1. Feeding studies in Atlantic salmon evaluating GM soybean (Roundup Ready) showed spleenomegali and possible impaired spleen function, as evaluated from increased counts of young erythrocytes in circulation (Hemre *et al.*, 2005). Hemre *et al.* (2007) concluded that Atlantic salmon postsmolts (150 g) fed GM maize (MON810) as carbohydrate source (15 and 30% inclusion levels) for 82 days resulted in similar growth rates and feed utilization as fish fed its near-isogenic parental line and commercial Suprex maize. Fish health was considered normal, as evaluated from neglectable mortality, normal relative organ sizes and normal blood chemistry. A minor concern was, however, related to changes in intestinal glucose transporter mechanism in GM fed fish. A transgenic lupin meal, with higher methionine content than meal from traditional non-GM crops was successfully used in diets for red sea bream, *Pagrus auratus* (Glencross *et al.*, 2003).

According to international food and feed legislation all GM products are risk evaluated and approved before they are introduced to the market. The European Food Safety Authority (EFSA) is responsible for the science-based assessment of GM food and GM feed in Europe. Further, the European Community demands labeling of products containing more than 9% of approved GM ingredients, and $\leq$5% for non-approved GM ingredients (Regulation EC 829/2003, 2003). Today, a major part of selected plant feed stuffs are GM, while parts of the aquaculture industry are reluctant to use GM products due to the demanded product labeling of feed (GM labeling) and the market demands on traceability of farmed fish. Clearly there are differences between the single GM and non-GM products to consider before the products can also be regarded as sound in feed for fish, even if they are evaluated safe for other animals and humans. This holds especially for use in feed for carnivorous fish species; since they are not naturally adapted to complex plant feed stuffs.

Macro zooplankton as alternative marine feed ingredients

Marine organisms harvested at a lower trophic level, such as macro zooplankton (krill, amphipods) and cuttlefish are shown be highly nutritious for fish (Hertrampf and Piedad-Pascual, 2000). Despite the great potential of global macro zooplankton resources in the seas, these sources are scarcely exploited (Storebakken, 1998; Suontama, 2006). Inclusion of krill and amphipod meals in

feed to Atlantic salmon, cod and halibut seems to be comparable to high quality fish meal, even though the content of chitin from the exoskeleton contributes to an elevated ash and lower energy content when used as a major protein source (Suontama, 2006). A mild diarrhea has been observed in fish fed at elevated inclusion levels, probably related to the content of chitin. Studies show, however, that selected macro zooplankton meals may exceed the present upper safe limits for fluorine, copper and cadmium given in the EU feed legislation (EC Directive 32/2002, 2002), which may restrict their use in animal feeds. Since the undesirable elements did not accumulate in the fish (Moren *et al.*, 2006), research is needed to support a risk evaluation of the use of such meals for fish health, especially for use at higher inclusion levels.

15.2.3 Development of explanatory experimental models

When diseases of unknown origin occur, measures are taken to restrict or stop the development at local level, depending on the currently operating health and welfare biosecurity infrastructure of the farm and local authorities. The need for rapid counteractions both for welfare and economic reasons is obvious, but most often this is not achievable since the etiology is unknown. A final solution can only be completed if an explanatory cause-relationship is sorted out. The history of nutritional maladies in aquaculture tells us that the way to a complete solution of a given disorder may be long and cumbersome. Recent examples here are the histidine-related cataract in Atlantic salmon smolts, reviewed by Bjerkås *et al.*, (2006) and Breck (2004); and backbone (spine) deformities in farmed salmon (Kvellestad *et al.*, 2000; Witten *et al.*, 2006), both of which are discussed later in this chapter. However, as long as the visual symptoms are reduced to a minimum by measures taken (such as over-nutrition or reduced feeding) or even for unknown reasons, it is often accepted that one may have to live with 'a basic level' of a given disorder.

An ideal situation is to scientifically identify detailed mechanisms behind observed cause-relationship for a disorder, and then be able to verify this relationship in an experimental model based on identified risk factors and established early warning markers for disease development. As an example, selected stress proteins studied in fish appear to be good biomarkers responding to different kinds of stressors or pollution (Iwama *et al.*, 1998; Martinez-Alvarez *et al.*, 2005).

15.3 Nutritional and environment-related bone disorders in farmed fish

Bone deformities in farmed fish may occur as developmental errors initiated by external stressors or suboptimal nutrition at early life stages (Cahu *et al.*, 2003), as late effects of such errors, or as a response to more situation-determined stressors, such as nutritional deficiencies or toxicities, later in life (Brown and

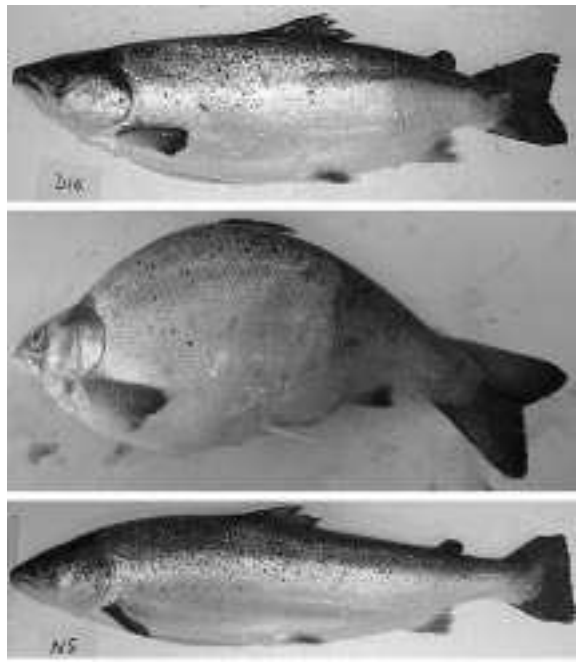

Fig. 15.2 Examples of visual backbone deformities appearing in Atlantic salmon; Upper 'short tailed' salmon with shortened posterior spinal column, Mid: 'humpback' with severely shortened anterior spinal column, as compared to a normal salmon (bottom). Photos extracted from Ørnsrud (2003).

Nunez, 1998). The expressed symptoms can severely affect the visual appearance of the fish, and common names as humpbacks (anterior), short-tails (caudal) and star-gazers (neck) are used (Fig. 15.2), while expressions like lordosis, xiphosis and scoliosis expresses major directional deviations of the spine. Bone disorders may be systemic, to influence all bone tissues or for some reason restricted to certain bones or areas of the spine, and includes conditions like platyspondylia (compressed vertebrae), fused vertebrae, deviation in single vertebrae (hyperdense appearance) and abnormal inter-vertebral spaces (Kvellestad *et al.*, 2000; Witten *et al.*, 2006). Such disorders are mostly not fatal; however, the severity clearly depends on the nature of the causal impact factor at time of damage. In this context, imprinting means that adverse changes induced at early life stages may develop into disorders at a later stage, especially in situations when it is difficult to meet and restore adverse changes by adequate measures.

When developed into visible malformations, bone disorders affect swimming behavior, feed uptake, energy expenditure, and resistance to infectious diseases, and represent an ethical, esthetic and economic problem for the farming industry (Waagbø *et al.*, 2005). Variation in body size and condition factor in a moderately affected population, however, allows, sorting and removing of diseased individuals.

A multitude of environmental and management factors have been associated with the development of bone disorders in farmed fish, such as elevated temperatures during egg incubation and juvenile rearing, light regimes (for smolt production), water quality parameters (water flow, gas saturation, salinity) (Martens *et al.*, 2006), use of feed supplemented antibiotics (Toften and Jobling, 1996) and vaccination regimes (Berg *et al.*, 2006). The vertebrate column in salmonids seems to display a regional growth pattern, with increased longitudinal growth in caudal and trunk regions during and after smoltification (Fjelldal *et al.*, 2005). By comparing under-yearling (0+) and yearling (1+) smolt, it was shown that rapid growth negatively affected mineralization and the mechanical strength of the vertebrae, especially in some spine regions. Thus, smoltification and the successive rapid growth in the sea seem to be a critical period for developing bone deformities. Table 15.2 summarizes risk factors and conditions leading to bone deformities in farmed Atlantic salmon and Atlantic cod in Norway according to Waagbø *et al.* (2005).

The role of nutrients in bone metabolism and pathology was recently reviewed by Lall and Lewis-McCrea (2007). Table 15.3 shows an overview of nutrients that have a critical role in bone formation and health in fish according to their roles in cellular growth and differentiation, bone mineralization and

Table 15.2 For commercially farmed Atlantic salmon and Atlantic cod, several risk factors are associated with the development of bone disorders (summarized from Waagbø *et al.*, 2005)

Species Risk factors	Possible aetiology
Atlantic salmon, *Salmo salar* L.	
Unfavorable water quality (inorganic elements, oxygenation, ion strength)	Toxicities, cell membrane oxidations, osmoregulation
Fast growth	Increased/organ specific nutrient requirements
Husbandry	Handling stress, inflammation
Under yearling (0+) smolt with subsequent fast growth in sea	Genetics, less flexible physiology
Egg incubation and juvenile rearing temperatures	Developmental
Vaccination type and temperature at vaccination	Inflammation; type of immune response
Imbalanced or suboptimal mineral nutrition (Ca, P, Zn)	Suboptimal mineralization; reduced bone strength
Atlantic cod, *Gadus morhua* L.	
Unfavorable water quality (inorganic elements, gas saturation, oxygenation, ion strength)	Developmental disorder
Unbalanced mineral nutrition (Ca, P, Zn)	Suboptimal mineralization; reduced bone strength

Table 15.3 Nutrients associated with reduced bone health in fish. Different parts of bone formation can be affected, from cellular development and function, bone mineralization and formation of the supporting extra cellular organic matrix

Nutrient	Roles in bone formation	References
Cellular growth and differentiation		
Vitamin A	Bone tissue cell development and differentiation	(Ørnsrud *et al.*, 2002, 2004; Hernández-H *et al.*, 2006)
Vitamin D metabolites	Blood calcium and P regulation (absorption, homeostasis and excretion)	(Lall, 2002; Lall and Lewis-McCrea, 2007)
Bone tissue mineralization		
Calcium	Major bone mineral	(Baeverfjord *et al.*, 1998; Lall, 2002; Lall and Lewis-McCrea, 2007)
Phosphorus	Major bone mineral	
Magnesium	Bone mineral/mineralization process/enzyme cofactor	
Manganese	Bone mineralization/enzyme cofactor	
Zinc	Bone mineralization/enzyme cofactor	
Copper	Enzyme cofactor	
Extra cellular organic matrix formation		
Ascorbic acid	Hydroxylation of proline and lysine, cross-linking of collagen in connective tissue	(Sandnes, 1991; Sandnes *et al.*, 1992; Li and Robinson, 2001)
Vitamin K	Bone Gla-protein formation like osteocalcin; (mineralization)	(Udagawa, 2001; Lall and Lewis-McCrea, 2007)
Unknown causes		
Tryptophan	Not defined; lack of serotonin	(Wilson, 2002)

extra cellular matrix formation. In line with this, several nutritional deficiencies have been linked to reduced bone health in juvenile fish, of which the most striking historical example is related to vitamin C (ascorbic acid) deficiency (Meier and Wahli, 1990). Like primates and humans, most fish species lack the ability to synthesize ascorbic acid (Moreau and Dabrowski, 2001; Mæland and Waagbø, 1998), and vitamin C deficiency in juvenile fish results in deformed vertebrae with severe scoliosis and lordosis (Meier and Wahli, 1990; Sandnes *et al.*, 1992), see Fig. 15.3. Ascorbic acid participates in post-translatory hydroxylation of protein bound proline and lysine moieties to their respective hydroxyl amino acids, which is essential for the cross-linking of collagen peptides in connective tissues. Since the introduction of stable and bioavailable ascorbic acid phosphate derivatives (Sandnes *et al.*, 1992), likelihood for vitamin C deficiency in modern aquaculture is reduced. Nevertheless, this can represent a problem if unstable chemical forms for some reason (price, availability, or ignorance) are still in use.

Fig. 15.3 Vitamin C deficiency in Atlantic salmon juveniles showing severe lordosis (upper) and scoliosis (lower) (own photo).

Besides numerous metabolic roles of calcium and phosphorus in cells and tissues, they are quantitatively essential as bone minerals in fish as in other vertebrates in a Ca/P ratio between 1.5 and 2.1 (Lall, 2002). In contrast to land-living animals and humans, farmed fish normally experience excess of calcium from the water and feed (Shiau and Tseng, 2007), while phosphorus deficiency is more likely to occur, since both fresh and sea water contain relatively low concentrations and the supply is solely through diet. It should normally be easy to correct or balance dietary P by supplementation the diets with suitable P salts. However, the main focus on P has rather been on local environmental pollution of aquaculture activity, with eutrophication by excess feed, fecal P and urine P, especially in fresh water (Ketola and Harland, 1993). As much as 80% of feed P may enter the local environment in soluble or solid forms. Consequently, marginal P supplementation has been used in the feed. Another complicating factor is the very variable bioavailability of P from feed ingredients of both marine and vegetable origins, with digestibility also depending on dietary level (Sugiura *et al.*, 1998; Lall, 2002). However, increased replacement of marine feed ingredients with plant materials means larger proportions of phytate P, which is even less available for farmed fish than fish meal P (Denstadli *et al.*, 2006). Novel strategies by use of enzymes such as phytase have been examined in relation to use of plant ingredients as fish meal replacers in fish feed (Cao *et al.*, 2007; Cheng and Hardy, 2002). Phytase treatment not only releases phytate P, but may also makes other phytate bound nutrients (minerals, protein amino acids) more digestible and available (Storebakken *et al.*, 1998), thus representing a cost and environment friendly alternative with respect to feed and P utilization. Since phytase is heat labile, technical solutions must include phytase pre-treatment of feed stuffs or coating onto pellets after extrusion (Cao *et al.*, 2007; Vielma *et al.*, 2004).

For marine species with small offspring, egg and larvae production are bottlenecks for further success (Mangor-Jensen *et al.*, 2007). The sensitive production of offspring depends on the use of live feed and weaning regimes to formulated diets (Hamre *et al.*, 2007; Mangor-Jensen *et al.*, 2007). Live feed like *Artemia* and rotifers vary in nutritional quality and the outcome of developmental disorders like lack of eye migration, mal-pigmentation and skeletal

malformation are high, relative to the use of wild caught zooplankton. Nutrient enrichment procedures have been used to improve their quality, with local zooplankton as the gold standard (Hamre *et al.*, 2007).

15.3.1 Methods to reduce malformation in farmed fish

With the main reasons for malformation in farmed fish mostly of unknown origin, it is as difficult to suggest countermeasures. However, the focus should be on identified risk factors at sensitive stages and as far as possible reducing the risk potential by adequate handling, such as:

- nutritional measures in the form of adequate bone minerals supply;
- environmental conditions should be kept within safe margins, such as egg and juvenile incubation temperatures and water gas saturation levels;
- reconsideration of disinfection types and regimes;
- focus on recommended vaccination routines, including type of vaccine and time, temperature and physiological status of the fish at vaccination;
- focus on possible biological limitations of light manipulated fish, like 0+ smolt.

15.4 Nutritional and environmental conditions related to cataract development in fish

Cataracts are a class of disorder characterized by opacifications of the eye lens, eventually leading to impaired vision and reduced feed uptake of farmed fish. The lens represents a highly specialized optical organ that grows more or less in line with the somatic growth and remains transparent throughout life. According to its structure, the lens cells often show the same changes to external and metabolic stress, regardless of the cause. These changes include lens fiber swelling, cell destruction and epithelial proliferation (Bjerkås *et al.*, 2006). The visual damage causes light scattering and loss of transparency as illustrated in Fig. 15.4.

For the farmer, all cataracts look the same, while by using a slit-lamp bio microscope for closer examination of anaesthetized fish, it is possible for a trained person to distinguish types of cataracts, as well as quantify the changes by cataract scores ranging from 0–8 for both eyes (Wall and Bjerkås, 1999). Nutritionally linked cataracts in salmonids have most often been reported in trout species, related to sub-optimal levels of thiamine (Hughes, 1985), riboflavin (Hughes *et al.*, 1981), methionine (Poston *et al.*, 1977; Cowey *et al.*, 1992) and tryptophan (Poston and Rumsey, 1983; Akiyama *et al.*, 1986). Cataracts induced by zinc deficiencies have been described both in trout and salmon species, caused either directly by low dietary concentrations or, as a secondary consequence of reduced zinc availability due to formation of chelates in the intestine or competitive uptake of other minerals (Ketola, 1979; Shearer *et al.*, 1992; Maage and Julshamn, 1993). In general, these nutrient-related cataracts were more often seen when salmonid farming was in its infancy (Table 15.4).

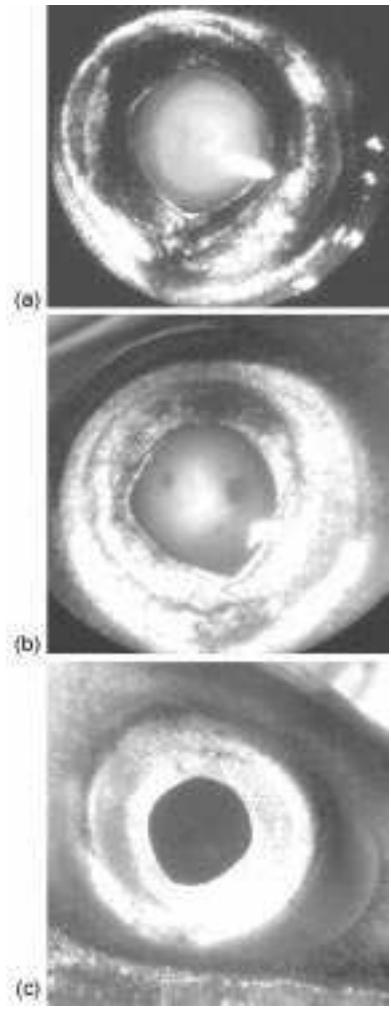

Fig. 15.4 (a) Photo of a mature cataract (Score 4) causing blindness in Atlantic salmon. For comparison eyes with (b) an osmotic cataract and (c) a normal clear lens (Score 0). Photos are from Bjerkås *et al.* (2006) with kind permission from CABI Publishing.

The role of nutrition in the development of cataracts in farmed fish was recently reviewed by Bjerkås *et al.* (2006). Besides classical nutritional deficiencies leading to cataracts, several environmental and nutrition interaction aspects more relevant for modern aquaculture were discussed, among others the use of novel fish meal and fish oil replacers in fish feed. Pro-oxidative conditions from selected nutrients (minerals and lipids) and water oxygenation and seem to affect cataract development in farmed Atlantic salmon (Waagbø *et al.*, 2003b, 2008). A confounding aspect in the cataract outbreaks in field and in experimental studies is that the groups of fish with cataracts often show the best growth. The relationship between growth and cataract development has been

Table 15.4 Environmental, toxicological, nutritional or infectious causation factors for the development of cataracts have been identified or proposed in several farmed fish species

Fish species	Potential risk factor(s)	References
Rainbow trout	Riboflavin, Zn	(Barash *et al.*, 1982)
	Riboflavin deficiency	(Hughes *et al.*, 1981)
	Tryptophan deficiency	(Poston and Rumsey, 1983)
	Zn deficiency	(Ketola, 1979)
	Mn, Zn and riboflavin deficiency	(Yamamoto *et al.*, 1983)
	Eye fluke	(Sharif *et al.*, 1980)
Atlantic salmon	Pharmaceuticals	(Fraser *et al.*, 1989)
	Methionine deficiency	(Barash *et al.*, 1982)
	Nutritional status; dietary oxidants incl. lipid level	(Waagbø *et al.*, 1996, 1998, 2003b)
	Zn deficiency	(Barash *et al.*, 1982)
	Genetically determined (triploids)	(Wall and Richards, 1992)
	Omission of blood meal	(Breck *et al.*, 2003; Wall, 1998)
	Suboptimal histidine levels	(Breck *et al.*, 2005a)
	Water temperature (fluctuations)	(Bjerkås and Bjørnestad, 1999; Bjerkås *et al.*, 2001; Bruno and Raynard, 1994)
	Rapid growth	(Bjerkås *et al.*, 1995; Breck and Sveier, 2001)
Arctic char	Vitamin E	(Heitz, 1984; Simmons *et al.*, 1999)
	Methionine	
Lake trout	Zn deficiency	(Barash *et al.*, 1982)
	Gas supersaturation	(Krise and Smith, 1993)
Carp	Riboflavin deficiency	(Takeuchi *et al.*, 1980)
Atlantic halibut	Unknown	(Williams *et al.*, 1995)
Tilapia	Riboflavin deficiency	(Lim *et al.*, 1993)
Dogfish	UV irradiation	(Zigman *et al.*, 1991)
Chum salmon	Trypthophan deficiency	(Akiyama *et al.*, 1986)
Chinook salmon	Ca, P, Zn, phytate	(Richardson *et al.*, 1985)

confirmed in separate studies (Bjerkås *et al.*, 1996; Breck and Sveier, 2001). Whether this reflects lens-specific nutrient deficiencies or limitations in lens physiology in periods of elevated growth rates needs to be investigated. The lens nutrition is complicated since it is mediated through the production of aqueous humor and not though blood supply directly.

During the last two decades, development of permanent cataracts has been described mainly in European Atlantic salmon farming (Midtlyng *et al.*, 1999; Wall, 1998), constituting major ethical concerns and variable economic losses for the industry (Menzies *et al.*, 2002). An epidemiological survey performed in

1998 in 49 sea farms along the Norwegian coast found a cataract prevalence of 82% (Ersdal *et al.*, 2001). Even though cataract was acknowledged to have a multifactorial etiology, including environmental, physiological, toxicological and genetic factors, a link to nutrition was often suggested. Outbreaks of the salmonid cataract seemed to coincide with the omission of mammalian blood meal as ingredient in fish feed; this was a prevention measure towards spreading of BSE (bovine spongiform encephalopathy) in the mid 1990s (90/667/EEC, 1990). Indeed, later studies confirmed the cataract-mitigating effect of blood meal (Breck *et al.*, 2003), more specifically defined to its elevated content of the essential amino acid histidine (Breck *et al.*, 2005a). Breck *et al.* (2005b) suggested that histidine (His) is not only essential for lens protein synthesis, but also constitutes an important osmolyte in the chemical form of N-acetyl His (NAH). The lens is normally kept in a dehydrated state to sustain transparency, and high concentration of NAH is thought to be important for the water homeostasis in fish lens, as well as in nervous tissues (Baslow, 1998). The concentration and synthesis of lens NAH clearly depends on dietary His, as well as the parr-smolt transformation process where the salmon prepares for life in sea water (Breck *et al.*, 2005a,b). The lens NAH concentration increases and daily turnover is upregulated during the period of smoltification and in sea, provided sufficient dietary His supply. The role of His and NAH in lens osmoregulation includes the ability of rapid osmolyte efflux to equalize any osmotic disturbances, since the lens easily endures in hyper- and hypo-osmotic environments by shrinking and swelling, respectively (Breck, 2004). Consequently, any physiological state that impact lens osmoregulation or put osmoregulatory stresses to the lens may be cataractogenous, while elevated dietary His may counteract such osmotic disturbances. Since His and His related compounds (imidazoles) also take part in the cellular integrated antioxidant system, muscle pH buffering system, and function as anti-glycating agens, the roles of His in cataract prevention in salmon is not completely understood. In humans, eye drops containing the His compound acetyl-carnosine are marketed to prevent and even heal cataract development in humans; argued to be related to the special antioxidant properties of the imidazole carnosine (Babizhayev *et al.*, 2001, 2004).

The introduction of alternative lipid sources as fish oil replacers in aquaculture feeds have in some cases shown interaction between dietary lipids and cataract development in adult fish (Waagbø, 2006). In humans, age-related cataract has been related to dietary fat intake, and Lu *et al.* (2005a) concluded in one study that elevated intakes of 18:2 n-6 and 18:3 n-3 may increase the risk for nuclear opacities, while another prospective study from the same group concluded that higher intakes of n-3 PUFA from fatty fish consumption may contribute to cataract prevention (Lu *et al.*, 2005b). In a full life cycle feeding experiment with Atlantic salmon, the fatty acid composition of the lens was partly determined by the dietary lipid source, a blend of plant oils or a fish oil. While lens n-3 PUFA was relatively similar among these two groups, n-6 PUFA differed significantly (Waagbø *et al.*, 2004). The latter may be associated with

the dramatically higher severity of cataracts among fish fed plant oil than fish fed diets based on fish oil in this study.

Genetically linked predisposition to cataract formation in different fish species, including Atlantic salmon has been reported (Kincaid, 1989; Wall and Richards, 1992). Breeding experiments with aquarium-raised tilapia (*Sarotherodon mossambicus*) indicated that the observed development of bilateral cataract may be inherited (Noga *et al.*, 1981). This was also indicated by occurrence of skeletal abnormalities in the affected individuals, and this suggests that these disorders may be related. A genetic aspect was also demonstrated in the His related cataract in farmed Atlantic salmon discussed above (Breck *et al.*, 2005a).

15.4.1 Methods to reduce cataracts in farmed fish

Cataract development in a fish population may progress rapidly during a few weeks. In Atlantic salmon, periodically feeding of elevated dietary His has been shown to effectively mitigate cataract development both experimentally and in field. However, feeding elevated dietary His during critical periods is probably to be considered as a temporary solution since the basic mechanism for cataract development is not fully understood.

The lens possesses mechanisms of repair which include sealing off damaged areas after terminating or reducing the impact factor (physical, oxidative or toxic factors). This allows the rest of the cells to return to normality. This phase of repair could be stimulated through diet, like, for example, by antioxidants (Waagbø *et al.*, 2003a) or histidine compounds (Breck *et al.*, 2005a).

In summary, measures to reduce cataracts in farmed fish include:

- elevated dietary inclusion levels of histidine (or other His compounds in complete feed ingredients);
- elevated dietary inclusion levels of antioxidants (vitamin C, vitamin E and astaxanthin);
- if possible, control of husbandry routines towards stable environmental conditions relative to water temperature, oxygen saturation, salinity and stress;
- if possible; prevent of periods with extreme growth rates.

15.5 Other production-related diseases

15.5.1 Nutrition, stress, reduced immune competence and unspecific mortalities

New diseases with mass mortalities occur occasionally in aquaculture (Lillehaug and Skrudland, 2007). Normally, first action is to seek for infectious agents and take action to prevent further escalation of the disease. In case of considering non-infectious disorders, environmental and nutrition-related disorders often represent acute and chronic situations, respectively that have to be sorted out. Unfavorable changes in water chemistry and exposure to waterborne pollutants

are normally more acute and lethal than similar exposure through diet (Handy, 1996). This holds for toxic metals, as well as organic contaminants and pollutants such as, for example, the organochlorine pesticide endosulfan (Petri *et al.*, 2006). Most often the cause of an incidence of mortality is the result of many interacting factors, including daily management and the limitations set by the farming conditions, such as, for example, restricted area and type of feed. Diagnosis of non-infectious disorders can be evaluated at several organizational levels (at population, individual, organism, tissues, cellular or at sub-cellular levels). Leatherland *et al.* (1998) proposed a stepwise diagnostic approach to sort out a possible cause-relationship based on indicators at these biological organization levels in wild and farmed fish, and adequate countermeasures. Similarly, Hardy (2001) presented sample keys to sort out nutritional deficiencies based on primary clinical signs found in salmonids.

It is commonly accepted that fish experience and react to stress in similar ways as land living animals (Wendelaar Bonga, 1997; Tort *et al.*, 2004). Stressors in aquaculture include fish rearing conditions such as fish density and physical handling stress, and environmental factors like unfavorable water quality parameters such as salinity, water gases (oxygen and carbon dioxide), algae and pollutants. For this reason, simple stress resistance tests in fish research include measures of resistance or recorded physiological responses following a defined stressor. Today traditional as well as molecular biomarkers among stress proteins are used to identify potential negative health effects of different stressors (Iwama *et al.*, 1998; Martinez-Alvarez *et al.*, 2005); this may include potential impact of environmental factors such as, for example, water oxygenation (Olsvik *et al.*, 2006) and physiological effects following feeding of GM containing feeds (Sagstad *et al.*, 2007). In commercial farming, stress-induced immunosuppression is one of several factors that can lead to secondary infections by favoring growth of opportunistic pathogens and outbreak of diseases, such as the development of winter ulcer in Atlantic salmon (see Section 15.5.2; Poppe *et al.*, 2007a).

Immune deficiencies and increased susceptibility to infectious diseases in fish have been observed following suboptimal nutrition, including toxicological conditions, as reviewed by Waagbø (2006). Vitamin C is among the most studied nutrients relative to stress and disease resistance in deficient fish, since it is among the most labile vitamins that is easily lost during feed processing and storage (Sandnes, 1991). On the other hand, excess vitamin C in the feed, up to 50 times the minimum requirement, has been examined for its positive immune modulatory properties, stress ameliorating effects and its role in protection against waterborne pollutants; as reviewed by several authors (Norrgren *et al.*, 2001; Li and Robinson, 2001; Sandnes, 1991; Waagbø, 2006; Fletcher, 1997). Results from such studies are often contradictory, probably related to diverging physiological directions of the responses to stress and immunity in the fish relative to time and biological priority. The response to stress aims to adapt the organism to a threatful state induced by a stressor and to recover homeostasis as soon as possible. The neuro-endocrine response to stress facilitates immediate

life sustainable physiological actions to gain rapid homeostatic control (Wendelaar Bonga, 1997), while the immune system is regarded to be of less priority in this state and time frame; immunity may even be temporarily suppressed by the stress hormones (Schreck, 1996).

In the search for new and sustainable feed resources, imbalanced fatty acid composition, phytosterols, antinutrients and contaminants may all have an impact on fish physiology and health. By using immunological markers and stress indicators at organ and sub-cellular level it is possible to locate and detect responses at an early stage. The intestine is the first barrier for feed borne stressors and feeding studies has shown that mucosa cell integrity, cell turnover and markers of local inflammation can be used as early indicators for such stress (Berntssen *et al.*, 2004; see also examples in Table 15.1).

15.5.2 Skin and gill disorders

Winter ulcer is a term used on infectious (*Moritella viscosa)* skin disease, traditionally occurring during winter–spring season in Atlantic salmon, ranging from superficial lesions with scale loss to deep wounds with exposure of the underlying muscle, and mortalities (Lunder *et al.*, 1995). While mortality is not dramatic relative to other infectious diseases (normally <5–10%), ethical and not at least esthetic aspects call for attention and action. Experimental challenges with *Moritella viscos* infection have also shown that marine species such as juvenile Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) are sensitive to the bacteria to a different degree (Gudmundsdottir *et al.*, 2006). Even with protection of vaccination, problems with ulcers are considered extensive (Poppe *et al.*, 2007a).

Although defined as infectious, predisposing causes for outbreaks of winter ulcer as well as other skin disorders seem to be multidisciplinary, and often include a combination of primary mechanical skin lesions that promotes opportunistic infections. These may arise from tank or pen conditions, physical handling in daily management, low biomass density or suboptimal feeding regimes with aggressive behavior like fin biting, eye snapping, etc. Factors affecting the coagulation system (Salte *et al.*, 1994) and osmoregulation (Rørvik *et al.*, 2001; Evelyn *et al.*, 1998), including extreme water temperatures have been associated with ulcer formation. It is important, however, to sort out the cause-relationship here, since bacterial infections and ulcers in themselves impact intravascular coagulation and disturbances in homeostasis. On the other hand, selected nutrients, such as n-3 fatty acids (Waagbø, 1994) and feed additives, like urea (Rørvik *et al.*, 2001) may mitigate the disease symptoms by modulation blood clotting and osmoregulatory capability, respectively.

Bone deformities, skin lesions, wounds, bleedings and anemia are all pathology associated with ascorbic acid deficiency in fish, related to its many essential roles (Sandnes, 1991). Consequently, excess ascorbic acid supply above the minimum requirement (up to 1000 mg/kg diet depending on species) have been examined and found to be beneficial against disease development and

for tissue repair after trauma in several farmed species (Halver, 1972; Lim and Lovell, 1978; Wahli *et al.*, 2003). Today, excess ascorbic acid is often supplied to health promoting diets for farmed fish.

Several nutrient deficiencies in fish affects the gill tissue with degeneration or fusions of lamellae, like essential fatty acids, magnesium, vitamin E, vitamin C, biotin and pantothenic acid (Tacon, 1993; Halver and Hardy, 2002). The tissue damage seems to be related to a reduced membrane integrity caused by changes in membrane composition, reduced antioxidant defense, or reduced vitamin coenzymes in energy production needed for the highly active osmoregulation in the gills. Even though estimates for requirements are given for most nutrients, there may be difficulties to reach target feed levels when main feed ingredients are exchanged in fish feeds. Bell and Waagbø (2008) illustrated with an example that partially replacing fishmeal with a plant protein source like soybean meal means changes in nutrient supply, content of antinutrients, as well as nutrient bioavailability; these aspects should be focused in future feeds to prevent sub optimal micro nutrient supply. Besides use of novel feed ingredients, classical nutritional interactions should not be forgotten. For example, ten per cent replacement of fish meal with spray dried hen egg white resulted in biotin deficiency with severe gill pathology (fusion and thickening of gill lamellae) in Atlantic salmon juveniles (Mæland *et al.*, 1998). Raw hen egg white contains the protein avidin that binds strongly to biotin and biocytin, making the vitamin compounds unavailable for absorption.

15.5.3 Cardiovascular diseases

Coronary lesions and heart disorders are among disorders frequently observed in farmed salmonids and these may be of infectious origin, such as, for example, in pancreas disease (PD) and the recently discovered heart and skeletal muscle inflammation disease, HSMI (Kongtorp *et al.*, 2006; Lillehaug and Skrudland, 2007), or represent production-related disorders of more unknown origin (Iwama and Farrell, 1998; Seierstad, 2008; Tørud and Hillestad, 2004). HSMI affects heart and red skeletal muscles (Kongtorp *et al.*, 2004). The disease may start as a subclinical inflammation in the heart, escalating to clinical disease affecting other organs during months in the sea, with variable mortalities up to 20% (Kongtorp *et al.*, 2006). As for many production disorders, stress may predispose the fish for this disease. Outbreaks of another heart disorder, cardiomyopathy syndrome (CMS) in adult Atlantic salmon and rainbow trout in sea water have occurred regularly since 1985, with moderate mortalities (Ferguson *et al.*, 1990). The syndrome represents myocardial degeneration and necrosis, and may end in acute mortalities as a consequence of rupture of the atrial wall. High incidences observed in brood fish populations cause moderate mortality, but it is not known to what degree a sub-clinical syndrome impacts sexual maturation, the quality of the offspring, and brood fish health (Tørud and Hillestad, 2004).

Farmed fish seem to have smaller relative heart sizes with a rounder appearance than wild fish of similar body size. During periods with high and

stressful activities at the farms (transportation, sorting and other treatments), sudden mortalities have been ascribed to failure in the heart and circulation system (Tørud and Hillestad, 2004; Poppe *et al.*, 2007b). The authors in their report point to infectious, environmental, genetic and nutritional backgrounds as risk factors for chronic heart disorders. The characteristic smaller heart in farmed salmonids is often related to rapid growth as in light manipulated (0+) smolts, and is associated with reduced staying power and stress resistance of the heart (Poppe *et al.*, 2007a). In addition, developmental disorders such as missing *septum transversum* and abnormal location of the heart (*situs inversus*) were earlier frequently seen in salmonid farming as a consequence of using suboptimal high or accidentally fluctuating incubation temperatures for egg and juvenile stages of Atlantic salmon, to reduce production time or synchronize production (Ørnsrud *et al.*, 2004; Takle *et al.*, 2005). Ørnsrud *et al.* (2004) found that elevated egg incubation temperature was the main factor, while elevated egg vitamin A concentration also could be associated with development of deformities. In line with smaller heart sizes, such malformations make the fish less resistant to production stress throughout the life cycle.

Arteriosclerosis, with degeneration in the coronary arteries and myocardial disease have also frequently been observed in adult wild salmonids related to sexual maturation (Iwama and Farrell, 1998; Farrell, 2002; Saunders *et al.*, 1992). Like other production related disorders discussed above, the occurrence of coronary lesions in earlier stages seems to be related to rapid growth, body size or age of farmed fish (Farrell, 2002; Seierstad, 2008). Interestingly, coronary arteriosclerosis seems to be absent in non-salmonid teleosts, even during sexual maturation (Farrell, 2002). It is considered a chronic disorder that reduces blood circulation due to thickening and loss of elasticity of the arterial wall (Fig. 15.5). Experimental, clinical and epidemiological studies in humans and human animal models suggest that dietary n-3 PUFA prevents development of cardiovascular diseases (Bell and Waagbø, 2008). In line with human research, dietary lipids have also been suggested to be part of the etiology for atherosclerosis-like disorders in farmed fish (Farrell *et al.*, 1986; Moore *et al.*, 1976), where elevated blood cholesterol as well as low density lipoprotein (LDL) in salmonids was associated with development of coronary intimal thickening. In contrast to mammals, however, lipid and calcium accumulation, or inflammatory cells have not been observed in coronary lesions in fish, except the finding of inflammatory related MHC class II^+ cells in the intima of a few individuals of Atlantic salmon with cardiovascular changes (Seierstad, 2008). External modulation of membranes in coronary smooth muscle cells with n-3 and n-6 PUFAs, and changes in synthesis of respective eicosanoids seem to impact muscle cell mitosis in salmonids (Gong *et al.*, 1997). This is suggested as initial steps in the observed intimal thickening and atherosclerosis. Bell *et al.* (1991) found other types of cardiac lesions after feeding Atlantic salmon a sunflower oil-based diet for 16 weeks, manifested as thinning of ventricular wall and muscle necrosis. They suspected a low n-3/n-6 fatty acid ratio to be responsible for these health impacts. In a recently published lifespan feeding

Fig. 15.5 Changes of the coronary artery characterized by large intimal change covering more than 75% of the vessel circumference. The vascular change is characterized by a disrupted and reduplicated inner elastic lamina. The underlying medial layer is absent and underlying adventitia with loose connective tissue stretched into the affected intima. Photo from Seierstad *et al.* (2008) with kind permission from Wiley Blackwell.

study with pure marine or plant lipid regimes, early histological signs of lesions in the coronary artery were already observed in juvenile Atlantic salmon prior to smoltification (Seierstad, 2008). The lesions developed significantly with time, to include patchy intimal thickening in the proximal main part of the artery during the growth period in sea. There was, however, no difference in lesions related to the dietary lipid regimes. In line with this, Farrell (2002) demonstrated a clear positive correlation between Atlantic salmon length and prevalence and severity of cardiac lesions, and that the lesions progressed with age.

Since dietary lipids are considered a risk factor for chronic development of cardiovascular diseases, the results of life lasting feeding studies are important for a successful and safe replacement of fish oil with plant oils in diets for fatty fish species. Clearly, focus should also be on safe oil types and blends of plant oils for use.

15.5.4 Digestion disorders

Introduction of new feed ingredients and feed production technologies are likely to affect feed intake and digestion in farmed fish. Symptoms related to severely disturbed feed digestion have been observed in both fish larvae and adults. For marine fish larvae, this has mainly been associated with their immaturely developed intestine and the lack of phospholipids in the live feed regimes (Cahu and Infante, 2007; Geurden *et al.*, 1998), probably disturbing intestinal lipid micellar formation and lipid transport from enterocytes to the peripheral tissues. In adults, several other feed aspects may conflict with digestion, like lipid and

antinutrients components in plant feed ingredients (Table 15.1). Besides, several infectious diseases impact liver and pancreas function (like pancreas disease) and thereby nutrient digestion and uptake.

A special feeding disorder is the abdominal distension syndrome (bloat) which is characterized by a water filled gastrointestinal tract in farmed salmonids. The disorder seemed to appear along with the introduction of high energy extruded diets with elevated dietary lipids and available carbohydrates (Fig. 15.1; Staurnes *et al.*, 1990). Reduced physical water stability of extruded pellets was shown to cause oil-belching in rainbow trout exposed to fluctuating water salinities between fully seawater 34 g L^{-1} and brackish water at 17 g L^{-1} (Baeverfjord *et al.*, 2006). The authors suggested that the oil separation in the stomach, and regurgitation may represent early events in the disturbed lipid digestion seen in the abdominal distension syndrome. Recently, Anderson (2006) proposed a mechanism for the development of the water distension syndrome, involving factors of digestion and osmoregulation. A combination of high feed intake of energy-rich diets, rapid disintegration of pellets in the stomach, hormonally regulated slow release of chyme into the intestine, water loss (increased with stress, elevated water temperatures and low oxygen levels), increased thirst and subsequent drinking seem to escalate the symptom of water-filled stomach. Proposed measures to counteract development of this disorder included reduced feeding, change in dietary composition and management for reduced stress (Anderson, 2006).

In Norway in 2002–2004 another disorder related to feed or lipid digestion was observed in farms with Atlantic salmon held their second year in sea, mostly appearing at elevated water temperatures. It was called 'Floating feces' syndrome and was characterized by white/yellowish lipid rich (>70% lipid) creamy-like 'feces' covering the water surface of the net pens. Besides the concerns of fish welfare in these incidents, this also constituted an environmental problem. Intestinal morphological changes in the fish showed thickening of the pyloric, however, with no special pathology of internal organs. The problem had occasionally been observed in other salmonid producing countries, as well. Since the problem disappeared in the years thereafter, it was suggested to be linked to selected feed batches, seasonal algae blooms, occurrence of certain jelly fish species, or unknown factors disturbing the lipid digestion.

15.6 Conclusions

Since major infectious diseases in aquaculture today are often correctly diagnosed and met by effective preventive management strategies and vaccines, the fish farming industry has increased focus on non-infectious diseases related to production conditions, environment and nutrition. Indeed, fish welfare issues are increasingly important from both ethical and economic viewpoints, and the industry and research have common aims in improving welfare and health of farmed fish by eliminating risk factors for production diseases, reducing stress

and improving disease resistance. The market asks for ethically produced seafood, and has in line with this initiated use of own markers and standards for such production. This chapter focused on some important non-infectious disorders with a nutritional origin or with a possible interaction with nutrition, and how to cope with these in short- and long-term perspectives. While visual symptoms of the various disorders give indications on sensitive organs, we do not know if the symptoms just reflect 'the tip of an iceberg' of even more complex disorders. Most production-related disorders reflect a reduced quality of life and, if left untreated, they progress to worse conditions, with reduced feed intakes, increased susceptibility to secondary diseases, and mortalities. Even though many of the disorders are thought to result from a multi-factorial etiology, the search for exact disease mechanisms is an important step towards elimination of the disorders by correct counteractions.

15.7 Future trends

According to recent estimates global farming steadily increases in gross production and in introduction of new species (Tacon *et al.*, 2006). The aquaculture industry operates on a global market for both feed ingredients and seafood production. Major challenges lay in obtaining sustainable, stable and safe feed ingredients for continuous growth for both high and low value farmed fish species. Several historical examples have demonstrated considerable differences among fish species in optimal environmental variables, feed availability and dietary composition, with subsequent development of disorders and abnormalities (Brown and Nunez, 1998). Consequently, care should be taken in introducing new feed ingredient alternatives. On the other hand, introduction of new fish species represents a valuable flexibility in aquaculture production, both relative to resistance to occurring infectious diseases and to use of available feed ingredients. Irrespective of species, fish health aspects need to be carefully considered when major changes in feed composition and feed types are introduced to prevent feed-related stress and development of disorders.

Feed and feeding are clearly key factors in health and disease of farmed fish. Besides fulfilling the requirements for essential nutrients, this includes active roles of single nutrients in disease prevention, by reducing the rate and severity of infectious diseases, and improved recovery, restoration of nutritional imbalances, increased immune competence prior to, during and after vaccination, reduction of vaccine and pharmacological side-effects, as well as to reduce toxicity by pollutants and contaminants.

15.8 Sources of further information and advice

Besides the literature cited in the text, further information and advice on the topic of nutritional disorders in farmed fish can be found in the following key literature:

- *Fishery resources as feed inputs to aquaculture* (Tacon *et al.*, 2006)
- *Fish nutrition* (Halver and Hardy, 2002)
- *Nutrition and fish health* (Lim and Webster, 2001)
- *Non-infectious disorders in fish* (Leatherland and Woo, 1998)
- *Feeding and disease resistance in fish* (Waagbø, 2006)
- *Stress physiology in fish* (Iwama *et al.*, 1997)
- *Advances in Norwegian aquaculture research 2000–2005* (RCN, 2007)

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15.10 References

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16

Microbiological quality and safety of farmed fish

B. T. Lunestad, National Institute of Nutrition and Seafood Research (NIFES), Norway and J. T. Rosnes, Norconserv AS, Norway

16.1 Introduction: microorganisms, quality and safety

As long as humans have existed, microorganisms and their activity in foods have created challenges by their ability to cause quality reduction or cause disease. On the other hand, microorganisms are beneficial in preparation or preservation of foods.

An adequate microbiological evaluation of the quality and safety of fish and fish products requires skills and experience. In addition to analytical results for relevant parameters, information on the origin and history of the fish or fish product under examination, as well as the intended usage should be taken into consideration.

Knowledge in microbiology will enhance the safe production of fish as food, and is a strict requirement to maintain high quality of fish and products of fish for sufficiently long periods necessary for harvest, processing, transport and storage. Taking into account that marine fish recourses are limited, an increased value adding must come as a result of better quality, which in turn is strongly influenced by the activity of microorganisms.

In this chapter we will discuss how microorganisms may reduce the quality of farmed fish and fish products, and also consider possible health risks posed by such organisms if present in seafood (Fig. 16.1).

16.2 Microorganisms affecting fish spoilage

The spoilage of foods must have been a constant challenge to the early humans, and even today, with access to sophisticated processing and chilling

Fig. 16.1 Fish and fish products represent high quality nutrients for humans, and for microorganisms. Due to their numbers and activity, bacteria may give a strong quality reduction during storage. In some cases bacteria or virus contaminating fish products may cause foodborne infectious disease in humans.

technologies, a regular supply of high quality fresh fish is challenging. On a global basis it is estimated that between 10 and 50% of all produced foods have to be rejected due to post-harvest or post-slaughter spoilage. Furthermore, it has been estimated that up to 25% of all fresh food spoilage is due to microbial action (Anon, 1985a). Historically the oceans were considered limitless and thought to harbour enough fish to feed an ever-increasing human population. Now aquaculture production – fish and shellfish farming – has grown rapidly to address the shortfall in capture of the fisheries. However, there are advantages in using farmed fish compared to wild catches as a result of controlled farming and processing conditions.

Fish and other seafoods are considered to be particularly prone to spoilage. Fresh fish are very perishable with a neutral pH (6.5–7.0), a high protein content and high water activity ($a_w > 0.95$). Enzymes in fish from cold waters are also adapted to low temperatures, and autolytic processes may easily accelerate post harvest if the temperature increases.

Fish spoilage starts with enzymatic and chemical reactions as a slight loss of freshness attributes, commonly known as autolysis. Later in the shelf-life microorganisms are the major limiting factor. The bacterial flora on newly caught fish depends on the environment in which it is found, rather than on the fish species it self. Fish caught in cold, clean waters carry low numbers, whereas

fish caught in warm waters have higher bacterial counts. On live fish in temperate waters, bacteria are present in the slime layer of the skin (10^2–10^7 cfu/cm^2), the gills (10^3–10^7 cfu/g) and the intestine (10^7–10^9 cfu/g). Many bacteria on fish from temperate waters are able to grow with a high spoilage rate at refrigerated temperatures. This is different compared with bacteria from agricultural origin, often adapted to higher temperatures. The microorganisms in temperate waters may be classified according to their temperature range as either psychrotrophs (growth at 0 ºC and optimum growth about 20 ºC) or psychrophiles (growth at 0 ºC and optimum about 15 ºC).

After death, the immune system of fish collapses and bacteria from the skin start to invade and degrade the previously sterile flesh. It has been shown that only a limited number of bacteria actually invade the flesh during storage on ice, with no difference in the invasive pattern of specific spoilage bacteria and non-spoilage bacteria. Thus, it is likely that spoilage largely occurs by bacterial enzymes diffusing into the flesh and nutrients diffusing to the fish surface. The parameters affecting the growth of microorganisms in fish have been categorised into two general groups: intrinsic (inherent qualities of the fish) and extrinsic (qualities of the fish environment).

The first and most obvious handling technique for preserving the quality of fish is to keep them alive for as long as possible before cooking and consumption. This has been done for thousands of years in China for carp, using long-established techniques. Today, a large number of species are kept alive in fish farms, which preserve quality prior to processing or consumption.

A number of methods are used to preserve fish, e.g. techniques based on temperature control, using ice, refrigeration or freezing, or techniques based on the control of water activity which include drying, salting, smoking and freeze-drying. Techniques may rely on the physical control of microbial fish loads, such as through heating or on chemical control of microbial activity and loads by adding acids, for example, to fish products. Techniques are also used that are based on lowering the redox-potential, such as by vacuum packaging and during application of modified atmosphere packing (MAP) (Fig. 16.2). Often a combination of different techniques is used to preserve fish.

The further processing of fish into a wide variety of value-added products is now common with the increase in demand for food products that are ready to eat or require little preparation before serving.

16.3 Specific spoilage organisms

Not all bacteria growing on fish will lead to the production of objectionable characteristics. A minority of the species are often associated with the majority of the spoilage. The concept of specific spoilage organism (SSOs) is not new and has been used for foods like yogurt and cheese. For fresh fish, it has been known since 1940 that the bulk of microbial population on newly caught fish does not cause off-flavour and off-odours. During the 1970s, work with

Fig. 16.2 In order to suppress bacterial growth, and thereby retain a high quality and safe product throughout the shelf-life, several preservation techniques may be applied. The picture show salmon fillets packed in plastic trays under a modified atmosphere.

inoculated sterile fish blocks resulted in identification of specific micro-organisms, which produced the characteristic spoilage compounds of the fish (Herbert *et al.* 1971; Miller *et al.* 1973). These organisms are described as potential spoilers, but only if or when they reach numbers capable of producing sufficient spoilage compounds to effect rejection do they become the SSOs of the product, Table 16.1 (Gram and Huss, 1996).

The spoilage of a product is strongly influenced by the conditions under which the product is held. The characterisation of spoilage of each product must therefore be made before the identification of responsible spoilage organisms.

It is particularly important to predict effects of product characteristics and storage conditions, due to the short and variable shelf-life of many types of seafood. Such predictions of shelf-life are done by predictive shelf-life studies and microbial modelling described in Section 16.7. Bacteria identified as being associated with the spoilage process of fresh and preserved fish are discussed below.

16.3.1 *Pseudomonas* spp

The pseudomonads represent a large and poorly defined group of micro-organisms. The family *Pseudomonadaceae* are Gram-negative rods, motile with polar flagella, oxidase-positive, catalase-positive, and they are obligate respiratory bacteria. There are many marine species among the pseudomonads. The spoilage compounds associated with the growth of psychrotrophic *Pseudomonas*

Table 16.1 Specific spoilage microorganisms in fresh and preserved fish products (temperate waters)

Storage conditions	Marine fish	Freshwater fish
Aerobic, on ice	*S. putrefaciens* *S. baltica* Other *Shewanella* sp.	*Pseudomonas* spp.
Anaerobic	*S. putrefaciens* *S. baltica* *P. phosphoreum* Lactic acid bacteria	Lactic acid bacteria
MAP packing (CO_2)	*P. phosphoreum*	Lactic acid bacteria *Brochothrix thermosphacta*
Highly salted fish	Halophilic bacteria Osmotolerant yeast	
Lightly preserved fish (low salt)	Lactic acid bacteria Enterobacteriaceae	Lactic acid bacteria Enterobacteriaceae
Mild heat treated (Sous vide)	*Bacillus* spp.*	*Bacillus* spp.*

* Psychrotrophic *Bacillus* spp. may contaminate product by addition of ingredients as spices.
Modified from Gram and Huss (2000).

spp. on fish are diverse and in many cases species-specific. *Pseudomonas* spp. mediated spoilage is characterised by 'fruity', 'oniony' and 'faecal' odours from the production of biogenic amines, ketones, aldehydes, esters and non-H_2S sulphur-containing compounds, such as methyl sulphide. Members of the genus are able to produce pigments and proteolytic and lipolytic enzymes that may affect the quality of fresh and, more especially, processed (e.g. frozen) fish products.

The spoilage of unprocessed fish is generally ascribed to the growth of *Pseudomonas* spp., however, only few conclusive studies have determined their absolute role as SSOs.

16.3.2 *Shewanella putrefaciens*

Shewanella putrefaciens has been recognised as the main spoilage bacteria on iced cod from temperate waters. The type culture (ATCC 8071) was first described in 1931 and it has changed genus name four times. In many cases *S. putrefaciens* constitutes 1–10% of the total flora of newly caught fish from temperate marine waters. It is also present in fresh waters, and thus may play a role in the spoilage of fresh water fish. The importance of *Shewanella putrefaciens* to the spoilage of fresh marine fish has been recognised since the 1940s, although only since the late 1960s has it been realised that the metabolites produced by *S. putrefaciens* during growth may be used as

indicators of spoilage. *S. putrefaciens* is able to reduce TMAO (Trimethylamine oxide) which is naturally found in some fish, especially gadoid fish species, to TMA (Trimethylamine). It is also able to produce hydrogen sulphide (H_2S) and a range of other off-odour compounds. *Shewanella* may grow in the absence of oxygen using alternative terminal electron acceptors, although, like members of the *Pseudomonadaceae*, it is strictly respiratory.

16.3.3 *Photobacterium phosphoreum*

Photobacterium phosphoreum is a bioluminescent microorganism and the taxonomy and phylogenetic relations of the organisms are well characterised. *P. phosphoreum* has been recognised for some time as being present on spoiled marine fish. An increased interest of the organism was noted when it was found that reduction of TMAO to TMA limited the shelf-life of modified atmosphere packaged (MAP) cod fillets (Dalgaard *et al.*, 1993). Since no other TMAO-reducing bacteria were present in sufficient numbers to produce the quantities of TMA that were related to rejection, it was proposed that this organism, owing to its large cell size and activity, was the main spoilage organism in high CO_2 concentrations. Research has shown that approximately 10^7 cfu/g of *P. phosphoreum* were required for 50% of taste panellists to reject a sample, whereas > 10^8 cfu/g *S. putrefaciens* was required. In 50% N_2/50% CO_2 MA packaged cod, at the time of rejection, a population of *P. phosphoreum* sufficient to cause spoilage was found, but *S. putrefaciens* was not present in such numbers.

16.3.4 *Brochothrix termosphacta*

Brochothrix thermosphacta is a well characterised psychrophilic spoilage organism of meat. Growing evidence also suggest a role of *B. thermosphacta* of some MAP fish. Recent studies have investigated the dominance of *B. thermosphacta* on spoiling fish packaged in a 40% CO_2/30% N_2/30% O_2 modified atmosphere. Acetate production has been reported as a good indicator of spoilage by this organism. MAP studies have also demonstrated this organism's sensitivity to oxygen and have shown that they are also inhibited by high CO_2 concentrations.

16.4 Microbiological safety issues related to farmed fish

The vast majority of microorganisms in nature and in foods are harmless and in general terms beneficial. In fact, several microorganisms are applied actively in the production of foods such as bread, alcoholic beverages, cheese, yoghurt or fermented fish and meat products. However, some organisms are of significance with respect to food safety. In this section the most important pathogenic microorganisms that may be encountered in seafood from farmed fish will be

discussed. The following groups of agents will be discussed: prions, virus, bacteria and fungi. It should be stressed that there are profound regional differences in the prevalence of microorganisms and their corresponding foodborne diseases.

Microbial diseases associated with intake of food may be divided into two major classes: intoxications and infections. Intoxications may be defined as diseases caused by the action of preformed toxins found in food. When certain microorganisms, mainly Gram-positive bacteria such as *Bacillus* sp., *Clostridium* sp. and *Staphylococcus* sp., grow in foods, toxins may be produced as a part of their metabolism. The formation of such toxins may be a problem if the foodstuffs have been stored under improper temperature or time conditions. Most intoxications are characterised by a rapid onset of disease, often within hours or less after food intake and may for some toxins give rather dramatic symptoms including fever, stomach pain, vomiting or diarrhoea. Other foodborne intoxications may give more diffuse symptoms, appearing over time as seen for the botulinum toxin. Foodborne infections, on the other hand, occur when a microorganism itself gives a disease when ingested. Infections may give a disease with varying severity from mild symptoms from transient microorganisms found in the gastrointestinal system to severe diseases with microorganisms penetrating the lining of the intestine and giving infections in several organs.

To give reliable estimates on the importance of foodborne diseases from different countries is difficult due to large variation in the accuracy of national reporting systems. Statistics from the European countries and North America shows that infections with Norovirus, *Salmonella* sp. and *Campylobacter* sp. dominate, whereas in other parts of the world other infective agents are of higher significance. In Taiwan, for example, *Vibrio parahaemolyticus* seems to be the agent giving the highest number of outbreaks (Blackburn and McClure, 2002). In USA it has been estimated that foodborne diseases cause 76 million cases, 325 000 hospitalisations and 5000 deaths each year (Mead *et al.*, 1999). It should be emphasised that the true number of foodborne infections in any country by far exceeds the number found in official statistics. Table 16.2 gives an overview of some relevant indigenous and non-indigenous bacteria in seafood that could pose hazards.

Appropriate handling of microbial hazards must be based on knowledge of some key characteristics for relevant organisms such as minimum growth or toxin production temperature, minimum pH and water activity (a_w) for growth and information on the relationship to oxygen. Examples of such information for some bacteria are given in Table 16.3.

16.5 Prions and viruses

Prions are proteinous particles able to give infection in mammals including humans. Infections by prions are collectively known as ‘transmissible spongiform encephalopathy’ or TSEs, and are histopathologically characterised by

Table 16.2 Bacterial health hazard associated with seafood products

Indigenous		Non-indigenous	
Toxin preformed in product	Infection	Toxin preformed in product	Infection
Clostridium botulinum non-proteolytic type B,E and F Psychrotolerant histamine producing bacteria (photobacteria, *Morganella prychrotolerance*)	*Listeria monocytogenes* *Vibrio cholerae* *Vibrio parahaemolyticus* *Vibrio vulnificus* *Aeromonas hydrophila* *Plesiomonas shigelloides* *Shewanella alga* *Mycobacterium marinum* *Erysipelothrix rhusiopathiae*	*Staphylococcus aureus* *Clostridium botulinum* proteolytic type A and B Mesophilic histamine producing bacteria (*Morganella morganii* *Proteus* spp. *Klebsiella* spp.)	*Salmonella* spp. *Shigella* spp. *Escherichia coli*

Adapted from Huss (1994).

degenerative changes in the brain of the infected individual. Typically infected brain tissue shows a vacuolised grey substance resembling a sponge, thus the term 'spongiform'. Infections with TSEs give neurological symptoms and are always fatal. The time of incubation for TSEs may be extremely long, reaching many years. In contrast to other infective agents, prions do not give an immune response in affected persons. Normal prions are a constituent of healthy brain tissue and are located on the membrane of the cells. The exact function of these proteins is largely unknown, but it has been suggested that they might be involved in binding and regulating copper levels. These normal prions may, however, in rare cases revert to a pathologic, form which do not show normal turnover times in the brain, but resist proteases and therefore accumulate in the tissue giving the typical structural changes. Such reverted prions may in turn interact with normal prions catalysing their transformation into the pathologic form. Prions may spread from animals to humans via food, and introduce the transformed prion variety that is able to cause disease. A remarkable feature of prions is their stability to physical or chemical treatments. These infective proteins will maintain infectivity after heat treatment, irradiation and exposure to disinfective agents that would otherwise inactivate all other known contagious agents, including virus bacteria and even bacterial endospores.

Some TSEs have been known for many years among animals (scrapie in sheep and goats) or humans (Kuru), but more focus were put on these diseases in connection with the 'Mad-cow disease' scientifically termed Bovine spongiform encephalopatia (BSE) (Belay, 1999). If transferred to humans via infected bovine meat, the prion causing BSE is shown to cause a contagious variety of an otherwise sporadically occurring neurological disease termed Creutzfeldt-Jakob disease (vCJD). Even though the number of patients suffering the vCJD is comparably low, the economic losses due to decimation and destruction of infected animals were very high. In addition such incidents profoundly reduce consumer confidence in food safety.

Table 16.3 Some relevant pathogenic bacteria and their growth limits

Microorganism	Minimum temp. (ºC)	Minimum pH	Minimum a_w	Aerobic/ anaerobic	Typical food item and environmental reservoirs
Bacillus cereus	4	4.3	0.95	Facultative	Rice, spices, vegetables, eggs, diary products, heat-treated fish products
Clostridium botulinum (mesophilic, proteolytic)	10	4.6	0.93	Anaerobic	Meat, fish, vegetables, soil, sediments
C. botulinum (psychrotrophic, non-proteoloytic)	3	5.0	0.97 (or $\geq$ 5.5% NaCl)	Anaerobic	Seafood (type E), meat (type B, F)
C. perfringens	12	5.0	0.95		Heat-treated meat and fish products, soil, aquatic sediments
Escherichia coli	7	4.4	0.95	Facultative	Meat and fish products, intestine of warm-blooded animals, faecal contaminated water and soil
Listeria monocytogenes	0	4.3	0.92	Facultative	Seafood, meat, vegetables, non-pasteurised diary products, soil, water, plants, sewage drain
Salmonella sp.	7	4.0	0.94	Facultative	Poultry, egg, spices, animal feeds, dried ingredients
Staphylococcus aureus	6 (10 for toxin)	4.0 (4.5 for toxin)	0.83 (0.9 for toxin)	Facultative	Recontaminated heat-treated foods Humans and warm-blooded animals
Vibrio cholerae	10	5.0	0.97	Facultative	Seafood, human intestine, faecal polluted water
Aeromonas hydrophila	0	4.0	0.97 >5% NaCl	Facultative	Fish and shellfish, some red meats (beef, pork, lamb) and poultry
V. parahaemolyticus	5	4.8	0.94 (moderately halophilic)	Facultative	Seafood, coast and brackish water

Adapted from European Chilled Food Federation (ECFF, November 1996). *In*: Martins, T., *Harmonization of safety criteria for minimally processed foods*. Inventory report FAIR concerted action FAIR CT96.1020, September 1997.

Fish are shown to have DNA coding for production of prion proteins and thus do fulfil the general precondition to develop prion diseases (Oidtmann *at al.*, 2003). The fish prions, however, are different from mammalian proteins in key sites, and it has been considered most unlikely that transmission of possible reverted prions could occur between fish and mammals (Rivera-Milla *et al.*, 2003).

16.5.1 Viruses

Viruses are non-living particles consisting of a proteinous coat containing nucleic acids as either RNA or DNA. These nucleic acids carry the necessary genetic information for the production of new viral particles. Some viruses are supplied with a lipid envelope, but this is not common among the foodborne types. Pathogenic viruses in food are usually of the RNA type. The replication of viruses must actually be carried out by the cell in which the virus is parasitising. In contrast to bacteria, viruses are totally inert outside living cells and are therefore not able to multiply in foods. Infections with viruses rank among the ten most important causes of disease in humans (Cliver, 1994, 2001; Koopmans, 2002). Among viral infections in humans, gastroenteritis has been estimated to be second in frequency after the common cold (Jay *et al.*, 2005). When seafood is involved in foodborne infections, the product in question has in the majority of cases been shellfish and in particular oyster (Lees, 2000). In principle any food, including farmed fish, could be a carrier of viral contamination from the environment or from infected food processors or handlers. The methods for detection of viruses are laborious and require expensive equipment and this may explain why the true numbers of cases in any country is probably much higher than those reported in official statistics.

Foodborne viruses are generally more resistant than bacteria to environmental factors such as heat, disinfective agents and low pH, and may retain infectivity for several months in water or foods. The infectivity of virus is not dramatically reduced during freezing. One obvious problem in this respect is the fact that several viruses of relevance for food have a longer ‘survival’ in the environment than the commonly applied indicator organisms such as coliforms and enterococci.

Important foodborne viruses include, amongst others, Norovirus and hepatitis A virus. The Norovirus, formerly termed Norwalk virus, are classified into the family *Calisiviridae*, and are small (30–38 nm) single-stranded RNA viruses. The transmission of the disease is by the faecal-oral pathway and the dose of infection may be as low as 10 to 100 particles. It should be noted that the virus may be shed in the faeces up to 10 days after infection, thus by persons feeling fully recovered. The typical time of incubation for Norovirus infections varies from 24 to 48 hours and common symptoms are vomiting, stomach pain, diarrhoea, headache and mild fever. Norovirus infections affect persons of all ages and typically last some days, and the course of the illness is usually mild and self-limiting. Due to its highly contagious nature, spreading via faeces and

vomiting aerosols, Norovirus is typically involved in large outbreaks in health care institutions, schools and kindergartens. A large range of foods has been associated with Norovirus outbreaks, and includes oyster, cockles, butter cream and contaminated water. Fish products have also been expected as possible vectors of the virus (de Wit *et al.*, 2003).

The hepatitis A virus is classified into the family *Picornaviridae* that comprise small (22–30 nm) single-stranded RNA viruses. The transmission of the disease is by the faecal-oral pathway and the dose of infection is as low as 10 to 100 particles. Persons of all ages may be infected by hepatitis A, but the severity generally increases by the age of first contact with the virus (Koopmans, 2002). If repeatedly exposed to the virus during childhood, few symptoms may be observed and the infection will not be detected. The incubation period is relatively long, and may vary from 14 to 50 days. The symptoms observed are more unspecific than for Norovirus, and includes fever, headache, nausea, stomach pains and vomiting, followed by hepatitis later in the course of the disease. A large range of foods have been associated with hepatitis A outbreak, and includes oyster, clams, caviar, mineral water, orange juice, sandwiches and lettuce. Hepatitis A virus has been found on the gills and in the intestine of farmed fish (Gershy-Damet *et al.*, 1987).

Foodborne pathogenic viruses infect by ingestion and are shed by faecal materials, or even by vomit. The prophylactic measures against viral infections are therefore similar to those relevant for most other infective agents. They include good hand and kitchen hygiene, prevention of food contamination by infected food processors, selection of unpolluted farming sites for aquaculture or alternatively depuration of shellfish grown in non-optimal sites. Furthermore adequate heat-treatment of foods and chlorination or UV-treatment of potable water are important hygiene measures.

16.6 Bacteria, fungi and mycotoxins

16.6.1 *Listeria monocytogenes*

The genus *Listeria* includes at present six species, *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*. Of these species only *L. monocytogenes* and *L. ivanovii* have been recognised as human pathogens. The importance of *L. ivanovii* as a foodborne pathogen is unclear, thus only *L. monocytogenes* will be discussed in the following text. *L. monocytogenes* is a Gram-positive short rod not able to produce spores. The bacterium is able to grow under both aerobic and anaerobic conditions, and at tempertures normally found during refrigeration. *L. monocytogenes* has been described as a bacterium with a wide distribution, and may therefore be detected regularly in many foods, including farmed fish. The reported incidences in seafood products vary from 0–75% in the literature. Unprocessed or frozen fish is reported to harbour this bacterium in 0–50% of examined samples, the corresponding reported figures for processed seafood products are 0–26%,

scrimps 0–20%, shellfish 0–7.5% and smoked salmon or trout 0–75% (Ben Embarek, 1994).

L. monocytogenes has been shown to cause the disease listeriosis mainly affecting sheep, but also humans. The first verified human case occurred in a soldier during the First World War. Even though the number of listeriosis cases is relatively low, the fatality rate is as high as 20–30% (Paoli *at al.*, 2005). Owing to its potential fatal outcome, the presence of *L. monocytogenes* in foods is of great concern among consumers and food safety authorities. Listeriosis in humans has been associated with the consumption of a number of different foods including green salad, cheese, cold cuts, mussels, cold-smoked fish and fermented fish and meat products. The first documented case of listeriosis with fish as the most likely source was reported in 1989 (Facinelli *et al*., 1989).

During recent decades a general increase in the numbers of diagnosed cases of human listeriosis has been observed. The reasons for this increase are multiple, including better diagnostic tools, increasing population at risk, change in production and conservation technologies and new eating habits favouring lightly processed foods. The number commonly observed in most Western countries is 2–4 cases per million inhabitants. In the vast majority of human cases, listeriosis occurs among individuals with a lowered immune competence, such as the foetus, infants, the weakened elderly, drug addicts, HIV-positives or patients under immune depressing therapy. Listeriosis may involve symptoms of varying strength, from a mild flu-like picture to severe infection in the central nervous system (meningitis), bacteria in the bloodstream (septicaemia) or multi-organ infection. *L. monocytogenes* has shown itself able to invade cells, penetrate mucosal linings and to be transmitted from mother to foetus. Infections among pregnant women may give mild symptoms in the mother, but could in many cases be fatal for the baby. In several countries pregnant women are advised to avoid eating products at risk such as ripped cheese, cold cuts, cold-smoked fish and fermented fish and meat products.

Survey programmes for *L. monocytogenes* in foods are performed on a regular basis by several national food control authorities. The findings from such examinations show large year to year variations even for the same product and the same country. The general tendency is that the fraction of positive samples and the number of bacteria in these samples increases during processing of products with no or minimal preservation. Thus the raw materials generally contain less *L. monocytogenes* than the ready to use product, e.g. smoked salmon. This reflects the ability of the bacterium to establish in food processing environments and to grow under refrigerated temperatures.

Several international bodies that deal with food safety issues have addressed *L. monocytogenes*, and work to establish acceptable numbers in different types of foods is currently being undertaken. It has been scientifically documented that products containing less than a certain number of bacteria (100 to 1000 bacteria per gram) are not involved in disease among healthy adults. Some national food control authorities have given recommendations for certain groups of consumers (children, pregnant and immunocompromised) to avoid products where there is a

risk for *L. monocytogenes* contamination. Such products may include smoked vacuumed fish products and cold cuts.

Under fish processing in cold and temperate areas where the general incidence of foodborne pathogenic bacteria is low, *L. monocytogenes* may be considered one of the most challenging bacteria with relevance for fish.

16.6.2 *Vibrio* species

Bacteria belonging to the genus *Vibrio* are Gram-negative, oxidase-positive, non spore forming and typically appear as slightly curved rods. In contrast to most other bacteria of importance for seafood safety, members of this group are widespread in aquatic habitats at different salinities. These bacteria are common in marine and estuarine environments, and on the surfaces of marine plants and animals (Baumann *et al.*, 1984). They also occur naturally in the intestinal content of marine animals (Sakata, 1990), and some *Vibrio* species are also found in freshwater (West, 1989). There is no correlation between the occurrence of vibrios and bacteria of faecal origin, and common indicator organisms do not give information on presence of potentially pathogenic vibrios. Vibrios are not robust bacteria and are sensitive to heating, freezing, drying and several other preservation techniques, as well as the low pH in the stomach of humans.

More than twenty *Vibrio* species have been described as being able to cause disease in animals, while twelve species have so far been reported to be pathogenic to humans. The latter species are *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus, V. fluvialis*, *V. alginolyticus*, *V. damsela, V. furnissii*, *V. hollisae, V. mimicus*, *V. cincinnatiensis*, *V. metschnikovii* and *V. carchariae*. Eight of these species have been reported as associated with foodborne infections, of which *V. cholerae, V. vulnificus* and *V. parahaemolyticus* are considered to be predominant as human pathogens. The pathogenicity of *V. cholerae* and *V. parahaemolyticus* is highly correlated to the presence of specific virulence factor genes. An overview of the pathogenic *Vibrio*-species associated with human infections is given in Table 16.4.

The incidence and density of human pathogenic vibrios in the environment and consequently in seafood products are highly dependent on the ambient temperatures. These bacteria are occurring in considerably larger numbers at high seawater temperatures (Baffone *et al*., 2000; Høi *et al*., 1998; Oliver and Kaper, 2001; O'Neil *et al*., 1992; West, 1989; Dalsgaard, 2002). Human pathogenic vibrios may also be detected in seafood organisms in temperate waters as in the Nordic countries during the summer months, but their abundance is comparatively low. Foodborne infections with vibrios are most common in Asian countries, and less common in Europe especially countries having a cold climate (Sutherland and Varnam, 2002).

Infections with *V. cholerae*, termed Asiatic cholera or epidemic cholera, have historically been associated with large outbreaks involving several countries and a large number of persons, i.e. pandemics. Cholera pandemics have occurred

Table 16.4 Overview of pathogenic *Vibrio* species associated with human infections

Vibrio species	Site of infection						
	GI tract	Wound	Ear	Primary septicaemia	Bacteraemia	Lung	Meninges
1. *V. cholerae* O1/O139	++	(+)	?	?	?	?	?
V. cholerae non-O1/O139	++	+	+	(+)	(+)	?	(+)
2. *V. parahaemolyticus*	++	+	(+)	?	(+)	(+)	(+)
3. *V. vulnificus*	+	++	?	++	+	(+)	(+)
4. *V. fluvialis*	++	?	?	?	?	?	?
5. *V. alginolyticus*	?	++	+	?	(+)	?	?
6. *V. damsela*	?	++	?	?	?	?	?
7. *V. furnissii*	(+)	?	?	?	?	?	?
8. *V. hollisae*	++	?	?	(+)	?	?	?
9. *V. mimicus*	++	+	+	?	?	?	?
10. *V. metschnikovii*	(+)	?	?	(+)	?	?	?
11. *V. cincinnatiensis*	?	?	?	?	(+)	?	(+)
12. *V. carchariae*	?	++	?	?	?	?	?

GI tract: gastro intestinal tract; ++ : most common site of infection; + : other sites of infection; (+) : rare sites of infection; ? : infection remains to be established. Information in table from West, 1989 and Oliver and Kaper, 2001.

since ancient times, and dehydrating diarrhoeic diseases fitting the symptoms of cholera have been described by Hippocrates and in Sanskrit writings. Epidemic cholera was described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India. The mode of transmission of cholera by water was first shown in 1849 by the London physician John Snow. In 1883, Robert Koch successfully isolated the bacterium from the intestinal discharges of cholera patients (Todar, 2004). The largest recent cholera pandemic occurred in South America in the early 1990s and involved more than 400 000 cases. This outbreak was at least partially caused by a contaminated ceviche, a raw fish product (Huss *et al*., 2004).

Infections with *V. cholerae* seem to occur only in humans, and the source for bacteria during epidemics is the faeces from infected persons. Strains within the species *Vibrio cholerae* may be divided into more then 130 serotypes based on the O antigens in the cell envelope. The epidemic capability of *V. cholerae* is linked to the presence of genes for cholera toxin (CT) production, and the main serotypes having such genes are designated O1 and O139. Cholera outbreaks are often related to natural disasters or war-like situations where the general hygienic and sanitary conditions, including drinking water quality, are poor. The infective dose of *V. cholerae* is rather high (10^8–10^{10} cells) and the time of incubation may vary from approximately six hours to some days. In its classical form, symptoms are dominated by profuse diarrhoea with rice water-like stools, eventually leading to a failure in circulation and death due to loss of water and electrolytes. An effective treatment includes replacement of lost water and electrolytes by excessive intravenous administration. The mortality in untreated cholera may reach 60%, whereas good treatment reduces the mortality to a few percent. *V. cholerae* strains not possessing the cholera toxin genes (non O1, non O139) may also cause disease in humans, but symptoms are generally milder. However, in some cases of non-epidemic cholera infection, the severity of the disease may reach what is seen for O1 and O139 types.

V. parahaemolyticus typically gives a disease characterised by rapidly emerging abdominal pain, nausea and explosive diarrhoea, sometimes followed by mild fever, vomiting and headache. Infections have exclusively been linked to consumption of seafood, especially raw or undercooked products. An obvious problem in this respect seems to be the cooling of heat-treated seafood products with non-treated seawater during production. As for *V. cholerae*, the dose of infection is high, and typical is reported to be approximately 10^6 cells/gram food. The time of incubation lasts from some few to 24 hours, and the duration of the disease is usually 2–3 days. The fatality rate is low. In some cases a more severe dysenteric form for infection occurs. At least four haemolytic products from *V. parahaemolyticus* are known.

Patient isolates often carry genetic material coding for the toxin thermo stabile direct haemolysin (Vp TDH) or a TDH-related toxin (TRH). TDH positive strains give typical haemolysis on agar plates supplied with human blood, and are termed Kanagawa positives. Kanagawa positive strains are seldom found when isolating bacteria from the environment. *V. parahaemolyticus* is able to

grow rapidly under mesophilic temperature conditions. If cooling is inadequate (>5 ºC) the numbers may increase to high numbers during storage (Huss *et al.*, 2004).

V. vulnificus is a pathogen causing disease in humans and a number of marine animals. In humans this bacterium is an agent of wound infection or invasive infection after ingestion of contaminated food. In both cases infections are most often associated with handling and consumption of raw oysters, predominantly by males above 40 years having a predisposing medical condition such as liver dysfunction, high blood iron content or alcohol abuse. The mortality in this group may reach 60%.

There has been some discussion among different food safety authorities regarding how to handle detection of the most common pathogenic vibrios, *V. cholerae, V. parahaemolyticus* or *V. vulnificus.* The common reaction form in countries with low prevalence of these bacteria is rejection or mandatory heat-treatment of the product in question.

16.6.3 *Bacillus cereus*

B. cereus is an important spore former of relevance for food microbiology. *B. cereus* strains are facultative anaerobic, Gram-positive spore forming bacteria, widely distributed in the environment and have been isolated from a wide variety of foods, especially of plant origin, but also from meat, fish, and dairy products. It is a part of the *B. cereus* group, including also *B. anthracis*, *B. mycoides* and *B. thuringiensis*. There have also been a number of reports of food poisoning caused by other *Bacillus* species such as *B. subtilis, B. licheniformis*, and *B. pumilis*. *B. cereus* may cause different infections and intoxications. The two types of food poisoning, emetic and diarrhoeal disease, are caused by very different virulence factors.

Most strains are mesophilic and are able to grow in low-acid foods at temperatures down to 15 ºC and up to 55 ºC (optimum 30–40 ºC). During the last decade, psychrotrophic strains of *B. cereus* have been recognised that are able to grow at temperatures down to 4 and 6 ºC. (maximum 30–35 ºC). *Bacillus* species has been isolated from *sous vide* cod fillets at 5 ºC (Ben Embarek, 1994) and in many other *sous vide* products (Nissen *et al.*, 2002). Food containing more than 10^4 *B. cereus* cells per g may not be safe for consumption.

Mixed fish products, e.g. fish-pudding and fish cakes containing spices, milk and flour ingredients, may contain low levels of *Bacillus* spp. Many products only have a mild heat-treatment, enough to safeguard the product against spores of *Clostridium botulinum*, but not for the more heat-resistant spores of *Bacillus cereus*. Control of *Bacillus cereus* is efficiently obtained by chilling, except for the few psychrotrophic strains.

16.6.4 *Clostridium botulinum*

Clostridium botulinum are anaerobic spore formers of great importance for food safety. *C. botulinum* is a Gram-positive, rod-shaped bacterium (Cato *et al.*,

1986) naturally found in soil, sediments and water on a world-wide basis (Fach *et al.*, 2002; Hielm *et al.*, 1998; Huss, 1980; Gram, 2001). The bacterium has even been detected in sea-salt intended for fish salting (Fenicia *et al.*, 2002). Most strains within the species of *C. botulinum* are able to produce very potent proteineous toxins during growth, and the presence of such bacteria is consequently of great concern in the assessment of food safety.

The species *C. botulinum* may be divided into seven types (A to G) based on the serology of the toxins produced. These toxins are thermally unstable, and will generally be inactivated at temperatures above 85 ºC for five minutes. However, bacterial toxins in general, including botulinum toxin are stable at high salt concentrations and low pH (Huss and Rye Pedersen, 1980). Any toxin present or preformed in the raw material could be carried over to the final product, and strict growth control from harvest to consumption is necessary (Huss *et al.*, 2004). Human botulism, i.e. infections or intoxications associated with *C. botulinum*, are in the vast majority of cases associated with the types A, B, E and rarely F (Austin, 2001). Types C and D causes botulism in animals, and type G has so far not been shown to cause any disease.

The type E *C. botulinum* together with the types G and F are classified as psychrotrophic, and thereby able to grow at relatively low temperatures. At otherwise optimal conditions *C. botulinum* type E may grow and produce toxins at temperatures down to 3.3 ºC, and in products containing up to 5% NaCl (Gram, 2001). Optimal temperature of growth is reported to be 18–25 ºC, and the minimum water activity (a_w) required is 0.97.

Several authors have reported on the prevalence of *C. botulinum* in seafood (Cann *et al.*, 1966; Fach *et al.*, 2002; Gram, 2001; Hielm *et al.*, 1998; Huss, 1980; Hyytiä *et al.*, 1998; Hyytiä-Trees, 1999). When found in seafood products from cold-water areas such as Scandinavia, Canada, Alaska, Russia and some parts of Japan, *C. botulinum* type E is reported to be the most prevalent type (Huss, 1994). Based on the widespread distribution reported in the present publication, the author considers *C. botulinum* type E to be a true aquatic organism.

The amount of toxin necessary to cause human illness is very small. Thus, if an environment favourable to germination and growth of spores from any of the seven types of *C. botulinum* known to produce neurotoxins exists in fish before or during processing, there is a potential risk of illness.

Telzak *et al.* (1990) reported that out of 32 outbreaks by *C. botulinum* type E reported from 1899 to 1977, 31 could be traced to marine products. The spores are reported to be found at highest prevalence in the viscera of fish (Badhey *et al*., 1986; Telzak *et al.*, 1990).

In the period from 1899 to 1990 a total of 962 cases of human botulism outbreaks were registered in the United States (Solomon and Lilly, 2001). These outbreaks involved 2320 cases of which 1036 were fatal. Two outbreaks of botulism could be traced back to the product ‘kapchunka’, a whole, smoked and brined whitefish preparation (Anon, 1985b, 1987). In 1981 a Californian man was sickened and in 1985 two Russian immigrants died of botulism poisoning from commercially prepared ‘kapchunka’ in New York. Furthermore six Israelis

contracted botulism from fish that had been sent from New York as a gift. In the period from 1996 to 1999 no cases of botulism were reported in the Netherlands.

The incidence of botulism in Norway is comparatively low. The first verified case was described in 1934, involving consumption of home-made cured ham. From 1961 to 2002, a total of 62 cases have been reported. Under Norwegian conditions botulism is, in the vast majority of foodborne cases, linked to the consumption of non-vacuumed, lightly salted and fermented trout from freshwater sources ('rakfisk'), or from home-produced lightly salted and dried ham. 'Rakfisk' is produced on a small-scale local basis, and has at present no export value.

Provided anaerobic conditions, strains of *C. botulinum* possess the ability to grow at low temperatures and in the presence of relatively high concentrations of NaCl. Consequently these organisms have the potential of growth and toxin production in chilled and lightly preserved seafood products with an extended shelf-life.

The sodium chloride concentration is an important factor in controlling the outgrowth and toxin production of *C. botulinum*. It is generally accepted that the inhibitory concentration for nonproteolytic *C. botulinum* is 5.0% water-phase salt (WPS), equivalent to a water activity of 0.97. A 10% WPS concentration is necessary to inhibit the proteolytic strains. The hurdle principle will apply in such a situation, and it should be stressed that these NaCl limits apply under optimal conditions for bacterial growth, and that other factors such as the presence of oxygen, low temperature and high or low pH will reduce the salt tolerance of *C. botulinum* in a practical situation. Huss (1994) concluded that in fish products stored at temperatures below 10 ºC, a water phase salt concentration of 3% is sufficient to inhibit the growth of *C. botulinum* for at least 30 days. In the traditional salting process applied for herring in Norway, the salt content will within one week reaches at least 12–13% in the water phase through the fish. This may give a time interval where growth may occur, if the temperature is favourable for bacterial growth. Due to strict temperature control during this step, botulism from salted herring is not considered a problem.

However, Eklund *et al.* (1982) reported on the difficulty of achieving uniform salt concentration in large batches of fish or in sections of an individual fish during the brining operations. The dynamics of brining of fish does not seem to have been studied in detail; however, it would be expected that water drawn osmotically from the fish would be a rapid process, with the diffusion of sodium chloride into the fish being a slower process, especially for ungutted fish. To facilitate salt penetration, the Norwegian code of practice for salted herring recommends that each fish should be 'nibbed' or 'gibbed' before submersion into brine. This practice involves removal of gills and parts of the viscera, removal of gills only or the mechanical destruction of the skinny tissue below the ventral parts of the gills. Such treatment is performed on a routine basis during the production of salted herring in Norway.

Eklund *et al.* (1982) also cautioned that the nonproteolytic nature of type E organisms would not result in the development of odours indicative of spoilage.

Thus toxin could be formed with little evidence to the consumer that the fish was spoiled and possibly unsafe. In a 1963 outbreak described by these authors, only 3 of 16 affected people reported any unusual flavours or off odours.

16.6.5 *Clostridium perfringens*

Clostridium perfringens is widely distributed in the environment where it may be found at levels of 10^3–10^4 per gram soil. These anaerobic, Gram-positive spore-forming bacteria are also isolated from water and sediments and from faeces of healthy individuals.

C. perfringens does not grow at chill temperatures, and minimum growth temperatures are often referred to 15 ºC, and the growth rate is slow below 20 ºC. The vegetative cells are sensitive to acid (minimum pH of 5), salt (maximum 6%) and they do not grow at water activities below 0.95. Suitable growth conditions in chilled fish products are therefore only found sporadically, and growth control is not complicated. Controlling proper time-temperature conditions and avoiding cross-contamination to heat-treated foods is essential.

Approximately seven annual cases of *C. perfringens* are reported in the US with links to seafood and it is estimated that approximately 200 seafood-caused cases occur every year (Feldhusen, 2000). Food poisoning may occur if high levels of cells are eaten. Then a number may survive the gut passage, sporulate in the small intestine and produce an enterotoxin. This results in nausea, abdominal pain, diarrhoea and sometimes, vomiting 8–24 h after ingestion.

16.6.6 *Escherichia coli*

Escherichia coli belongs to the group commonly designated as coliform bacteria. *E. coli* like the other coliform bacteria is facultative anaerobic, Gram-negative rods capable of fermenting lactose with gas production within 48 hours at 30–37 ºC. The coliform bacteria are common in the gut and intestine of warm-blooded animals and humans, and are therefore often used as an indicator for faecal contamination. The coliforms include several geni from *Enterobacteriaceae* and some strains are psychrotrophic capable of surviving and even growing at chilled temperatures (4 ºC). Since the coliforms not necessarily have a faecal origin, they are used an indicator of improper production hygiene.

Most of the *E. coli* strains are considered harmless commensals in the intestine, but some strains are pathogenic and can cause disease. Pathogenic *E. coli* are currently divided into groups depending on virulence and clinical symptoms. Important groups are: enteropathogenic (EPEC) and enteroaggregative *E. coli* (EaggEC) both involved in diarrhoea among children, enterotoxigenic *E. coli* (ETEC) that give the common trawlers diarrhoea, enteroinvasive *E. coli* (EIEC), diffusely-adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC) that cause haemorrhagic colitis and Haemolytic uremic syndrome (HUS) (Smith and Fratamico, 2005).

The main sources of *E. coli* infections are contaminated water and infected food handlers that contaminate the products. Outbreaks of EHEC have been

connected to many food items including meat, milk, vegetables, fruit juices and fish (Jay *et al.*, 2005). Neither of the *E. coli* types are typical in temperate waters or on products from temperate waters. Poor hygiene, cross contamination by food handlers or contaminated water may, however, transfer the organism.

The use of Good Hygienic Practice (GHP) with emphasis on clean water and good personal hygiene will help in the control of these organisms. As all are sensitive to heating, the GHP-programme must be particularly strict when ready-to-eat foods are processed.

16.6.7 *Salmonella* species

Bacteria in the genus *Salmonella* belong to the family *Enterobacteriaceae*, are Gram-negative and oxidase negative rods. Even though *Salmonella* sp. represent typical intestinal bacteria, they may survive and even multiply in food or feed if the conditions are favourable. *Salmonella* types may grow both aerobically and anaerobically in the temperature span between 5 and 46 ºC, and at a pH ranging from 3.8 to 9.5. As for other bacteria in the *Enterobacteriaceae* family, the optimal growth temperature is 37 ºC. The heat resistance of salmonellae is strongly influenced by the surroundings in which the bacterium is found, and increases by decreasing water content or increasing fat content in the product in question. In frozen or dried products and products rich in fats as fish feed, *Salmonella* sp. may survive for months or even years.

The taxonomy of *Salmonella* sp. is rather complicated, and has been a subject of scientific debate for many years and is still evolving (Bell and Kyriakides, 2002; Andrews and Bäumler, 2005). Presently the *Salmonella* species are arranged in two species: *S. bongori* with 19 serovarieties mainly found in heterothermic animals, and by far the most numerous species *S. enterica* with six subspecies and the majority of serovarieties.

Salmonella species has been known for more than 100 years as the cause of infection among animals and humans. On a global basis *Salmonella* sp. is one of the most important causes of infections related to foods. The reported number of salmonellosis cases per 100 000 inhabitants in industrialised countries varies from 0.8 to 258 (Stan Bailey and Maurer, 2001). The problem is generally less in the Nordic countries due to the cold climatic conditions, decentralised population and the low prevalence of *Salmonella* sp. in food-producing animals. Transmission of salmonellosis is by the faecal-oral route, possibly after multiplication in foods. The range of foods involved in transmission of salmonellosis is large, and also includes seafoods such as fish, shellfish and crustaceans (Huss *et al.*, 2004). Owing to the heterothermic properties of seafood animals, *Salmonella* is not a constituent of the normal flora of these animals. Contamination of such products is therefore a result of faecal contamination via polluted rearing water, from infected food handlers or from cross-contamination during production or transport. Compared to other foods, farmed fish is not considered a main vehicle for *Salmonella* transmission.

The many *Salmonella* serotypes may give infections of highly varying

severity, ranging from a lack of symptoms or mild and self-limiting infections to a fatal outcome. Depending on the serotype in question, the properties of the contaminated food and the person to person variations in susceptibility, the dose of infection may vary from 10 to 10^6 *Salmonella* cells. The time of incubation are typically one to three days, but may also be as short as 12 hours or more than a week. The majority of *Salmonella* serotypes give a self-limiting intestinal infection lasing typically up to one week. The symptoms include diarrhoea, stomach pain, fever and occasionally vomiting. In some cases accompanying symptoms (sequelae) occur, and may be joint pains, meningitis, urinary tract infections or infections in the heart linings (Bell and Kyriakides, 2002). Depending on the form, some sequelae may last for months after the disappearance of initial symptoms. One additional complicating food safety factor for salmonellosis is the carrier condition that some recovered patients acquire. Even though the number of *Salmonella* in faeces usually declines after recovery, some persons may shed bacteria for a considerable time, and may therefore represent a source of contamination if involved in food production or preparation.

The most important *Salmonella* serotypes in terms of number of infected persons through the world are *S.* Typhimurium and *S.* Enteritidis, which account for approximately 70% of reported cases. *S.* Typhimurium has established among wild living birds and mammals, and is a frequent serotype involved in large outbreaks sometimes reaching many thousand cases (Stan Bailey and Maurer, 2001). *S.* Enteritidis is also a very common cause of infection transferred by many foods, and has amongst others shown itself able to infect eggs if the parent bird is infected.

Feed intended for aquaculture species may in principle be contaminated by any type of *Salmonella*; however, some serotypes dominate when found in these types of products. Common serotypes in vegetable or animal components for production of fish feed, or in ready to use feed are *S.* Agona, *S.* Senftenberg, *S.* Montevideo, *S.* Livingstone, *S.* Bloemfontain, *S.* Johannesburg, *S.* Lexington, *S.* Anatum, *S.* Cerro, *S.* Worthington, *S.* Lille and *S.* Oranienburg.

A recent study by Lunestad *et al.* (2007) summarises the prevalence of *Salmonella* in samples of compound feed, feed materials and environmental samples from feed producers in Norway during the years from 2000 to 2004. During this period, the prevalence of *Salmonella* in ready to use compound fish feed was shown to be 0.3%, and the most common serovars were *S.* Senftenberg, *S.* Agona, *S.* Montevideo and *S.* Kentucky. The prevalence in feed materials varied from 0.14–0.33%, and the prevalence in environmental samples was found to be 3.78%. The predominant serovars found in fish meal were *S.* Senftenberg and *S.* Montevideo. The same serovars were dominating in isolates from the production environment, and could in these cases be considered 'house strains'. Under natural rearing conditions for farmed Atlantic salmon in Norway, and with low concentrations of *Salmonella* in the feed, the risk of transmission to humans via fish products is minimal (Nesse *at al*., 2005). This assumption is supported by the finding that the most common serovars in Norwegian fish feed ingredients, fish feed and fish feed factories count for approximately 2% of

clinical *Salmonella* isolates from domestically acquired cases in Norway. There is no known direct association between the isolates recovered from feed and any human cases in Norway.

Even though *Salmonella* sp. has not been shown to establish or multiply in the intestine of cold water fish, the safety for handlers of fish feed and the possible dissemination of new *Salmonella* serotypes among wild homoeothermic animals such as birds and rodents have been of some concern.

16.6.8 *Staphylococcus aureus*

The *Staphylococcus* genus comprises several species of which especially *S. aureus* is associated with foodborne disease. *S. aureus* has a lower growth limit at 6 ºC and for toxin production 10 ºC. They are rarely found on fresh fish from temperate waters, but may be isolated from newly caught fish in warm waters. Most bacteria are transferred from food handlers with hand infections and poor personal hygiene. Investigations have shown that many food handlers, 6% and 12% according to hand and nasal sampling, carry enterotoxigenic *S. aureus*. This is documented in that some investigations has found 2–10% *S. aureus* in fresh fish and bivalves, while 24–52% are found in some vulnerable cooked products (Jablonski and Bohach, 1997).

The staphylococci are Gram-positive cocci with their primary habitat in the skin, glands and mucous membranes of warm-blooded animals including humans. Infected sores and scratches are often harbourage sites for *S. aureus*. Outside the body, *S. aureus* is one of the most resistant non-spore forming human pathogens and can survive for extended periods in a dry state. Staphylococcal food poisoning ranks as one of the most prevalent food causes of gastroenteritis worldwide. It results from ingestion of one or more preformed Staphylococcal enterotoxins in staphylococcus-contaminated food. Due to the mesophilic growth, some sort of temperature abuse must precede intoxication, and a level of about 10^6 cfu/g is needed to form toxic doses. The bacterium is very salt tolerant and levels and toxins may be produced in up to 10–15% NaCl. Growth and toxin production may occur in products handled by man such as cooked and peeled crustaceans, warm smoked fish or fish products which are ripened, like sardines (Arkoudelos *et al.*, 2003).

Prevention and control of staphylococcal toxin food poisoning include proper chilling and avoidance of cross contamination of both raw and cooked products. The toxins are heat stable and may resist boiling temperatures.

In addition to the bacteria mentioned so far, several other species are of relevance as foodborne pathogens. The most significant ones are *Campylobacter*, *Yersinia* and *Shigella*, but they do not seems to be a particular problem in farmed fish or fish products in cold and temperate waters.

16.6.9 Fungi and mycotoxins

A diverse range of moulds is able to produce toxic metabolites, and there are three genera that are especially important in foods: *Aspergillus, Penicillium*, and

Fusarium. In terms of acute toxicity the mycotoxins most commonly encountered in food are much less toxic than the botulinum toxins and many of the algal toxins. However, long-term chronic toxicity is of special concern, since several of these mould metabolites are carcinogenic or may have other toxic effects. The production of each mycotoxin usually is limited to a small range of fungal species or even strains within a species. Many foods are susceptible to mould growth when stored under inappropriate conditions, furthermore a number of foods have evolved in which mould growth is an essential part of their manufacture, e.g. mould-ripened cheese and sausages. Mycotoxins may be formed in foods, raw materials for food production, or animal feeds.

Most moulds can grow at a_w down to 0.8 indicating that they are able to grow at high salt concentrations or in dry products. Traditional smoked fish, e.g. salmon and mackerel, may be vulnerable to mould growth if stored under suboptimal conditions over time.

16.7 Predictive models for seafood safety and shelf-life

The objective of predictive food microbiology is to mathematically describe the growth or reduction in numbers of foodborne microbes, or spoilage bacteria under specific environmental conditions and thereby make it possible to predict the safety and shelf-life of products. It is assumed that growth or decline of a particular microorganism is governed by the environment it experiences. This environment includes intrinsic factors as pH, and water activity (a_w) and extrinsic factors (temperature, gaseous atmosphere). A large number of factors affect the microorganism; however, in most foods only a few exert most of the growth-limiting activity. Several presumptions are made during modelling, e.g. that the effect of one factor is assumed to be independent of whether the microbe is found in a broth or present in food.

16.7.1 Safety models

The earliest predictive models for food safety were the ones developed for canned foods during the 1920s. Theses models described thermal processes sufficient to destroy 10^{12} spores of *Clostridium botulinum* type A, as a model organism. Such treatment has later been referred to as a 'botulinum cook' (Whiting and Buchanan, 2001; McMeekin *et al.*, 2002). The models described production processes with a very large safety margin and, whilst that probably accounts for their continued use, it perhaps also inhibited their widespread recognition as predictive models.

The origin of 'modern' predictive microbiology can be traced to the 1960s and 1970s when kinetic models were used to address food spoilage problems (Spencer and Baines, 1964; Olley and Ratkowsky, 1973a,b), followed by the use of probability models to address food poisoning problems, particularly botulism and other intoxications (Genigeorgis, 1981; Roberts *et al.*, 1981).

More precise models have been developed during the later years, and they are often classified based on the population behaviour that they describe, e.g. growth models, limit of growth models or inactivation models. Predictive microbiology provides invaluable information for the production of safe food with adequate shelf-life, and it can predict effects of several environmental factors combined.

16.7.2 Spoilage models

Mathematical growth models are now available for predicting the shelf-life of fish at different storage conditions, like temperature, atmosphere, and salt concentration (often recalculated to water activity a_w). Shelf-life prediction by kinetic models requires some information such as: (i) the SSOs and their spoilage domain in the specific product, (ii) the initial numbers of the SSOs, (iii) a relevant spoilage criterion such as the level of SSOs at sensory rejection, and (iv) recording of relevant conditions, e.g. temperature of storage (Dalgaard *et al.*, 1997). Kinetic models that predict both growth of SSO and shelf-life accurately, i.e. within $\pm 25\%$ can be developed on the basis of laboratory experiments using liquid media. This, however, requires careful selection of SSO, media, growth systems, and experimental protocols.

Different models are available such as: (i) kinetic models for growth of SSOs at different ambient conditions, (ii) empirical models for relative rates of spoilage, and (iii) simple models for linear relations between indices of spoilage and remaining product shelf-life at different temperatures. The effect of storage time and temperatures has been determined to be cumulative and this has allowed various predictive models to be used for time-temperature integration.

Some mathematical models for shelf-life predictions are included in computer software, which allows users without detailed mathematical knowledge to apply models and thereby obtain information from the usually large amount of data collected during model development. Model for *S. putrefaciens*, *P. phosphoreum*, *Pseudomonas* spp. and *B. thermosphacta* are now available.

Certain models are available free of charge, and are downloadable from the internet. As examples The Danish Institute of Fisheries Research (DIFES) in Lyngby, offers software with a Seafood Spoilage and Safety Predictor (SSSP) from their homepage http://www.dfu.min.dk/micro/sssp/), USDA in Philadelphia, have developed the Pathogen Modelling Program (http://ars.usda.gov/Services/docs.htm?docid=6786) and Institute of Food Research, UK offer Growth Predictor (http://www.ifr.ac.uk/Safety/GrowthPredictor). The Growth Predictor is based on data generated in various laboratories in the UK, under the funding of the Food Standards Agency, UK.

16.8 Future trends

It has for many years been a trend among consumers to favour products that are mildly preserved, thus appearing as close to the fresh counterpart as possible. It is estimated that prepared meal consumption in Europe and America will double

in ten years, to exceed US$40bn by 2009, and strong opportunities are predicted in the crossover between health and convenience (Anon, 2005). For many products this can be met by maintaining the nutritional value through minimal processing. Their taste and texture are in many cases comparable to those of freshly prepared foods. The convenience aspect they offer has also led to increasing demand by quality conscious consumers in retail markets. These products, however, have a limited shelf-life and require chilled distribution.

The definition of shelf-life is not obvious. Most chilled raw or partly processed food products packaged in modified atmosphere (MAP) will have a limited shelf-life with good quality, then chemical and biochemical processes together with microbiological spoilage will decrease the sensory quality. After the period of good quality, a period with regular or even poor quality may follow, without introducing safety hazards to the consumer. Future development in preservation should focus on prolonging the good quality life span of MAP products, as well as providing safe products. Most processes or preservations used together with MAP do not prolong the high quality period. An exception to this are low temperature and superchilling treatments, which may inhibit both microbial spoilage and biochemical reactions.

Time is the most endangered commodity of modern life. On-the-go consumers are forever searching for meal solutions that are quick to fix, nutritious, tasty and safe.

16.9 Sources of further information and advice

The following books may be recommended for general information on safety and quality issues, including microbiology of relevance for fish.

- Allan Bremner, H. (2005). *Safety and quality issues in fish processing*, CRC Press, Woodhead Publishing Ltd., Cambridge. ISBN 1 85573 552 0.
- Steele, R. (ed.). (2004). *Understanding and measuring the shelf-life of food*. CRC Press, Woodhead Publishing Ltd., Cambridge. ISBN 1 85573 732 9
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- Huss, H.H., L. Ababouch and L. Gram (2004). *Assessment and management of seafood safety and quality*, FAO Fisheries Technical Paper No. 444, Rome. Also available free of charge at the web pages of FAO (http://www.fao.org/docrep/).

The following two websites can be recommended for information in food microbiology and food safety:

- http://vm.cfsan.fda.gov/~mow/intro.html. This website contains the 'The bad bug book' describing major foodborne pathogens.
- http://www.cdc.gov/ncidod/diseases/food/index.htm Centre for Disease Control and Prevention, USA. This web ite gives an overview and updated information on foodborne pathogens.

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17

Parasites in farmed fish and fishery products

A. Levsen and B. T. Lunestad, National Institute of Nutrition and Seafood Research (NIFES), Norway and B. Berland, University of Bergen, Norway

17.1 Introduction

Parasitism is a highly successful way of life as it has evolved independently in almost every phylum of protists, animals and plants. Thus, most animals have on or within their bodies at least one – often several – species of parasites, sometimes totalling hundreds or thousands of individuals. This also includes all species of fish which, on a worldwide scale, are a major and important part of the human diet. By definition, parasites have lost the ability to live on their own, i.e. their survival depends upon one or more other species, the hosts. Thus, parasitism is a symbiotic relationship where one partner lives exclusively at the expense of the other. However, the degree to which parasites may affect their hosts is highly variable, ranging from an apparently uncomplicated co-existence with low virulence to situations where they may strongly affect entire host populations, under both natural or aquaculture conditions. In many parasite groups, but especially among aquatic ones, there are intricate and complex life cycles. These generally involve one or more intermediate hosts where larval development or asexual multiplication occurs, and a definitive host in which to breed and from which to shed their offspring in order to ensure dispersal (Fig. 17.1).

In and on the commercially most important fish species and stocks in temperate seas (the main areas covered by this chapter) there is a variety of parasites that are of concern to the aquaculture, fishery and fish processing industry. Comparatively few but widely distributed parasites of fish are considered zoonotic agents, i.e. they may be transferred to and cause disease in man.

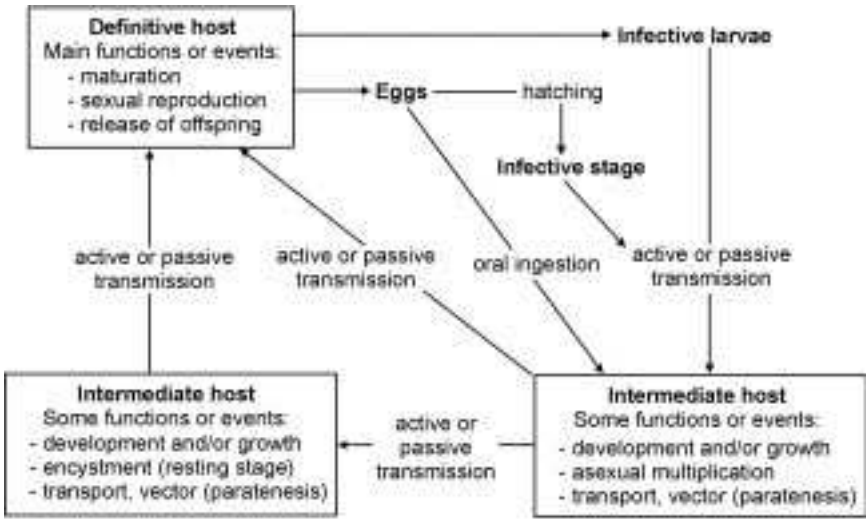

Fig. 17.1 Some possible life cycle pathways of indirectly transmitting helminth fish parasites.

Besides the species of concern to seafood safety, other frequently occurring parasites are primarily affecting the food quality as they may severely reduce the aesthetic appearance of the product. Thus, a number of different fish parasites may have severe economic impact, either related to their pathogenicity, often resulting in substantial losses under aquaculture conditions, or due to their importance regarding the quality and safety of fish and fishery products.

The continuously growing awareness by consumers and public food authorities as to the possible presence of parasites or parasite-related quality defects in seafood emphasises the importance of providing the fish processing industry with up-to-date knowledge on the occurrence, detection and control regarding the most important parasite species of fish. This is further underlined by the increasing popularity of Asian-inspired seafood based on semi-processed or even raw fish meat. Thus, this chapter primarily focuses on those parasites which are most frequently associated with substantial food quality defects and/or pose a direct consumer health risk. In this respect, the larvae of at least three species of roundworms (nematodes) are among the most frequently occurring parasites in virtually all of the commercially exploited fish stocks in temperate seas around the globe. Besides the considerable quality-reducing effect of these parasites, they are of direct human health concern, especially regarding the consumption of undercooked, brined, marinated or even raw fish meat. Additionally, the potential of these worms – both dead and alive – to induce hypersensitivity reactions in humans has recently received increased attention. Therefore, this group of parasites will be particularly addressed.

17.2 Types of parasite

The majority of parasites discussed here belong to the animal kingdom, including the myxosporidians, flukes, tapeworms, roundworms and sea lice. Only the microsporidians and the 'tumbling disease' agent (*Ichthyophonus hoferi*) belong to the fungal and protozoan kingdom, respectively. A brief overview of their basic systematics is given below.

17.2.1 Kingdom Fungi: phylum Microsporidia

Microsporidians are strictly intracellular parasites of many invertebrate and some vertebrate groups including fishes. Microsporidians develop small unicellular spores, 3–10 μm in length. They contain a single infective germ, the sporoplasm, and a hollow polar tube serving to inject the sporoplasm into the host cell. Some actual fish parasitic species stimulate the infected host cells to increase enormously in volume (called xenomas), often reaching 10 mm or more in diameter and which are easily seen as whitish knobs or nodules in or on the affected tissue.

17.2.2 Kingdom Protozoa: subphylum Choanozoa; *Ichthyophonus hoferi*

According to recent molecular studies, *Ichthyophonus* belongs to an unusual group of protists, evolutionarily close to the animal-fungal boundary. The parasite occurs worldwide in many species of marine and freshwater fishes. Its life cycle is not yet fully elucidated but fish seem to become infected by ingestion of spores which are thick-walled, spherical in shape and contain multiple nuclei. The stage most often observed in the host tissue is a resting spore which is roughly circular in shape and highly variable in size, measuring 10–250 μm in diameter.

17.2.3 Kingdom Animalia: phylum Myxosporidia

All myxosporidia are obligate endoparasites. The characteristic myxosporean structure – the spore – consists of one to several shell valves, at least one amoeboid infective germ (sporoplasm) and one to several polar capsules, each containing a single more or less coiled extrusive filament, which has solely an anchoring function. Recent studies have demonstrated that this group seems to have molecular and structural affinities with the cnidaria (anemones, jellyfish and corals). Myxosporean spores from fish vary greatly in shape and size, ranging from round or ellipsoid to quadrate or stellate, some even carry appendages. The spores of most species measure 8–30 μm in length or diameter. The myxosporean life cycle is complex and still not fully understood for many species, especially the marine ones. However, it has become clear that many, if not all, require an alternative host (e.g., polychaets or bryozoans) for successful completion of the life cycle.

Phylum Platyhelminthes – the flatworms
Flatworms are the only bilaterally symmetrical animals that lack any type of body cavity. A digestive tract is either incomplete with only a single opening for food uptake and waste excretion, or even absent.

Trematoda – the flukes
Digenean flukes are obligate endoparasitic flatworms; most adults parasitise vertebrates. The typical digenean has two suckers, one at the anterior end surrounding the mouth/anus opening, while the second larger one is located ventrally at mid-body. Their body size is usually 1–8 mm. Most digeneans have complex life cycles, often involving two intermediate hosts. Fish may serve as second intermediate or as definitive host while a mollusc acts as first intermediate host.

Cestoda – the tapeworms
Adult tapeworms occur in the intestine of various vertebrates including fish. Their bodies are typically long and flat, consisting of many segments called proglottids, where each is a gamete-producing factory. In contrast to trematodes, the body surface of tapeworms is covered with microvilli, which are tiny projections that formidably increase the surface area. As a digestive tract is completely absent, the microvilli facilitate absorption of nutrients from the host. At the anterior end, there is an attachment apparatus – the scolex – consisting of suckers and/or hooks. The life cycle of tapeworms typically includes the egg, one free-living larval stage, a procercoid and a plerocercoid in the first and second intermediate host, respectively, and the adult in the definitive host. Fish may serve as second intermediate host, definitive host, or in some species as both.

Phylum Nemathelminthes: Nematoda – the roundworms
Nematodes comprise many free-living and parasitic species; some are important parasites in man as well as wild and farmed animals including fish, and in agriculture. Their bodies are cylindrical, i.e. round in transverse section, with a rather rigid outer cuticle lining the body. Nematodes possess a complete digestive tract commencing at an anterior mouth and terminating at a posterior anus. There are separate sexes and five distinct stages in the life cycle. Small roundworms like the common pinworm (*Enterobius*) seldom exceed 10 mm in length, while large ones like *Ascaris*, the common intestinal roundworm of man, may reach 30 cm or more. However, there is great intraspecific variability in adult nematode body size, largely depending on state of maturity, age and host size. Fish parasitic nematodes are mainly found as adults in the digestive tract or as encapsulated larvae on and in the visceral organs and to some extent in the flesh.

Phylum Arthropoda: Copepoda – the sea lice
The morphology of parasitic copepods is highly variable. Some groups still resemble their free-living relatives, with the typical copepod appearance largely

intact, while others are reduced to sac-like structures that perfectly illustrate an almost complete adaptation to a parasitic way of life. Copepods have separate sexes and the size of species parasitising fish varies considerably, ranging from small ones, a few mm in length, to 'giant sacs' reaching 8 cm or more. Two parasitic copepods are among the most important economic loss factors in northern European marine aquaculture. Other species occurring on wild fish may have significant impact on the dynamics of their host populations. The parasitic copepod life cycle includes at least one free-living nauplius stage, one or more free-living copepodid stages, one or two pre-adults and, finally, the adult stage. There are several modifications of this general life cycle. Thus, in some important groups there exist several parasitic chalimus stages between the infective copepodid and the pre-adult stage.

17.3 Reducing fish quality

17.3.1 Microsporidia and *Ichthyophonus*

Some commonly occurring microsporidian species from commercially important NE Atlantic fishes induce clearly visible whitish knobs or nodules (xenomas) in various host tissues including the fillets. There are several species belonging to at least three genera that may catch the attention of the consumer while processing whole fish or preparing a fish meal. A species within the genus *Glugea*, *G. stephani*, infects both wild and maricultured plaice and turbot in coastal European waters that regularly reach at least 15 ºC. The xenomas are usually small, not exceeding 1 mm in diameter, and located in the connective tissue layer of the intestine. In massive infections, the xenomas may give the intestinal wall a chalk-white pebbled appearance. Although the fillets may not be directly affected, heavily parasitised fish are not fit for human consumption. Other microsporidians with actual quality-reducing effects are *Pleistophora* spp., forming elongated white nodules or even large subcutaneous swellings, sometimes exceeding 10 cm in size, within the body musculature of long rough dab, common sole or wolf-fish. In cultured turbot, the small xenomas of *Tetramicra brevifilum* may aggregate to form clearly visible whitish nodules in the body muscle. Besides the unpleasant appearance of the nodules themselves, *Tetramicra* has also been associated with severe muscle degeneration in infected fish.

Ichthyophonus is a common parasite in many cultured or wild fish species including Atlantic salmon, plaice, herring and mackerel. The most frequently infected organs are the heart, liver, kidney, spleen and skeletal muscle. In heavy infections, the parasite often appears as clearly visible whitish or yellowish nodules caused by a strong granulomatous host response surrounding groups of spores. *Ichthyophonus* does not represent a human health threat but if many nodules are found in the fillets, the fish may not be marketable. For comprehensive information on fish parasitic microsporidian and protozoan parasites, the reader is referred to Lom and Dyková (1992).

17.3.2 Proteolytic myxosporidia – the 'soft flesh' inducing parasites

Many myxosporidians are known from various wild and cultured fish species worldwide. Most species seem to be rather non-pathogenic to fish and apparently do not reduce the quality of the final product. However, within the genus *Kudoa* there are a number of species that may seriously affect the fillet quality of several important wild or maricultured fish stocks around the globe. For example, the species *K. thyrsites* is of concern to the marine fish aquaculture industry along the west coasts of North and South America, Europe, South Africa and in temperate waters around Japan and Australia. In infected fish, this species induces the notorious condition of post-mortem muscle degradation, or 'soft flesh'. The parasite has so far been reported from more than 30 different fish species worldwide (Moran *et al.*, 1999).

K. thyrsites represents a serious problem for the Atlantic salmon aquaculture industry in British Columbia, Canada, as the reduced quality of affected fish may cause significant loss of revenue. This is not only attributed to the downgraded quality of the product, but also due to the misconception in the marketplace that farmed Atlantic salmon from certain areas represents an inferior product. Another isolate of this *Kudoa* species, formerly assigned to *K. histolytica*, may become a serious quality problem for the lucrative fisheries of Atlantic mackerel in the Northeast Atlantic (Levsen *et al.*, 2008).

Upon death of infected fish, the actual *Kudoa* species releases powerful proteolytic enzymes which cause a rapid softening of the flesh. The site of origin of the proteolytic enzymes within the parasite's different developmental stages has not yet been identified. In heavy infections, the entire muscle may within hours turn into a viscous jelly-like mass, even if stored cool after catch (Fig. 17.2). If frozen immediately, the enzymes involved seem to be largely deactivated but may continue the proteolytic process as soon as the fish is defrosted. While still alive, infected hosts are apparently able to control the parasitic enzyme release by antagonistic means since seriously infected fish still have a normal healthy appearance shortly after catch. Thus, the spoiling

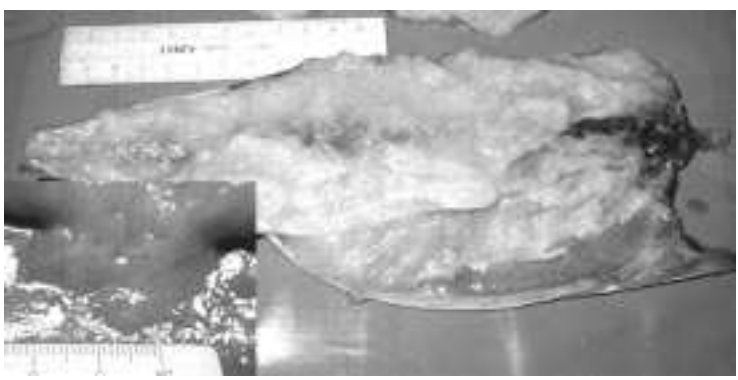

Fig. 17.2 Severe post-mortem myoliquefaction ('soft flesh') in Atlantic mackerel, 48 h post catch, caused by the myxosporidian parasite *Kudoa thyrsites*.

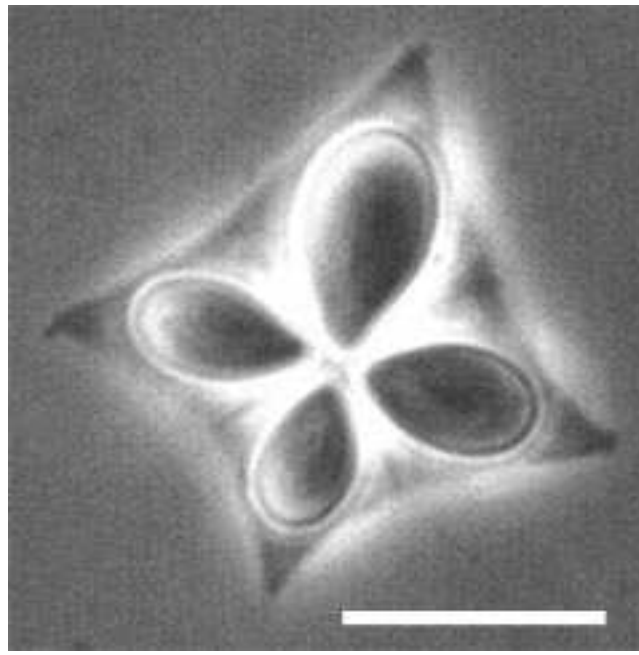

Fig. 17.3 A single spore of *Kudoa thyrsites* at high magnification phase contrast microscopy. Scale bar 5 μm.

symptoms may first appear after arrival at the final marketplace. If the problem is prominent in lots of specimens, the market value of the actual shipment may be considerably reduced, possibly putting the reputation of the dealer/exporter at stake since 'soft flesh' is easily misinterpreted as spoilage due to poor storage or transport conditions. In fish showing severe post-mortem 'soft flesh' symptoms, thousands or millions of spores are pervading the body musculature. The spores are small, measuring 12–15 μm in diameter, stellate in shape, and have four shell valves, each containing a pear-shaped polar capsule (Fig. 17.3).

Kudoa infections in maricultured fish can neither be chemically treated nor prevented by other means. Since the life cycle of this group of myxosporidians has not yet been resolved, ecological control of the fish infective stage, e.g. by eliminating the possible alternative host organisms from the surroundings of actual aquaculture facilities, is not an option either. Moreover, *Kudoa* spores seem to be rather resistant to physical destruction as they may survive daily temperature fluctuations between −18 ºC and +21 ºC. However, rapid and prolonged deep-freezing (below −18 ºC) as well as heating over 70 ºC will kill them.

17.3.3 Flukes and tapeworms

The digenean fluke *Cryptocotyle lingua* is – like all flukes – an endoparasite, but one of its life cycle stages, the metacercaria, is commonly found in the skin of many wild and net-pen reared marine fish species. When present in large

Fig. 17.4 Head region of Atlantic cod (*Gadus morhua*) with heavy infection of *Cryptocotyle lingua* metacercariae ('black spot disease'). (Courtesy of Dr Egil Karlsbakk.)

numbers, the parasite causes 'black spot disease'. The metacercariae lodge in the fish skin where they produce tiny clear cysts around themselves while the fish produces a second outer connective tissue capsule with black pigment. The black spots are easily visible, and by removing pieces of fish skin the clear cyst can be seen at low magnification (Fig. 17.4). Fish-eating birds are the normal definitive hosts in which the worms reach maturity and their eggs are voided with the birds' faeces. *Cryptocotyle* is often used as textbook example to illustrate the general fish digenean life cycle. From the eggs shed into sea water tiny ciliated larvae hatch and swim in search of a periwinkle (*Littorina littorea*) which is the first intermediate host. The larvae bore into the snail and migrate to the digestive gland where they undergo asexual multiplication processes that eventually give rise to large numbers of tailed larvae called cercariae. These will leave the snail and swim off searching for a fish to infect. *Cryptocotyle* develops to maturity mainly in various fish-eating birds, but also in fish-eating mammals. The fluke does not affect human health but fish on display with heavy black spot disease are not likely to find eager buyers. Marine net-pen reared fish may be at risk to acquire large numbers of metacercariae if the pens are located in close proximity to the shoreline allowing the cercariae to easily find a fish host. However, by keeping the periwinkle-population around the culture facilities low, a potential quality problem arising from heavy *Cryptocotyle*-infections can be largely prevented.

Tapeworms occur as larvae in the viscera and flesh or as mature worms in the intestine of fish. Members of the genus *Eubothrium* commonly occur in several important salmonid fish species including Atlantic and Pacific salmon, rainbow trout and charr in both fresh- and seawater. The species *E. crassum* and *E.*

salvelini are of concern to the aquaculture industry as they frequently occur in cultured salmon, trout and rainbow trout. The adult worms attach themselves with the scolex in the pyloric caeca, but as they can grow to a length approaching 1 m, they may almost fill the intestine. When cultured fish is gutted and dressed for export they are removed with the viscera so most customers will never see these worms. However, as they need energy to grow, they absorb important nutrients from the fish which may become stunted and even weigh 10–25% less compared to uninfected siblings (Bristow and Berland, 1991).

Another group of tapeworms which is of both quality and consumer health concern belongs to the genus *Diphyllobothrium*. The broad tapeworm of man – *D. latum* – has plerocercoids that are found encapsulated or free as clearly visible white ribbons in the viscera and flesh of trout, charr and other fishes. Its life cycle involves a procercoid, in a copepod first intermediate host while various fish serve as second intermediate host. In the intestine of various carnivorous mammals including man, *D. latum* plerocercoids grow and reach maturity. Details on the consumer health aspect of *Diphyllobothrium* are discussed in Section 17.4.2.

17.3.4 Sea lice and other copepods

Several species of copepods are fish parasites. The caligiform types are flattened, the fore body forms a disc-shaped sucker and the limbs grip the fish skin. Their hind body is more rod-like and the mature females typically carry a pair of long trailing egg strings. The caligids can move about on the fish host, they can swim off and transfer to another fish. Their planktonic larvae attach to a suitable fish by literally gluing itself to it, and subsequently going through a series of chalimus stages during which they grow and eventually become adult males and females. They feed on host mucus and skin; the females also drink blood. In heavy infections they may cause open skin wounds, even exposing the underlying muscles. Thus, several caligid species are of great concern to the marine aquaculture industry worldwide since heavily affected fish, even if the lice themselves have been removed, will not be marketable.

The salmon louse (Fig. 17.5a), *Lepeophtheirus salmonis*, is a large species specific to marine salmonids. This is a common parasite on salmon in the Northeast Atlantic, along the Pacific coast of Canada and around Japan. Before the onset of modern large-scale marine aquaculture, salmon lice used to be present in the winter months on salmon at sea. Today, however, farmed salmon may be infected at all times. In heavy infection, the lice may cause bleeding, open sores and in extreme cases even expose the skull bones (Fig. 17.5b). Such fish may suffer from severe osmotic stress as well as risking secondary infections, rendering them unfit for sale. Salmon coming off the high seas on their spawning run are infected, but the lice do not survive for long during the hosts' upstream migration in rivers. Lice on river salmon indicate that the fish arrived just recently. The outward bound smolts entering the fjords and coastal waters in spring are challenged by hordes of infective copepodids, which in the

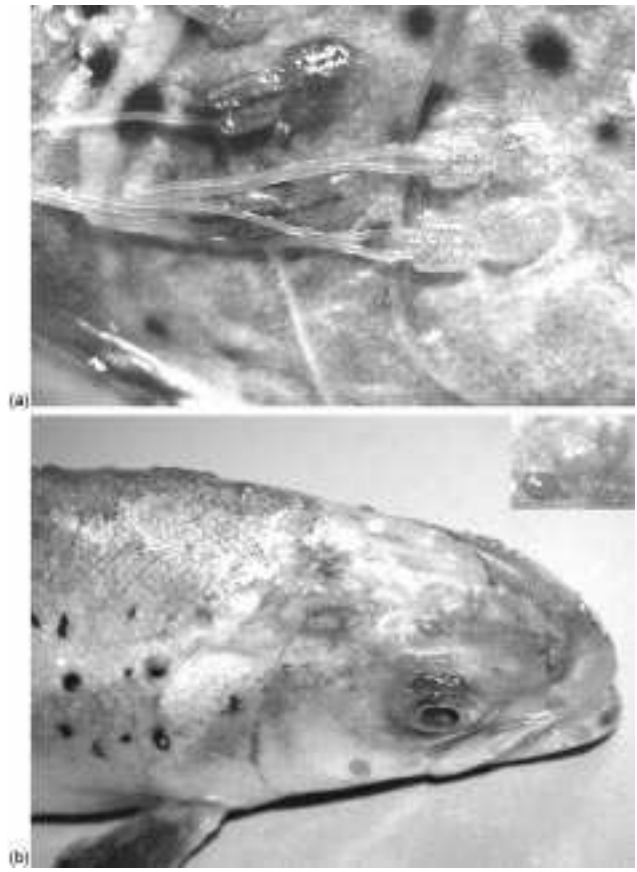

Fig. 17.5 Salmon louse (*Lepeophtheirus salmonis*) on farmed Atlantic salmon (*Salmo salar*). (a) Mature females *in situ*. Note the egg strings extruding from each female's hind body. (b) Head region of Atlantic salmon smolt with severe skin lesions caused by the parasite. Note the numerous juvenile lice on and around the lesions. (Courtesy of Mr Lars A. Hamre.)

course of a month or two reach maturity, and if more than a few are present, the smolts may succumb. In marine aquaculture the salmon lice browse on the fish, and their larvae re-infect the penned fish. Much effort has been directed at getting rid of the lice, any treatment should be carried out simultaneously in an area. Escaped salmon are outside control, and their lice represent a source of re-infection. Salmon lice may move freely on the fish skin, sometimes seen on fresh whole fish at display, or even after overseas export of fresh salmon packed on ice.

Another commercially important fish louse is *Caligus elongatus* which parasitises a number of different fish species, both wild and farmed ones including various salmonids, in the Northeast Atlantic. Interestingly, *C. elongatus* seems to be the most important crustacean parasitic problem in the marine aquaculture

industry around the British Isles, in contrast to Norwegian coastal waters where the salmon louse, *L. salmonis*, is the most prominent and harmful caligid species on both wild and farmed salmon. Other important *Caligus* species on cultured salmonids include *C. clemensi* along the Pacific coast of Canada, *C. orientalis* around Japan, while *C. rogercresseyi* and *C. teres* are the most troublesome caligids along the coast of Chile. There exists a vast body of literature on fish lice in general and on fish pathogenic and quality-reducing species of farmed salmonids in particular. For more information on this important group of fish parasites, the reader is referred to Johnson (1998).

A very large S-shaped copepod, *Lernaeocera branchialis*, is relatively common on haddock and both wild and net-pen reared Atlantic cod. This lightly red-coloured parasite is firmly attached to a gill arch; its 'neck' follows the blood vessel to the heart, where its head forms a four-pronged anchor. The large S-shaped red body is without segmentation, and carries two white tangled egg strings. Once this parasite has found a host, its body is transformed to a sac; it feeds on host blood and has an impressive output of eggs. This copepod has a complicated life cycle: their pelagic larvae search for a flatfish, commonly flounder, attach themselves to the gills, and grow into young females and males. After copulating, the males leave and die, while the fertilised females swim off in search of a new cod to infect. Due to its size and conspicuous appearance, this parasite is easily seen during gutting and filleting, and thus, the final product will normally not be affected by *L. branchialis*. However, net-pen reared cod infected with this parasitic copepod may experience stunted growth and generally lowered resistance against other infections.

17.3.5 Common nematode larvae in marine fishes

Species of the genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Phocascaris* live as adult parasites in the stomach of fish-eating mammals and birds. Their third-stage larvae lodge in fish preyed upon by their respective definitive host, and, when arriving in the warm stomach they grow and moult twice, reaching the fifth stage, which subsequently grow into sexually mature male and female worms. The eggs produced are voided with the host's faeces into the sea. Development to young larvae takes place in the shed eggs; in shallow waters the eggs/larvae fall to the sea floor where they may be ingested by invertebrates, in the open sea by various plankton, which again my be preyed upon by the next higher levels in the food web, and so on until they reach a suitable fish, which becomes a transport host. In the fish host, the larvae bore through the wall of the digestive tract into the viscera and body cavity. The host's immune system produces a connective tissue capsule around the larva, which remains 'imprisoned for life'. If an infected fish or the viscera of a gutted fish thrown into the sea is eaten by another fish, the encapsulated larvae become digested free and the fish-cycle is repeated. Large and old carnivorous fish may harbour hundreds, or even thousands, of encapsulated larvae. These larvae are all in their third stage, they all have a so-called boring tooth on the head, there

are no reproductive organs but characters in their digestive tracts permit them to be identified to genus or type.

Anisakis spp., variously known as the herring or whale worm, commonly occurs in virtually all commercially important fish species in many seas worldwide. The best known species is *A. simplex* – however, it has been shown that it actually represents a complex of closely related siblings in many areas of the North Atlantic and adjacent areas. In fish, the majority of the *Anisakis* larvae are encapsulated as flat tight spirals in and on the visceral organs, mesenteries and peritoneum (Fig. 17.6). However, a minor proportion of the larvae usually migrate from the visceral cavity into the flesh. This behaviour is largely to blame for the attention the parasite receives from consumers and food safety authorities. Besides the considerable quality-reducing effect of *Anisakis* larvae, they are of direct human health concern, especially regarding the currently increasing interest in Asian-inspired seafood dishes based on undercooked, brined or marinated, or even raw fish meat (e.g., sushi, sashimi). Most of the flesh-invading larvae seem to reside in the belly flaps, some, however, may penetrate deeply into the dorsal musculature of their fish host. When liberated from the capsule the worm, 20–30 mm long, moves vigorously. Whales, and less commonly seals, act as definitive hosts which may harbour thousands in their stomach. In some whale species such as the minke whale, the adult worms may reach 20–30 cm in length. The eggs/larvae voided with the whale's faeces are strained out mainly by krill which are the main first intermediate hosts. Plankton-feeding fish, such as herring and in particular blue whiting, become infected with *Anisakis* larvae, and as described above, carnivorous fish, such as large cod may accumulate large numbers of encapsulated larvae (Fig. 17.7).

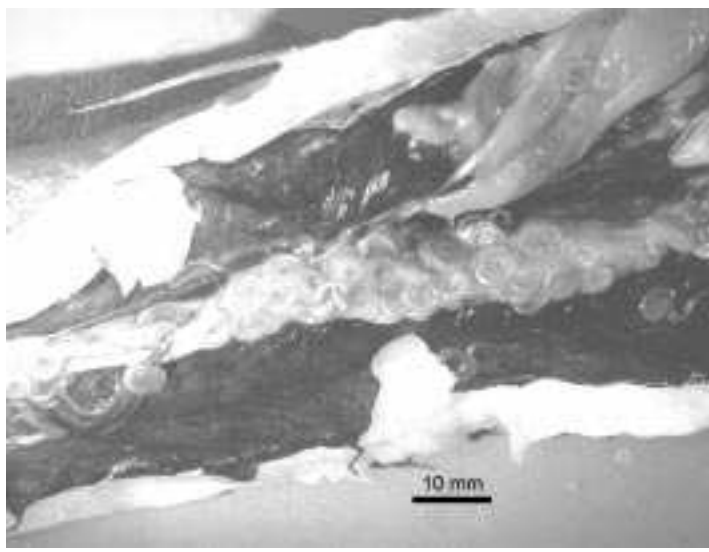

Fig. 17.6 *Anisakis simplex* third-stage larvae in the abdominal cavity of blue whiting (*Micromesistius poutassou*) from the Northeast Atlantic.

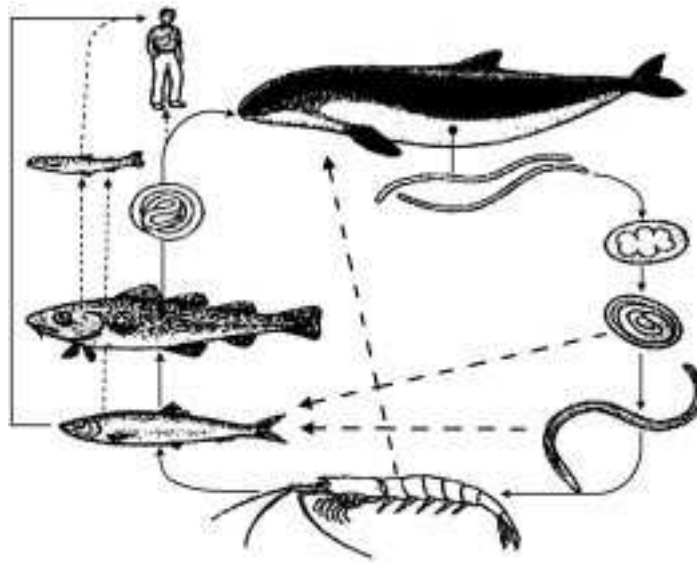

Fig. 17.7 The life cycle of *Anisakis* sp. in the North Atlantic oceanic ecosystem (modified after Buchmann, Bresciani and Beyerholm 2001, Parasitic Diseases of Freshwater Trout, DSR, Copenhagen). Possible alternative transmission routes are indicated as dashed lines.

Pseudoterranova decipiens larva, variously known as cod or seal worm, is larger than the former, is yellow-brown in colour, and most often occurs in the flesh of cod and other coastal fishes. It is stouter than *Anisakis*, and its intestine has an anterior caecum. The life cycle is similar to that of *Anisakis*, but the definitive host is seal. Where coastal seals are common this worm is also common in commercial fishes. The larvae in the flesh are aesthetically unpleasant, and heavily infected fish may be discarded or the worms have to be manually removed from the fillets, which is costly. The cod worm is a serious problem in Atlantic Canada, a lesser problem in Iceland, and as seals are protected in the EU and most associated states, it may become increasingly important in coastal northern Europe as well. Wild-caught young cod placed in net-pens to grow to market size (capture-based aquaculture), may carry along nematode larvae which they acquired during their free-ranging life. *P. decipiens* was until recently believed to be one species with a worldwide distribution in colder seas. In the North Atlantic there are three species; the 'true' *P. decipiens* which mainly occurs in the harbour seal, the recently described *P. krabbei* from grey seal, while the third species, *P. bulbosa*, mainly occurs in the bearded seal, also in the North Pacific.

Nematode parasites and farmed fish

Several studies have been conducted to examine the presence of potentially human pathogenic nematodes in different farmed fish species using several available methods. Such examinations have been performed on a large number

of fish including species as Atlantic salmon (*Salmo salar* L.), coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*) and rainbow trout (*O. mykiss*). In a comprehensive study by Lunestad (2003) a total of 1180 samples of muscle and viscera from Norwegian farmed salmon were examined for the presence of nematode larvae. These samples represented all salmon-producing counties in Norway.

In all studies reported so far, farmed fish has been found free from nematode larvae of known human infective potential. This apparent absence is probably due to the widespread use of industrially produced dry-feed which does not contain viable nematode larvae. Although the main components of such fish-feed are of marine origin, any larvae possibly present in the raw material are killed during the processing of the feed. Thus, whenever unprocessed, raw marine fish products are to be consumed, farmed fish is to be preferred. However, the use of fresh, unprocessed pelagic fish (e.g., herring or capelin) for feed in capture-based marine aquaculture may result in transmission of nematode larvae from the feed to the actual cultured fish.

17.4 Fish parasites and consumer health considerations

Fish parasites from temperate waters do normally not pose any health risk to the consumer. However, some important exceptions exist which are discussed below. For comprehensive information regarding zoonotic parasites of fish in tropical and subtropical regions, the reader is referred to Murrell (2002).

17.4.1 Anisakidosis

In the 1950s a 'new' human affliction, anisakidosis, caused by consumption of a very lightly salted herring speciality, was reported from the Netherlands (Van Thiel *et al.*, 1960; Berland, 2003). The culprit turned out to be live *A. simplex* third-stage larvae, which in the human gut did what they usually do in their natural whale host – they bored into the intestinal mucosa eliciting an immune reaction which again may bring about considerable distress. The clinical manifestations of anisakidosis include epigastic pain, nausea, vomiting, diarrhoea and urticaria. The condition may resemble several other gastro-intestinal disorders, such as gastric ulcer, tumours, acute appendicitis or rectal carcinoma (Bouree *et al.*, 1995; Pinkus *et al.*, 1975; Sakanari and McKerrow, 1989).

When the Dutch experience was published, the symptoms turned out to be well known in Japan, but not the cause. The disease has since been frequently reported from Japan (Higashi, 1985), but also from other countries including France (Petithory and Marty, 1988) and the US (Deardorff *et al.*, 1991). A few years ago close to 35 000 human cases of anisakidosis were reported worldwide, most of them from the Far East where people have a long tradition of eating raw fish. After the Dutch enforced a mandatory deep-freezing step for such fish

products, anisakidosis is no longer considered a problem in the Netherlands. This perfectly illustrates the efficacy of countermeasures specified in various national and international legislations (see also Section 17.5). In northern European countries, anisakidosis is only rarely diagnosed as the traditional ways of preparing fish meals, e.g. proper cooking, frying, salting, drying or hot smoking, will kill any nematode larvae that might be present in the fish flesh. However, the emerging interest for Asian-inspired products based on lightly processed or raw fish thus represents a challenge regarding up-to-date legislation in order to minimise the consumer health risk as to fish-borne zoonoses including anisakidosis.

17.4.2 Diphyllobothriosis

Human diphyllobothriosis, primarily caused by *Diphyllobotrium latum* – the broad tapeworm of man, seems to be most abundant in certain areas of Eastern Europe and Russia, but also occurs regularly in North America, especially around the Great Lakes and Alaska, and recently, it has also been reported from South America. Human infections can always be linked to consumption of raw or under-processed fish. For example, recent outbreaks of the disease in Brazil were traced to the consumption of semi-raw seafood (sushi, ceviche) based on salmon produced by the aquaculture industry in southern Chile (Cabello, 2007).

In northeastern Europe (i.e., Finland and parts of Russia) the worm is a serious human pathogen which may reach 10 m in length. It absorbs vitamin B12, leading to pernicious anaemia. However, in spite of its formidable length, *D. latum* is normally not considered a serious threat to the consumer, although cases of peripheral and spinal nerve degeneration due to toxic worm secretions have been described (Rausch *et al.*, 1998). As for the fish parasitic nematodes, prolonged deep-freezing or proper cooking or frying prior to consumption of wild-caught or outdoor-cultured freshwater fish will kill all plerocercoids that might be present in the fish flesh.

17.4.3 Fish parasites and allergies

In addition to their infective capabilities, fish parasitic nematodes were shown to have an allergenic potential. Thus, several authors report on allergy associated with the ingestion of nematodes from fish. Audicana *et al.* (1995, 2002) and Pozo *et al.* (1996) report cases where allergic reactions occurring after ingestion of fish could be traced to allergens from nematodes. It has also been demonstrated that occupational exposure to *Anisakis*-allergens among fish-processing employees leads to increased levels of allergic airway hyperactivity and dermatitis (Nieuwenhuizen *et al.*, 2006). The allergic reaction was in these cases shown to be triggered by an IgE mediated response. So far at least five allergens have been identified, but there is reason to believe that several other also exist (Kobayashi *et al.*, 2007). The substances in question are shown to retain their allergenic potential even after freezing and heating. Hypersensitivity

reactions, i.e. non-IgE mediated, are also seen for allergens from fish parasites. Parasite excretion or metabolic waste substances left behind in the flesh may be potent allergens as well. Other muscle lodging parasites, e.g. *Kudoa* spp., appear also to induce IgE-mediated responses in the consumer (Martínez de Velasco *et al.*, 2007).

17.5 Legislation and control

If parasites in their larval stage are present in underprocessed seafood when consumed, they may represent a human health hazard. This hazard is reflected in several national and international guidelines or regulations specifying preventive measures against food-borne parasitics. Several treatments have been shown to be sufficient to kill human pathogenic parasites in fish. Heat treatments giving a core temperature above 60 ºC and deep-freezing below −20 ºC for at least 24 hours will kill all parasites commonly found in fish. Several factors may influence the effect of freezing such as the fat content of the fish and the type of parasite present. In general, the resistance to freezing decreases from flukes to tapeworms to nematodes. Brining may reduce the parasite hazard, but the process is not easily standardised and the method is not considered optimal. Since parasites are adapted to stomach conditions, low pH does not affect them significantly and pickling is therefore not a proper means of destroying parasites in fish. The effect of using hydrostatic pressure to kill parasites is presently under investigation.

Parasites in fish are also covered by the EU legislation (Anon., 2004). According to these regulations, all fish intended for consumption in a raw or semi-raw state should be frozen to a core temperature of at least −20 ºC for at least 24 hours. Other public control authorities such as the US Food and Drug Authority (FDA) have a more detailed and stepwise approach to minimise the parasite problem in seafood. Such approaches are often based on the control principles described in Hazard Analysis and Critical Control Point Analysis (HACCP) in which possible health hazards are identified and corresponding countermeasures are determined. In the FDA control guidance a hazard analysis worksheet is described. This sheet presents procedures to identify potential hazards and their significance, helps to identify relevant control points and gives examples of various control strategies.

17.6 Future trends

Consumers expect parasite-free fish and fishery products. For the time being, however, fish seems to be the only industrially produced food which is at risk of carrying parasites when put on the market. As with other important semi-wild animal stocks used as food (e.g., sheep, boar, deer or fowl), thorough quality inspection prior to market release of farmed fish and fishery products in general

seems to be mandatory in order to maintain consumer confidence regarding the safety of fish and fishery products. However, one has to keep in mind that – for the time being – it seems impracticable due to economic and technological constraints, to detect and subsequently remove all the parasites that might be present in the fish flesh. These aspects along with the new culinary trends including raw or semi-processed seafood, challenge both national and international food legislative authorities to regularly update the actual rules and regulations regarding the quality and safety of various fishery products.

Moreover, the currently observed global climate change may eventually result in a significant rise in water temperature in many productive temperate seas and coastal areas around the globe. This again may represent a serious challenge as to the possibly increasing problems with already existing parasites in given areas, or the dissemination of originally non-endemic parasite species to these areas. For example, the 'soft flesh'-producing myxosporidian species *Kudoa thyrsites* may spread northwards, along with infected wild fish such as Atlantic mackerel, to the Norwegian Sea and adjacent coastal areas; many of which house important and vulnerable aquaculture sites. Thus, parasites in general may become increasingly important since future consumer demands will probably push the limit of what is considered acceptable with respect to the occurrence of parasites or parasite-related quality defects in fish and fishery products.

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18

Colouration and flesh quality in farmed salmon and trout

S. J. Davies, University of Plymouth, UK

18.1 Introduction

The prominent colouration of seafood with respect to reddish to orange/yellow hues is due to deposition of carotenoid pigments found in nature and their accumulation via the food chain into the tissues, organs and flesh of many species. Of particular note is the characteristic colouration of the flesh of salmon and trout, the integument of marine fish such as red sea bream and the exoskeleton and muscular epithelium of lobsters, crabs, shrimp and other crustacean carapaces as well as in the hepatopancreas and gonadal tissues of molluscan organisms such as sea urchins and starfishes.

The use of carotenoids as pigmenting feed additives in aquaculture has received much attention in the media with considerable emphasis on their necessity and safety, with statements suggesting that synthetic pigments, colourants or indeed amongst the misinformed that dyes are employed to achieve the desired colour in mainly salmon and trout.

The colouration of salmonid fish has attracted the most attention in research and development as it can be appreciated that the typical pink-red colour of salmon is symbolic of quality and value, with retailers demanding specific criteria for farmed fish such as Atlantic salmon (Moe, 1990). It is this attribute that will form the key topic for this review on salmonid flesh quality in relation to colour (Meyers, 1994; Baker and Günther, 2004).

Carotenoids belong to a very complex family of xanthophylls and carotenes which vary in their chemical structure to provide the host of yellow to orange red and pink colours found in nature. The main carotenoids of importance to salmon and trout are astaxanthin and canthaxanthin, which are specific in their

mode of activity and in their manner of metabolism (Storbakken *et al.*, 1985; 1986; 1987). Bell *et al.* (1998) reported the flesh lipid and carotenoid composition of Scottish farmed Atlantic salmon and the significance of astaxanthin in providing a source of pigmentation. Astaxanthin is recognised to be the predominant red carotenoid found in salmonids in nature with canthaxanthin also used commercially to a limited extent (Nickell and Springate, 1999). Salmon and trout do not possess the ability to *de novo* synthesise these pigments but instead require them within the diet as preformed molecules which are absorbed and subsequently deposited in tissues (Bjerkeng, 2000). This authoritative investigator has extensively reviewed carotenoid pigmentation in salmonid fishes outlining recent progress and the established concensus of learned scientific opinion.

18.2 Sources of pigmenting carotenoids for aquafeeds

Traditional use of commercially synthetic sources of astaxanthin in compounded feeds adds greatly to their costs and the value of the resulting products. The estimated market for astaxanthin in 1993 was in the region of US$90 million, of which over 90% is primarily the synthetic astaxanthin products of DSM Nutritional Products (Carophyll pink) and BASF (Lucantin pink) that dominate this important market. The annual sales of synthetic astaxanthin are estimated at more than $200 million for salmonid fish alone with the canthaxanthin market expected to reach $156 million by 2009. The total global carotenoid market will likely exceed 1$ billion by 2010 (Breithaupt, 2007).

Recently, however, there has been a growing demand for the production of farmed fish under less intensive conditions, and with more emphasis on flesh quality and the use of natural feed stuffs in the diet. This has led to promising research on novel additives and supplements to replace the fishmeal protein component, and especially the inclusion of pigmenting agents based on natural astaxanthin sources. A number of investigations have reported the feasibility of single cell products such as the red yeast *Phaffia rhodozyma* and *Haematococcus pluvialis* algae with respect to their pigmentation ability compared to the synthetic form currently available to the industry. Investigations have confirmed that *Phaffia* can effectively pigment salmonid fish but only few products are currently available for this purpose. Recently, Choubert *et al.* (2006) found differences with respect to the colour of rainbow trout fed *Haematococcus pluvialis* compared to synthetic astaxanthin with higher reported colour and flesh retention with earlier work by Barbosa *et al.* (1999) suggesting that algal astaxanthin from *H. pluvialis* was better utilised at higher dietary oil content for trout. There are conflicting results in the literature due to variations in experimental design, technological differences in astaxanthin production and product variability.

Nevertheless, consumer awareness of welfare and food issues has led to growth in the use of natural sources of feed ingredients (Johnson and An, 1991). Consequently, there is much interest in developing new pigment-rich natural

sources such as those obtained from algae and yeast products for potential applications in aquafeeds to achieve colouration.

Consequently, several commercial products are currently available based on this material being advocated for use in aquafeeds. In Hawaii, Cyanotech Corporation has for a number of years produced a high quality natural astaxanthin rich product namely (NatuRose®) derived from *Haematococcus pluvialis* grown in pond systems in Hawaii. Lorenz and Cysewski (2000) have reviewed the commercial potential for *Haematococcus* microalgae as a more natural source of astaxanthin for applications in aquaculture with reference to salmonids and other fish species.

Similarly Igene Ltd, produce high grade *Phaffia* yeast based astaxanthin product (AstaXin®) for use in aquaculture as well as other applications based on fermentation technology using mainly sugar cane as a substrate. Archer Daniel Midland (ADM) produce a similar product for use in Europe and USA called Ecotone. Other potential sources include krill meal and shrimp processing wastes (Kotik *et al.*, 1979; Scheidt *et al.*, 1985; respectively) and these are now finding much favour for applications in the organic fish farming market, although they are not a reliable material with respect to consistency of astaxanthin content or in terms of supply.

In general, some of these natural sources may show variable success mainly due to inconsistencies in the composition and quality of natural organic materials, or the complex biochemical arrangement of the astaxanthin derivatives and the physico-chemical properties associated with their cell wall structure. The prevailing consensus would indicate that the synthetic commercial astaxanthin is readily available for assimilation by fish, and is a more stable and consistent product. The alternative natural sources, however, have been evaluated in a number of recent investigations with rainbow trout as the model salmonid species with highly favourable results and with acceptable pigmentation levels consistent with retailer and consumer requirements. It should also be noted that effective integument pigmentation in respect of the generation of reddish-pink and orange colour is also favoured for certain fish that can deposit carotenoids or metabolic derivatives in the skin such as red sea bream (Gouveia *et al.*, 2002). This is also a characteristic of high value and acceptability to consumers who perceive this to be superior compared to un-pigmented fish.

18.3 Metabolism of carotenoids in fish and flesh deposition characteristics

The efficacy of flesh colouration by different carotenoids is a function of complex physiological processes followed by a series of biochemical events that involve metabolism primarily in the liver as well as the intestinal tract (Page and Davies, 2002, 2006; Page *et al.*, 2005).

Biochemical transformation of these compounds may then lead to a number of related molecules and metabolites that impart pigmenting properties and

potential pro-vitamin activities as earlier reported for salmonid fish by Torrisson *et al.* (1989).

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′dione) and canthaxanthin (β, β-carotene-4,4′ dione) are converted into zeaxanthin and to β-carotene respectively in fish. The physiological and biochemical processes involved in carotenoid metabolism and utilisation are of enormous interest and govern their efficiency with respect to eventual pigmentation. This has been the basis of recent studies which have attempted to ascertain the fate of ingested carotenoids in salmon and trout with a focus on gastro-intestinal and liver metabolism in order to predict the degree of pigmentation capacity relating to muscle retention. It is the subsequent metabolism and distribution of carotenoids to the muscle that dictates the final level of retained pigmentation.

Investigations have shown that rainbow trout seem to utilise dietary astaxanthin to a greater extent when compared to canthaxanthin (Choubert and Storebakken, 1989; Bjerkeng *et al.*, 1990; Storebakken and Choubert, 1991; No and Storebakken, 1992). This additional discrepancy between the two carotenoids may be due to differences in intestinal absorption as indicated by digestibility coefficients of either astaxanthin and canthaxanthin as affected by dietary level of carotenoid and influence of feed oil levels (Torrisson *et al.*, 1990; Choubert and Storebakken, 1996); concentrations of the two carotenoids in digestive tract segments (Torrissen, 1986, 1989) and transient plasma levels (Guillou *et al.*, 1992; Choubert *et al.*, 1994; Gobantes *et al.*, 1997). Conversely, recent evidence suggests that Atlantic salmon utilise canthaxanthin more efficiently than astaxanthin from feed (Buttle *et al.*, 2001; Kiessling *et al.*, 2003). However, Baker *et al.* (2002) reported that there was no evidence of interaction between graded inclusions of both astaxanthin and canthaxanthin for Atlantic salmon with flesh deposition linearly related to feed dose for each type of carotenoid up to 70 mg/kg of diet. Østerlie *et al.* (1999) reported the selective deposition of different geometrical isomers, i.e. all-E, 9Z and 13Z forms as well as the optical isomers of astaxanthin (3 and 3′ R/S) in rainbow trout fed diets containing this principal carotenoid source. This finding has important implications with respect to the absorption and metabolism of synthetic compared to natural astaxanthin sources in practical feeds. Bjerkeng and Berge (2000) ascertained the effect of digestibility of astaxanthin E/Z isomers in respect of their post-absorptive accumulation in the muscle of Atlantic salmon and comparisons with Atlantic halibut, (*Hippoglossus hippoglossus L.*) for comparative purposes.

The efficiency of carotenoid absorption from the digestive tract is one factor that influences the retention of carotenoids in salmonids (Bjerkeng *et al.*, 1997). Moreover, the salmonid intestine seems to be a major site of provitamin-A conversion of carotenoids (Schiedt *et al.*, 1985; Al-Khalifa and Simpson, 1988).

It should be noted that vitamin A is essential for a number of physiological processes, such as regulation of cell differentiation and proliferation; vision and reproduction. Indeed, Christiansen *et al.* (1994) reported the effect of astaxanthin and vitamin A on growth and survival in the initial feeding stages

of Atlantic salmon, demonstrating the significance of carotenoids and pro-vitamin A forms in fish health.

Several metabolic pathways for the conversion of carotenoids to vitamin A have been suggested in fish (Hata *et al.*, 1973; Barua and Goswami, 1977; Schiedt *et al.*, 1985, 1986; Al-Khalifa and Simpson, 1988; Guillou *et al.*, 1989, 1992; Yamashita *et al.*, 1996). Evidence from the research investigations in Plymouth by White *et al.* (2003a) suggests that a major fraction of absorbed astaxanthin is transformed into vitamin A in the intestinal tissue of rainbow trout and most likely salmon as well. It is apparent that the absorption of dietary carotenoid sources for salmonids is subject to complex processes involving differential uptake mechanisms within the intestine and subject to a post-absorptive metabolism after entering the systemic circulation. Evidence for such sequential physiological and post-absorptive factors as determinants of flesh pigmentation in salmonid fish was advocated by March *et al.* (1990). March and McMillan (1996) further compared muscle pigmentation and plasma levels of astaxanthin in rainbow trout, Chinook salmon and Atlantic salmon in response to different dietary levels of this carotenoid and found important associations. The role of the blood and liver in astaxanthin and canthaxanthin metabolism remains to be fully explored and recently Page and Davies (2003) conducted physiological experiments to validate the interaction of physiological and biochemical effects on pigmentation capacity in both trout and salmon. Davies (2005) reviewed aspect of biochemical and physiological parameters affecting salmonid pigmentation with particular emphasis on post-prandial absorption kinetics and retention efficiency.

There is also an optimum fish size effect, since only fish above 80 grams demonstrate the full capacity for carotenoid retention in the flesh. It is unclear as to whether this is related to an inferior gut absorption mechanism that is activated for fish above a size threshold or hormonal effects. Rainbow trout and salmon are likely to possess the necessary enzyme systems required for the release of astaxanthin from mono- and di-esters and various protein complexes found in their natural forms in red yeasts and algae (Bowen *et al.*, 2002). The free astaxanthin (commercial form) would be expected to be more available under similar dietary inclusion levels, resulting in higher flesh retention values, since there would be no prior need for esterase activity. This has been verified by the studies of White *et al.* (2002, 2003b) based on a series of laboratory experiments with rainbow trout.

Obviously, a further factor that must be considered is the level of incorporation and the duration of the pigmentation stage in the management of cultured fish or in experimental studies. At present, the acceptable levels range between 25 and 80 ppm in most commercial salmonid diets, and the overall efficiency of net retention is governed by the levels employed for evaluation. For this reason, a target of about 50 ppm is normally used in practice for comparative trials to evaluate different pigment sources and feeding regimes in practical feeding trials. Despite the variation in diet carotenoid levels and sources available, there is a finite limit to the deposition rate and eventual accumulation of pigment in fish flesh. Indeed, some species of Pacific salmon

and rainbow trout possess levels in muscle exceeding 20 mg/kg whilst levels in Atlantic salmon may only reach a maximum of 10–11 mg with farmed salmon having typical levels of only up to 7 mg/kg.

A number of abiotic and biotic factors are intrinsically involved in the rate of astaxanthin deposition in the flesh of fish. These are the rate of growth, as affected by temperature, strain and sex of the animal, water quality criteria and more importantly the nature of the feed and feed ingredients. With respect to the latter, a number of workers have shown that the level and type of dietary oil is a major factor in the absorption of carotenoids from the intestinal tract of trout and salmon.

The protein and energy level of the feed dictates the rate of biomass accumulation (lean tissue mass) in fast-growing fish. The rate of muscle fibre recruitment and development is known to influence the binding and hence retention of astaxanthin during the pigmentation phase. In a detailed review of the importance of muscle growth patterns in relation to fish flesh texture and colour, Johnston (1999) described the architecture of the main white (epaxial) muscle mass of salmonids in terms of fine structure and distribution. The complex organisation of white muscle in fish is quite different from mammalian and avian species with fibres traversing more complex pathways in various directions. Increasing muscle accretion can arise from phases of muscle fibre recruitment and hypertrophy. During freshwater growth, the number of white muscle fibres per myotome can be around 18 000 in freshwater stage to about 700 000 after 1-sea winter and above 2 million in a 2-sea winter fish. Since astaxanthin is bound in some manner to muscle fibre proteins, positive correlations have been observed between the DSM colour fan score and muscle fibre density accounting for 25–45% of colour variation in flesh although actual astaxanthin can be independent of fibre density. Density would appear to affect the light scattering process and this trait would be of importance in the perception of colour and could lead to superior use of pigmenting carotenoids in farmed salmon and trout. The importance of muscle ultra-structural characteristics and flesh quality traits for wild and farmed Atlantic salmon was thoroughly investigated by Johnston *et al.* (2006) in which the influence of several muscle-related physiological parameters were reported. These included colour visualisation per unit of pigment concentration being found to be superior in wild fish compared to farm-raised. Although actual astaxanthin levels were higher in farmed salmon, increased colour perception for wild salmon could be linked to the way astaxanthin is bound to the individual muscle protein complexes, such as actomyosin in the cellular organisation of muscle fibres. The distribution of which can vary within fish populations, resulting in significant differences in colour scoring and external perception.

18.4 Pigmentation and fish husbandry

The production of farmed salmon involves a complex life cycle and is dependent on a number of sophisticated management strategies for effective husbandry as

well as the formulation and manufacture of balanced diets using various feed ingredients, raw materials, diet supplements and additives. Modern aquaculture embraces a high degree of intensive production that must integrate the best types of fish farm operational procedures and feeding practice to ensure consistent growth, feed conversion ratios and fish health with increasing emphasis on fish welfare and of course end product quality in terms of consistency, texture, colour and eating qualities.

There are many production-related factors that can influence the degree of pigmentation of farmed salmonids. These include species, race or stock type, intra-population variation, age of fish, type and quantity of carotenoids ingested over specific period, seasonal effects, maturation, health and state of physiological stress, dietary formulation, regulated feed deprivation periods maintenance feeding, slaughter conditions, visualisation of colour, processing conditions: storage, smoking, cooking, etc. Nickell (1998) explained some of the problems relating to the pigmentation of rainbow trout, and Sinnot (2001, 2002, 2003) reported new pigmentation strategies for both farmed rainbow trout and salmon respectively.

The rate of growth and the influence of temperature are known to be major determining factors, as are salinity and physiological processes such as maturation. In salmon, this can extend to the choice of either S(1), S(2) or S(1/2) smolts prior to sea water transfer and the onset of grilsing after the first year. Bjerkeng *et al.* (1992) were able to study the pigmentation of rainbow trout throughout their entire production period from start-feeding to sexual maturation.

Storebakken and Choubert (1991) and Torrissen and Choubert (1991) examined the efficacy of astaxanthin and canthaxanthin pigmentation for rainbow trout raised in both freshwater and saltwater and found a significant difference in astaxanthin flesh content in freshwater held trout compared to those in seawater whilst no differences in overall retention of carotenoids. No and Storebakken (1992) also compared the efficacy of flesh pigmentation of rainbow trout raised in freshwater and seawater fed diets containing similar levels of both astaxanthin and canthaxanthin as pigmenting sources.

The high growth rates achieved in modern salmon production can result in a reduction of pigmentation and uneven distribution within the flesh. It is well known that considerable variations can occur in the ability of fish to retain carotenoids within the muscle and this can sometimes manifest as very low pigmentation or indeed excessive levels in regions. In particular, Olsen and Mortensen (1997) recorded the influence of temperature on flesh colour of Arctic char *Salvelinus alpinus.* It was found by these workers that char maintained at 8 ºC had a much higher pigmentation than at 12 ºC demonstrating the sensitivity of cold water fish to the metabolism and deposition of astaxanthin and the modulation effect of temperature. Indeed the fact that seasonal changes can modulate various selected muscle quality parameters including the degree of pigmentation in salmon was noted by Nordgarden *et al.* (2003). These investigators concluded that rapid period of growth achieved under continuous

lighting compared to natural photoperiod resulted in elevated growth of salmon and increased oxidative stress with marked reductions in fillet vitamin E levels (α-tocopherol) and astaxanthin.

Other environmental factors that may affect the efficiency of pigmentation would likely include water quality and flow rates that would interact to modulate growth and nutrient retention. Sinnot (2003) has extensively listed the main factors that can affect colouration efficiency of salmon. Stocking density and exercise are known to affect fish performance and optimum fish stocking densities will produce superior growth and feed conversion leading to improved fish quality. The SQA (Scottish Quality Assurance) directive is for limits of 20 kg per cubic meter. Similarly adequate flow rates and water exchange rates will promote firmer fleshed fish and may enhance pigmentation in fish under intensive production systems as described previously in relation to muscle quality.

Our knowledge of fish nutrition has expanded considerably in recent times and there have been numerous investigations that have addressed the effects of dietary levels of both astaxanthin and canthaxanthin separately or in combination on the flesh colouration of trout and salmon. The type of feed, level of feeding and matrix effects on carotenoid uptake will affect the degree of pigmentation in salmonid fish and can significantly alter the colour characteristics resulting in possible tainting with background colour.

It has been found that an optimum dietary level of about 65 ppm is preferable in general to achieve acceptable results and that a minimum size threshold exists to initiate pigmentation and maintain deposition during the initial freshwater stages of growth in fish of above 75–100 grams mean body weight. This extends with subsequent transfer as smolts to sea water with salmon fed 65 ppm total carotenoids either as astaxanthin or in combination with canthaxanthin with the latter not exceeding 25 ppm in the mixture (Sinnot, 2003). However revision of such levels is the basis of recent scientific activity to refine dietary levels. Over the last decade, feed manufacturers have been able to moderate the use of pigment in feed. Consequently, few farmers would now feed salmon with diets containing 75 ppm for the whole production period in seawater. Instead many feed manufacturers recommend a three-phase pigmentation strategy with post-smolts being fed pigment at a relatively high level of 60–75 ppm with a transition to an intermediate level of 40–50 ppm from a body weight of 2–3 kg before a final regime maintained on a finishing diet prior to harvest of 25–35 ppm of dietary carotenoids concentration (Sinnot, 2006). Such a strategy, possibly including a mixed pigment regime where astaxanthin and canthaxanthin were combined, can be efficient whilst reducing costs considerably. The further advantages are that an early colouration can be achieved to allow some salmon to enter harvest below any target harvest weight for the main cohort with an acceptable degree of pigmentation.

There are some scientific rationales for supporting higher pigment levels in the later stages of growth for the feeding of large adult fish. This is based on the view that larger salmon can pigment more efficiently than smaller fish. Although an important biological trait, it the advice presented to the fish farmers

is that since pigmentation develops quite rapidly from post seawater transfer, pigmentation generally slows down when fish get bigger, although the absorptive efficiency may increase. It is still therefore pertinent to promote a strategy based on using higher levels in early phase feeding with a reduction to a maintenance level in fish approaching harvest. Indeed, Wathne *et al.* (1998) proposed that Atlantic salmon could be fed astaxanthin in all meals or an alternating meal regime in order to achieve more efficient pigmentation. It has been concluded by these investigators that a more consistent effect resulted from frequent administration of carotenoids.

There is much research needed to refine the basis for an optimum feeding and nutritional programme to meet the challenges of the industry and meet the requirements of the processor and of course ultimately the consumer. To this end, Forsberg and Guttormsen (2006) have advocated their pigmentation model for farmed salmon: this nonlinear regression analysis of published experimental data focuses on dietary aspects and serves as an important basis for improving the efficiency and targeting of pigmentation towards harvest.

18.5 Pigmentation and diet

Fish feed formulators are aware that the composition of the diet can affect the pigmentation of fish due to the choice of raw materials and feed ingredients at their disposal. Lie (2001) has reviewed the significance of nutrition on flesh quality, including pigmentation. Care is practised accordingly to allow for these effects and thus formulate feeds to meet the nutritional requirements of the fish and achieve best performance in terms of flesh colouration and texture.

As stated previously, carotenoids, such as astaxanthin and canthaxanthin, are fat-soluble in nature and their properties as effective feed supplements will be governed by this characteristic. Since fish, such as salmon and trout, are fed balanced diets with a defined protein to energy content and oil levels ranging from 15–38%, it must be appreciated that diet composition both in terms of nutrient content and ingredients will play a major modulating influence on the efficacy of pigmentation. The oil in salmonid fish diets has been traditionally exclusively fish oil or related marine oils, but with increasing pressure on resources there is flexibility to include specific plant oils to replace fish oil at various stages of production and this is a further compounding factor. The degree of polyunsaturation characteristic of marine oil sources rich in EPA- and DHA-type omega fatty acids will be expected to have a marked affect on astaxanthin and canthaxanthin uptake and post-absorption transport and metabolism as noted in the previous section. Of particular interest is what effect increasing plant oils high in omega-6 fatty acids could have on carotenoid uptake and deposition in salmon and trout if high plant oils are used in the later stages of production prior to the use of finishing feeds where fish oils are restored. Choubert *et al.* (2006) have reported that the pigmenting efficacy of astaxanthin fed to rainbow trout was affected by the type of dietary oil present

and that olive oil differed to fish oil in relation to the deposition of flesh astaxanthin. However, some evidence suggests reduced pigmentation efficiency may result in fish fed diets with elevated plant oils over extended periods during growth. However, consumer tests seem to indicate that there are no significant differences in public perception regarding the overall appearance of salmon colour when fed diet regimes that include appreciable levels of vegetable oil sources to replace fish oils (Rosenlund *et al.*, 2003).

Another concern is that fish diets undergo oxidative changes that are more apparent when high energy dense or raised oil levels are employed (over 25%) and this can impede astaxanthin efficacy. Investigations have evaluated the protective role of vitamin E as α-tocopherol acetate in feed and other anti-oxidants whilst it has been suggested that ascorbic acid (vitamin C) can also be beneficial in reducing astaxanthin oxidation and enhancing flesh pigment stability in fish such as salmon. In this context, Akhtar *et al.* (1999) reported the positive association of dietary-induced pigmentation and deposition of α-tocopherol and carotenoids in rainbow trout muscle and liver tissue. Previously, Pozo *et al.* (1988) demonstrated that an elevation of the vitamin E content in the diet of rainbow trout resulted in enhanced canthaxanthin deposition in the flesh. More recent research has shown that selenium (Se), particularly in its organic form, is important in protecting muscle protein fibres from the changes associated with oxidative stress, and that astaxanthin as well as canthaxanthin can be protected from free radical induced oxidative insult (Surai, 2006). Likewise, zinc (Zn) has important properties as an essential trace element with potent specific anti-oxidant properties with a function that includes supporting muscle fibre integrity and therefore helping to stabilise the muscle protein-carotenoid complex from oxidative damage. The US Biotechnology company Alltech have been pioneers in the development of superior bio-complexed zinc and natural seleno-protein organic sources to improve fish flesh quality and pigmentation levels throughout production and post harvesting storage of salmon and trout products. Investigations and farm trials in Chile (De Lyons, 2006) have clearly established that fortification of the diet with natural anti-oxidant supplements such as SelPlex can greatly enhance pigmentation stability in Atlantic salmon with much longer storage times and improved product quality post harvest. Colour stability has important consequences in respect of fish storage. Indeed, colour stability of rainbow trout fillets during frozen storage was investigated by No and Storebakken (1992) and this would be clearly influenced by the pre-history of the fish in terms of husbandry, type of feed, source of pigmenting carotenoids and vitamin/mineral fortification. This area needs much more rigorous scientific exploration.

In practical terms, some feed companies have promoted special formulations that can enhance the 'visually perceived colour' of salmon flesh. A slaughter or harvest diet containing special ingredients was first tested on large rainbow trout with satisfactory conclusions. The effects were positive on visual colour scoring with an increase of 0.3–0.8 units of the DSM colour score although flesh astaxanthin levels remained the same between control and fish fed the special

feed. Practical trials with Atlantic salmon in full strength seawater confirmed these results with significantly higher colour scoring after six weeks of feeding with pronounced increases in perceived colour in the belly-flap region as well in the dorsal and ventral areas producing more even fish colour (Koppe and Roberts, 1998). Investigations with Chinook salmon in Canada have also been encouraging with more pronounced colouration compared to standard diet formulations.

18.6 Comparing commercial pigment sources

There have been a number of investigations to determine the efficacy of different sources of carotenoids to produce an acceptable degree of pigmentation in farmed fish species with most of the research focused on salmonid fish mainly rainbow trout and Atlantic salmon (Sommer *et al.*, 1991, 1992; Buttle *et al.*, 2001). Information tends to be scarce compared to the numerous studies conducted for synthetic pigments due to commercial and intellectual property considerations. However, the efficacy of several alternative natural astaxanthin sources has been reported in the scientific literature. Some of the more documented studies have been those involving the *Haematococcus* product (NatuRose) from the Hawaiian grown strain of this marine microalgal source rich in astaxanthin esters (Sommer *et al.*, 1991, 1992). Choubert and Heinrich (1993) had later compared the use of *Haematococcus* algal astaxanthin with both synthetic forms of astaxanthin and canthaxanthin in experiments with immature rainbow trout.

A more natural and more even pigmentation effect of salmon and trout flesh has been reported for such algal and yeast-based products and Lagocki (2001) and Bowen *et al.* (2002) detailed the use of NatuRose and derived astaxanthin esters as effective alternatives to synthetic astaxanthin for rainbow trout held under controlled experimental conditions. Earlier, Davies (unpublished data) evaluated *Haematococcus pluvialis* at comparable levels with Carophyll pink (DSM) for rainbow trout over a 12-week period in a succession of trials that determined digestibility, carotenoid retention as well as the flesh levels and perceived colour using the standard commercial chart colour scores, Minolta and direct HPLC measurements following the methodology of Skrede and Storbakken (1986). It was found that each of the pigment sources produced similar results with only slightly less astaxanthin deposited in the flesh without causing any appreciable reduction in the perceived colour.

More recently, investigations undertaken at the University of Plymouth, UK compared the efficacy of several commercial pigment sources to achieve flesh colouration in rainbow trout. Diets were formulated to contain equivalent carotenoid levels from various synthetic astaxanthin, canthaxanthin additives as well as several commercial *Haematococcus* and *Phaffia* type products now currently available at an inclusion level of 50 ppm.

It is recognised that the digestibility and absorption characteristics of commercial pigment products will depend largely on their matrix effects with

the synthetic forms being more simple in structure and consisting of free-carotenoids (10%) combined with protein and anti-oxidants enveloped in a protective shell of starch and silica. The natural forms being more complex such as *Haematococcus* with 2% total carotenoid content (70% astaxanthin mono-ester, 10% astaxanthin diester 5% free astaxanthin with beta-carotene, canthaxanthin and lutein also present). All commercial *Phaffia*-based products contain only unesterified astaxanthin at varying levels. The main limitation for algal and yeast products are their inherent cell wall structures that require sufficient cracking or rupture to obtain a viable product with adequate carotenoid availability. The drawback being that increased cell wall degradation may lead to elevated oxidative instability of astaxanthin.

Tables 18.1 and 18.2 show the typical degree of flesh carotenoid levels measured after a 16-week feeding trial in trout fillets for each determined two-week period and the overall final retention efficiency respectively. This particular farm-based study demonstrated that for rainbow trout, synthetic astaxanthin sources were better retained in epaxial white muscle compared to either canthaxanthin sources or the naturally derived algal and yeast forms tested. Values for retention are low for all sources ranging between 5.4 and 12.8% with synthetic astaxanthin carotenoids yielding higher flesh retention values.

Table 18.3 shows the DSM colour score index for the individual treatments and interestingly, despite the expected variations in the mean flesh pigment values, there were no major discernable significant differences in the perceived colour scores (12.07–13.7) for rainbow trout fed each dietary regime.

The Minolta colour determinations (Table 18.4) were consistent with these but did, however, provide more reliable independent measurements showing actual differences in colour.

The recent interest in promoting organic ranges of fish has prompted an interest in seeking other pigmenting sources for astaxanthin. Mori *et al.* (1989) compared krill meal as a means to effect acceptable colouration in Coho salmon and compared this natural astaxanthin di-ester with free astaxanthin supplemented diets. Although reasonable pigmentation is possible, krill varies appreciably in carotenoid concentration and availability and may not be considered a good sustainable source in future. Requirements could be better met from waste material from the shrimp and crab processing industries for such ‘organic’ or special diet needs in aquaculture.

18.7 Genetics and pigmentation

The influence of genetics in modulating pigmentation is less understood, but is a major factor that must be borne in mind underpinning the nutritional and husbandry considerations. The variations in the flesh carotenoid retention capacity for salmonids was identified by Torrisson and Naevdal (1988). Choubert and Blanc (1985) have evaluated the importance of genetic variation in the flesh colour of rainbow trout with respect to diploid and triploid strains.

Table 18.1 Rainbow trout tissue astaxanthin/canthaxanthin levels in mg/kg ($n = 3$) (U/D undetectable) ± SEM

Wk No	Pink BASF	Red BASF	Pink CWD BASF	Astaxin Igene	Carophyll Pink DSM	Carophyll Red DSM	Ecotone ADM	Naturose Cyanotech
0	0.37	U/D	0.37	0.37	0.37	U/D	0.37	0.37
4	1.12	0.86	1.15	0.98	1.12	0.81	0.88	0.86
12	3.76	2.21	2.88	2.29	3.16	1.97	2.83	2.10
16	5.44 ± 0.50	3.44 ± 0.27	5.42 ± 0.39	3.51 ± 0.06	4.91 ± 0.12	3.29 ± 0.09	3.07 ± 0.45	3.87 ± 0.31

Key: Dietary carotenoid source; BASF Pink (10% astaxanthin, beadlets); BASF Red (10% canthaxanthin); BASF CWD (Cold Water Dispersible astaxanthin); Carophyll Pink (10% astaxanthin beadlets DSM); Carophyll Red (10% canthaxanthin DSM); Astaxin (*Phaffia* yeast astaxanthin, Igene Corp.); Ecotone (*Phaffia* yeast astaxanthin, ADM); Naturose (*Haematococcus pluvialis* Cyanotech Inc.).

Table 18.2 Net apparent retention of astaxanthin and canthaxanthin ($n = 3$) ± SEM

Diet	Pink BASF	Red BASF	Pink CWD BASF	Astaxin Igene	Carophyll Pink DSM	Carophyll Red DSM	Ecotone ADM	Naturose Cyanotech
%Ret	10.24 ± 1.00	8.19 ± 0.86	12.09 ± 0.78	6.64 ± 0.13	10.29 ± 0.21	8.73 ± 1.00	5.41 ± 0.64	6.84 ± 0.20

Key: See Table 18.1.

Table 18.3 Roche colour card score for each pigment source (n=3) ± SEM

Wk No	Pink BASF	Red BASF	Pink CWD BASF	Astaxin Igene	Carophyll Pink DSM	Carophyll Red DSM	Ecotone ADM	Naturose Cyanotech
16	13.70 ± 0.06	12.47 ± 0.16	13.65 ± 0.05	12.68 ± 0.25	13.57 ± 0.04	12.68 ± 0.25	12.07 ± 0.09	12.87 ± 0.09

Key: See Table 18.1.

Table 18.4 Minolta score (a* values indicates redness) ($n = 3$)

Wk No	Pink BASF	Red BASF	Pink CWD BASF	Astaxin Igene	Carophyll Pink DSM	Carophyll Red DSM	Ecotone ADM	Naturose Cyanotech
4	13.03	11.10	11.83	10.19	12.69	10.89	5.81	9.98
12	21.12	15.94	11.56	17.49	18.89	15.95	13.61	16.35
16	24.17	20.06	24.84	20.31	24.72	19.67	17.67	22.17

Key: See Table 18.1.

The dynamics of dietary canthaxanthin utilisation in sexually maturing female rainbow trout was compared to triploid fish by Choubert and Blanc (1989) and it was evident that triploid fish deposited more carotenoids in their flesh than diploid trout. More recently, Quinton *et al.* (2005) have undertaken trials with salmon to identify the influence that genetics can play in influencing the absorption of dietary carotenoids, metabolism and the efficiency of flesh deposition. These workers in Canada evaluated the genetic parameters in different year classes of Atlantic salmon and at harvest, determined sexual maturation levels and associations to colour score, astaxanthin, canthaxanthin, fat and moisture contents of muscle. Positive genetic correlations were found between body weight and the carotenoid pigments. There remains the long-term goal and potential to explore various strains of salmon and trout in order to exploit more efficient stocks for pigmentation capacity using selective breeding programmes. There have been a number of commercial salmon hatcheries and private salmon growers as well as feed companies that have explored the potential to improve salmon colour through these means. However, due to the economic benefits and competitive market considerations, there is a paucity of scientific information readily available for assessment.

18.8 Legislation governing pigmentation of salmonid fish

Carotenoids are subjected to the same safety and efficacy standards associated with all dietary feed supplements and additives used in the animal feed industry including aquafeeds. There are strict guidelines based on safety and toxicological data with the situation under constant review and update.

The major constraints that have impeded the use of natural product-based astaxanthin sources have been the legislative and regulatory control measures associated with government agencies such as the FDA, EU and DEFRA and Foods Standards Agency in the UK.

Indeed there is an established maximum allowable level of astaxanthin in diets for salmonid fish of 100 ppm and for canthaxanthin this is only 25 ppm in Europe due to concerns that high accumulation in humans can be toxic (Commission Directive 2003/7/EC: Council Directive 70/524/EEC). In the EU, the use of both astaxanthin and cathaxanthin is barred from fish below six months of age and therefore can only be included in diets for larger fish. The EU-SCAN (Scientific Committee on Animal Nutrition) directive provides details concerning the use of synthetic pigments in animal nutrition including salmon and trout.

Although NatuRose is available for use in the USA, it is currently not permissible for use in EU member states until further validation is made to its efficacy and safety although currently approved in all animal feeds in Japan, United States FDA for salmonids (21 CFR 73. 185), Canada CFIA for salmonids (Reg.#990535). The *Phaffia* produced by ADM (Ecotone®) is authorised for inclusion in aquafeeds in Europe whilst the Igene product is not yet available for

(1991) had previously evaluated the effects of frozen storage on rainbow trout fillets with respect to colour integrity over time. The significance of muscle texture as defined by muscle fibre cellularity and fibre density in both fresh and smoked fillets of Atlantic salmon was related to pigment concentration, colour perception (*Salmo*Fan^{TM}) and lipid content in a comprehensive study by Johnston *et al.* (2000). Visual colour score (but not astaxanthin concentration) was found to be significantly correlated with muscle fibre density in early and late maturing strains of salmon accounting for up to 44% of colour variation in fillets.

Given the attention to new advances in food technology and expansion of different product lines for salmon and trout, then it is clear that more research is performed in this area to define standards and meet retailers' requirements.

In terms of feed costs it is well known that the added costs of including astaxanthin in feeds amounts to an extra 10–15% which manifests as an additional 4–6 pence per kilogram produced, i.e. a cost of £40 000–60 000 per 1000 tonnes of production. In Europe, it is common practice to pigment rainbow trout to a defined level at portion size, although this is not so usual in the United States where un-pigmented (white) fleshed rainbow trout is more favoured by the consumer. In Scandinavia and especially in Norway, rainbow trout are often raised in seawater conditions similar to salmon and therefore the effects of salinity in respect to pigmentation efficacy become an issue. Likewise Arctic char is a very popular fish in these countries and can become deeply pigmented. There are few publications for this species (Olsen and Mortensen, 1997) but is an area worthy of attention due to the fact that this species is becoming quite popular in Scandinavia for export. The costs of pigmenting rainbow trout towards harvest have been evaluated by several feed companies with strategies for more economic approaches advocated by several feed companies and fish farming operations. The relative cost of pigmentation in rainbow trout was investigated by Struksnaes (1996). Since canthaxanthin is relatively cheaper than astaxanthin, trials have demonstrated that it is more expensive in the longer term to use canthaxanthin to pigment trout, since it is less effective in its degree of red pigmentation compared to astaxanthin based on the DSM colour chart index system. This would effectively add an extra 5% cost to the producer. Therefore it is seen that canthaxanthin is the most expensive pigment to use for trout, although there is no significant difference in costs if a mixed pigmentation strategy were to be applied. These differences in costs would be considerable when scaled to a 1000-tonne production standard. The cost benefit of an optimum pigmentation strategy for salmon is based on the same basic principles but must consider the important biological differences between salmon and trout and the fact that astaxanthin is less well utilised in salmon compared to canthaxanthin at similar inclusion levels in feed (Page and Davies, 2006).

The increasing market for 'freedom' raised fish and the organic market presents unique additional challenges and opportunities for developing a new strategy for fish production and the question of pigmentation will undoubtedly arise.

use in the European Union member states (SCAN report, 2002) but can be used in feeds in other countries including the USA. These make practical comparisons difficult unless direct data is available from controlled experiments.

One of the most important aspects has been to fully comprehend the internal physiological and metabolic processes that govern the potential for different carotenoids to exert their functional and pigmentation properties in fish and in particular salmonid species. Such work will help to establish the efficacy and safety of novel carotenoids based on sound scientific evidence.

18.9 Fish colouration, market and consumer acceptance

It is the requirements of the consumer and retailers that are driving the agenda and generating the need for more information in this area with attention to colouration. The question of producing a 'pink' fleshed salmon in the same manner as a wild salmon accumulates pigment is a widely accepted principle advocated by a number of market surveys in which the pink flesh colour of salmon scores consistently higher as a desirable factor, second only to fish freshness. Nickell and Springate (1999) stated that colour is reported to be of prime importance for trained consumer panels when considering the preference of salmon and trout products whilst un-pigmented salmon appeared to taste no different to trout.

To meet this desire, the original Roche (DSM) Colour Score (RCC) chart was designed to obtaining a suitable guide for pigmentation levels in salmon and trout for obtaining satisfactory results at harvest and subsequent processing into a variety of products for the consumer. Large retail organisations have found such indices invaluable in order to maintain uniform quality and reliability of salmon colouration. Although subjective, it is a fairly cheap method although a more refined technique using the Minolta Colorimeter is frequently used in the industry for direct and rapid measurements of the colour parameters of importance. This system measures the key components governing colour; i.e. the L (lightness) a (red-greeness) b (yellow-blueness) components. The angle of hue or colour is identified from mathematical transformation and the meter records the composite of the chroma and angle of hue which correlates non-linearly to the flesh astaxanthin concentrations in salmon. The details are well described by Robb (1999) with regards to measurement of fish flesh colour. The most recent study in this area (Forsberg and Guttormsen, 2006) has modelled the relationships between chemical muscle astaxanthin concentration and visual colour perception using the DSM developed *Salmo*Fan^{TM} score with the variables of dietary astaxanthin level and the effect of fish size. These were reported as providing the basis for a more integrative model to predict how these parameters can influence the degree of flesh colouration to optimum effect for farmed salmon as stated previously. The importance of colour stability in relation to product storage and shelf life in relation to processing such as freezing or smoking should not be underestimated and No and Storbakken

The growth in the organic food market sector has been accelerating since 2000 and this has been reflected in the rise of several specialised fish farms in Europe and the UK producing trout and salmon under more natural conditions. One of the main criteria that must be fulfilled for organic certification is the type of feed and constituents fed to fish. This is especially valid for salmonid species where the aspect of pigment source is a direct attribute for authentication of quality and appearance.

One such example of innovation in this area has been the development of a novel and natural pigmenting supplement in diets for salmon employed by the Loch Duart salmon company in Scotland with much success (Joy, 2007, pers. com). This company has had considerable success with its 'more natural' salmon production approaches. The Soil Standards Association in the UK is the body that certifies organic status for foods and has specific criteria for the use of pigmenting agents in the food chain and especially for fish.

Such bodies, together with large retailers and public opinion regarding bio-security issues such as food safety, fish welfare and the promotion of sustainable farming based on natural and renewable commodities, will increasingly dominate the social and political agenda in the future setting of criteria for farmed fish production.

The question of fish colouration will always be a controversial and emotive topic since it is easily visualised and can be measured. The aquafeed industry and fish nutritionists must be responsive and embrace innovative products and concepts to meet these expectations with confidence and be aware of the wider practical and ethical considerations.

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18.11 References

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19

Off-flavour problems in farmed fish

Z. G. Papp, Research Institute for Fisheries Aquaculture and Irrigation, Hungary

19.1 Introduction: off-flavour problems in farmed fish

Flavour is a property of food and it is perceptible both in the mouth (taste) and in the nose (smell). Off-flavour is the presence of objectionable tastes and/or odours in food. Unfortunately, consumers consider that farmed fish tainted by different flavours is unacceptable. Unpleasant and unacceptable tastes and odours, 'off-flavours', in water and fish are a worldwide problem and existed well before reports began appearing in the scientific literature. Off-flavours in farmed fish of either salt or freshwater origin might be caused by food ingredients or natural foods, post-mortem oxidative rancidity or odorous compounds absorbed from the environment (Whitfield, 1999).

This chapter will discuss the most important problems that exist regarding off-flavour tainting in farmed fish, such as sources, uptake and removal, detection, and avoiding its formation, and problems in particular fish species. As off-flavour problems have been reported since the 19th century, we should begin with a short history.

As cited in the historical review of Persson (1995), Conrad Gesner (1558/1670) stated that the flesh of tench (*Tinca tinca* L.) may acquire a taste like that of bottom mud (originally published in Latin in 1558). However, although several papers were published on the tastes and odours in drinking water during the 19th century as Persson cited (1995) in his historical review (Farlow, 1883; Garret, 1893; Jackson and Ellms, 1897, etc.), scientific studies on the 'off-flavours' in fish only began in 1910. In the first scientific paper on off-flavours, Léger (1910) described the muddy flavour of rainbow trout with correlation to mats of *Oscillatoria tenuis* growing in the ponds. The origin of the muddy-

earthy taint, an odoriferous species of Actinomyces, was described for the first time in the 1930s (Thaysen, 1936; Thaysen and Pentelow, 1936).

19.2 Sources and characterisation of off-flavours in farmed fish

19.2.1 Off-flavour sources

Odorous compounds responsible for pre-harvest off-flavours may be accumulated from the water or diet, although flavour problems of dietary origin are uncommon in aquaculture (Tucker, 2000). Environmental off-flavours may originate from artificial or natural sources. Several odorous compounds can be absorbed by fish from the environment and cause disagreeable flavours in the flesh. Post-harvest off-flavours can be the result of post-mortem oxidation (rancidities).

The most common artificial pollution is caused by petroleum products and pesticides. Petroleum products are extremely liphophilic, so they are rapidly absorbed from the environment by fish and then slowly eliminated (Tucker, 2000). Most components of crude or refined petroleum are highly bioconcentrated from the water into the fish, but this type of tainting has been found mainly in fish caught from natural waters (Tucker and Martin, 1991). Pesticide off-flavours have a variety of characteristics depending on the source of pollution but are usually identified by their unique odour (van der Ploeg, 1991). Untreated effluent from pulp mills contains several substances that cause off-flavours, such as polymeric linings, phenol compounds, mercaptans, terpenes and other residues (Tucker and Martin, 1991). However, artificial or anthropogenic off-flavours might cause problems mainly in fisheries, because fish culture facilities are usually located where they can be supplied with unpolluted water; although some facilities, such as open-water net pens, are susceptible to unpredictable types of pollution that may cause fish to accumulate off-flavour compounds.

The most common causes of off-flavour in cultured fish are odorous metabolites of different forms of aquatic microorganisms. Pteropods, blue-green algae, benthic bacteria, polychaetes, and marine and freshwater macroalgae have been shown to be sources of distinctive natural flavours in freshwater and marine fish, prawns and shrimps (Whitfield, 1999). Table 19.1 shows several off-flavours of natural origin that might be caused by metabolites of different microorganisms (Tucker and Martin, 1991).

For example, the source of petroleum- and blackberry-like flavours in salmon and cod is a metabolite dimethyl-β-propiothenin of some marine algae such as *Polysiphoria fastigata* or *P. nigrescens*, or pteropods (shell-less molluscs) such as *Clione limacina* and *Limacina helicana*. Enzymic cleavage or cooking the contaminated muscle causes the dimethyl-β-propiothenin to decompose and form the dimethyl sulphide compound, which is responsible for this odour (Whitfield, 1999). Dimethyl sulphide has an odour threshold concentration (OTC) of $0.33\,\mathrm{ng\,g^{-1}}$ in water (Buttery *et al.*, 1971).

Table 19.1 Examples of compounds isolated from cultures of aquatic microorganisms (adapted from Tucker and Martin, 1991)

Compound	Odour	Organism	Reference
Geosmin	earthy	Streptomyces	Gerber (1968)
2-methylisoborneol	musty	Streptomyces	Gerber (1969)
β-cyclocitral	tobacco	Mycrocystis	Juttner (1984)
2-isopropyl-3-methoxypyrazine	musty	Streptomyces	Gerber (1979)
furfural	putrid	Streptomyces	Gerber (1979)
cadin-4-ene-1-ol	woody	Streptomyces	Gerber (1971)
dimethylsulfide	fishy	Asterionella	Juttner and Muller (1979)
β-ionone	floral	Synura	Juttner (1981)
isopropylmercaptan	onion	Mycrocystis	Jenkins *et al.* (1967)
1-octen-3-one	mushroom	Anabaena	Mohren and Juttner (1983)
hexanal	lettuce	Stephanodiscus	Juttner (1981)

Bromophenols are responsible for an iodoform-like flavour. Certain Australian species are occasionally affected by this flavour including the benthic carnivore *Namadactylus douglasii*, the diverse omnivore *Acathopagrus australis* and the restricted omnivore *Girella tricuspidata*. Studies have shown that the bromphenols are concentrated in the animal's gut; hence the source of the iodoform-like flavour is the animal's diet, for example polycates or marine algae (Whitfield *et al.*, 1995).

Muddy-musty-earthy odours and taste in fresh water fish are the most common off-flavours. Most scientific and technological reports have been on the study of these flavours, especially their high intensity in farm-raised channel catfish (*Ictalurus punctatus*) from the US aquaculture industry (van der Ploeg, 1991). However, other farmed freshwater fish often have this flavour, which is frequently described in books on the culinary aspects of fish as being characteristic of freshwater fish. A muddy odour is strongly associated with extreme eutrophication (Persson, 1982).

Some algae and actinomycetes species in aquatic systems produce muddy-musty off-flavour compounds that accumulate in the bodies of fish and, even in small concentrations, adversely affect the flavour of fish meat. These muddy-musty taste and odour problems are caused mainly by two isoprenoid compounds (Fig. 19.1), 2-methylisoborneol (MIB) and geosmin (GSM), which are synthesised in the pond water and sediment by different microorganisms. These compounds are primarily produced by *Anabaena*, *Oscillatoria* and *Microcystis* blue-green algae and by *Streptomyces* and *Nocardia* actinomycetes species, and can be found in the water and also in the sediment during or after algal bloom and/or during the intensive breakdown of organic materials (Tucker and Martin, 1991). MIB and GSM are found mainly in the sediment of natural water bodies or fish culture systems. Table 19.2 summarises the published data about the microorganisms that produce the earthy-muddy flavour (Robin *et al.*, 2006).

Geosmin and 2-methylisoborneol taint the taste of fish meat most

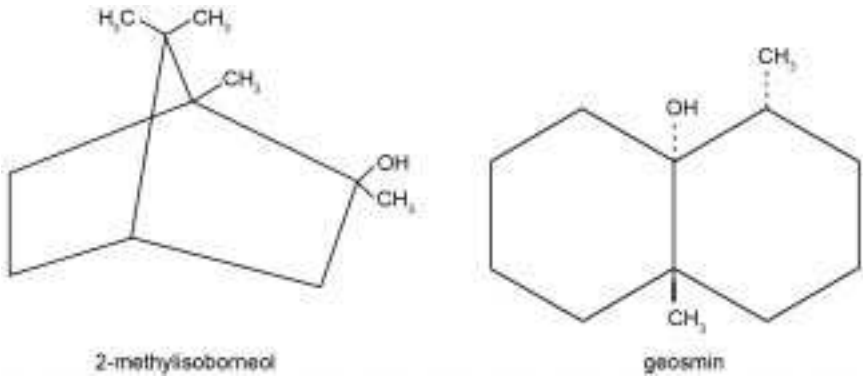

Fig. 19.1 Structure of 2-methylisoborneol and geosmin.

significantly in late summer or early autumn in temperate zones, like the USA (Martin *et al.*, 1986; van der Ploeg and Tucker, 1994), Hungary (Lipták *et al.*, 1998), and France (Robin, *et al.*, 2006). It is thought that different fish species in the same pond may assimilate different amounts of GSM and MIB from the water, mainly through the gills or the digestive tract and partially through the skin (Howgate, 2004), and that off-flavour contents are correlated to the lipid content of fish tissues (Tucker and Martin, 1991; Tucker, 2000). However, tainting may be caused by benthic sources of the odour compound, such as a cyanobacteria mat, or actinomycete-algal debris, which are directly ingested, for example by the bottom-feeding omnivorous common carp (*Ciprinus carpio*), and absorbed through the gut and gills (Gy. Papp *et al.*, 2007). Muddy-musty tainting of fish fillets shows a general relationship with the feeding habits of different fish species.

MIB and GSM can be detected by humans at an odour threshold concentration (OTC) as low as 0.01 $\mu g\,L^{-1}$ in water (Persson, 1980a). Detection thresholds for fish will not be the same as those for water for several possible reasons (Howgate, 2004). Detection thresholds for MIB and geosmin are affected by the lipid content of the fish. The flavour of the fish has a smaller effect on masking the muddy-musty flavours. The OTC values of GSM for the flesh of bream (*Abramis brama*), pike (*Esox lucius*) and rainbow trout (*Salmo gairdneri* or *Oncorhynchus mykiss*) are 0.90, 0.59 and 6.5 $\mu g\,kg^{-1}$, respectively (Persson, 1980a) and 8.5 $\mu g\,kg^{-1}$ for channel catfish (Lovell *et al.*, 1986). As Persson (1980b) reported, the OTC of MIB in bream, pike, pikeperch (*Lucioperca lucioperca*) and trout are 0.095, 0.085, 0.075 and 0.55 $\mu g\,kg^{-1}$ respectively.

Off-flavours originating from storage develop as a result of microbial growth and oxidation affecting the degradation of the fish tissues. Short-chain alcohols, carbonyls and esters, trimethylamine, hydrogen sulphide, methylmercaptan, dimethyl disulfide and dimethyltrisulfide are among the most volatile compounds produced in degrading tissues and might cause spoilage odours in fish fillets (Olafsdottir *et al.*, 2004).

Table 19.2 Review of the published data on off-flavour events, with details about the cyanobacteria or actinomycetes implicated and the odorant compound identified (adapted from Robin *et al.*, 2006)

(a) Cyanobacteria

Country	Species	Off-flavour metabolite
USA	Oscillatoria chalybes	2-MIB
Norway	Oscillaotria brevis	Geosmin
USA/Japan	Oscillatoria tenuis	2-MIB
Japan	Oscillatoria limnetica, Oscillatoria geminata	2-MIB
Japan	Oscillatoria splendida	Geosmin
USA	Oscillatoria limosa	2-MIB
USA	Oscillatoria perornata	2-MIB
Japan, USA	Phomidium tenue	2-MIB
USA	Pseudanabaena sp.	2-MIB
Taiwan, USA	Anabaena sp.	Geosmin
Japan	Anabaena macrospora	Geosmin/cadiner
USA, Australia	Anabaena circinalis	Geosmin
Taiwan	Nostoc sp.	Geosmin/MIB
USA	Lyngbya sp.	Geosmin
Japan, USA	Anabaena flos-aquae	Geosmin
Europe	Aphanizomenon gracile	Geosmin

(b) Actinomycetes

Country	Species	Off-flavour metabolite
USA	Streptomyces halstedii	Geosmin
UK	Streptomyces albidoflavus	Geosmin
USA	Streptomyces tendae	Geosmin
USA		Geosmin
Japan		Geosmin, MIB
USA	Streptomyces sp.	Geosmin, MIB, IPMP
Japan		MIB
Japan	Myxococcus sp.	Geosmin
USA	Nocardia sp.	Geosmin, MIB, IPMP
USA	Actinomadura sp.	Geosmin, MIB, IPMP
USA	Microbispora rosea Geosmin,	MIB, IPMP
Japan	Actinomycetes biwako	Geosmin, MIB

MIB: 2-methylisoborneol; IPMP: 2-isopropyl-3-methoxypyrazine

19.2.2 Relationship between off-flavour contents of fish tissues and different properties; uptake and removal of the different off-flavours

Bioaccumulation and bioconcentration in fish have been found to be affected by several factors such as the lipid content of tissues, water temperature and feeding habits. Off-flavours of natural origin may develop within a matter of hours if the level of odorous metabolites in the water rises suddenly, for example during the sudden die-off of odour-producing algae (van der Ploeg, 1991). The rate of off-

flavour removal is much slower than its uptake and fish are not expected to purge geosmin and 2-methylisoborneol flavours in less than about five days. Thus elimination of the off-flavours is relatively slow from lipid-rich tissues (Tucker and Martin, 1991).

Strong positive correlations have been found between MIB concentrations and the fat content of channel catfish (Johnsen and Lloyd, 1992). The fatter fish (>2.5% muscle fat) accumulated nearly three times more MIB than the lean fish (<2% muscle fat). However, higher average MIB concentrations were found in the fat of leaner fish than in the fat of the fatter fish. Purging off-flavours from fish tissues in clean water indicated that leaner fish depurate faster than fatter fish. Uptake and depuration models containing temperature by time interactions suggested increased MIB uptake and depuration at increasing temperatures in experiments carried out by Johnsen *et al.* (1996). In addition, there will be differences in uptake rates and elimination of off-flavours for different species and size of fish (Howgate, 2004).

19.2.3 Characterisation of off-flavours

The characterisation of off-flavours is often difficult for several reasons:

- there are many chemical compounds responsible for unpleasant flavours;
- the same compound can be described differently by different people;
- several flavours may be produced by the presence of more than one chemical compound;
- variations in the concentration of an odorant may cause changes in the flavour characteristic rather than in the flavour intensity (van der Ploeg, 1991).

Various descriptors are used for the flavours in different fish species. Sources of odorous compounds in aquatic systems where fish are raised include algae, microorganisms that decompose vegetation, fish waste products and pollutants such as diesel fuel or pesticides. There are many other unidentified specific compounds which cause unacceptable flavours in fish. Thus most flavour descriptors are referred to by the name of commonly known materials and scans with odour characteristics similar to the off-flavour. Flavour descriptors have been clustered in different categories depending on the species, country, etc. For example, six main categories (acceptable, blue-green algae, chemical (diesel fuel), decay, vegetable and fishy) are used in the US catfish industry (van der Ploeg, 1991) whereas seven reference terms (global off-flavours, earthy-musty, waste water, chemical (phenols), woody, chlorophyll/grassy and sour) are identified in France for trout (Robin *et al.*, 2006). The common flavours encountered in catfish have been summarised in a flavour wheel, (van der Ploeg, 1991).

19.3 Detecting off-flavours in farmed fish

Numerous methods have been developed for the determination of off-flavours. However, for several reasons, the measurement required for food samples is

often not able to be readily defined or easily accomplished. Taints and off-flavours in fish might be detected with sensory and instrumental analysis.

19.3.1 Sensory analysis

Quantitative chemical assays are time- and cost-consuming methods of screening fish for flavour quality before harvesting to avoid marketing fish with environment-related off-flavours. While only a limited number of odorous compounds can be detected by instrumental methods, sensory analysis (taste and odour testing), which treats trained 'testers' (panel) as analytical instruments, is much more effective for the routine evaluation of fish flavour quality. The fish taste testing method is modelled on the Flavour Profile Analysis procedure. A well-trained panel can detect odorous compounds at very low levels and make a distinction between the types of off-flavours described by flavour wheels (van der Ploeg, 1991) as well as flavour intensities. Fish must be tested in constant conditions (temperature, light, etc.), and in an environment free from odours (cigarette smoke, smell of food, perfumes, chemicals, and other strong odours) that can interfere with sensory evaluation. Flavour intensity may be quantified on different scales, depending on species, country etc. (Table 19.3 and Fig. 19.2). Fish should be cooked either by microwaving or by steaming over boiling water. Cooking time depends on the method used or size and number of fish analysed simultaneously (van der Ploeg, 1991). Testers have to have time between each sample to rinse their mouth with water and eat a piece of bread or something else with a neutral flavour to limit the contamination by too strong an off-flavour sample of the following one (Robin *et al.*, 2006). Despite several experimental protocols for determining off-flavour thresholds having been described in the scientific and technical literature on sensory assays, there is no generally applied standard procedure for their determination; although, as cited by Howgate (2004), the International Organisation for Standardisation (2002) has issued a standard for the determination of sensory thresholds based on the 3-Alternative Forced (3-AFC) procedure.

Table 19.3 Fish flavour intensity scales

Sensory class channel catfish (MIB) by van der Ploeg (1991)	Intensity scale	Sensory class rainbow trout (GSM) by Robin *et al.* (2006)	Sensory score
No off-flavours	0	On-flavour/non tainted	<3
Threshold	T		
Very slight	0.5	Very slight tainted	≥3 and <4
Slight	1		
Slight to distinct	1.5	Slightly tainted	≥4 and <5
Distinct	2		
Distinct to strong	2.5	Tainted	≥5 and <6
Strong	3	Strongly tainted	6

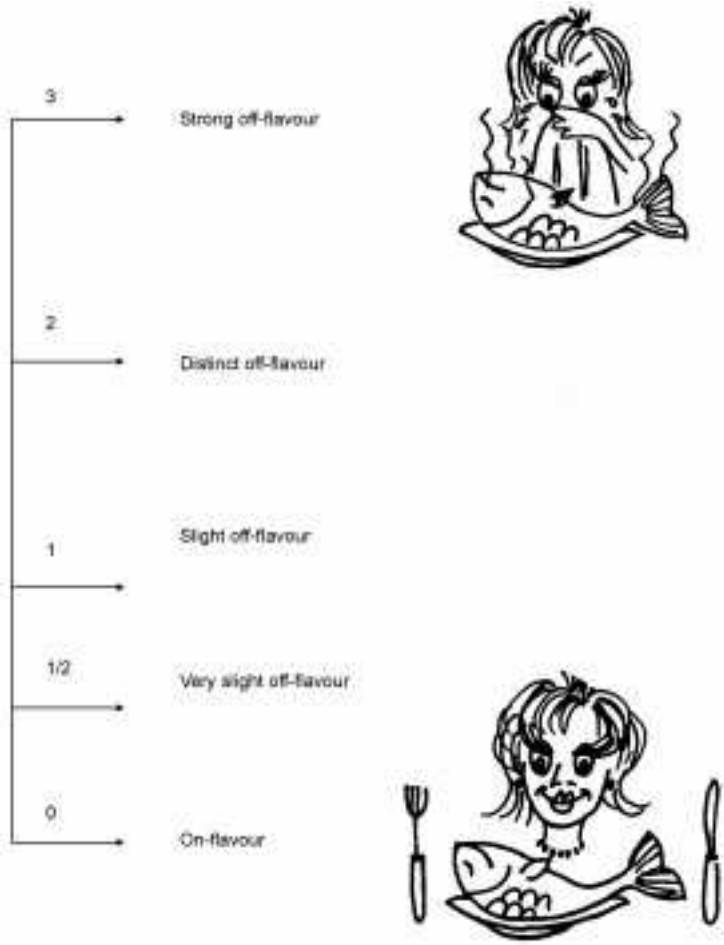

Fig. 19.2 Flavour intensity scale.

Worthy of note is that the muddy off-flavour of catfish might be detectable by trained dogs (Shelby *et al.*, 2004). At the lowest concentration tested, 10 ng L^{-1} of 2-methylisoborneol, the mean correct responses for the dogs were between 37 and 67%. However, on-flavour samples were correctly identified with 96% accuracy for all dogs and samples, therefore dogs may provide a practical method for the early detection of off-flavour problems in catfish ponds.

19.3.2 Instrumental detection of taints and off-flavours

As muddy-earthy-musty flavours are the most frequent problem in farmed fish, several instrumental methods have been developed for MIB and GSM determinations in water, sediment, fish fillet and fish food, including colorimetric (Miller *et al.*, 1999), ELISA (Chung *et al.*, 1990, 1991), gas chromatographic

(Johnsen and Kuan, 1987; Wood and Snoeyink, 1997), different GC-MS (Lloyd *et al.*, 1998; Zhu *et al.*, 1999; Zhang *et al.*, 2006) and electronic nose (Olafsdottir *et al.*, 2004) methods, however analyses have not been standardised as yet.

The colorimetric method was developed for 2-methylisoborneol and geosmin determination in water and fish flesh by Miller *et al.* (1999). Filtered water samples or fish flesh purged in a microwave oven (Conte *et al.*, 1996) were pumped through a solid phase device (Sep-Pak C_{18}) and the off-flavours eluted from the device with toluene. The eluted solutions were combined with 1 per cent vanillin in concentrated sulphuric acid and agitated for 30 minutes to produce a colour reaction. The extraction of a 1 litre water sample resulted in a sensitivity of 1 $\mu g\,L^{-1}$, thus any colorimetrically measurable level of MIB and GSM would result in taste problems in fish flesh. Despite interference effects from other alcohols, organic acids and phenols being possible, the test has the advantages of being simple, inexpensive and relatively fast, and is the main one used for screening off-flavours.

Direct competitive enzyme-linked immunosorbent analysis (ELISA), using antibodies to MIB and GSM linked to colorimetric development, has been developed for water samples (Chung *et al.*, 1990, 1991), but sensitivities are about 1 $mg\,L^{-1}$. Although ELISA provides a quick field test, in practice these methods are not suitable for the detection of off-flavours in aquaculture because of the low sensitivities of the assays.

Off-flavour analysis by chromatographic methods requires careful, often laborious, *sample preparation*. Off-flavour compounds taint water, sediment and fish by several different mechanisms, and samples have different matrices and so each sample type requires different handling strategies. As off-flavour compounds are present in low concentrations in the water, sediment or fish samples, analysis might be carried out on extracts of samples (Wilkes *et al.*, 2000). The off-flavour compounds present in the fish tissue might be extracted by liquid-based methods, like a distillation followed by extraction with methylene chloride in a separator funnel (Yurkowski and Tabachek, 1974). However, over the last decade, sample preparation techniques have greatly improved. Presently the most frequently used sample preparations of fish and sediment are carried out using microwave-assisted distillation with helium purge (Zhu *et al.*, 1999; Grimm *et al.*, 2004). Off-flavour compounds from condensates of sediment and fish fillet are collected in a chilled water bath or original water samples (salted with NaCl) might be extracted by the heated headspace method with solid phase micro-extraction (SPME) using a divinilbenzene-carboxen-PDMS fibre. The carboxen coating is excellent for trapping and releasing MIB, while divinilbenzene is excellent for the larger geosmin compound. The 2 cm fibre length permits detection of values as low as 1 $ng\,L^{-1}$ in water (Supelco, 1998) and 0.001 $\mu g\,kg^{-1}$ in fish flesh (Gy. Papp *et al.*, 2007).

The determination of MIB and GSM contents by *capillary gas chromatography* (GC) can be performed in a gas chromatograph equipped with a flame ionisation detector (FID) and different types of 60 m long wide-bore capillary columns for separations. The method requires a programmed oven temperature

from 100 ºC to 250 ºC. Quantitative analysis can be performed by using dodecane or bornyl acetate internal standards (Lipták *et al.*, 1998). The disadvantage of capillary GC is that some unknown compounds might contaminate the MIB or GSM peaks, so both compounds might be overestimated.

Gas chromatography can be combined with sensory analysis using special equipment (gas chromatography olfactometry (GCO)). The method described by Acree (1997) has been improved in our laboratory. Eluent emerging from the gas chromatographic column is split using an outlet splitter and the off-flavour components identified by single-sniff analysis. The gas chromatograph is equipped with FID and the separations made on a packed analytical column with temperature programming of the column oven. A well-practised tester identifies peaks of the off-flavour compounds.

The most sensible and effective qualitative and quantitative analysis of extracted samples might be carried out by using capillary gas chromatography/mass spectrometry GC-MS (Lloyd *et al.*, 1998; Zhu *et al.*, 1999; Zhang *et al.*, 2006) with different chromatographic conditions, for instance: Column: HP-5MS 60 m × 0.25 mm ID, 0.25 μm film, Injector: 250 ºC splitless, closed 3 min; Oven: initial temperature 60 ºC, 1 min hold, then programmed at 15 ºC/min to 250 ºC, Carrier: helium, 35 cm/sec, MS: m/z 75–180 at 0.6 sec/scan with 3 min solvent delay; quantification: m/z 95 (MIB) and m/z 112 (GSM). The calculation might be carried out by the addition of the MIB and GSM standard mixture directly to the water samples, before the microwave distillation of sediment and fish samples (Gy. Papp *et al.*, 2007), or by the use of internal standards, like cis-decahydro-1-naphtol (Zhu *et al.*, 1999).

Gas sensor array systems, also known as 'electronic noses', respond to specific or groups of volatile compounds and may be used for the evaluation of food spoilage. These measurements require little sample preparation and the analysis time is relatively short. Different gas sensors are available including electrochemical, metal oxide and organic polymers. The responses of electrochemical and conducting polymer sensors have been shown to correlate with other objective measures of quality in fresh seafood. However, the stability of correlation between sensory data and electronic nose response still represents a problem for the practical application of gas sensors in seafood shelf-life evaluation (Olafsdottir *et al.*, 2004).

19.4 Avoiding the formation of off-flavours in farmed fish

19.4.1 Avoiding the formation of off-flavours with different technological solutions (pre-harvest methods)

Several techniques are known to reduce the incidence of off-flavour in fish-raising systems or ponds and most of them have been developed in US catfish farming to prevent muddy-earthy off-flavours caused by blue-green algae. Three approaches might be appropriate for the reduction or prevention of off-flavour tainting:

- harvest around off-flavour episodes
- attempt to prevent or reduce the incidence of off-flavour taints
- try to remove the off-flavour from fish after it has developed.

Evaluation of different techniques shows the advantages and disadvantages of the possible off-flavour management methods (Tucker and van der Ploeg, 1991). Treatment with the selective blue-green algicide, potassium rinecolate, did not reduce the incidence or percentage of blue-green algae in phytoplankton communities and did not reduce the off-flavour in fish. The incidence and severity of odour tainting was not affected by flushing or dyes. Plankton-eating fish might substantially reduce the amounts of odour-producing microorganisms; however, neither blue tilapia (Tucker and van der Ploeg, 1991) nor silver carp (Tucker, 2006) polycultured with channel catfish decreased the off-flavour in fish tissues.

A strategy has been described for blue-green off-flavour management in Mississippi pond-raised catfish by van der Ploeg *et al.* (2001). As this study demonstrates, off-flavour removal might be achieved in two ways:

- the fish can be transferred from the tainted pond to clean water; or
- the organism that is producing the off-flavour compound can be killed (copper-based products).

Off-flavours in fish will disappear only when the fish are no longer exposed to the odorous compounds and sufficient time is then provided for the compounds to be completely purged from the fish. Purging of the geosmin-caused off-flavour in clean water might also be partially successful in common carp (Gy. Papp *et al.*, 2005). GC-MS analysis detected some remaining geosmin in the fish tissues, but it was below the human odour threshold concentration (OTC). Treatment with an algicide to kill the earthy-muddy off-flavour-producing blue-green algae might stop MIB production without having to move fish to another pond. Although several questions surround the use of copper sulphate and certain other copper-based products, these are the only algicides registered for use in catfish ponds against the algae (*Oscillatorica chabella*) responsible for MIB production. Figure 19.3 shows the strategy for off-flavour management developed by van der Ploeg (1991).

Some other methods might have a role in preventing natural origin off-flavours. The use of polyculture filter ponds combined with intensive ponds in Hungarian carp and African catfish farming (Gy. Papp *et al.*, 2005) has been studied. The pond recycling system (Fig. 19.4) consisted of production fishponds where fish were stocked in high densities and a filter pond where the water treatment and nutrient removal took place (Gál *et al.*, 2003). African catfish and common carp were fed in the intensive ponds (I_1 and I_2) with commercial fish feed. Supplementary grain feeding was applied in the filter pond. The filter pond of the pond recycling system was partially effective against off-flavour (Fig. 19.5). Two to five times higher GSM concentrations were found in the flesh of common carp originating from the filter pond than those that were found in the flesh of fish in productive ponds in autumn.

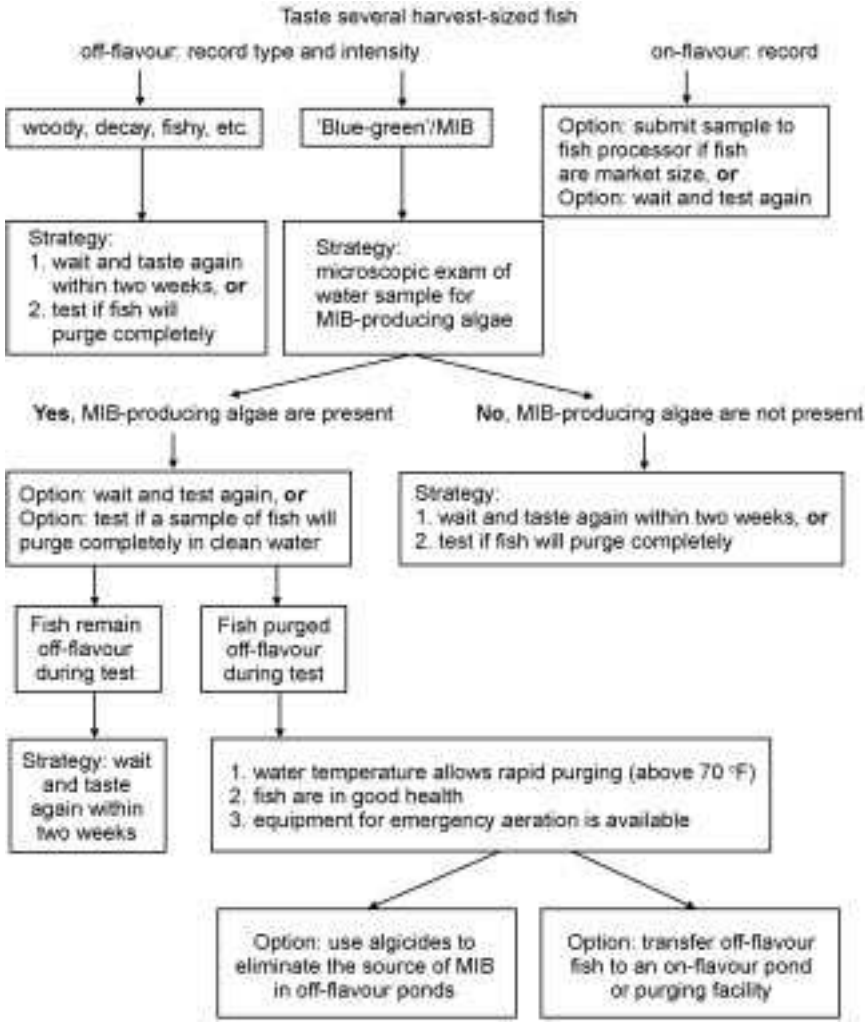

Fig. 19.3 Strategy for off-flavour management (adapted from van der Ploeg *et al.*, 2001).

Effluent-fed fishponds (Fig. 19.6) were utilised for intensive aquaculture (flow-through African catfish farm) effluent treatment and fed daily with 300 m^3 discharged water (Kerepeczki *et al.*, 2003). The effluent-fed fishpond1 (EFP1) received the nutrient load directly and the overflow water was introduced to effluent-fed fishpond2 (EFP2). There was no feeding applied in the fish ponds; the fish consumed the food sources present in the ponds. Off-flavour concentrations in the water and fish of both systems were lower than those found in the traditional fish pond (Fig. 19.7). In conclusion, the application of polycultured fish ponds as filters might be a promising biological control against naturally originating off-flavours.

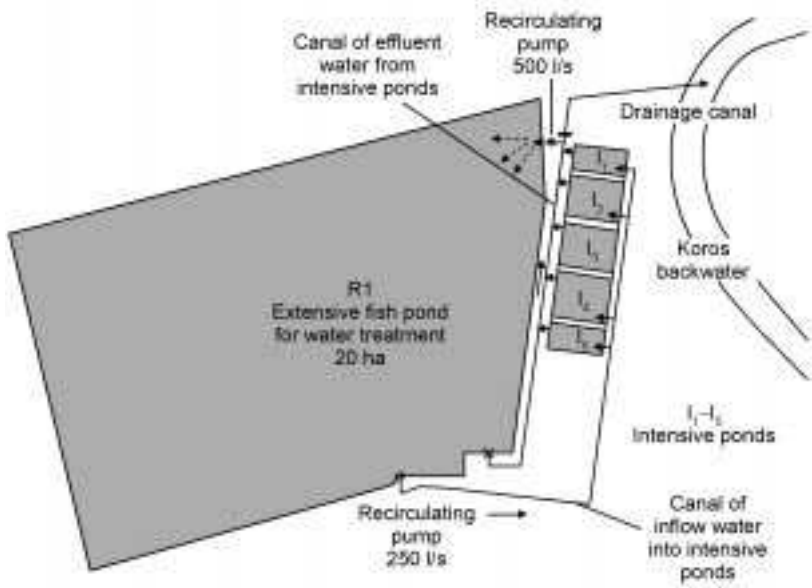

Fig. 19.4 Design of the combined intensive-extensive experimental pond system (adapted from Gál *et al.*, 2003).

Several methods have been used or developed for intensive recycling in fish culture systems, like filtering with active carbon, ozonation and the development of feeding practices in the French trout industry (Robin *et al.*, 2006). Photocatalysis with titanium dioxide is a promising process for the removal of the off-flavour compounds such as MIB and GSM (Lawton *et al.*, 2003). The

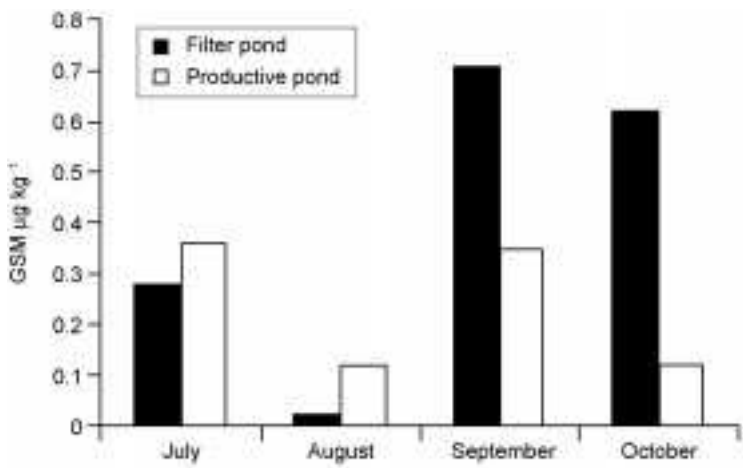

Fig. 19.5 Effects of filter pond on GSM concentrations of common carp (*Cyprinus carpio*) flesh in 2002.

Fig. 19.6 Scheme of effluent-fed fish ponds (H1 and H2) and constructed wetland ponds (W1 and W2).

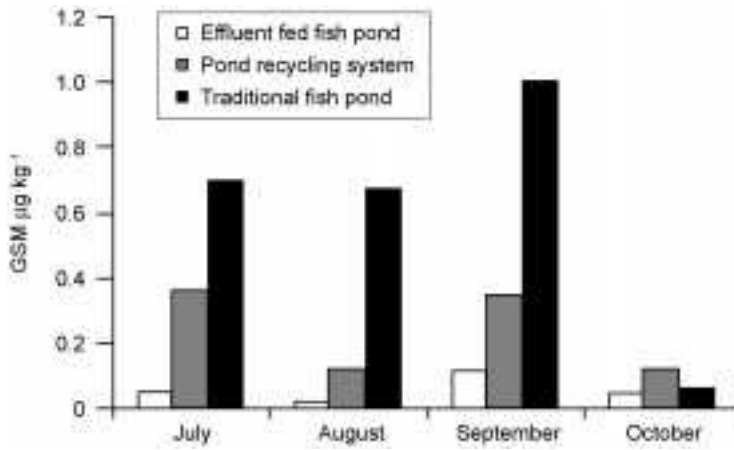

Fig. 19.7 Geosmin concentrations of common carp flesh in different aquatic systems in August and September 2002.

experimental process proved effective in the laboratory with the complete destruction of MIB and GSM being achieved within 60 min. It will be necessary to develop suitable reactor configurations; however, the process will only be feasible as a recycling loop system in closed-tank fish farms such as those operated in Scandinavia.

19.4.2 Post-harvest elimination or masking of the off-flavour compounds

Methods for the reduction of off-flavour compounds in tainted fillets deal mainly with the elimination or masking of muddy-earthy odours caused by MIB or GSM. Hungarian fishermen say that sometimes the earthy-muddy flavours of common carp can be masked by the use of garlic. However, this is a good method for masking earthy-muddy flavours together with roasting.

There are some other treatments but each have their disadvantages. For example, as 2-methylisoborneol and geosmin are semi-volatile cyclic alcoholic compounds, food-grade acids, like citric acid combined with vacuum tumbling, may be useful in the processing of off-flavoured fish (Forrester *et al.*, 1999). Ozone may be a way to reduce or eliminate MIB and GSM tainting in fresh fish fillets; however the efficiency of this method depends on the off-flavour concentration (Xi and King, 2001).

19.5 Off-flavour problems in particular fish species

Some fish that are caught have specific off-flavours and in certain localities this is a common phenomenon, but it is mainly some intensive or extensive pond-raised species that are affected by this serious problem in fish farms. As fish food is usually under the producer's control, off-flavours of environmental origin, mainly cause by microorganisms, are the most common in farmed fish species. The three most common fish which may occasionally be tainted with natural origin odours, in different countries, are common carp (*Cyprinus carpio*), channel catfish (*Ictalurus puctatus*) and trout (*Oncorhynchus mykiss*). However, off-flavours of environmental origin may be found in cultured species, like Atlantic salmon (*Salmo salar*), (Farmer *et al.*, 1995) and Nile tilapia (*Oreochromis niloticus*) (Yamprayoon and Noomhorm, 2000).

19.5.1 Channel catfish (*Ictalurus puctatus*)

The largest aquaculture industry in the United States is that of channel catfish (*Ictalurus puctatus*), although 387 918 tonnes were produced across the world in 2004 (FAO, 2004). Several off-flavours have been described for pond-cultured channel catfish such as sewage, stale, rancid, metallic, mouldy, petroleum, weedy, but the earthy-musty flavour caused by MIB and GSM is generally found to be the most intensive and this odour causes the largest problem in the culture and marketing of this species. Producers of channel catfish consistently identify environment-related off-flavours as their major production problem. Earthy-muddy off-flavours are the most prevalent in catfish farming as first described by Lovell and Sackey (1973) and Maligalig *et al.* (1973). As off-flavour incidence is episodic and dynamic it can be difficult to trace its cause (Tucker and Martin, 1991). Fish flavours have seasonal trends. For example, on a commercial farm in west-central Mississippi, off-flavours were strongest from July to September when fish were tainted by 2-methylisoborneol in 76% of its ponds and the MIB off-flavour could be correlated with the presence of a MIB-producing cyanobacterium *Oscillatoria chalybea* (van der Ploeg and Tucker, 1994). During the winter and spring, ponds were affected by woody and decay off-flavours, but at a lower intensity than that of the earthy-muddy off-flavour in summer and early autumn. The proportion of off-flavoured catfish might be very different from one pond to another and these variations are unpredictable (Gautier *et al.*, 2002). The between-pond variance in the proportion of off-flavour tainted fish was greater than the within-pond variance.

19.5.2 Common carp (*Cyprinus carpio*)

Common carp (*Cyprinus carpio*) is one of the main fish produced in the world (total production 3 387 918 tones in 2004), especially in Asia but also in Europe (FAO, 2004). In Europe, carp is traditionally consumed during the winter, mainly at Christmas time. However the common carp has a negative image: a big fish with a muddy-earthy taste, numerous bones and a low filleting yield.

Aschner *et al.* (1967) provided the first complete description of the earthy off-flavours produced by cyanobacteria in fish. Their studies showed that the earthy off-flavours of the common carp growing in the fish culture ponds of Israel were caused by a blue-green algae *Oscillatorica tenuis.* They also reported that the problem could be solved either by killing the problem organism with an algicide (copper sulphate) or by moving the off-flavoured fish to clean water. The muddy-earthy taste of common carp might be connected to their relatively high fat content and also to the feeding habits of this species, as shown in our own field study. Five different fish species raised in a variety of aquaculture systems were studied in Hungary (Gy. Papp *et al.*, 2007). Results showed that off-flavour was caused mainly by geosmin and it was always lower in fillets of carnivorous African catfish (*Clarias gariepinus*) and herbivorous grass carp (*Ctenopharyngodon idella*) than in species with other feeding habits in the same aquatic system. Geosmin concentrations were usually higher in the fillets of bottom-feeding omnivorous common carp than those found in all species with other feeding habits in the same aquatic system on the same sampling day. As common carp roots out food from the sediment, results suggest that this species may take up more geosmin via ingestion and less through the gills during respiration. Overall, geosmin tainting of fish fillets showed a general relationship with the feeding habits of these species. Therefore human consumption patterns of common carp might be understandable. Compounds causing the earthy-muddy taste are purged from the fish during the late autumn when water temperatures are below 15 ºC and no off-flavour-producing microorganisms can grow in the water of fish ponds.

19.5.3 Rainbow trout (*Oncorhynchus mykiss*)

Earthy flavours have been found in rainbow trout (*Oncorhynchus mykiss*) cultured in prairie pot-hole lakes in Central Canada. Geosmin and 2-methylisoborneol produced by cyanobacteria were isolated and identified as the causes of the disagreeable odours (Tabachek and Yurkowski, 1976). Muddy-earthy off-flavours are also well known in European trout farming, for example in France (Robin *et al.*, 2006). Tainted fish have been found in sites where water recirculation is practised in summer and early autumn. Recirculation increases the water temperature and the augmentation of the organic load, therefore increased trophity can cause important modifications to the phytoplankton community structure.

19.6 Future trends

Off-flavour management has to be environmentally and consumer friendly in the future. Strategies against off-flavours must be both efficient and cost-effective. However, these solutions might be different for intensive and pond fish cultures.

One of the most important recommendations that can be made is for the management of water quality in order to limit the increase of the organic load

when recycling intensive fish-raising systems. Filtering methods must be developed to control the concentration of suspended matter and limit the development of off-flavour-producing microorganisms. Filtering procedures using active carbon and ozonation are technically efficient and inexpensive (Robin *et al.*, 2006). A photocatalysis process for the destruction of the off-flavour compounds using, for example, titanium dioxide is another promising method and a novel approach (Lawton *et al.*, 2003).

Changes in feeding practices in intensive and extensive fish culture could possibly be another important method of limiting the amount of uneaten pellets and thus reducing the organic load. The automatic feeders currently used in many recycling systems cause overfeeding. A more prevalent use of demand feeders could be a good way to limit overfeeding (Robin *et al.*, 2006). Several fish, like trout over 50 g, can be readily trained to feed themselves.

As occurrences of off-flavours of natural origin happen most frequently in fishponds, technologies are needed to prevent eutrophication by decreasing the nutrient content of the incoming water, as well as the water and sediment of ponds. Future strategies in pond fish culture have to be based on environmentally friendly and cost-effective technologies. Combined aquatic systems such as a pond recycling system (Fig. 19.8) might be good, prospective solutions. Extensive filter ponds stocked with polyculture can remove high amounts of nutrients, which might prevent the growth of off-flavour-producing microorganisms. Another promising environmentally friendly biomanipulation tool might be to use water treatments with constructed wetlands combined with fishponds (Fig. 19.9) to control natural-origin off-flavours (Gy. Papp *et al.*, 2005).

The quality control of water and fish during the pre-harvest period might have a key role in limiting the proportion of tainted fish that reaches the market. Although the quality control of fish during the pre-harvesting phase has been well-studied and developed for channel catfish farming in the USA (Gautier *et al.*, 2002), systematic quality control of the other species like trout or common carp needs to be developed. The localised, temporary and mostly unforeseen nature of off-flavour episodes makes them difficult to study. New, mainly in-

Fig. 19.8 Pond recycling system.

Fig. 19.9 Water treatments with constructed wetlands combined with fishponds.

the-field methods need to be developed to quickly define the key sources of each episode.

Last but not least, methods for informing and educating fish farmers need to be developed to improve their understanding of the perceptions, habits, management and determination of off-flavours.

19.7 Sources of further information and advice

As off-flavours have a really large literature base, further information may be found in books or in thousands of scientific and technical publications. Several institutions and some societies have web pages. Although the space in this chapter is too small to list all of them, the reader might find some more information about off-flavours from the books, institutions, societies and web pages listed below:

19.7.1 Books

- *Taints and off-flavors in food* edited by Brian Baigrie
  http://www.woodheadpublishing.com/en/book.aspx?bookID=405
- *Off-flavours in drinking water and aquatic organisms*. Proceedings of the 3rd IAWPRC International Symposium held in Los Angeles, USA, 3–8 March 1991
  http://www.elsevier.com/wps/find/bookdescription.cws_home/30016/description#description

- *Flavour science: Recent developments* edited by A J Taylor, University of Nottingham and D S Mottram, University of Reading, UK http://www.woodheadpublishing.com/en/book.aspx?bookID=686

19.7.2 Institutions

- Research Institute for Fisheries, Aquaculture and Irrigation, Szarvas, Hungary: http://www.haki.hu
- Southern Regional Aquaculture Center, Stoneville, Mississippi USA: http://www.msstate.edu/dept/srac

19.7.3 Societies

- IWA International Water Association: http://www.iwahq.org/
- IWA Publishing: http://www.iwapublishing.com/

19.7.4 Other web pages

- The Maryland Aquafarmer Newsletter Issue-2004-03 (produced quarterly each year by the Cooperative Extension Service, University of Maryland, College Park with support from the Maryland Sea Grant College). http://www.mdsg.umd.edu/programs/extension/aquaculture/Aquafarmer/Summer04/#1
- Aquaculture Network Information Center: http://aquanic.org/beginer/catfish/catfish.htm

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20

Husbandry techniques and fish quality

B. Damsgård, Nofima Marine, Norway

20.1 Introduction

Human food consumption in Europe has changed drastically in the last century. Our grandparents' generation was mostly concerned about the amount of food, our parents' generation to a larger extent about food quality, while our generation has a complex relationship with food. During the last decades, an increasing focus on human health and lifestyle has changed our expectation of food products. These changes might be looked upon as a development from a nutrient regime to a sound food regime and to food choices based more on feelings. In a society with enough food resources, people are generally more concerned about potential problems and benefits of the different food components, including how our food species have been treated during production. These ethical and animal welfare issues include both how the animals are treated during their lifetime, including the fulfilment of basic needs, and questions about how the animals are treated before and during slaughter.

20.1.1 The relationship between humans and other animals

The moral status of non-human species is not a new discussion, but a very old philosophical question that suddenly took on a new charm of novelty. The way we understand animals is clearly related to how we understand ourselves as humans. According to the Greek philosopher Aristotle in the 4th century BC, non-human species were without reason ('*logos*') and belief ('*doxa*'), and they were thus far below humans because of their alleged irrationality. Despite extensive behavioural descriptions of several animal species, Aristotle stated

that animals were created and unchanged. This interpretation was the guiding principle not only for how humans understood animals, but also for how they treated them. The view had a strong influence on Western Christianity, represented, for example, by the view of Augustine in the early 5th century AD, and the view of the French philosopher René Descartes in the 17th century who argued that animals had no soul or conscious mind, and therefore could not think or feel pain. This view of Aristotle existed for two thousand years, until Jean-Batiste Lamarck, John Stuart Mill and others developed ethology as a scientific field during the 18th and early 19th centuries. This issue was based upon the need to 'understand' animals, and especially to describe individual differences in behavioural traits. The term 'animal welfare' was used by the British philosopher Jeremy Bentham in the early 19th century, who argued that animals should be treated well because they could suffer. After the scientific paradigm shift caused by Charles Darwin's evolutionary theories in the late 19th century, many biologists started to show an increasing interest in animal behaviour. It soon became clear that animals indeed had a complex range of reasonable behaviours. During the early 20th century, Ivan Pavlov focused principally on individual instincts as the driving forces for behaviour, while Konrad Lorenz and Niko Tinbergen developed animal behaviour in the direction of understanding behaviour in an integrative way, studying animal behaviour in their natural environments. The debate has now taken several directions, and one of them is the development of the use of evolutionary methods in behavioural ecology in order to describe how the behaviour of an individual animal can be understood with both proximate models of regulatory mechanisms and ultimate fitness models in an evolutionary time scale.

This historical development is based on the question of what are the differences between humans and other animals. The most distinct difference is perhaps human consciousness or self-awareness, which some authors have termed a human 'I-am-ness'. Such consciousness may be defined as a sense of the 'I' and how this 'I' relates to the environment. The term 'sentience' usually refers to the ability to respond to and perceive external stimuli, but the relationship between sentience and consciousness is still poorly understood. Non-human species may have a reasonable behaviour and they may communicate, but many people will still say they lack the linguistic syntax to discuss who they are. With such moral background, most humans feel we have the moral right to culture, kill and eat other animals. The discussion of sentience and consciousness in animals, including fish, may change the future moral status of some animal species.

The question of moral status is general, but the discussion of animal welfare is mainly limited to farming conditions, where animals are held under our control. Humans have reared animals from prehistoric times, and the possibility that we have to show compassion and care for non-human species may be an important part of our social instinct. One might say that humans become hosts for other species in non-symmetric relationships. At the same time, the cruelty to farmed animals was one of the main starting points for the increasing debate

over animal welfare in modern times. Ruth Harrison's book, *Animal machines* (1964), resulted in a report to the British Parliament on animal welfare in agriculture, known as the Brambell Committee, in 1965. The report stated that welfare should be fulfilled with the so-called 'five freedoms', including freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury or disease, freedom to express normal behaviour, and freedom from fear and distress.

20.1.2 Why such concern about fish?

Fish have been reared for a long time in Europe, but only during the last decade has the focus on fish welfare emerged and it may significantly change the way we treat and produce fish for food. There are several reasons for this increasing interest.

The moral status of fish seems to be increasing. Apparently, such status cannot be explained merely as an animal's evolutionary position, what is often referred to as 'lower' and 'higher' species. Teleost fish have an unclear position in such classification and might be considered the boundary between higher and lower animals. In biological classification, however, such separation is misleading as the evolution is developing into several lines, and fish cannot be regarded as a simpler form of higher animals, but as a highly successful and diverse group of animals. New knowledge about fish biology has demonstrated that functions in fish are not so different from other animal groups.

Many tend to believe that the negative focus on farmed fish must be a consequence of an increasing urban lifestyle far removed from food production. This view may be understood as that fish farmers always know what is best for the animals, while consumers have a romantic and unrealistic view about the food they eat. The general picture, however, is much more complex, and includes elements of changes towards more intensive fish farming, combined with megatrends in lifestyles and political changes in Europe. Many people, urban or not, are generally more concerned about the fish they eat. The quality aspects of seafood now extend from taste, risk factors and health effects to aspects of sustainability, husbandry methods and slaughtering, the so-called ethical qualities. There are not necessarily links between the muscle quality and the ethical quality of a food product, since our food choices are based largely on feelings. An egg from a free-range hen does not necessarily have to taste better or be healthier than an industrially produced egg, but the consumer feels better because our biological needs for food have less negative effects on other animals or the environment.

There are several reasons why this trend developed later for fish than other species. Compared with terrestrial farm animals, many farmed fish have a relatively short evolutionary history of farming. Most of the other terrestrial farmed species developed thousands of years ago, while large-scale salmon farming in Europe has a history of far less than a hundred years. Biologically, the number of terrestrial farm animals is quite low, and very few new candidates

have emerged since the start of agriculture. Fish farming in Europe is currently based largely on fish species such as Atlantic salmon, rainbow trout, sea bass, sea bream, carp species, eel and turbot, and we may expect emerging species such as Atlantic cod, halibut and Arctic charr. The present farmed fish species have mostly been evaluated for high growth rate and market price and not to a large extent whether a fish species is acceptable for welfare reasons. The scientific background for the knowledge of fish is generally based on wildlife biology, developing into experimental studies of fish biology. Such studies have focused on optimisation of growth and solving of practical problems for the industry, and despite the large number of salmon studies, for example, there has been little focus on behavioural needs and fish welfare until the last decade. Thus, the knowledge of fish as a farm species is far less than for other animal production, especially concerning how we understand the behaviour of farmed fish. The method used is also challenging. Behavioural studies are based on how we as humans interpret what we observe. One of the methodological problems with the development of fish welfare is that fish expressions are generally very different from human expressions, and behavioural differences may be overseen or wrongly interpreted.

20.2 Terms and definitions

Only a decade ago, farmers and scientists seldom used the terms fish welfare and ethical aquaculture, while these questions are now basic issues in the development of European fish farming. The terms, however, are poorly understood and defined, and aquaculture stakeholders use them differently.

20.2.1 How do we understand and assess fish welfare?

Animal welfare as a scientific term is not regarded as a research field on its own, but rather as a cluster of scientific areas based in several research fields. There are definitions of animal welfare based on biological functions, subjective perception or species-dependent behavioural needs (Duncan and Fraser, 1997). In biological terms, animal welfare is regarded as a coping mechanism, defined, for example, as the 'individual's subjective experience of its mental and physical state as regards its attempt to cope with its environment' (Anon., 2005), which is developed from a definition by Broom (1986). The definition indicates that welfare is a property of the individual animal, and that it deals with this individual's subjective experience of its state as the balance between positive and negative perceptions (Braastad *et al*., 2006). The term 'mental state' thus includes emotional and cognitive elements that are affected by stimuli and memories of previous experiences with similar stimuli. The term 'physical state' includes physiological states that potentially influence mental states, and 'environment' includes the physical environment (rearing conditions, water quality), social interactions and all other biotic factors such as pathogens, parasites and predators, in addition to interactions with humans.

Coping with physiological, immunological and behavioural functions means that an animal is functioning well biologically with a low stress level, adequate growth and reproduction and good health, which in sum might be termed 'a good quality of life' (Broom, 1986, 1991). According to Broom's definition, homeostasis is the ultimate goal, and coping comprises the biological mechanisms an animal utilises to handle a significant threat to its stability or homeostasis and to regain control.

One of the five freedoms in the Brambell Committee document was the right to express normal behaviour. It is easy to think that what is 'normal' for an animal must also be the best for its welfare. This is not always true for farmed animals. For example, aggression and fighting are normal behaviours in nature, but are probably not behavioural needs. They are also unwanted behaviours in culture. Welfare is not a question of imitation natural habitats, and most fish welfare scientists address welfare issues as a coping strategy. Being in a tank or sea cages is clearly not natural for a fish, but welfare issues are not about making the system as natural as possible, but rather to understand how the fish is coping with this unnatural situation. A farmed fish has good welfare if it can cope with its farming conditions, including factors such as the water environment (e.g., temperature), physiological needs (e.g., food), social intraspecific interactions (e.g., aggression), and interactions with humans (e.g., handling).

Behavioural models in fish welfare are often based on a cost-benefit analysis. For example, a fish will be aggressive when the benefit of performing the behaviour (e.g., more food) outweighs than the cost of the behaviour (e.g., more energy utilisation and risk of wounds). Such models are based on the assumption that a fish may make a 'decision'. A behavioural decision does not have to be a cognitive choice, but is simply a way to say that an individual fish has a number of alternatives, and these in some way have a consequence for survival and fitness. Some of these decisions are frequent, such as should the fish feed or not or should it fight or not, while others, often called life-history strategies, happen only once or very few times. Habitat shifts such as seaward migration and mating are examples of evolutionarily important decisions, including a long pre-adaptive period before and a short vulnerable period during smoltification and reproduction, respectively.

The other definitions of welfare go beyond the question of coping, and address issues such as the animal's subjective experience or the possibility for an animal to utilise its full behaviour repertoire for which it is genetically pre-adapted. According to the definition of an individual's subjective experience, a good quality of life requires that there be no mental suffering (Duncan, 1993), such as negative emotions related to stress, fear and pain. According to the definition by Spruijt *et al*. (2001), animal welfare is 'the balance between positive (reward, satisfaction) and negative (stress) experiences or states'. Dissatisfaction, or even frustration, is the result of an inability to achieve this homeostasis. Another alternative definition of animal welfare is based more on the natural life and suggests that farmed animals should live in an environment as close to their natural habitat as possible (Kiley-Worthington, 1989). This

principle is often used as a platform for organic farming, including organic aquaculture.

The use of the term 'ethical' provides a wider definition than animal welfare, and goes beyond biological regulation and coping. Handling of fish before slaughter may thus be regarded as a welfare issue, while the killing itself is not necessarily a regulatory welfare question, but might still be an important ethical issue. On a higher level, animal welfare and ethical questions are parts of what is termed 'sustainable aquaculture'. Together with environmental sustainability, welfare forms the biological platform of fish sustainability, being parallel to economic and social sustainability of aquaculture.

The term 'Welfare Indicator' usually refers to a parameter that may be used to measure welfare. An assessment may be based upon a list of needs of a farmed species, for example measuring the non-fulfilment of these needs, or be based upon the deviation from normality. The assessment can be deduced by how far an individual animal has deviated from what is normal for animals in that environment. Normality is not necessarily that is natural for the fish, and an assessment of such deviation must be based upon base line studies covering the complexity between the individuals and their rearing environment. Any welfare indicator can be directly linked to the biology of single individuals, to a population of fish (e.g., all fish in a tank or a seacage) or indirectly to the whole fish farm. The term 'Operational Welfare Indicator' (OWI) usually refers to a parameter that may be used in practical farming. Welfare indicators may be measured by the farmers or by the authorities, for example as a part of a fish health assessment. If each indicator is estimated within a defined range, e.g. from 0 to 10, a 'Welfare Index' may sum up the result from several indicators, weighting the indicators in comparison to each other. A 'Welfare Assessment System' (WAS) is an aggregation of welfare indicators, including a total evaluation of the farm. The term welfare indicators is, however, used in multiple ways by various aquaculture stakeholders.

20.2.2 Wild versus farmed fish

Farming of fish is based on biological materials either caught in natural waters or bred in culture. Most farmed species are domesticated throughout a number of generations, often in breeding programmes aiming to increase growth or minimise production problems. Behavioural traits are both genetically and environmentally determined. Farmed fish often have similar behaviour as in nature, but domestication may increase or decrease the expression of the behaviour. Some species have a higher behavioural plasticity and are thus better able to cope with a changing environment during domestication.

There are several important differences between nature and a fish farm. In the sea, space is not limited, but the habitats may still be small and fish density variable. In culture, space is limited and the density is more constant and higher. In nature, the environment and food qualities and quantities are highly spatial and temporally variable compared with less variation in a farm. During short

periods in nature, food might be in excess, but limited food resources and competition for food is much more pronounced in nature. Nature is 'cruel', and most fish in natural habitats have to fight for food in situations that might look like lifelong suffering with hunger and pain. Fish in nature also have more predators and parasites compared with cultured fish, and the natural survival rate is much lower than in fish farms. On the other hand, farmed fish have no possibilities to escape from adverse situations and thus may be less able to cope with stressors. The consequences of chronic stressors may be physiological stress effects and reduced immune function, increasing disease susceptibility and heath-related problems. However, it is irrelevant to ask what is best or worst of nature and culture because according to legislation, we are responsible for the welfare of the fish as soon as we bring them into culture, and also because the definition we use for welfare is based on how the fish cope with culture, not on how they would have lived in a natural environment.

20.3 Fish farmers and consumers seeking a common destiny

Several large national and pan-European NGOs work actively with welfare questions related to fish, and they play important roles in the development of legislation and regulation of fish farming. The interest in fish welfare and ethical considerations of aquaculture varies amongst European countries, and these differences apparently do not depend on the volume of fish production or fish consumption, but rather a combined effect of several driving forces, including cultural views in different countries.

The aquaculture industry and farming organisations have generally shown divergent views on welfare issues. Many fish farmers expect national and international welfare regulations to increase production costs, for example with a reduction in maximum fish density. Producers also fear that regulations in their own country may reduce European competitiveness compared with countries without such regulations, selling cheap and less ethically produced fish into the European markets. Some farmers also think that fish farming is already ethically sound and does not really need national and international regulations, simply based on the fact that farmers would not do anything that would harm their own animals. At the same time, production intensity has increased in the farming industry. In Norway today, compared with 20 years ago, a smolt farmer often produces three to four times more fish per year, and despite the fact that the average fish today is much bigger than it was 20 years ago, the farmer uses only approximately one-tenth of the running freshwater. The main reason for this change is simply better technology for oxygen regulation in the water, and a more effective industrial intensive production. On the other hand, many producers also believe that welfare improvements and expenses may be repaid in terms of higher production rate, less variation in size, fewer health problems and better fish quality. The ethical issues of fish farming are now high on the agenda in most fish farming organisations.

European consumers, retailers and consumer organisations are increasingly aware of ethical issues of fish welfare in general. In some countries, farmed fish is an important protein source, while in Europe, the demand for a particular fish product is regarded more as a luxury product with an elastic demand. The more a product costs and the richer the consumers are, the more likely it is that they will buy something else if a product is regarded as unhealthy or unsound. The importance of ethical questions in consumer behaviour studies has been addressed during recent years, probably as a part of a general megatrend in the way consumers perceive food (Fig. 20.1). Generally, the interest in how and where our food is produced seems to be increasing, but there is great variation amongst countries and amongst segments of the market within a country.

Consumers' concerns about aquaculture included a range of factors, including a divergent taste and texture of farmed fish compared with wild fish, fear of contamination from feed, depletion of natural marine resources used in fish food, adverse environmental impact associated with pollution and interaction with wild stocks, and ethical concerns over intensive production and slaughter. Most consumers do not distinguish amongst these issues, but probably respond negatively to farmed fish for a variety of reasons. For example, the relationship between the sustainability of wild-caught and farmed fish is complex. Some consumer groups presumably do not distinguish between wild and farmed fish. Environmentally oriented consumers are often negative towards wild-caught fish because of depletion of the natural fish population, and even see the possibilities that farming of Atlantic cod, for example, will decrease the fishing pressure on wild cod stocks.

In all European countries, there are consumer groups who prefer certified organic products, such as 'ecological salmon'. The factors determining such ecological products are mainly based on sustainability and environmental

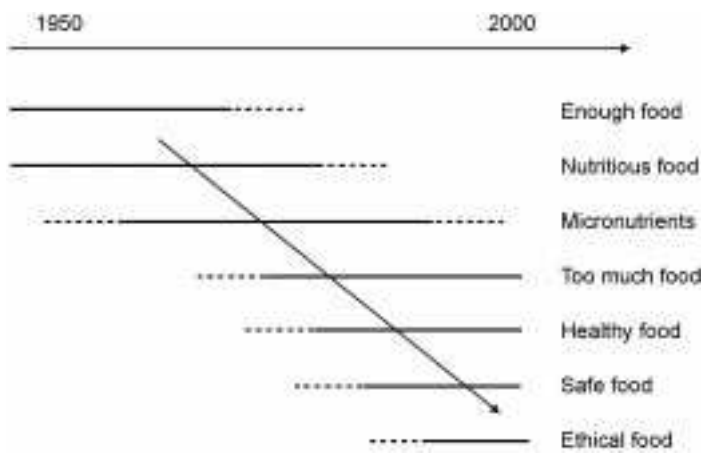

Fig. 20.1 Food megatrends during the last 50 years. Each line represents the relative importance of fish food quality traits, moving towards healthy and safe seafood, produced in an ethically responsible way.

effects, and only to a minor extent on direct welfare issues. Organic fish produced without the use of health treatment on outbreaks of diseases may even have poorer welfare than non-organic fish. The eco-labelling is still an important driving force for the development of alternative production regimes for farmed fish, and may in the future include fish welfare to a greater extent.

Most European consumers have relatively good knowledge about the positive effects of eating fish and other seafood products. Too much information about welfare aspects may have negative consequences for the consumption of fish. Consumer behaviour may be both irrational and inconsistent, and low price is probably more important than any other factor in determining fish preference. However, consumers do not necessarily directly determine the minimum ethical level of fish production. Large retail organisations probably have the knowledge necessary to set production standards, and may thus strongly affect consumer behaviour.

Legislation and policy-making around fish farming in general consist of a large variety of international regulations and recommendations, in addition to national laws and farming regulations. Over the last decade, an increasing amount of this legislation includes welfare issues, and the number of such issues is expected to rise in the future. England was the first country with welfare legislation in 1822, followed by Norway in 1935, while China has the most recent law enacted in 2005. Norway aims to be a leading country in the development of animal welfare, especially regarding fish production. A White Paper on animal husbandry and welfare (Anon., 2003) states that animals, including fish, have an 'intrinsic value' and should be treated with respect to the species' natural needs. Few other European countries have welfare acts that clearly include fish farming, but welfare-related aspects may be nationally regulated by other legislation. The EU strategy for sustainable aquaculture development focuses on the availability to consumers of products that are healthy, safe and of good quality, as well as promoting high animal health and pan-European welfare standards.

Several large animal protection groups have included fish, and especially farmed fish, in their areas of interests. These groups focus mainly on a small number of welfare-related problems, such as pain and fish density. The questions are not necessarily the most important welfare issues, but they are easy to understand and may thus attract public attention.

20.4 Welfare during the production cycle

Welfare issues cover the entire lifespan from egg to brood stock or slaughter, and each factor is size- and life-history dependent and varies between species and systems. The husbandry and water quality requirements during intensive rearing of juvenile fish are good examples of the complexity in fish welfare issues.

20.4.1 Intensive production systems and rearing technology

European farmed fish are reared in a large range of production systems, from extensive carp farming with low energy input to highly intensive industrial production of Atlantic salmon or turbot, with fully controlled water recirculation. In extensive systems, the farmer has no possibility to control the water quality and few means to avoid suboptimal levels of, for example, oxygen concentration. Intensive industrial farming, on the other hand, includes high fish density produced with less water, high energy feed and fast growth, short generation time and season-independent production. In these systems, the fish farmer may easily monitor and change water quality traits, but the systems face the challenge of finding a balance between what is economically optimal for the farmer and the limits for acceptable fish welfare. The present knowledge about lethal levels of different water quality parameters is not adequate to evaluate the welfare consequences of suboptimal rearing conditions. Each fish species has developed a set of minimums and maximums for tolerance for each environmental factor, and the ranges may be wide or narrow, developing the species into opportunistic generalists or specialists. The plasticity of the opportunistic generalists is the key to the evolutionary success of the fish, and enables individual fish to cope with a changing environment, including large changes in water temperature and other water quality traits. Compared with warm-blooded terrestrial animals, fish are poikilothermic and have few possibilities to regulate their body temperature. They have developed a life strategy aiming to meet a changing and unpredictable environment.

Relatively minor factors during early life stages may have severe effects in later stages. Complex causal relationships exist amongst water quality requirements, stress, growth and immune function, and chronic factors may have long-term effects on both coping strategies and on general quality traits. The physiological stress responses are similar between fish and mammals. The primary stress responses include release of stress-related hormones (e.g., adrenalin, cortisol), leading to a secondary stress responses that stimulate oxygen uptake, mobilisation of energy substrates, and finally reallocate energy away from growth and reproduction. The stress response is mainly adaptive, but chronic exposure to stressors will be maladaptive when the adaptive capacity is exceeded. Farmed fish that are exposed to suboptimal rearing conditions over time will experience irreversible consequences for welfare, health and mortality, including maladaptive stress responses such as behavioural changes, reduced feed intake and growth, and decreased immune function. Several experiments of the effects of chronic exposure to intensive rearing conditions in juvenile Atlantic salmon demonstrate a clear relationship between the degree of intensive rearing and the negative effects afterward on growth and susceptibility to diseases after seawater transfer (Toften *et al.*, unpubl., Fig. 20.2).

Most people tend to think that fish density is the most critical parameter in fish production, and maximum density is often suggested as a key issue to maximise fish welfare. Several studies, in both freshwater tanks and sea cages, have revealed that density *per se* is probably neither the most critical factor nor

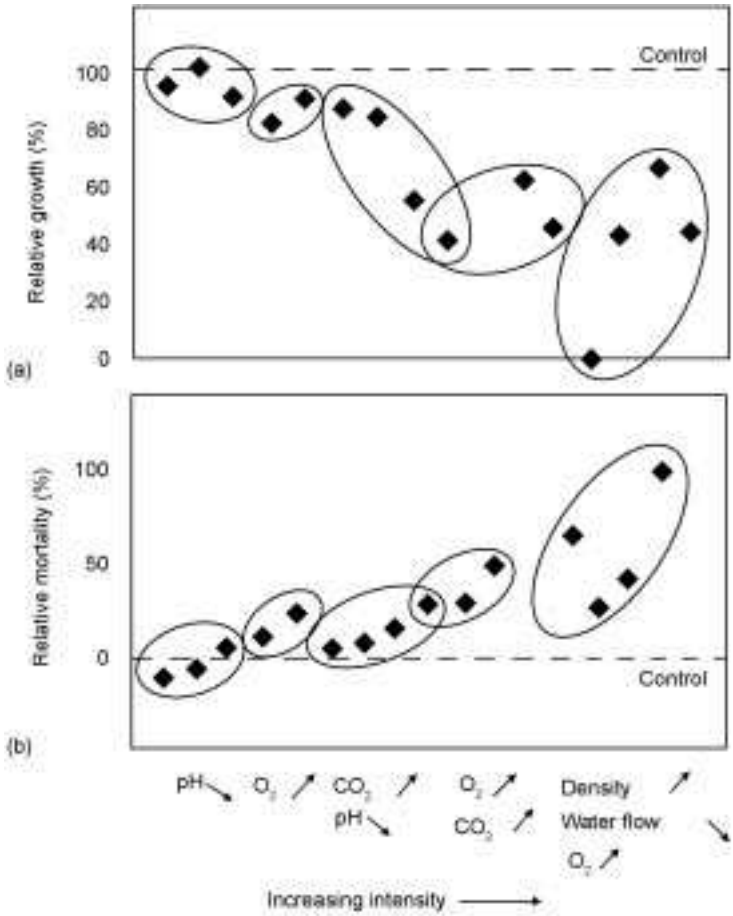

Fig. 20.2 Relative growth (a) and relative mortality due to IPN virus susceptibility (b) as a function of the degree of intensive rearing in juvenile Atlantic salmon. Each circle represent an experiment with different water quality treatments, and each point represent an average of the experiment groups estimated as a percentage of the control groups (dashed line). All treatments are within the normal range of salmon farming, and the treatments within each experiment range from optimal (left side) to suboptimal (right side). Toften *et al.*, unpubl.

the best indicator of welfare. On the contrary, many species reared in low density may increase the aggression amongst the fish when the fish start to scramble for food beyond a certain fish density. In addition, fish do not usually distribute equally in a tank or a cage, and actual density may be much higher then average density. At high density, there might be greater variation in water quality, but as long as the water quality and water current are adequate, the density itself affects the fish only to a minor extent. Independent of the density, a reduction in the oxygen concentration or an increase in the carbon dioxide concentration, however, may seriously affect the welfare of the fish.

20.4.2 The effects of high carbon dioxide on fish welfare

Some carbon dioxide (CO_2) is present in all types of fresh or sea water, but the large increase in CO_2 commonly measured in fish farms is mainly due to the metabolites from the fish's respiration. The carbon dioxide reacts with the water and creates carbonic acid (H_2CO_3), whereas the HCO_3 fraction leads to a reduction in water pH and will thus change all pH-dependent responses in the fish. With high fish density or low water flow, the CO_2 concentration may build up in the farming unit, leading to hypercapnea (high CO_2), which starts a stress response and is known to affect gill functions. The Atlantic salmon responds to elevated CO_2 levels with decreasing feed intake and growth (Toften *et al*., 2006), an increase in general stress parameters like cortisol, leading to higher post-smolt mortality (Fivelstad *et al*., 2003), and impaired immune functions and increasing susceptibility to diseases after transfer to sea water (Toften *et al*., 2006).

A study on the effects of hypercapnea in juvenile Atlantic cod revealed that intensive rearing of cod might have similar effects as reported for Atlantic salmon (Toften *et al.*, unpubl.). In the study, cod weighing approximately 100 grams were reared in high fish densities (60 kg $\times$ m^{-3}) with either a high specific water flow (SWF; 0.84 litre water $\times$ kg $fish^{-1}$ $\times$ min^{-1}) or low SWF (0.17 litre water $\times$ kg $fish^{-1}$ $\times$ min^{-1}). The two groups were compared with a control group reared at low density (30 kg m^{-3}) and high SWF (0.84 litre water $\times$ kg $fish^{-1}$ $\times$ min^{-1}). The oxygen concentration was held constant at 85%, while the low water flow resulted in an increase in CO_2 and a decrease in pH in the water, leading to an increase in the partial pressure of CO_2 and HCO_3 in the blood plasma of the cod. The most intensive group (with high density and low SWF) had a significantly lower growth rate compared with the two other groups (Fig. 20.3). The fish in this group also developed nephrocalcinosis due to

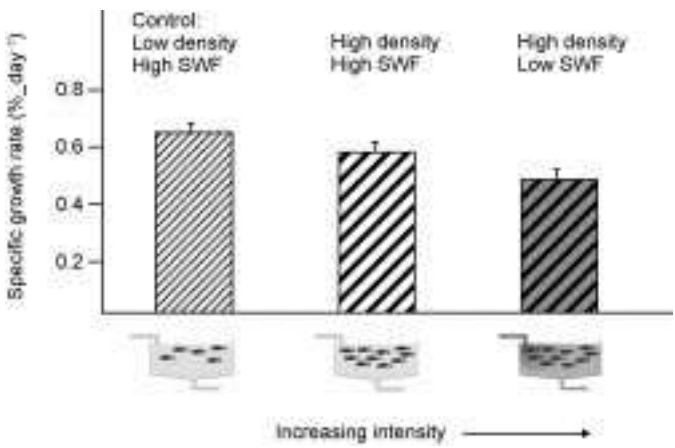

Fig. 20.3 Specific growth rate of juvenile Atlantic cod reared in groups of increasing intensity. The control groups had low fish densities (30 kg $\times$ m^{-3}) and high specific water flow (SWF; 0.84 litre water $\times$ kg $fish^{-1}$ $\times$ min^{-1}). The treatment groups had high fish densities (60 kg $\times$ m^{-3}) and high SWF or low SWF (0.17 litre water $\times$ kg $fish^{-1}$ $\times$ min^{-1}). Toften *et al.*, unpubl.

calcium secretion in the kidney, also an indication that intensive rearing challenges the physiological coping mechanisms in cod and affects the welfare of the fish.

20.5 Welfare during slaughter of farmed fish

The slaughter period includes the effects of pre-slaughter treatment such as transportation, handling and stunning, in addition to the killing of the fish. Most fish species in Europe are starved for a period before slaughter. During starvation, the fish will lose biomass as they mobilise energy stores such as lipids, but after a number of days, the biomass reduction will slow down as the fish become hypometabolic and downregulate their metabolisms. Depending on the production system, the fish is then caught, stunned and killed or transported by some means to a slaughter site. This last period in the fish's production cycle is probably the time when various welfare issues most strongly affect general muscle quality traits. In addition, the killing of an animal is easily understood by the consumers as either 'cruel' or 'humane', and information about slaughter methods may thus significantly affect consumer behaviour and human fish consumption.

The slaughter of fish is largely linked with the debate over pain and suffering in fish. Most people have problems distinguishing between the terms 'pain' (or nociception) and 'pain perception'. The question whether 'the fish feels pain' is partly irrelevant as there is no scientific doubt that the fish can feel the pain, but it is still an open question to what extent it perceives pain similarly to humans. Fish have nerve ends dedicated to pain nociception, and have a nociceptive neuronal pathway similar to mammals to communicate nociception from the body to the brain. Some scientists argue that fish and other animal groups cannot perceive pain because they lack a neocortex, the most important area for pain perception in mammals (Rose, 2002). On the contrary, scientists argue that other brain structures may also have the same functions as the neocortex (Braithwaite and Huntingford, 2004), and there are clear indications that fish have numerous pain receptors and show long-term behavioural indicators when exposed to pain stressors (Sneddon *et al*., 2003). Suffering is an even more complex question than pain perception, focusing more on the animal's consciousness and cognitive skills. The debate over pain and suffering will probably continue, and the way we understand these questions may have a large impact on how we handle and slaughter farmed fish.

20.5.1 Stunning of fish

Stunning is applied to render a fish unconscious and insensible until death, without avoidable excitement, pain or suffering (Van de Vis *et al*., 2003). Adding carbon dioxide (CO_2) to a fish tank has been a common way of stunning Atlantic salmon, for example. This leads to a drop in blood pH and a disruption

in the central nervous system, followed by immobility. The method leads to asphyxia under stressful conditions and is no longer recommended as a stunning protocol. Cooling of fish prior to or during stunning has also been used to calm the fish and improve muscle quality. Cooling slows down the behavioural responses of the fish, but does not result in loss of consciousness and sensibility without avoidable excitement for fish species such as Atlantic salmon, rainbow trout, sea bream, sea bass, eel and African catfish. Even though the fish moves more slowly, cooling before death will probably only prolong the time period of poor welfare before killing. For example, African catfish during live chilling had an increase in heart rate from 70 to 300 beats per minute in a conscious animal (Lambooij *et al*., 2006), indicating a poor welfare status.

One of the other alternatives is electrical stunning (Van de Vis *et al*., 2003), which will probably be commonly used in European fish farming in the future. The scientific criterion for electrical stunning or any other new methods must be an immediate loss of consciousness and sensibility to pain stimulus. Behavioural measures are insufficient for assessment of loss of consciousness and sensibility. Sensibility is required, since unconscious fish should not be aroused during gutting, for instance. Because of exhaustion or paralysis, a conscious fish may not be able to show spontaneous behaviour and responses to administered stimuli. In contrast to warm-blooded animals, the spinal cord in fish controls a major part of the coordinated movements. Unconscious fish can be classed as conscious and sensible. Therefore, the use of EEG recordings, as well as evoked responses on the EEG, is recommended for an unequivocal assessment of the level of brain function in animals. The ECG can be used to establish whether heart failure occurs or whether a slow stunning method may cause stress to the animals. For an instantaneous electrical stun, sufficient current should be passed through the brain of the fish. The applied voltage across the head of the animal or electrodes in water only is not a sound criterion for guaranteeing immediate loss of consciousness and sensibility. For example, when sea bass were exposed to 50 V with electrode plates at 50 cm distances in seawater (conductivity 52 mS $\times$ cm^{-1}), the animals were immediately stunned. When the animals were exposed to the same electric field (1.0 V $\times$ cm^{-1}) in freshwater (conductivity 1 mS $\times$ cm^{-1}), the animals were not stunned (Lambooij and Van de Vis, unpubl.). Several studies have demonstrated that fish may not be killed by the electricity. This could be because permanent heart failure cannot be induced by electricity. In order to prevent recovery of a stunned fish, a killing method has to be applied. The use of electricity, on the other hand, may lead to fractures and haemorrhages, which can be due to strong muscle stimulations, but carcass damage may have several causes and a further optimisation is needed to minimise or prevent damages.

As an alternative stunning method in carp, electrical stunning has been tested (Van de Vis *et al*., 2006). Common carp reared in ponds are usually slaughtered after 30 minutes' asphyxia in air followed by a manually applied blow to the head. Asphyxia is used to exhaust the carp before killing it. It is known that the asphyxia period is stressful for the fish, and a manually applied blow is often

inaccurate and not hard enough to result in immediate loss of consciousness. In the SEAFOODplus experiment in Poland, carp from the pond were drained and crowded into a canal, where the fish farmer caught the fish with a hand net. The fish were stunned by applying electricity and immediately chilled on flake ice or a slurry of ice and water.

The effectiveness of electrical stunning was monitored by EEG registration, and the muscle quality changes were measured by analysis of colour and muscle pH. The EEG recordings revealed that carp lost consciousness immediately using a current density of at least 0.14 A $\times$ dm^{-2} with 50 Hz AC for one second in water with conductivity 0.2 mS $\times$ cm^{-1}. The fish would not recover from stunning after five seconds of electricity combined with chilling. The experiment demonstrated that carp stunned with electricity had a significantly higher pH value in the fillets during a seven-day period after slaughter (Fig. 20.4). The results conclude that electrical stunning in carp leads to a more humane slaughter and may improve muscle quality.

For comparison, carp have also been stunned mechanically using an instrumental blow to the head. EEG recordings revealed the appearance of theta, delta waves and spikes, which were proceeded by no brain activity on the EEG providing evidence of unconsciousness and insensibility. In 18 of 20 fish, an iso-electric line was observed after an average of 16 $\pm$ 12 seconds. However, two carp responded to pain stimuli at 0.5 minutes and one carp also at 3 minutes post stunning, demonstrating there is no certainty for instantaneous loss of consciousness and sensibility (Lambooij *et al*., 2007). The percussive gun was characterised by using a high-speed camera (5000 frames $\times$ $second^{-1}$), and the head was X-rayed to show the damage to the scull as a result of the blow. The displacement of the nylon cylinder in the percussion pistol, which delivered the blow on head, reached a maximal velocity of 19.13 $\pm$ 0.76 m $\times$ $second^{-1}$. The resulting kinetic energy at maximal velocity was 10.99 $\pm$ 0.88 J.

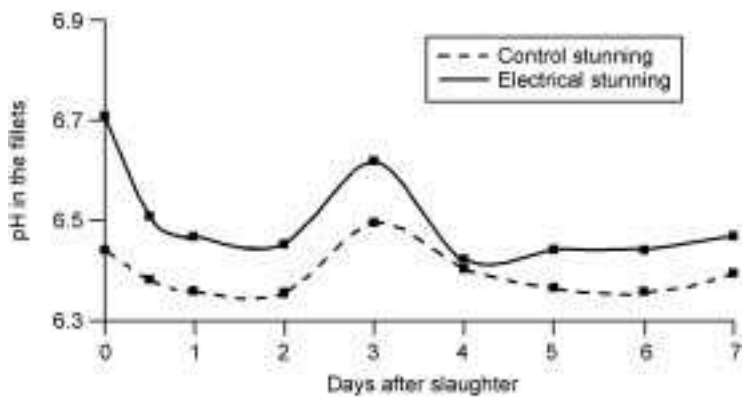

Fig. 20.4 pH in the fillets of common carp slaughter by electrical stunning followed by chilling compared with control stunning (asphyxia followed by a blow to the head). Van de Vis *et al*. (2006).

20.6 Monitoring ethical qualities in farmed fish

In order to improve the welfare of farmed fish, some measurements of welfare are needed to move towards defining protocols and standards of fish husbandry. A set of rapid, inexpensive and non-invasive screening methods may be used as welfare indicators. The most obvious indicators are related to feed intake, growth, health, injuries, damages or stress, while indicators of motivational and emotional states are much more difficult to identify and validate. Measurements and monitoring of fish welfare will probably have to include sets of integrated measures, perhaps combinations of broad indicators affected by multiple stressors, and more specific physiological and behavioural indicators.

In most experimental fish welfare studies, one or several physiological samples are analysed to measure the level of biological coping, such as measurements of blood plasma cortisol to indicate the general stress level. However, it is difficult to obtain blood samples fast enough without stressing the fish and such measurements will only give a glimpse of the situation. There is no single physiological parameter showing sensitive and linear responses to all factors that could cause potential distress to the fish.

The development of online, non-invasive methods enables continuous measurements of fish welfare in free-swimming fish. This approach requires knowledge of the reference levels of the measured welfare parameters and that the relationship amongst measured response and relevant types of acute and chronic distress are established in a series of experimental validation studies. For experimental validation, this may be done before, during and after exposing the fish to potential stressful episodes.

20.6.1 Breathing patterns as a welfare indicator

Several laboratory studies suggest that measurements of breathing patterns in fish are a promising indicator of a wide range of important welfare factors. Ultimately, fish breathe to supply oxygen and remove waste. If the animal is stressed, oxygen requirements increase and this is reflected in the breathing pattern. Likewise, the gills also represent the major arena of interaction between the fish organism and its external medium. If environmental quality changes, the breathing pattern may also change in order to maintain functional homeostasis. The fish's breathing pattern results from integrated processing in its central nervous system and is generated through nervous innervation of the different breathing muscles. The physiological and affective statuses of the fish are thus continuously processed and incorporated into a breathing pattern that reflects the status and needs of the organism as a whole relative to the changing qualities of its external medium. As such, breathing pattern is known to be significantly affected by a variety of factors such as hypoxia (low O_2), hypercapnea (high CO_2), changes in water pH or metabolite levels, toxic or subtoxic levels of metabolites, anaemia and diseases, as well as during the general stress response and factors that presumably also include psychological responses such as shelter, fear and pain. For example, the breathing pattern in Atlantic cod changed as a

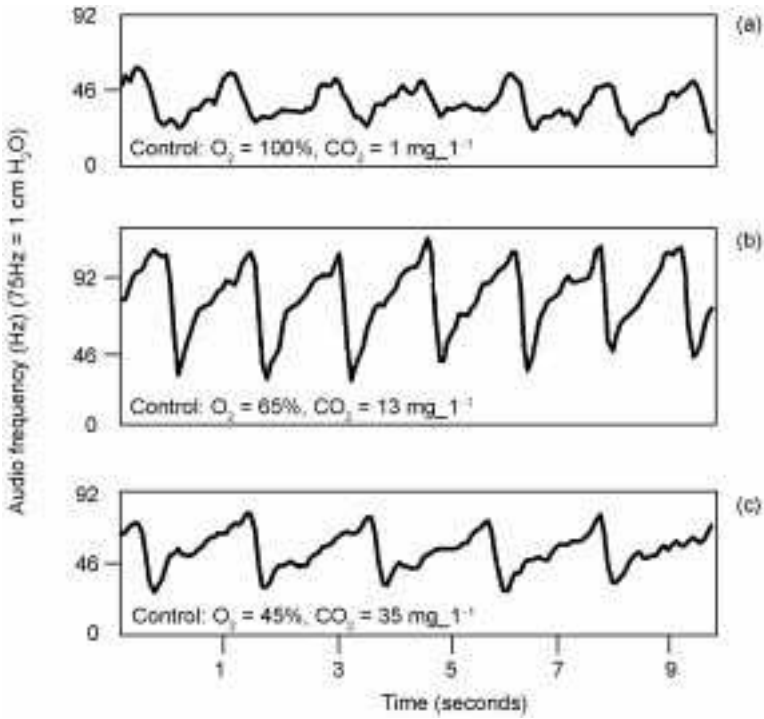

Fig. 20.5 Breathing patterns in Atlantic cod in a swim tunnel, measured with the SmartTag. The audio frequency represents the pressure (in cm H_2O) in the buccal cavity. Fish from the control group (a) with high oxygen and low CO_2 concentration with a breathing frequency on 38 min^{-1} and an amplitude of 0.8 cm H_2O, from the partial hypoxia/hypercapnea group (b) with 65% oxygen and 13 mg $\times$ l^{-1} CO_2 with a breathing frequency on 42 min^{-1} and an amplitude of 1.7 cm H_2O, and from the hypoxia/hypercapnea group (c) with 45% oxygen and 35 mg $\times$ l^{-1} CO_2 with a breathing frequency on 31 min^{-1} and an amplitude of 1.1 cm H_2O. Aas-Hansen *et al*., unpubl.

response to both low oxygen and high CO_2 in a swim tunnel experiment, affecting both the frequency of breathing and the amplitude of each breath (Aas-Hansen *et al.*, unpubl., Fig. 20.5).

The use of breathing pattern as a welfare indicator has been tested, including the development of a 'SmartTag' technology (Aas-Hansen and Damsgård, 2006) together with the R&D company THELMA (www.thelma.no). The transmitter contains a differential pressure sensor that measures the water pressure inside the buccal (mouth) cavity of the fish relative to the surrounding water pressure. The output from the transmitter is a continuous frequency-modulated acoustic signal in the ultrasound range (50–120 kHz). The acoustic signal from each tag is picked up by a hydrophone, amplified, and displayed and stored on a computer. The relationship between the frequency-modulated acoustic signal and actual water pressure is constant for each tag and determined through simple calibration. The online screenshot thus shows the actual changes in water pressure inside the

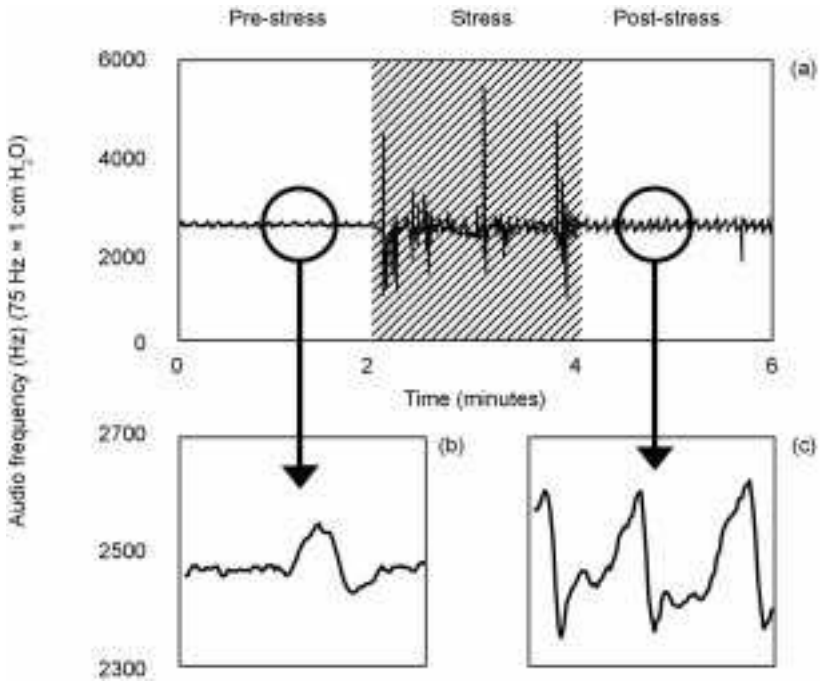

Fig. 20.6 Breathing pattern in Atlantic cod in a sea cage, before, during and after a two-minute disturbance (a) measured with the SmartTag. The audio frequency represents the pressure (in cm H_2O) in the buccal cavity. Typical breathing patterns before and after the stress period are enlarged in (b) and (c). Aas-Hansen *et al*., unpubl.

mouth cavity (i.e., the breathing pattern), with additional online calculation of the breathing frequency and changes in breath amplitude during inspiration and expiration. The present tag weight is six grams in water with an estimated battery life of 25 days. The tag has been tested in various situations during the production cycle, including short-term handling stress and changes in water quality traits such as a decrease in O_2 concentration or an increase in CO_2 level (Fig. 20.5). These factors affected both breathing frequency and amplitude, indicating that the breathing pattern may be a suitable candidate as a welfare indicator. The tags have been tested in full scale farming, for example to measure the effects of short term handling in sea cages (Aas-Hansen *et al*., unpubl., Fig. 20.6).

20.7 Future trends

The further development of welfare issues in Europe depends on a compromise amongst various driving forces. The interest of ethical questions is expected to rise further in the future and the variation amongst European countries is expected to decrease. The increasing focus on animal welfare will strongly affect our everyday lives, our food preferences and how we treat animals. The

consumers and the market have and will always have a strong impact on fish farming and are expected to be the most important driving force for fish welfare. Consumers and food producers are equally dependent on each other's trust. Even small changes in food confidence may have large market consequences. European farmers are in a unique position to be able to market a fish that is not only tasteful, safe and healthy to eat, but also a traceable seafood product that is produced in a way that accommodates the welfare of the animals.

Finding a compromise between the current trend towards intensive rearing of fish and the increasing demand for healthy, high-quality seafood produced in an ethical husbandry system will be the main challenge of sustainability. Given the development of questions regarding welfare issues during the last decade, the future of fish welfare may take several directions.

The first scenario is a further development of legislation and regulation of fish farming, both nationally and within the EU, leading step-by-step to a change in the way fish is produced. The consumers will be increasingly aware of ethical issues and will include these questions in a general quality discussion, separating the market in segments of consumers who are price- or quality-oriented. Traceability may become an important tool to communicate ethical qualities of fish products. On the other hand, if farmers experience an increasing focus on the reduction of production costs in order to meet a price pressure, we might expect few positive effects of an increased ethical development. In such case, welfare will be more of a problem than a possibility for the farmers.

Another and much more drastic scenario may develop into an ethical disaster for fish farming. Consumer awareness of fish welfare may increase together with general food awareness, but the fish farmers may not be able to meet these questions by scientifically based knowledge and improvements in the production. In such case, we might expect incidents in the farming industry that will hit the headlines and be actively used by anti-aquaculture groups to establish a picture of farmed fish as unsound and unethical, ultimately leading to a drastic decrease in the consumption of farmed fish and fish in general.

20.8 Sources of further information and advice

More information about European fish farming can be found on the website of the European Aquaculture Society, EAS (http://www.easonline.org), and the Federation of European Aquaculture Producers, FEAP (http://www.feap.info). The World Organisation for Animal Health (OIE) has focused on animal welfare during recent years (http://www.oie.int). The European Food Safety Authorities, EFSA, publish expert opinions on welfare of European fish species, including a report on welfare aspects of animal stunning and killing methods, and forthcoming reports on the welfare of various European farmed fish species (http://www.efsa.europa.eu). Several EU projects have developed websites with information about fish welfare, including SEAFOODplus, project ETHIQUAL, 'Ethical quality traits in farmed fish' (http://www.seafoodplus.org), WEALTH,

'Welfare and health in sustainable aquaculture' (http://wealth.imr.no), FASTFISH, 'On farm assessment of stress level in farmed fish' (http://fastfish.imr.no), BENEFISH, 'Evaluation and modelling of benefits and costs of fish welfare interventions in European aquaculture' (http://www.benefish.eu), CONSENSUS, 'Sustainable aquaculture in Europe' (http://www.euraquaculture.info) and the COST Action 827, 'Welfare of Fish in Europe Aquaculture' (http://www.fishwelfare.com). Other scientific Internet pages on fish welfare include a website developed by Stirling University, UK (http://www.fishwelfare.net), and the report from FSBI, 2002. 'Fish Welfare. Briefing Paper 2', the Fisheries Society of the British Isles, Granta Information System, Cambridge, UK (http://www.le.ac.uk/biology/fsbi/welfare.pdf). More information on the SmartTag method can be found on THELMA's website (http://www.thelma.no). A strategic programme on fish welfare in Norway is presented in the report by Damsgård *et al*., 2006, 'Welfare in Farmed Fish', which can be downloaded from the website of Fiskeriforskning, NO (http://www.fiskeriforskning.no).

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21

HACCP and other programs to ensure safe products and for sustainable fish farming

M. Jahncke and M. Schwarz, Virginia Seafood AREC, Virginia Tech, USA

21.1 Introduction

Section 21.2 discusses the status and importance of aquaculture to the worlds' fishery supplies. It addresses the importance of international trade and that it is incumbent upon aquaculture producing countries to ensure that all fishery products are safe and wholesome. Exporting countries must understand the regulatory requirements of importing countries and use appropriate risk management tools such as Hazard Analysis Critical Control Point (HACCP) and Good Aquacultural Practices (GAqPs) to ensure safe/wholesome products. Sections 21.3–21.7 discusses the use of HACCP principles as a risk management tool to control shrimp viruses in aquaculture ponds and to control parasites in freshwater aquacultured catfish. A specific example on the use of HACCP principles as a risk management tool to protect the environment from possible disease, parasites and germ plasm introduction from an oyster hatchery used to hold and breed non-native oyster species is also presented. Section 21.8 provides an overview of appropriate books, websites and organizations that are suggested as additional reference materials.

21.2 Aquaculture, food safety and HACCP systems

According to statistics, wild capture fisheries are at the maximum sustainable yield and future increases are unlikely. Wild captured commercial fisheries

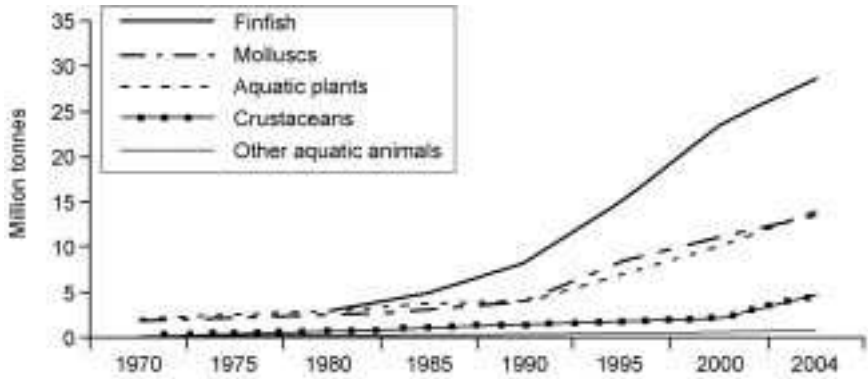


Fig. 21.1 Trend of world aquaculture production by major species groups (FAO, 2006).

cannot continue to meet the increasing worldwide demand for high quality/safe fishery products (FAO, 2004; Martin, 2002). Aquaculture will, on the other hand, help to meet the world's future protein requirements (Martin, 2002). Aquaculture production with an annual growth rate of 8.8% since 1970 is the fastest growing food supply sector (Fig. 21.1). Although recent indications indicate a leveling off of production (FAO, 2006), the Food and Agriculture Organization (FAO) estimates that one in three fish eaten is aquaculture produced with approximately 90% of all aquaculture fishery products being produced in Asia (FAO, 2004). In 2004, 59.9 million tonnes of aquacultured fishery products including aquatic plants were aquacultured with a value of US$70.3 billion (FAO, 2006).

China is leading the world in aquaculture production representing 69.6% of the total quantity produced and over half of the global value (Fig. 21.2) (FAO, 2006). Much of the production in China and in developing countries is for domestic consumption, but increasing amounts are being raised for export to the USA, Europe and Japan.

The value of international trade in fishery products increased from US$15.4 billion in 1980 to US$71.5 billion in 2004 (FAO, 2006). In eight out of eleven countries that were studied, international trade had a positive impact on food security in these countries (FAO, 2006). The international trading of seafood products is a complex issue, and expectations by countries and their citizens are that fishery products are safe and are high quality. In that regard, more and more countries and regional customs organizations are taking steps to control food safety hazards to an acceptable level of protection (Garrett, 2002). Numerous countries have implemented food control management systems such as Hazard Analysis Critical Control Point (HACCP) to ensure food safety (Garrett, 2002). Hazard Analysis Critical Control Point is a science-based food safety management system developed by the Pillsbury Food Company USA in the late 1960s to ensure the safety of food for astronauts during the USA NASA Apollo Moon Program. Since that time, it has been accepted by countries around the world as a science-based risk management tool to help

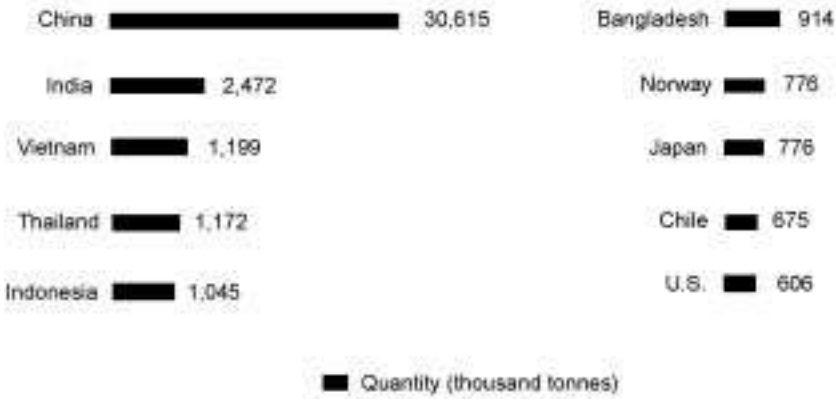


Fig. 21.2 Aquaculture production: major producer countries 2004 (FAO, 2006).

ensure food safety from production to consumption (Lima dos Santos, 2002). Recently its use has been expanded to include control of potential human, animal and environmental hazards associated with aquaculture (Jahncke and Schwarz, 2002; Lima dos Santos, 2002).

Countries around the world are detaining and rejecting fishery products that are contaminated with pathogens such as *Salmonella* spp., or contain chemicals such as antibiotic residues (Anonymous, 2005b, 2006, 2007; Garrett *et al.*, 1997, 2000). Several exporting countries of fishery products have had their fishery products placed on detention without physical examination (DWPE) by the USA and other countries based on past history of problems with pathogens and chemical contamination. In response, countries such as Vietnam, Thailand, China and others, are implementing strict testing protocols of their fishery products to help ensure their ability to export their products to countries such as USA, Japan, Europe, Russia, etc. (Anonymous, 2005b, 2006, 2007).

A survey conducted by the United States Food and Drug Administration (USFDA) in 1998, showed that 6.4% of the imported aquacultured seafood was found to contain *Salmonella,* while less than 1% of wild captured fishery products were contaminated with *Salmonella* (Koonse, 2008). Between 2000 and 2003, the USFDA analyzed 1744 samples of imported raw shrimp, primarily from aquaculture operations. Approximately 10% of these samples were positive for *Salmonella* and were detained (Koonse, 2008). Antibiotic residues found in many aquacultured fishery products are also a major reason for detention and rejection by importing countries (Anonymous, 2005a). Disease outbreaks in aquaculture operations are a common occurrence. Unfortunately, many aquaculture farmers turn to the indiscriminate and inappropriate use of antibiotics to address disease issues at their aquaculture farms. For example, the use of antibiotics to treat shrimp viral diseases is not appropriate, since viruses cannot be successfully treated with antibiotics. In addition, most countries have regulations concerning approved use of specific antibiotics, appropriate use levels, withdrawal times that can be used under the supervision of a veterinarian

or equivalent professional, to treat specific diseases and specific species (FDA, 2005; JSA, 1997, 2004). Exporting countries must know and understand the regulatory requirements of the importing countries concerning the proper and accepted use of chemicals and chemotherapeutics to treated aquacultured species, or their products will be detained and rejected by the importing country. The key to reducing use of antibiotics in aquaculture is to integrate proper use of drug applications with Good Aquaculture Practices (GAqPs) (Jensen and Greenless, 1997). Application of HACCP principles can also be used to control pathogens and chemicals in aquacultured products (JIFSAN, 2007; Jahncke and Schwarz, 2002).

The FAO has been instrumental in providing training programs on the use of HACCP principles in aquaculture in countries around the world. Several aquaculture farms in Brazil, with encouragement from the government, introduced HACCP principles to control chemical contaminants, food additives, veterinary drugs, pesticides, heavy metals, and pathogenic bacteria (Lima dos Santos, 2002). The government in Chile developed guidelines on the Control of Veterinary Drug Residues in Aquacultured Products (SERNAPESCA, 2000). Several countries in South East Asia are applying HACCP Principles and Best Management Practices (BMPs) to control drug and chemical use and to protect the environment (Suwanrangsi, 1997; Tookwinas and Suwanrangsi, 1997; Lima dos Santos, 2002; Koonse, 2006). It has also been used by the FAO in Laos, Vietnam and Cambodia to successfully control infestations of freshwater aquacultured carp (*Puntius goniotus)* fish by the parasite *Opisthorchis viverrini* (Khamboonruang *et al.*, 1997; Lima dos Santos, 1994).

The seven principles of HACCP have also been applied for shrimp aquaculture operations to control pathogenic shrimp viruses such as Taura syndrome virus (TSV) (Picornaviridae), yellow head virus disease (YHV) (Baculorviridae), and white spot syndrome baculovirus complex (WSSV) (Jahncke *et al.*, 2002). The USFDA Joint Institute of Food Safety and Applied Nutrition (JIFSAN) recently developed a Good Aquaculture Practices (GAqPs) Train-the-Trainer course to help reduce the use of chemicals and antibiotics in aquaculture. The use of HACCP principles as a risk management tool to control the use of antibiotics in aquaculture was identified in the training workshop as an effective method to control chemotherapeutic use in aquaculture (JIFSAN, 2007). Training is also being offered by the Pennsylvania Sea Grant and the US Fish and Wildlife Service USA on the use of the general principles of HACCP to control the spread of invasive aquatic species into the environment (Faulds, 2007).

21.3 Implementing a HACCP system in aquaculture: a case study

The use of HACCP principles as a risk management tool for aquaculture is a two-step process. The first step is to assemble a team of individuals with knowledge and expertise about the aquaculture operation. First the team

develops a flow diagram listing each step of the entire aquaculture operation from stock acquisition through production and sale of product. This is followed by on-site verification of the flow diagram (Garrett *et al.*, 2000; Jahncke *et al.*, 2002). The second step is for the HACCP team to apply the following seven principles of HACCP.

Principle 1 – Conduct an analysis of the potential public, animal and environmental hazards associated with the aquaculture operation. As hazards are identified for each step of the aquaculture operation, justifications on the importance of controlling these hazards to protect public, animal and environmental safety are developed, followed by determination of appropriate control measures to eliminate, prevent, or reduce to an acceptable level the identified aquaculture hazards (Jahncke *et al.*, 2002).

Principle 2 – The step(s) of the aquaculture operation where it is essential to control the identified hazards to protect the public, animal and/or the environment is identified as a Critical Control Point (CCP). A CCP is defined as a step at which control must be applied to prevent, eliminate, or reduce a hazard to an acceptable level.

Principle 3 – At each CCP a Critical Limit (CL) is selected. A CL is a maximum and/or minimum value to which a biological, chemical or physical parameter must be controlled at a CCP to prevent, eliminate or reduce to an acceptable level possible public, animal and/or environmental hazards from aquaculture (Garrett *et al.*, 2000; Jahncke *et al.*, 2002).

Principle 4 – The CLs are monitored in 'real-time' by trained personnel. Monitoring activities include a planned sequence of observations or measurements to assess whether or not a CCP is under control and to produce an accurate record for future use in verification.

Principle 5 – When real-time monitoring indicates that a CL is violated, specific Corrective Actions (CAs) are implemented to bring the operation back under control. Corrective actions are procedures to be followed when deviations occur. Corrective action reports are written and kept on file.

Principle 6 – The HAACP program undergoes periodic verification to ensure that the program is working and being followed. Verification are those activities used to determine the validity of the HACCP program and to ensure that the HACCP system is working according to the written plan.

Principle 7 – Records are kept on file addressing monitoring activities, corrective actions and verification activities (Garrett *et al.*, 2000; Jahncke *et al.*, 2002).

The following example developed for the Kauffman Aquaculture Center, USA, applies HACCP principles to control introduction of potential animal and environmental diseases into the environment from a hatchery holding non-native oysters (Tables 21.1–21.9). The HACCP Plan is separated into the following three sections: (1) Oyster broodstock harvest and shipment (Tables 21.1–21.3); (2) Oyster receipt and quarantine in a Level 1 biosecure facility (Tables 21.4–21.6); and (3) Grow-out of F_1 in the hatchery (Tables 21.7–21.9).

The use of HACCP principles is not a stand alone program. In addition to the HACCP Plan, this facility must have written Biosecurity Guidelines for the facility and for personnel, written Standard Operating Procedures (SOPs) that address all the operational aspects of the hatchery, including cleaning and sanitizing of the hatchery, maintenance of the HEPA filters, monitoring of incoming water and effluent water, appropriate record keeping procedures, and employee practices, etc. An Emergency Action Plan is also required (not included in this chapter).

21.4 Information needed to support a HACCP program

1.0 General information required from the facility or country providing oysters

1.1 Description of biosecurity protocols at the facility or harvest site: provide a copy of the facility or harvest site biosecurity protocols.

1.2 Description of all other aquatic species held at the facility or harvest site and any pathogens associated with these species for the past two years: provide a list of all aquatic species held at the facility or harvest site and any confirmed pathogens associated with these species.

1.3 Analytical testing protocols and reports of analytical test results for routine surveillance disease monitoring of oysters at the facility or harvest site for the past two years: provide analytical test results of routine surveillance disease monitoring of oysters at the facility or harvest site for the past two years. Include information on sampling protocols and sampling frequency, sentinel test results, etc.

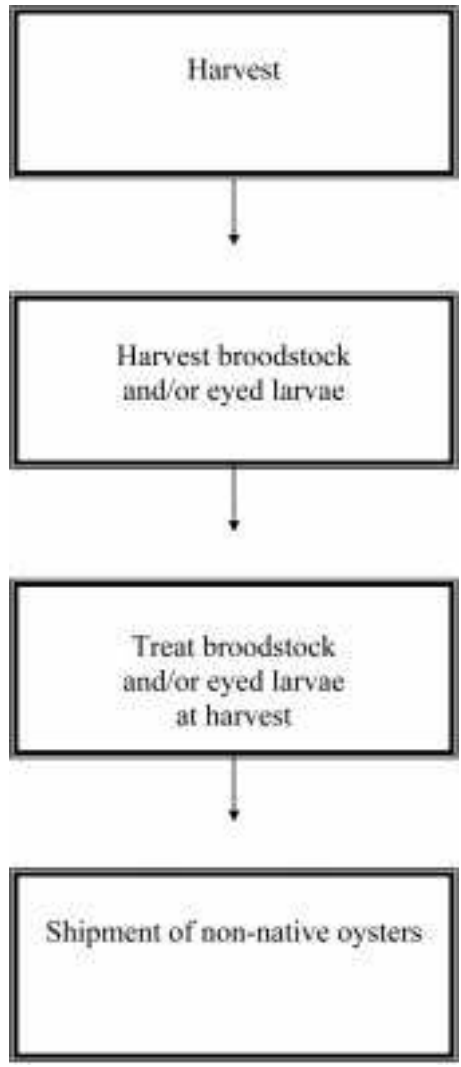
1.4 Analytical testing protocols and reports of analytical test results for disease outbreaks and routine disease monitoring of oysters for the past two years: provide analytical laboratory reports for any documented disease occurrences at the harvest site, holding or culture facility (See Section 3.0).

1.5 Description of disinfection protocols used to address disease outbreaks within the facility within the last two years: provide the written protocols for disinfection of the facility following confirmed disease outbreaks within the past two years.

1.6 Descriptions of disposition of oysters following confirmed disease occurrence during the past two years: provide the written protocols for disinfection of the facility following confirmed disease outbreaks within the past two years.

1.7 Analytical testing results for certification of known disease status: provide analytical data to verify the known disease status of oysters. Basic information that is required includes identification of the testing laboratory, a listing of the pathogens tested, a listing of the specific analytical tests performed, procedures for conducting the tests, sampling protocols and testing frequency, etc.

Table 21.1 General flow diagram – oyster broodstock collection, treatment and shipment



2.0 Oyster acquisition

2.1 Source history

2.1.1 Description of the geographic source of oysters: provide a geographic description, including the longitude and latitude, of the location(s) where the oysters were harvested or held.

2.1.2 Description of any disease occurrences within the past five years in the region or past two years in any facility from which the oysters were

Table 21.2 Hazard analysis – oyster broodstock collection, treatment, shipment and receipt

ID potential hazards	Significant	Justify	Preventive measures	CCP
Harvest	Yes	Harvest area may contain parasites, viruses, bacteria, etc.	Selection of harvest area	Yes
Treatment	Yes	Broodstock and eyed larvae may contain parasites, viruses, bacteria, etc.	Scrub shells (broodstock only) to remove fouling organisms, rinse w/25 ppm chlorine, and dip in 50 ppm iodine for 1 hr	Yes
Shipment	Yes	Shipment containers may leak fluid from oysters	Ensure shipment containers are double walled, lined with plastic and are leak proof	Yes

collected or held for any period of time: list all documented disease occurrences for oysters that occurred in the region or at the facility, where the oysters were harvested, held, reared or hatched. Include documentation on any confirmed disease outbreak for any aquatic species from the region during the past 2 years.

2.1.3 Description of transport procedures for oysters (e.g., any intermediate transfer from original source): describe how the oysters were packaged and transported. If the oysters were temporarily held at intermediate location during harvest and transport, include information on the facility, or intermediate holding site (See Section 1.0).

3.0 General Analytical Laboratory Requirements

General questions that need to be addressed before selecting an analytical laboratory are the following:

1) Is the laboratory qualified to conduct the analyses?
2) Are the laboratory personnel proficient in the analytical tests?
3) Does the laboratory have the appropriate facilities, equipment and methodology to properly conduct the analyses?
4) Are the proposed analytical methods accepted by the scientific community? and
5) Is the laboratory accredited?

3.1 Laboratory protocols: general questions that need to be addressed before selecting an analytical laboratory are the following:

1) Does the laboratory have written protocols?

2) Does the laboratory have an internal quality assurance program to ensure adherence to written protocols? and
3) Are there adequate procedures in place for sample receipt, handling and retention of the samples?

3.2 Analytical testing results and description of specific tests: before selecting an analytical laboratory, the following question must be addressed: are comprehensive reports provided that document sample identification, data, methods, and interpretation of results?

3.2.1 Specific analytical tests: the testing laboratory should provide a list of the specific pathogens that they have the capability and expertise to analyze. Written SOPs should also be provided that describe the analytical test procedures used for each specific pathogen.

4.0 Oyster production facility

4.1 Production facility protocols: see Section 1.0.

4.2 Description of visitor policy procedures: written protocols need to be developed concerning visitors to the oyster production facility. These protocols should at a minimum include identifications of building and other locations that are off limits to all visitors. Restrictions must be in place for any visitors that work at, or have recently visited hatcheries or other aquatic facilities.

4.3 Oyster receipt procedures: see sections 1.0 and 2.0.

4.4 Disease monitoring and testing protocols: written SOPs are needed to identify the specific diseases, and a description of sampling and testing protocols for these diseases in oysters, water, etc. If analytical testing for specific pathogens is conducted at the facility, descriptions of sampling protocols, sampling frequency, analytical testing procedures, and analytical test results are also needed.

4.5 Effluent water treatment protocols: describe the effluent water composition and location, and develop written protocols and SOPs on water effluent disinfection/treatment procedures at the production facility. The treatment protocols must be validated to determine their effectiveness, and results of the validation studies must be kept on file. SOPs also need to include information on routine monitoring procedures to ensure the effectiveness of disinfection/treatment protocols. Records of monitoring results must be kept on file.

4.6 Employee policies: all employees should receive training on biosecurity protocols. Access to the oyster facility should be limited to essential personnel only. Written protocols and employee training programs are needed to restrict movement of employees within and between buildings. Specific SOPs are also needed for equipment use and disinfection, use of employee showers, use of footwear covers, required clothing changes, etc., before admittance into biosecure areas.

4.7 Equipment policies: develop written procedures for the use, storage, and

Table 21.3 Aquaculture HACCP plan form – oyster broodstock harvest, treatment and shipment

(1) Critical Control Point (CCP)	(2) Significant hazards	(3) Critical Limits for each preventive measure	(4) Monitoring			(7)	(8) Corrective action(s)	(9) Verification	(10) Records
			What	How	Frequency	Who			
Treatment of broodstock and eyed larvae at harvest	Possible parasites, viruses, bacteria, etc.	Reduce or eliminate external shell contamination	Number of broodstock	Count broodstock and eyed larvae	Every batch	Biologist	Reconcile differences	Broodstock and eyed larvae are counted	Records to indicate number of broodstock and eyed larvae
			Outside shell contamination of broodstock	Scrub outside shell of broodstock to remove fouling organisms, rinse with 25 ppm chlorine and dip in 50 ppm iodine for 1 hr	Every batch	Biologist	If fouling is found, clean shells	Visual observation to verify no fouling organisms present on outside shell of broodstock	Records checklist indicating broodstock shell was scrubbed and rinsed with 25 ppm chlorine and dipped in 50 pm iodine for 1 hr
			Outside contamination of eyed larvae	Rinse eyed larvae with 25ppm chlorine and dip in 50 ppm iodine for 1 hr	Every batch	Biologist	If concentrations are inadequate, retreat	Chlorine concentration verified using test strips Iodine concentration verified using test strips	Records checklist indicating eyed larvae were rinsed with 25 ppm chlorine and dipped in 50 ppm iodine for 1 hr
Shipment of non-native oysters	Leakage from shipping container	No leakage from shipping container	Leakage from shipping container	Visual inspection of shipping container	Every shipment	Biologist	If containers leak, change packages	Visual inspection of shipping containers	Shipping container condition records

disinfection of equipment, tanks, nets, buckets, forklifts, carts, etc. In some instances, these objects can be color coded to control cross contamination between facility operations. SOPs also need to be developed that include information on routine monitoring procedures to ensure the effectiveness of disinfection/treatment protocols. Records of monitoring results must be kept on file.

4.8 Building access: color-coded signs may be placed on buildings to control access by unauthorized personnel into biosecure areas. Written protocols should be developed to restrict and control employee traffic in production buildings.

4.9 Building disinfection protocols: written SOPs are needed describing how to disinfect buildings after a confirmed disease outbreak, and how to verify that the disinfection procedures were effective.

4.10 Facility maintenance protocols: written SOPs are needed to ensure that all buildings, equipment, etc. are maintained on a regular basis to ensure that they are in good condition. A pest control program should also be in place to control all facets of pest control within the production buildings and on the outside grounds.

21.5 General biosecurity guidelines: an example

1. Entry into the Level 1 quarantine area by visitors is strictly forbidden.
2. Restrict entry of any visitors to the Level 1 quarantine area. When entering the Level 1 quarantine area, change clothes and put on disposable boots, spray 400 ppm quaternary ammonia sanitizer on Level 1 quarantine entrance floor (or use footbath). When exiting the Level 1 quarantine area, remove clothes, and place disposable boots in receptacle, shower and change clothes prior to exiting the facility.
3. Disinfect shoes, or put on disposable plastic boots, prior to entering the non-native hatchery area using footbath containing 400 ppm quaternary ammonia sanitizer. Disinfect shoes, or place disposable plastic boots in the receptacle, prior to leaving the non-native hatchery area using a footbath containing 400 ppm quaternary ammonia sanitizer. Wash hands prior to leaving the non-native hatchery area.
4. Entry into the non-native hatchery area by visitors is strictly forbidden unless pre-authorized by management.
5. All visitors must fill out a visitor's logbook that includes name, association, and reason for visit and any facility recently visited.
6. Develop and implement standard operating procedures (SOPs) to address employee and operational activities.
7. Develop written biosecurity protocols concerning cleaning and disinfecting vehicles and equipment at the facility. Such protocols may include disinfecting tires and equipment, and keeping the vehicles and equipment clean of all visible dirt at all times.

Table 21.4 Level 1 flow diagram

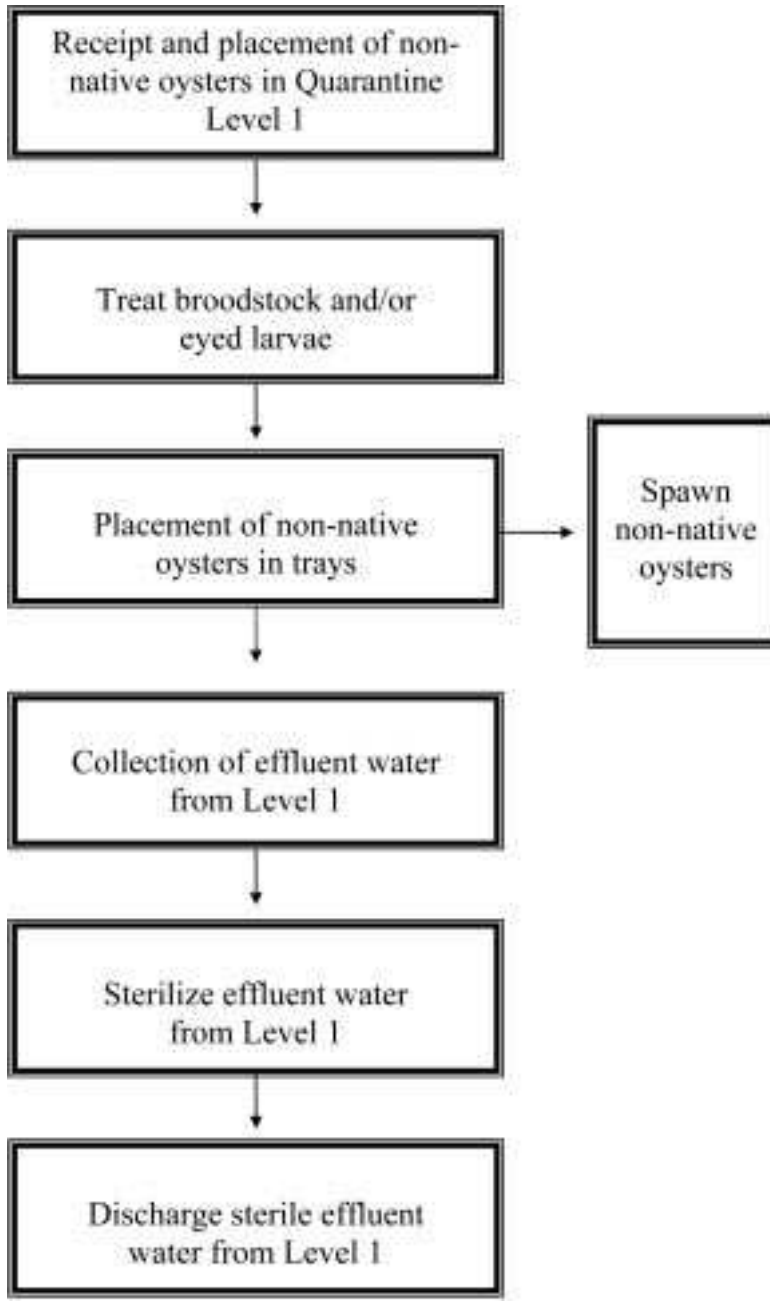


Table 21.5 Hazard Analysis – Level 1 quarantine

ID potential hazard	Significant	Justify	Preventive measures	CCP
Receipt Level 1 Quarantine	Yes	Shipment containers and oysters may contain parasites, viruses, bacteria, etc.	Ensure all oysters are placed in Level 1 quarantine, sterilize or incinerate or take to sanitary landfill all shipment containers	Yes
Treatment	Yes	Broodstock and eyed larvae may contain parasites, viruses, bacteria, etc.	Rinse oysters with 25 ppm chlorine, and dip in 50 ppm iodine for 1 hour	Yes
Place non-native oysters in trays				No
Collection of effluent water from Level 1 quarantine	Yes	Level 1 effluent water may contain parasites, viruses, bacteria, etc.	Initial collection of Level 1 effluent water into storage tank 1	Yes
Sterilize effluent water from Level 1 quarantine	Yes	Level 1 effluent water may contain parasites, viruses, bacteria, etc.	Sterilize Level 1 effluent water at 121 ºC for 15 min in retort, and transfer sterilized water to storage tank 2	Yes
Discharge sterile effluent water from Level 1 quarantine	Yes	Level 1 effluent water may not have been adequately sterilized	Verify sterilization times and temperatures (i.e., 121 ºC for 15 min) prior to cooling and discharge of sterilized water from storage tank 2	Yes

22.6 Standard operating procedures: an example

Employee Level 1 Biosecurity Protocols

Controls and Monitoring

a. Employees entering the Level 1 biosecure area must change clothes and put on disposable boots. Prior to entering the biosecure area, a 400 ppm quaternary ammonia disinfectant is sprayed on the floor at the entrance to the Level 1 secure area (or use footbath with 400 ppm quaternary ammonia disinfectant). **Monitoring frequency: daily**.
b. The biologist in charge exiting the Level 1 biosecure area must remove their disposable boots and place them in the receptacle. The employee must also remove clothing and place clothing in the locker. The employee must

Table 21.6 Aquaculture HACCP plan form – quarantine Level 1, hatchery

(1) Critical Control Point (CCP)	(2) Significant hazards	(3) Critical Limits for each preventive measure	(4) Monitoring			(7)	(8) Corrective action(s)	(9) Verification	(10) Records
			What	How	Frequency	Who			
Receipt and Level 1 quarantine	Non-native oysters and shipment containers may contain parasites, viruses, bacteria, etc.	All oysters and shipment containers are counted and oysters and containers are decontaminated	Number of broodstock or eyed larvae and shipment containers	Count broodstock or eyed larvae and shipment containers	Every shipment	Biologist	Reconcile differences	Count number of broodstock and eyed larvae	Records indicate numbers of oysters
			Decontamination or disposal of shipment containers	Shipment containers are incinerated or taken to a sanitary landfill	Every shipment	Biologist	Dispose of containers	Number of shipment containers	Shipment containers disposal records
			Decontamination of outside of broodstock or eyed larvae	Rinse with 25 ppm chlorine and dip in 50 ppm iodine for 1 hr	Every shipment	Biologist	If sanitizer concentrations are inadequate, retreat	Chlorine concentration verified using test strips	Chlorine concentration check list
								Iodine concentration verified using test strips	Iodine concentration and dip time check list

Treatment of broodstock and eyed larvae	Possible parasites, viruses, bacteria, etc.	Reduce or eliminate external shell contamination	Number of broodstock	Count broodstock and eyed larvae	Every batch	Biologist	Determine reason for inaccurate numbers	Broodstock and eyed larvae are counted	Records to indicate number of broodstock and eyed larvae
			Outside shell contamination of broodstock	Scrub outside shell of broodstock to remove fouling organisms, rinse with 25 ppm chlorine and dip in 50 ppm iodine for 1 hr	Every batch	Biologist	If sanitizer concentrations are inadequate, resanitize	Visual observation to verify no fouling organisms present on outside shell of broodstock	Records checklist indicating broodstock shell was scrubbed and rinsed with 25 ppm chlorine and dipped in 50 ppm iodine for 1 hr
			Outside contamination of eyed larvae chlorine and dip in 50 ppm iodine for 1 hr	Rinse eyed larvae with 25 ppm	Every batch	Biologist	If sanitizer concentration is inadequate, retreat	Chlorine concentration verified using test strips Iodine concentration verified using test strips	Records checklist indicating eyed larvae were rinsed with 25 ppm chlorine and dipped in 50 ppm iodine for 1 hr
Initial collection of effluent water from storage tank 1 – Level 1 quarantine	Effluent water may contain parasites, viruses, bacteria, etc.	All initial Level 1 effluent water is collected in storage tank 1	Initial transfer to Level 1 effluent water to storage tank 1	Visual inspection	Every batch	Biologist	If water is not transferred to storage tank 1, transfer water to storage tank 1	Visual check storage tank 1	Storage tank 1 records

Table 21.6 *Continued*

(1) Critical Control Point (CCP)	(2) Significant hazards	(3) Critical Limits for each preventive measure	(4) Monitoring			(7)	(8) Corrective action(s)	(9) Verification	(10) Records
			What	How	Frequency	Who			
Sterilize effluent water from storage tank 1 – Level 1 quarantine	Effluent water from storage tank 1 may contain parasites, viruses, bacteria, etc.	Sterilize effluent water at 121 ºC for 15 min	Retort time and temperature	Retort digital records (i.e., 121 ºC for 15 min)	Every batch from storage tank 1	Biologist	If records show inadequate time and temperature, resterilize water at 121 ºC for 15 min	Verify effluent water sterilized at 121 ºC for 15 min	Retort time-temperature digital records
Discharge sterile effluent water from storage tank 2 – Level 1 quarantine	Incomplete sterilization of Level 1 effluent water	Sterilized effluent water at 121 ºC for 15 min	Retort time and temperature	Visual confirmation of digital records	Every batch	Biologist	Resterilize water, clean and sanitize storage tank 2, repair retort, hold all water in raceways, storage tank 1 and storage tank 2 resterilize all water at 121 ºC for 15 min prior to cooling and discharge	Verify effluent water sterilized at 121 ºC for 15 min	Retort digital time and temperature records

Spawning	Contamination of larvae with broodstock bacteria and viruses	No contamination of larvae with bacteria and viruses (SPF)	Placement of SPF larvae into refrigerator	Place SPF larvae in refrigerator	Every spawn	Biologist	Destroy broodstock	Verify removal and destruction of broodstock	Broodstock removal and incineration records
			Destruction of broodstock	Remove and incinerate broodstock	Every spawn	Biologist	Reconcile differences and place SPF larvae in refrigerator	Verify SPF larvae placed into refrigerator	Checklist for placing SPF larvae into refrigerator
			Decontamination of Level 1 quarantine	Clean and sanitize quarantine room	Every spawn	Biologist	If inadequate sanitizer concentrations resanitize Level 1 area	Check chlorine and iodine concentrations	Chlorine and iodine concentration records

shower after exiting the Level 1 biosecure area. **Monitoring frequency: daily**.

c. On a weekly basis, the biologist in charge will remove and launder Level 1 clothing at an off-site location. On a weekly basis, the biologist in charge will remove from the premises in a secure plastic bag, all used disposable boots. **Monitoring frequency: weekly**.
d. Red color-coded buckets and other equipment will only be used in the Level 1 quarantine area. **Monitoring frequency: daily**.
e. All air in the Level 1 biosecure area must be filtered through HEPA filters. Maintenance and condition schedule for the HEPA filters will be determined by the manufacturer. **Monitoring frequency: monthly**.

Corrections:

a, b, c. If audits of records indicate non-compliance with the SOPs, the employee will receive training and instructions on the importance of the protocols. The director and staff will assess the risk associated with the breach in protocol and take appropriate documented corrective actions.
d. If red color-coded equipment and/or supplies are found outside the Level 1 quarantine area, the area where the equipment and supplies are found must be cleaned and disinfected. Any other equipment and supplies located near the area must also be cleaned and disinfected. (Note: phenols or quaternary ammonia compounds may be suitable). The red color-coded equipment and/or supplies must then be returned to the Level 1 quarantine area where it will be cleaned and sanitized. Employees will then receive additional training on the importance of following Level 1 SOPs. The director and staff will evaluate and assess the risk and take appropriate documented corrective actions.
e. If the HEPA filters need replacement, or are not functioning properly, the manufacturer must be called immediately.

Records:

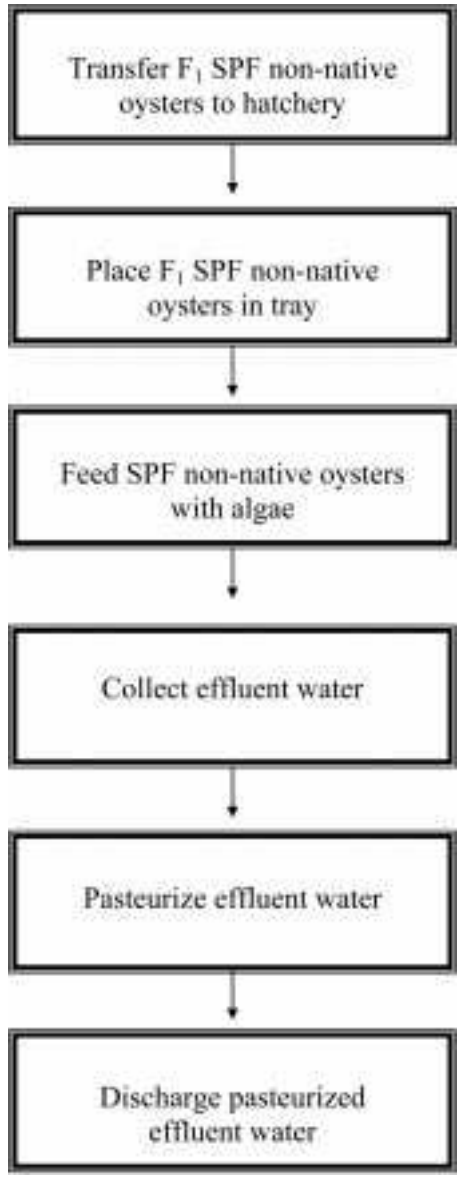
a and b. Daily check sheet indicating spraying of 400 ppm quaternary ammonia disinfectant on the Level 1 floor entrance (or footbath containing 400 ppm quaternary ammonia sanitizer) use test strips to verify quaternary ammonia concentration. Daily check list indicating employee changed clothes and used disposable boots before entering and after exiting the Level 1 biosecure area, and showered after exiting the Level 1 biosecure area.
c. Weekly check sheet indicating laundering of clothing and proper disposal of boots.
d. Daily check sheets indicating the location and condition of the red color coded equipment and supplies.
e. HEPA filter maintenance and condition records.

2. Sterilization of effluent water from Level 1 quarantine

Controls and Monitoring:

a. Effluent water from Level 1 raceways will be pumped to the storage tank 1.

Table 21.7 Flow diagram – non-native hatchery grow-out F_1 SPF oysters



Effluent water from the storage tank 1 will be pumped to the retort and sterilized to 121 ºC for 15 min. The sterilized effluent water will be transferred to the storage tank 2. Sterilization records will be reviewed and verified prior to cooling and discharge of effluent water from the storage tank 2. **Monitoring frequency: daily**.

Table 21.8 Hazard Analysis – hatchery grow-out F_1

ID potential hazard	Significant	Justify	Preventive measures	CCP
Transfer F_1 SPF* non-native oysters to hatchery	Yes	F_1 SPF non-native oysters are fertile	Ensure all F_1 SPF non-native oysters are placed in the hatchery	Yes
Place SPF non-native oysters in trays	Yes	F_1 SPF non-native oysters are fertile	Screen with a 45 micron mesh placed at the end of each raceway	Yes
Feed SPF non-native oysters with algae	No	Algae and algae water are sterile		No
Collection of effluent water from hatchery	Yes	Hatchery effluent water may contain viable gametes	Effluent water is screened with a 45 micron mesh and pasteurized	Yes
Pasteurize effluent water from hatchery	Yes	Hatchery effluent water may contain viable gametes	Pasteurize hatchery effluent water at 90 ºC for 1 min and transfer pasteurized water to storage tank 3**	Yes
Discharge pasteurized effluent water from hatchery	Yes	Hatchery effluent water may not have been adequately pasteurized	Verify pasteurization time and temperatures (i.e., 90 ºC for 1 min) prior to cooling and discharge of sterilized water from storage tank 3**	Yes

* SPF – Specific pathogen free
** Effluent water can also be treated with ozone or chlorine, however, studies must be performed to verify appropriate concentrations and contact time to ensure destruction of viable gametes

b. The retort unit will undergo regular maintenance and calibration certification as recommended by the manufacturer. **Monitoring frequency: monthly**.

Corrections:

a. If review of sterilization records indicates inadequate temperatures and holding times, the effluent water in the storage tank 2 will be returned to the storage tank 1 and resterilized. The storage tank 2 will then be cleaned and sanitized. If the retort unit is malfunctioning, incoming water will be stopped and effluent water will be held in the storage tank 1 until the problem is corrected.
b. If routine maintenance checks indicate a malfunctioning retort unit, effluent water will not be discharged until the retort unit is repaired.

Records:
a. Digital records indicating sterilization time and temperature.
b. Sterilization unit maintenance and certification records.

3. Employee hatchery area protocols
Controls and Monitoring:
a. Employees entering the non-native hatchery area must disinfect shoes or put on disposable boots. A footbath containing 400 ppm quaternary ammonia sanitizer is placed at the entrance. **Monitoring frequency: daily**.
b. Employees leaving the non-native hatchery area must wash hands, disinfect shoes or remove the disposable boots and place them in the receptacle. **Monitoring frequency: daily**.
c. Yellow color coded buckets and other equipment and supplies will only be used in the non-native hatchery area. **Monitoring frequency: daily**.

Corrections:
a. and b. If audits of records indicate non-compliance with the SOPs, the employee will receive training and instructions on the importance of the protocols. The director and staff will assess and evaluate the associated risk and take the appropriate documented corrective actions. If records indicate footbaths do not contain 400 ppm quaternary ammonia sanitizer, refill footbaths and provide training to employees.
c. If yellow color-coded equipment and/or supplies are found outside the non-native hatchery area, the area where the equipment and supplies are found must be cleaned and disinfected. Any other equipment and supplies located near the area must also be cleaned and disinfected. The yellow color-coded equipment and/or supplies must then be returned to the non-native hatchery area where it will be cleaned and sanitized. Employees will then receive additional training on the importance of following hatchery SOPs. The director and staff will evaluate and assess the risk and take appropriate documented corrective actions.

Records:
a. Daily check sheet indicating disinfecting of shoes, or use of disposable boots and washing of hands whenever exiting the non-native hatchery area. Daily check sheet indicating footbath is in place and use test strips to verify 400 ppm quaternary ammonia sanitizer.
b. Daily check sheet indicates employee washed hands when leaving non-native hatchery area.
c. Daily check sheet indicating location of non-native hatchery area yellow color-coded equipment and supplies.

4. Pasteurization of effluent water from hatchery area
Controls and Monitoring:
a. Effluent water from the raceways will be pasteurized at 90 ºC for 1 minute.

Table 21.9 Aquaculture HACCP plan form – hatchery grow-out F_1

(1) Critical Control Point (CCP)	(2) Significant hazards	(3) Critical Limits for each preventive measure	(4) Monitoring			(7)	(8) Corrective action(s)	(9) Verification	(10) Records
			What	How	Frequency	Who			
Transfer F_1 SPF oysters to non-native hatchery area	Incomplete transfer of F_1 SPF non-native oysters to hatchery	All F_1 SPF non-native oysters are transferred to hatchery	Number of F_1 SPF non-native oysters	Count F_1 SPF non-native oysters	Every transfer	Biologist	Reconcile any discrepancies	Review records documenting number of F_1 SPF non-native oysters	Records indicate numbers of F_1 SPF non-native oysters transferred to hatchery
Pasteurization of non-native hatchery area effluent water	Effluent water may contain viable gametes	All non-native hatchery effluent water is pasteurized at 90 °C for 1 min	Pasteurization of non-native hatchery area effluent water	Review of digital records indicating 90 °C for 1 min	Every batch	Biologist	If records show water is not adequately pasteurized, hold effluent water and repasteurize water at 90 °C for 1 min	Visual check pasteurization digital readout	Pasteurization digital readout records
Place F_1 SPF non-native oysters in trays	Non-native F_1 SPF oysters may be fertile	A 45 micron screen is placed at the end of each raceway	Prevent escape of viable gametes	Check condition and clear 45 micron screen placed at the end of each raceway	Every day	Biologist	If screens are missing or fouled, replace and clean screens	Daily visual check to verify screens are in place and are in good condition	Daily screen check and daily screen cleaning records Daily screen condition records

Collection of pasteurized effluent water in storage tank 3	If improperly pasteurized, effluent water in storage tank 3 may contain viable gametes	Pasteurize effluent water at 90 °C for 1 min 1 min)	Pasteurization time and temperature 90 °C for 1 min	Review of pasteurization digital records (i.e., 90 °C for	Every batch from storage tank 3	Biologist	If records show water is not adequately pasteurized, hold effluent water and repasteurize water at 90 °C for 1 min	Verify effluent water pasteurized at 90 °C for 1 min	Pasteurization time/temperature digital records
Discharge pasteurized effluent water from storage tank 3	Incomplete pasteurization of hatchery water	Pasteurize effluent water at 90 °C for 1 min	Pasteurization time and temperature of effluent water (90 °C for 1 min)	Visual confirmation of pasteurization time and temperature digital records	Every batch	Biologist	If records show water was inadequately pasteurized, retain all water in storage tank 3 and hatchery raceways Repair pasteurizer unit and repasteurize water prior to discharge Clean and sanitize storage tank 3	Verify effluent water pasteurized at 90 °C for 1 min	Pasteurization digital time and temperature records

The pasteurized effluent water will then be transferred to the storage tank 3. Pasteurization records will be reviewed and verified prior to cooling and discharge of effluent water from the storage tank 3. **Monitoring frequency: daily**.

b. The raceways will have a 45-micron screen at the end of each raceway. The screens will be checked for their overall condition, cleaned and disinfected on a daily basis. **Monitoring frequency: daily**.
c. The pasteurization unit will undergo regular maintenance and calibration certification as recommended by the manufacturer. **Monitoring frequency: monthly**.

Corrections:

a. If review of pasteurization records indicates inadequate temperatures and holding times, the effluent water in the storage tank 3 will be re-pasteurized. If the pasteurization unit is malfunctioning, incoming water will be stopped and effluent water will be held in the raceways or storage tank 3 until the problem is corrected.
b. If daily checks indicate damage to screens, the screens will be replaced.
c. If routine maintenance checks indicate a malfunctioning unit, effluent water will not be discharged until the pasteurization unit is repaired.

Records:

a. Digital records indicating pasteurization time and temperature.
b. Daily check sheet indicating condition and cleaning of raceway screens.
c. Pasteurization unit maintenance and certification records.

21.7 Disaster planning: the example of standard operating procedures for hurricanes

1. Approaching hurricane

Controls and Monitoring:

Weather information indicates a hurricane will arrive within two days.

Corrections:

a. Drain and sterilize all water in the system.
b. Place all non-native oysters into leak proof shipment containers and remove them to a secure off site location.
c. Clean and sanitize all raceways, floors, walls, etc.

Records:

a. Water sterilization records.
b. Checklist used to account for all non-native oysters.
c. Checklist to demonstrate all raceways, floors, walls, etc., were cleaned and sanitized.

2. Example of Hurricane Preparedness Plan

Introduction:

Although each hurricane threat is unique, these basic steps are to be followed when preparing the main laboratory building and its satellite buildings in the event of a hurricane. The Center Director or designee will determine if additional safety procedures are necessary and ensure their completion. Hurricane supplies, i.e., pre-cut plastic sheeting, tape, flash lights, etc., have been stored and labeled in each office and laboratory to be ready when needed (attachment 1).

Procedure for Level 1 quarantine area:

1. Drain and sterilize all water in the system.
2. Place all non-native oyster broodstock into leak proof shipment containers and remove them to a secure off site location.
3. Clean and sanitize all raceways, floors and walls inside the Level 1 quarantine area.

Procedure for non-native hatchery area:

1. Drain and pasteurize all water in the system.
2. Place all non-native oysters into leak proof shipment containers and remove them to a secure off site location. If the oysters cannot be removed, destroy non-native oysters, clean and disinfect the raceways, screens and floors.

Procedure for office areas:

1. Backup computers and store media in designated location.
2. Unplug all electronic equipment; move away from windows and off floor.
3. Secure any loose items and move away from the windows.
4. Cover desks, file cabinets, computers, and office equipment with plastic sheeting and secure with tape.
5. Wrap and label each CPU and peripherals and move to the designated location in the main building.
6. Conduct a physical search of each work area and/or office you are responsible for to locate hurricane supplies.
7. Ensure adequate plastic sheets are available to cover computers, equipment and furniture.

21.8 Future trends

There is increasing pressure on aquaculture producers to ensure that their operations are environmentally and socially sustainable and that all fishery products are safe and wholesome. Sustainable, environmental and socially friendly aquaculture is becoming the norm on a worldwide basis (Jahncke, 2002). It is less and less acceptable for aquaculture operations to negatively affect human health, environmental and animal health. Possible negative

impacts from aquaculture to public, animal and environmental health includes pathogens, chemotherapeutics, pesticides, disease transmission to wild stocks, coastal destruction, escapements of non-native species, etc. (Howgate *et al.*, 2002; Jahncke and Schwarz, 2002). These issues are being addressed on a worldwide basis, but it is a slow process, and as aquaculture expands, more and more emphasis is needed on developing sustainable aquaculture. The Food and Agriculture Organization's (FAO) Technical Guidelines for Responsible Fisheries Aquaculture Development states, 'it is essential for current efforts aiming at the future success of aquaculture in both developing and developed countries, that potential social and environmental problems are duly addressed in order to ensure that aquaculture develops on a sustainable basis' (FAO, 1997). 'Sustainable development is the management and conservation of the natural resource base and the orientation of technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. Such sustainable development (in the agriculture, forestry and fisheries sectors) conserves land, water, plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable' (FAO, 1997).

There are many countries and organizations developing Best Management Practices (BMPs) and/or Good Aquacultural Practices (GAqPs). These programs are essential components of sustainable aquaculture. Some individuals and organizations consider BMPs to have a wider focus compared with GAqPs, but in many instances these terms are used interchangeably. The use of GAqPs and/or BMPs can improve aquaculture practices and can help protect humans, animals and the environment from unintended consequences of aquaculture. These programs addresses issues such as: Proper Selection of Aquaculture Sites; Source Water; Proper Management of Hatcheries; Pond Management Principles and Practices; Disease Prevention; Appropriate and Proper Disease Treatment; Control of Exotic or Genetically Modified Organisms; Maintaining and Improving the Safety and Quality of Aquaculture Products; Development of Written Standard Operating Procedures; Good Manufacturing Practices for Handling, Packing, Storage and Transportation of Aquaculture Products; Good Employee Practices; Training of Employees in GAqPs and BMPs; Aquaculture Codes of Practice; Application of HACCP Principles as a Risk Management Tool, etc. (FAO, 1995; JIFSAN, 2007; Percy and Hishamunda, 2001). Training the aquaculture farmers, regulatory agencies, and other industry personnel in GAqPs and/or BMPs is also an essential part of the program.

More sophisticated aquaculture operations may also include Quality Management and Quality Assurance Programs such as the International Organization for Standardization (ISO). Many aquaculture feeds are already produced under ISO programs to ensure that they are free of chemical and antibiotic residues. Previous ISO standards addressed quality aspects and not safety, but on September 1, 2005, ISO 22000:2005 (Food Safety Management Systems-Requirements for Any Organization in the Food Chain) was published to ensure food safety (Flick, 2006). It is designed to allow companies to integrate

food safety management programs (e.g., HACCP) with quality management programs. Companies that already use ISO 9001 will be able to extend and integrate ISO 22000:2005 into their operations (Flick, 2006).

Procedures to trace the source of food products and ingredients from production through consumption are also becoming important as our food supply becomes more global in scope. In December 2004, the USA established the final regulations for traceability of food products stating that food supply chains and transporters of foods products must establish and maintain records to track and trace suppliers and buyers. The European Union (EU) implemented mandatory traceability requirements for all foods on 1 January 2005 (Regulation No 178/2002 of the European Parliament and Council and the General Food Law Regulation 178/2002) (Petersen and Green, 2006). Although, Canada and Japan currently do not have specific regulations concerning traceability of seafood products, regulations are in place addressing wholesomeness, packaging, labeling, safety, etc. (Petersen and Green, 2006).

Food traceability programs will help to ensure the safety and quality of all food products including those produced by aquaculture. Traceability programs help companies to identify the source and breadth of safety and/or quality issues of food products (Golan *et al.*, 2004). Rapid identification of food safety and/or quality issues will also help exporting countries reduce their risk of having their fishery products detained or rejected by importing country's inspection programs (Hobbs, 2006).

21.9 Sources of further information and advice

In addition to the information presented in this chapter, there are additional suggested references for readers to access on Quality Programs and HACCP Programs such as Howgate (1997); Howgate *et al.* (1997); Huss (1994); Reilly and Kaferstein (1997); Reilly *et al.* (1997); WHO (1999). Additional information on Codes of Conduct, Codes of Practice, GAqPs, BMPs, etc., are also available. The Office of International des Epizootics (OIE) is the World Health Organization's (WHO) program for animal health (Garrett, 2002; OIE, 2001). The International Council for Exploration of the Sea (ICES) has developed ICES Code of Practice for aquaculture on the Introduction and Transfers of Marine Organisms-2004 (ICES, 2004). The Global Aquaculture Alliance has developed a Responsible Aquaculture Program that encompasses Guiding Principles for Responsible Aquaculture; Codes of Practice for Responsible Shrimp Farming and Best Aquaculture Practice Standards (GAA, 2007). The Federation of European Aquaculture Producers currently comprises 23 European producers (FEAP) representing approximately 1.36 million tones of finfish production. They developed a Code of Conduct for European Aquaculture (FEAP, 2000). Countries such as Thailand and international organizations such as the FAO and others have also written Codes of Practice for Aquaculture (Marine Shrimp Culture Research Institute, 2003; FAO/NACA/UNEP/WB/WWF, 2006).

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22

Monitoring and surveillance to improve farmed fish safety

A. Maage, H. Hove and K. Julshamn, National Institute of Nutrition and Seafood Research (NIFES), Norway

22.1 Introduction: importance of monitoring to improve and document farmed fish safety

The world production of aquaculture products has grown rapidly in the last 50 years. According to FAO (www.fao.org/fi/website), the production in 2004 reached 59.4 millions tons (including sea plants) up from less than a million in the early 1950s. The value of the production also reached more than 70 billion USD. It is generally accepted that there is little room for an increase in traditional fisheries as most stocks are well or fully exploited. A vigilant growth in aquaculture production is required just to keep the per capita fish consumption on the present level worldwide.

The international trade with food, feed and feed ingredients has also grown rapidly in recent decades. However, different markets, and different consumers are increasingly focussing on the quality and safety of the products. In order to further increase the popularity of aquaculture products, the whole aquaculture production chain must keep up with a range of quality and safety regulations in the different markets.

The fish farming industry can be considered as a chain of value adding segments from the suppliers of feed ingredients through to the product the consumer buys in retail. There is a considerable physical and organisational distance along this chain. In order to satisfy the requirements for safety and quality documentation, a system should be established for the surveillance, monitoring and documentation of this food chain. The documentation includes data collection, integration, analysis, interpretation and data dissipation.

Different countries have different solutions on how to secure monitoring of the aquaculture production chain (Health Canada, 2001; US-GAO, 2005). This especially relates to organisation and how different tasks are divided by government and private sector. In this chapter we will use Norwegian data and experience as an example of a safety and quality monitoring system.

22.2 Production of fish feed

Fish feed is the first major step in the aquaculture production chain. In Europe, the production of fish feed is mainly targeted at highly predatory species such as the salmonids, cod, bass and bream. These carnivorous species require a high protein feed. In line with this, the feed receipts have traditionally contained fish meal and fish oil as the major ingredients. Carbohydrates can only be used in limited amounts for energy. Carbohydrates do, however, often also function as binder for the feed pellets. Small quantities of vitamin and mineral mixtures and other additives are used as well. These additives are important in a monitoring context as they are often regulated in terms of maximal upper limits.

The fish oil is a limited resource and it is anticipated that the world production will reach a maximum level of about 1.1 million tons. Other lipid sources, especially of plant origin, are therefore increasingly being used in aquaculture feed production. Thus an increasing number of 'new' ingredients will have to be monitored as part of monitoring the whole food chain.

The main protein source in fish feed has traditionally been fish meal. Due to the high price, also this source faces a growing competition from 'new' ingredients. A range of protein-rich meals of plant origin is therefore available.

22.3 Monitoring fish feed components and fish feed

Several countries have started regular monitoring of animal feed as part of controlling the whole production chain. This is presently especially the EU countries where certain regulations such as EU Directive 2002/32/EG mandates monitoring at the country level. Examples of monitoring reports can be found, for example, for the Netherlands (www.pdv.nl). We will as mentioned use the Norwegian system as an example of how a monitoring programme for fish feed can be undertaken.

A regular surveillance programme for fish feed has been in place in Norway since the early 1990s. The economic basis for the programme was initially a production tax for every ton of fish feed produced. From 1996, this surveillance was organised in a regular Food Authority surveillance programme, under the Directorate of Fisheries. After the major reorganising of the food safety authorities in 2004, the programme was continued under the new Norwegian Food Safety Authority (NFSA). The surveillance programme monitors whether fish feed produced in Norway is in compliance with the feed regulations in the EU, which are also implemented in Norwegian laws and regulations.

The ambition for the surveillance programme of fish feed has been that one sample of feed or feed ingredients should be sampled per 1000 tons feed produced. For 2008 this is to be reduced due to resource constraints. However, since the production of fish feed was estimated at approximately 900 000 tons in Norway in 2005, a volume of 900 samples of feed or feed ingredients was sampled in 2007. All samplings at the 10 fish feed factories in Norway is undertaken by staff from the NFSA. Analyses and reporting has been done by NIFES.

22.3.1 Fish feed monitoring programme parameters

Any surveillance programme designed today in order to monitor feed safety would have to include sampling and testing for microbiological parameters, a whole range of different contaminants, including heavy metals/undesirable elements, persistent organic pollutants (POPs) and natural toxins (i.e., mycotoxins) and different feed additives. In Europe, special attention is put on contaminants and additives for which upper limits have been developed and decided under the EU's legal framework.

The number of different contaminants determined in the programme has increased steadily since interest in food and feed safety issues has evolved. Heavy metals, or more precisely undesirable elements, have been analysed all along the programme period and as the set of background data increases and knowledge of expected natural levels increases, it has been more important to focus any increased analyses on other contaminants.

22.3.2 Level of contaminants found in fish feed

Inorganic elements

So far, the main focus in the programme has been on arsenic, cadmium, mercury and lead. Table 22.1 gives an overview of the levels of elements found in Norwegian fish feed in two selected years, 2000 and 2005. The table also shows the upper limits set for these elements in the feed.

The analysed mercury levels are normally well below the upper limit. Some studies have claimed to find high levels of mercury in fish feed (Choi and Cech, 1998) even though their values are within the upper levels. The upper limit in feed of 0.1 mg Hg/kg on 88% dry weight basis is in fact quite low compared to the upper limits in food for human consumption which is 0.5 or 1.0 mg/kg on a wet weight basis. Mercury can be carried over to fish fillet from feed (Berntssen *et al.*, 2004). Especially for methyl mercury a close surveillance of the feed is warranted.

The mean cadmium concentration in feed in year 2000 was 0.19 mg/kg (range: 0.09–0.54 mg/kg) and in 2005 the mean concentration was 0.33 mg/kg (range: 0.10–0.92 mg/kg) (Table 22.1). In 2005, feed contaminated by cadmium with an extreme value of 24 mg/kg feed was detected in the monitoring programme (see Case Study on pp. 554–5). Research has shown that the uptake of cadmium from feed in salmon is very low. Salmon feed which contained up to

Table 22.1 Monitored levels of undesirable elements and fluorine analysed in the Norwegian fish feed surveillance programme in 2000 and 2005. Data are on dry weight basis

Element	Analyses 2000	Analyses 2005	EU upper limit[c]
Arsenic, total (mg/kg)	4.3 (<0.1-8.7) $n = 83$	5.4 (2.6–10.1) $n = 24$	6.0
Arsenic, inorg (mg/kg)	–	0.024 (0.005–0.051) $n = 18$	–
Cadmium (mg/kg)	0.18 (0.08–0.54) $n = 83$	0.33 (0.10–0.92) $n = 22$ 2.7 (0.10–24.7) $n = 24$[b]	0.5/1.0[a]
Mercury (mg/kg)	0.046 (0.014–0.098) $n = 83$	0.070 (0.010–0.38) $n = 24$	0.1
Lead (mg/kg)	0.09 (0.025–0.34) $n = 83$	0.11 (0.022–0.30) $n = 24$	5.0
Fluorine (mg/kg)	30 (8–59) $n = 93$	40 (19–66) $n = 19$	150

[a]Limit for cadmium in fish feed was changed from 0.5 to 1.0 in 2005
[b]Including two contaminated feeds, see text
[c]88% dry weight

5 mg Cd/kg did not appear to be toxic to salmon in a three-month feeding experiment (Berntssen *et al.*, 2001). The EU maximum level for cadmium in fish feed was increased from 0.5 mg/kg to 1.0 mg/kg in 2006. Norwegian scientists have supported such an increase on the basis of experimental results (Lundebye *et al.*, 1999).

The EU has set a maximum upper level for lead in feed of 5 mg/kg. The mean concentrations of lead in fish feed, given in Table 22.1, were found to be less than 0.20 mg/kg, the highest lead concentration measured in fish feed in 2000 was 0.34 mg/kg feed ($n = 83$) and in 2005 was 0.30 mg/kg feed ($n = 24$). Feed ingredients of terrestrial origin are more susceptible to lead contamination than marine raw materials.

Arsenic is the only inorganic element that regularly has not been in compliance with the upper limits set by EU at 4 mg/kg feed and later adjusted to 6 mg/kg feed. The higher level is, however, not considered a toxicological problem for the fish nor for human consumption of fish, since the major form in marine samples is the organic form, arsenobetaine, and very little is inorganic (Table 22.1). This organic form has very low toxicity compared with the inorganic forms of the element.

The arsenic content in fish feed seems to have increased slightly in the period 2000 to 2005, with mean concentrations of 4.3 mg/kg in 2000 and 5.4 mg/kg in 2005. The range of arsenic concentrations in fish feed varied between approximately <0.1 and 9 mg/kg feed in 2000 and between 3 and 10 mg/kg in 2005. Arsenic concentrations in Norwegian fish feed sometimes has exceeded the EU

maximum upper limit level of 6 mg/kg feed. This maximum level does not allow for the naturally high arsenic content in marine raw materials, and more importantly does not account for the fact that most of this is in the form of arsenobetaine. It has been recommended by Norway to increase the maximum level of arsenic in fish feed to 10 mg/kg or alternatively to set the limits on the toxic inorganic forms. This recommendation has not been met, however, current regulations for undesirable substances in feed are under scrutiny by the European Commission. The naturally high arsenic content of seafood products has been recognised since Chapman reported the high arsenic content in shrimp in 1926. Farmed salmon has one of the lowest arsenic levels of the species examined in NIFES' Monitoring Programme (www.nifes.no/seafooddata), a fact that supports the acceptance of higher maximum level in feed. The concentration for inorganic arsenic in feed was low (Table 22.1). It was found to vary between 0.005 and 0.051 mg/kg with an average of 0.024 mg/kg feed ($n = 18$).

Fluorine in fish feed could become a future issue in fish feed monitoring. The present levels of fluorine in fish feed is not regarded as harmful for fish nor is it regarded a source for carry over to fish fillets (Julshamn *et al.*, 2004). However, probably the largest potential feed ingredients resource, the Antarctic krill, contains rather high levels of fluorine. If a feed contains more than 10% krill, the present limit of 150 mg F/kg feed (88% dry matter) will normally be exceeded.

Organic contaminants (POPs)

While the heavy metals in feed have been in focus since volume production of feed started in the 1980s (Maage, 1990), interest in the organic pollutants (POPs) in feed was first given high attention from the late 1990s. A boost in this interest came with the report from Hites *et al.* (2004) on the elevated levels of undesirable substances in farmed salmon compared to wild salmon. Gradually, an increasing number of these compounds have been included in regular feed monitoring programes all over the world. For some classes of compounds, such as dioxins, furans, di-PCBs and several pesticide compounds, upper limits for fish feed in aquaculture have been established.

As shown in Table 22.2 the levels of POP substances are either relatively constant or show a decreasing trend. Also feed ingredients have been monitored to document the flow of contaminants. From these analyses we can find that marine oils are the main contributor to organic contaminants. The plant oils have lower levels. There are some exceptions, for example the pesticide endosulphan, which is more abundant in plant oils than in marine oils.

Due to the large number of individual congeners, a complete analysis of PCB is difficult. Scientific consensus has been to measure seven individual congeners (known as the Seven Dutch or PCB_7). The concentrations of these seven congeners are summarised to give an indicator of total PCB contamination.

The mean-sums of PCB_7 concentration in complete feeding stuff brands sampled in 2000 and 2005, respectively, were 29 μg/kg with a range from 10 to 50 μg/kg, and 13 μg/kg with a range from 4 to 37 μg/kg (Table 22.2). Neither EU nor Norway have set upper limits for PCB in feed.

Table 22.2 Monitored levels of selected undesirable substances analysed in the Norwegian fish feed surveillance programme in 2000 and 2005. Data are on a dry weight basis

Compound	Year of analysis		EU upper limit[d] (mg/kg feed)
	2000	2005	
PCB_7 (μg/kg)	29 (10–50) $n = 32$	13 (4–37) $n = 46$	–
Dioxins (ng TEQ/kg)	1.5 (0.5–3.5) $n = 5$	0.54 (0.08–1.42) $n = 57$	2.25 (from 2005)
Sum dioxins and DL-PCBs	–	2.1 (0.7–2.1) $n = 57$	7.0 (from 2006)
Sum PBDE[a]	2.1 (0.5–4.6)[b] $n = 10$	2.4 (0.4–6.0) $n = 19$	–
Pesticides			
Sum DDT (g/kg)	22 (10–65) $n = 32$	24 (7–52) $n = 18$	50
Sum toxaphene (μg/kg)[c]	–	9.5 (6.5–31) $n = 18$	50
Dieldrin (μg/kg)	–	3.7 (0.5–9.9) $n = 18$	10

[a] PBDE congeners 28, 47, 99, 100, 153 & 154, 'upper bound' data
[b] Data from 2001, the first year these compounds were analysed in fish feed
[c] Toxaphene congeners 26, 50, 62 are used for regulations and for sum ('upper bound' data)
[d] 88% dry weight

Dioxins are often expressed as a 'sum of TEQ values', the sum of the polychlorinated dibenzo-para-dioxins (PCDDs) congeners and the polychlorinated dibenzofurans (PCDFs) congeners expressed in World Health Organisation (WHO) toxic equivalents, using the WHO-TEFs (toxic equivalency factors from 1997). The mean sum dioxins concentration for complete feeding stuffs sampled in 2000 was 1.5 ng TEQ/kg and in 2005 0.54 ng TEQ/kg. The EU has a maximum level for dioxins in fish feed of 2.25 ng-WHO/kg. All samples analysed both in 2000 and 2005 showed concentrations below that upper limit. From 2006 also the non-orto and mono-orto dioxin like PCBs were included in theses regulations with a new legal limit for these summarised compounds. Analyses of dioxin-like PCBs had not been started in 2000.

For several pesticides upper limits are also set for what is the legal content in fish feed and feed ingredients and some examples from the monitoring are given in Table 22.2.

22.3.3 Monitoring the levels of feed additives

Feed additives are used for their value in fish nutrition or for their effect on stability or texture of the feed. Additives such as the pigments increase the economic or nutritional value of the produced fish. In Europe, additives permitted in feed are given on a positive list indicating that substances not on the list are illegal to use.

Table 22.3 Monitored levels of essential minerals and vitamins in the Norwegian fish feed surveillance programme in 2000 and 2005. Data are on dry weight basis

Additive	Analyses 2000	Analyses 2005	EU upper limit[c]
Mineral			
Iron (mg/kg)	146 (64–203) $n = 49$	198 (83–353) $n = 23$	750
Zinc (mg/kg)	194 (109–329) $n = 80$	122 (31–254) $n = 23$	200[a]
Manganese (mg/kg)	54 (24–91) $n = 83$	30 (1–52) $n = 23$	100
Copper (mg/kg)	12 (5.4–18) $n = 83$	8.7 (2.5–15) $n = 23$	25
Selenium (mg/kg)	1.27 (0.55–2.5) $n = 95$	1.26 (0.44–2.9) $n = 23$	0.5[b]
Iodine (mg/kg)	4.5 (1.2–10.5) $n = 95$	3.6 (1.1–10) $n = 23$	20
Vitamins			
Vitamin A	6.3 (3.7–12)[c] $n = 24$	19 (5.3–69) $n = 20$	–
Vitamin D	0.25 (0.1–0.4)[c] $n = 24$	0.24 (0.1–0.4)[c] $n = 20$	0.075[b]

[a] Limit recently changed from 250 mg/kg to 200 mg/kg feed
[b] Limit is valid only when selenium is added, not based on natural ingredients
[c] 88% dry weight

For several permitted additives, such as selected minerals and vitamins (Table 22.3), legal limits have been established. Thus monitoring programmes should include additive surveillance. Some feed additives are monitored in Norway in order to establish background data for possible future legaslation.

For the essential minerals, the upper limits have decreased during recent years and the corresponding monitored values have reflected this decrease. This can be seen for the elements zinc, copper and manganese. The feed levels of other elements have not changed much. It is noticeable that for both selenium and vitamin A the content in marine feed ingredients is naturally so high that the limits are exceeded even without use of additives.

Of the other additives measured in the monitoring, there has been some focus on the pigments and antioxidants (Table 22.4). The latter are added to protect the long-chained marine fatty acids from oxidation and rancidity. Ethoxyquin is added to fish meal to prevent oxidising during long transports. Oxidising fish meal could otherwise be a fire hazard during transportation due to heat generated in the oxidation process.

22.3.4 Trueness in declaration, including legal feed components

A range of other analyses is also included in the surveillance programme. Some of these have an impact on food safety. This includes very different groups of compounds such as:

Table 22.4 Monitored levels of pigments and antioxidants in the Norwegian fish feed surveillance programme in 2000 and 2005. Data are on dry weight basis

Additive	Analyses 2002	Analyses 2005	EU upper limit[a]
Antioxdants			
BHA (mg/kg)	–	0.44 (0.07-2.9) $n = 10$	
BHT (mg/kg)	–	21 (0.01–80) $n = 20$	
Ethoxyquin (mg/kg)	12 (3.0–33) $n = 19$	38 (5.6–116) $n = 18$	
Sum antioxidants	–	58 (6.8–145) $n = 18$	150[b]
Pigments			
Astaxanthin (mg/kg)	42 (<1.2–100) $n = 204$	35 (1.4–74) $n = 54$	100
Canthaxanthin (mg/kg)	8.2 (<1.2–39) $n = 170$	<1.2 (<1.2) $n = 17$	25[c]

[a] 88% dry weight
[b] The limits is set for the sum of all synthetic antioxidants
[c] 25 mg/kg only if the sum of astaxanthin and cantaxanthin does not exceed 100 mg/kg

- Illegal protein sources (blood meal, bone meal).
- The use of tissue offal from the species for which the feed is intended.
- Some GMOs (Norwegian fish farmers and feed companies have had a self-imposed ban on GMO. This seems (as of 2007) soon to be abandoned).

The control of protein sources started in the aftermath of the mad cow disease and is given a high priority even though there have been questions about the scientific rationale for this action on fish feed.

For the control of fair labelling, several parameters are also regularly monitored by analysis. These include:

- protein content
- lipid content
- ash content
- dry matter.

As a curiosity, in 2007, there was an ongoing feed scandal from addition of the substance melamine to animal feed. By adding the substance, the analytical method for protein which is based on nitrogen analyses, is 'fooled' to believe that the protein content is higher than it really is. Thus it is a practice intended to fool customers and the monitoring system.

Case study: contaminated zinc sulphate

In the autumn of 2004 a cargo of about 16 tons of zinc sulphate intended for use as a feed additive was imported to Norway. The zinc sulphate was imported

from China to a company specialising in the production of mineral and vitamin premixes for animal feed, including fish feed.

At the end of March 2005 two feed samples from a fish feed company in Norway were analysed and showed values of 20.0 and 24.7 mg Cd/kg. The upper limit for Cd in fish feed at that time was 0.5 mg/kg. Just a few days later, on 4 April 2005, the Norwegian Food Safety Authority issued a regulation forbidding sales of the contaminated feed. The internal HACCP system in one of the affected feed companies had also detected the problem. Following this a total of 8500 tons of fish feed was destroyed.

The case shows the vulnerability of the international raw material trading system. But it also showed the effectiveness and usefulness of HACCP in companies and an active monitoring programme.

22.4 Monitoring of water quality and its relevance for food quality

This aspect will only be briefly touched upon. It is the author's view that this is of minor importance for the main bulk of European aquaculture. Most of the late phase aquaculture production, both in northern and southern Europe, is undertaken in sea water. The quality of the sea water supply does not have a great impact on the food safety issues as feed is the major source to the contaminants.

There might be exceptions and there are, of course, other important reasons to monitor water quality, both in fresh and sea water, with regards to such factors as oxygen level, pH and also for pathogens, but this is for the individual farmer more than for a governmental programme.

Any negative effect that fish farm management has on the sediment or sea water quality is outside the scope of this chapter.

22.5 Monitoring of food quality of aquaculture fish

In addition to monitoring the produced feed, and the feed ingredients, the safety of the produced aquaculture products is of great importance to monitor as well. Traditionally, the more general quality parameters of the fish have been monitored, but this activity had declined in the last decade. The documentation and assurance of quality is now considered to be the responsibility of the market through use of internal control regimes that include the HACCP principles. The challenges to food safety are considerable and the public focus on this topic is growing. The customers and markets of today require a level of activity and documentation previously unheard of.

22.5.1 Private sector activities

In the whole EU and in the US a HACCP (Hazard Analysis of Critical Control Points) based quality control and documentation system is mandatory in the food

sector including the fisheries sector. This has been further emphasised in the new food law and control regime in the EU. The role of the authorities in this system is now to audit the HACCP-based quality control systems, while it is the private companies' responsibilities to have a functional quality system and to have real control over their critical points.

22.5.2 The food authorities' monitoring of farmed fish

The activities in this sector were increased and harmonised in Europe with the arrival of Directive 96/23/EC. This directive describes mandatory measures to monitor certain substances for all farmed animal food products, including aquaculture products. The sphere of influence of this directive exceeds the EU and EFTA member countries. Also countries, like Australia, which want to export fish to EU, find it useful to use the national monitoring plans in accordance with this directive. The countries outside EU/EFTA do not have to report their monitoring results to the EU.

In Norway this directive has been implemented since 1998. The activity under Directive 96/23 replaced or complemented several national surveillance projects. The traditional fish industry fishing on wild fish stocks is not covered by this directive, and in Norway that fish is therefore covered by national surveillance programmes.

The scope of the Norwegian implementation of the 96/23 programme is, like the feed surveillance programme, defined by the volume of the total production two years previously. Thus the production statistics from 2005 define the scope for the 2007 surveillance programme. Up until now, the salmonids (salmon and rainbow trout) have dominated the production volume, even though the newly cultured species, such as cod, have a higher level of medication in the production per produced unit. For this reason the distribution formula of the sampling plan is up-weighted in favour of these species. Thus for the salmonids one sample is collected for each 100 tons fish produced and for the 'newer species' one sample is collected for each 25 tons. This situation is expected to continue for some years until suitable vaccination programmes for these species are developed and implemented.

The tissue monitored in the programme is mainly the fillet, since this is the most relevant tissue for human consumption. Some fish oil is monitored as well. The samples were analysed in compounded samples made from five fish, each from the same net-pen, one net-pen from each farm.

Table 22.5 gives the plan for the analysis for the 96/23 directive samples in 2005 in full detail. In the table the substances are grouped in classes as described in the directive. Group A compounds are substances prohibited for use in aquaculture. This group includes hormones and some compounds previously used in medication. The samples for group A analysis are sampled without pre-warning, at the fish farm. Any reliable detection of compounds in this group would be considered significant. There is no legal threshold level. Group B compounds are substances that can legally be used as medicine, and also include

Table 22.5 The scope of the surveillance of farmed fish in relation to EU Directive 96/23 in 2005, including the number of fish sampled and the number of chemical and microbiological analyses undertaken

Parameter group name		Parameter	No. of fish	Total number of analytical determinations
A (fillet)	A1	Stillebens	595	$118 \times 3 = 354$
	A3	Steroids	575	$115 \times 2 = 230$
	A6	Chloramfenikol	250	50
	Annex IV iv	Furazolidon, Furaltadon Nitrofurantoin, Nitrofurazon,	250	$50 \times 4 = 200$
Sum A			1670	834
B (fillet)	B2	Teflubenzuron	50	10
		Diflubenzuron	50	10
		Cypermethrine	250	50
		Praziquantel	200	40
		Fenbendazole	50	10
		Emamectin	300	60
		Ivermectin	50	10
		Deltamethrin	200	40
	B3a	HCB	150	30
		Heptachlor	70	$14 \times 17 = 238$
		Heptachlor-a		
		Aldrin		
		Dieldrin		
		Endrin		
		oxy-Chlordan		
		trans-Chlordan		
		Cis-Chlordane		
		α-Endosulfan		
		Endosulphane-sulphate		
		β-Endosulfan		
		Cis-Nonachlor		
		Trans-Nonachlor		
		Toxaphenes 26, 32, 50, 62		
		DDT compounds	80	$16 \times 6 = 96$
		Dioxins	150	$31 \times 29 = 899$
		Dioxin-like PCBs		
	B3b	Dichlorvos	75	15
		Azamethiphos	75	15
	B3d	Pb	560	$112 \times 4 = 448$
		Cd		
		Hg		
		As		
	B3e	Malachite green	500	100
	B3f	BHT	20	4
		Etoxyquin	15	3
Sum B (fillet)			2400	2078

Table 22.5 *Continued*

Parameter group name		Parameter	No. of fish	Total number of analytical determinations
B (liver)	B1	Oxolinic acid	0	Only done in cases of positive result from microbiological 'screening'
		Flumequine		
		Florfenicol		
		Tetracyclines		
		Sulphonamides		
		Microbiological Antibacterial Determination	1005	1005
Total sum liver			1005	1005
Total sum fillet			4070	2912

environmental pollution compounds. For some group B substances tolerable daily intake levels (TDI) have been established. Samples are taken from the slaughterhouses.

Table 22.6 lists the actual medical substances monitored under 96/23. In EU the use of medicines requires that medication of the animal husbandry including aquaculture is well regulated, scrutinously monitored and vigorously enforced. The knowledge about this has probably been one element in what is regarded as a successful policy as reflected in the monitoring data. Since the start of medical residue monitoring in the 1990s, no positive detection has been found in Norwegian-produced fish intended for human consumption. Positives have, however, been found in imported fish.

As mentioned, hormones are, in general, forbidden substances. Unlike for terrestrial farm animals there are few reasons to expect hormones to be used in aquaculture. Since 96/23 is a general directive, these compounds are included in the surveillance. No positives have been found since the start of this monitoring.

Table 22.7 presents data found for residues of synthetic anti-oxidants. These compounds are legal additives in the fish feed and some carry-over effect is expected. From the table it is also noteworthy that there are found quantities well above those of any environmental contaminant in the farmed fish samples. The variation between the samples is large. In Europe the control of these substances is done at the feed level. Recently, Japan has regulated the levels of residues of synthetic anti-oxidants in foods, including fish tissue, but the analysed levels in Norwegian farmed fish have been within the acceptable range.

Tables 22.8 and 22.9 present results of the monitoring of persistent organic pollutants (POPs) in fillets from farmed fish. The monitoring focuses on PCBs, PCDDs and PCDFs ('dioxins'), dioxin-like PCBs and pesticides. Since 2006 brominated flame retardants are also included in the programme.

The surveillance of dioxins and dioxin-like PCBs has received a lot of focus since these substances were analysed in some fish species to be rather close to

Table 22.6 Medical residues and hormones included in the surveillance programme in fillet of farmed fish

Compound	Grp	Analytical value Verdi	LOD* (μg/kg)	No. of compounded samples	Total number of fish
Chloramfenicol	A6	<LOD	0.3	50	250
Nitrofurantion	Annex iv	<LOD	1.0	50	250
Furaltadon	Annex iv	<LOD	1.0	50	250
Furazolidon	Annex iv	<LOD	1.0	50	250
Nitrofurazon	Annex iv	<LOD	1.0	50	250
Cypermethrin	B2a	<LOD	10	50	250
Deltamethrin	B2a	<LOD	15	40	200
Praziquantel	B2a	<LOD	50	40	200
Fenbendazole	B2a	<LOD	5	10	50
Teflubenzurone	B2a	<LOD	5	10	50
Diflubenzurone	B2a	<LOD	10	10	50
Emamectin	B2a	<LOD	2.5	60	300
Ivermectin	B2a	<LOD	25	10	50
Dichlorvos	B3b	<LOD	10	15	75
Azamethophos	B3b	<LOD	10	15	75
Malachite green	B3e	<LOD	1	100	500
Oxolinic acid	B1	<LOD	200*	–	1005
Flumequine	B1	<LOD	200*	–	1005
Florfenikol	B1	<LOD	200*	–	1005
Tetracyclines (several)	B1	<LOD	200*	–	1005
Sulphonamides (several)	B1	<LOD	200*	–	1005
Hexesterol	A3	<LOD	0.67	115	575
Ienesterol	A3	<LOD	0.62	115	575
Diethylstilbestrol	A1	<LOD	1.07	115	595
β-nandrolon	A1	<LOD	2.26	119	595
β-trenbolon	A1	<LOD	3.9	119	595

* LOD of screening method. (Microbiological assay). Chemical methods have lower LOD.

Table 22.7 Synthetic anti-oxidants measured in the fillet of farmed fish from the Norwegian monitoring according to EU 96/23 in 2005

Compound	Sample type	Mean value (μg/kg)	Max. value (μg/kg)	Min. value (μg/kg)	No. of compounded samples	Total number of fish
BHT	Fillet	2300	3800	100	4	20
Etoxyquin	Fillet	25.4	44.4	12.7	3	15
Etoxyquin dimer	Fillet	337	443	242	3	15
Sum etoxyquin	Fillet	362	487	255	3	15

Table 22.8 Chlorinated organic pollutants in the fillet of farmed fish from the Norwegian surveillance programme in 2005: PCB_7, PCB-105, PCB 156 (all in μg/kg wet weight), and dioxins, furans and dioxin-like PCBs (in WHO toxic equivalents as pg/g wet weight)

Compound	Mean value	Max. value	Min. value	LOQ	No. of compounded samples
PCB-28	0.21	0.35	0.02	0.06	16
PCB-52	0.72	1.2	0.22	0.09	16
PCB-101	1.3	2.7	0.07	0.09	16
PCB-118	1.0	2.2	0.04	0.09	16
PCB-138	2.3	5.5	0.06	0.12	16
PCB-153	2.4	5.9	0.05	0.09	16
PCB-180	0.61	1.5	0.02	0.15	16
Sum PCB-7	8.5	19.3	0.5	–	16
PCB-105	0.41	0.86	0.04	0.09	16
PCB-156	0.17	0.30	0.01	0.06	16
Dioxins:					
PCDD+PCDF	0.25	0.47	0.07		31
Dioxin-like PCB	0.95	1.77	0.30		31
Sum Total WHO Toxic Equivalents	1.20	2.24	0.37	–	31

what EU has established as a legal limit of 4 pg/g sum WHO-TEQ (wet weight) on sum PCDDs and PCDFs. This was the basis for implementing limits on acceptable content of these substances. The data from our monitoring and other analytical results as well, indicate that this limit is not a problem for Norwegian aquaculture products. A limit of 8 pg/g WHO-TEQ (wet weight) for the sum of dioxins and dioxin-like PCBs was established from 2006, with a stated intention of further reducing this limit by the end of 2008. Work is in progress to change the feed receipts to decrease the levels of this parameter in the feed. The feed is the source of this compound in farmed fish (Lundebye *et al.*, 2004) and stricter feed regulations on these compounds are expected to further reduce fillet concentrations (Isosaari *et al.*, 2004).

Many of the other POPs presented in Table 22.9 are measured very close to the method's quantification limits even though they are present in measurable quantities. It is possible to observe that for several of these compounds, the concentration levels are slowly decreasing as their use or production has stopped. The exposure to the environment of these are regulated and monitored on a world wide basis through the Stockholm Convention (see: www.pops.int).

Table 22.10 presents data on inorganic substances. Arsenic (As), lead (Pb), mercury (Hg) and cadmium (Cd) are the elements being monitored in this programme. The data indicates that these pollutants are not normally considered to be a problem in Norwegian aquaculture products. There are, however, some problems in relation to heavy metals in shellfish. Shellfish production is not a topic in this chapter, even though its farming is on the rise.

Table 22.9 Data on chlorinated pesticides (μg/kg wet weight) in the fillet of farmed fish from the Norwegian surveillance programme in 2005

Compound	Mean value	Max. value	Min. value	LOQ	No. of compounded samples
HCB	1.0	1.5	0.27	0.07	14
Heptaklor		<LOQ		2.5	14
Heptaklor-A		<LOQ		0.5	14
Aldrin		<LOQ		0.6	14
Dieldrin	1.2*	1.9	<LOQ	0.3	14
Endrin		<LOQ		1.0	14
oxy-klordan		<LOQ		1.3	14
trans-klordan		<LOQ		0.7	14
cis-chlordan	0.8*	1.4	<LOQ	0.5	14
endosulfan-a		<LOQ		0.3	14
Endosulfan-sulphate		<LOQ		0.5	14
Endosulfan-b		<LOQ		0.3	14
trans-nonaklor	1.0*	1.4	<LOQ	0.5	14
cis-nonaklor		<LOQ		0.7	14
toxaphene-26		<LOQ		2.5	14
toxaphene-32		<LOQ		1.5	14
toxaphene-50	–**	2.6	<LOQ	2.5	14
toxaphene-62		<LOQ		1.5	14
op-DDT	0.4	0.7	0.06		16
pp-DDT	1.4	2.3	0.06		16
pp-DDD	0.5	1.0	0.04		16
op-DDE	2.8	6.1	0.19		16
pp-DDE	6.3	11.8	0.15		16
Sum DDT	12.6	22.3	0.5		

*Upper bound mean value.
**Too few real numbers to give a valid mean value

Table 22.10 Arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) (mg/kg wet weight) in the fillet of farmed fish, and the EU legal threshold values (Group B3c)

	As	Cd	Hg	Pb
Mean value	2.0	<0.003	0.038	<0.012
Standard deviation	0.57		0.01	
Max. value	3.76		0.10	
Min. value	0.54		0.011	
No. of compounded samples	112	112	112	112
LOQ	0.009	0.003	0.009	0.012
EU upper limit		0.05	0.5	0.2

As for feed, monitoring programmes for fish for human consumption might include the speciation of several of the inorganic elements. It is well established that organic mercury (methyl-Hg^{+}) is more toxic than inorganic mercury. Also it is known that organic arsenic compounds are much less toxic than the inorganic arsenic. A major difference between terrestrial-based food and fish is that in fish most of the arsenic is found in organic molecules. Thus, in the future, inorganic arsenic instead of total arsenic and methyl mercury should be the preferred parameters to measure. Unfortunately, speciation analyses are often more difficult, expensive, time consuming and requires more advanced instrumentation than analyses of the total content.

The cadmium feed incident (see Case Study on pp. 554–5) showed the importance of a monitoring programme, and the importance of having established a knowledge base on the expected concentration range of each parameter of interest. A knowledge base is required to establish a 'baseline' to compare suspected incident data against.

This incident was further a powerful reminder that a surveillance programme should have a quick response time from sampling to analysis and report. This, however, might be in conflict with the advantage of a rational and efficient laboratory service resulting from large analytical series.

However, it also demonstrated the usefulness of storing historical samples so that one can go back and look for specific substances which might not have been analysed at the time, or to provide background values to compare against after a contamination from a 'new' species previously not focused on the monitoring.

22.6 Knowledge dissemination

The monitoring of feed ingredients, feed and seafood quality only has meaning if the data gathered are useful, sufficient and regarded as reliable by the industry, the authorities and consumers. In order to achieve this it is important that all parties have access to the data and that transparency in routines for the sampling and analysis is secured.

Such data only has value if open to the public, consumers, governments and industry. Most countries now publish such data on the internet. In Norway, the data has mainly been published on the Food Safety Authorities home page (www.mattilsynet.no). Also the data are found in reports on the institutions' websites, see: www.nifes.no.

22.7 Future trends

Europe experienced several food and feed scandals during the 1990s. In particular, the dioxin scandals and the outbreak of mad cow disease and spread of Creutzfeldt–Jakob disease showed food monitoring in a poor light. The new food laws and hygiene directives are answers to this. However, good laws are

not enough and it is expected that the the demand for reliable and independent food and feed monitoring data will increase.

It is difficult for consumers to gather real knowledge themselves, and even the consumer organisations lack the resources and know-how to fulfil this role. At the same time the trust in data provided by the business is decreasing. Therefore it is necessary that governments take on more of the responsibility for the surveillance and presentation of the data.

22.8 Sources of further information and advice

Some webpages from other countries with some aquaculture production and ongoing monitoring is listed.

Europe:
- European Food Safety Directives (http://ec.europa.eu/food/food/index)
- European Food Safety Authority (www.efsa.org)
- Denmark – Fødevarestyrelsen (www.foedevarestyrelsen.dk)
- Netherlands – Voedsel en waren autoriteit (www.wva.nl)
- UK – Food Standards Agency (www.food.gov.uk)
- Ireland – Food Safety Authority – (www.fsai.ie)
- Sweden – Livsmedeslverket (www.slv.se)

Norway:
- Norwegian Food Safety Authority (www.mattislynet.no)
- Norwegian Scientific Committee for Food Safety (www.vkm.no)

USA/Canada:
- Food and Drug Administration (www.fda.gov)
- Environmental Protection Agency (www.epa.gov)
- Health Canada (www.hc-sc.gc.ca)

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23

Confirming the origin of wild and farmed fish

M. Lees and F. Thomas, Eurofins Scientific Analytics, France

23.1 Introduction: the importance of confirming the origin of wild and farmed fish

Today's consumers are better informed and more discerning about the type of food products they buy. They are aware of the health benefits of eating fish, the problems of sustainability in fish production, and of the potential harm caused by eating contaminated fish. The media has in part responded to the latter concern and in part fuelled it through various reports and documentaries on food safety and adulteration. A well-known example of this is the study published in the review *Science* in January 2004 highlighting the toxic risks of eating farmed salmon (Hites *et al.*, 2004). The work focused largely on the elevated concentrations of organic contaminants which the authors of the study, conducted on about 700 farmed and wild salmon fillets purchased in 16 major cities in Europe and North America, found to be consistently and significantly more concentrated in farmed salmon than in the wild salmon. This work resulted in recommendations in the United States for a clear indication of the 'farmed' origin to be included on the fish label.

Legislators in Europe have also focused their work on consumer concerns in addition to ensuring a level playing field for the industry on the other hand. European Community fish labelling requirements, dealt with in the next chapter, now make it mandatory for all fish and fish-derived products to carry details of their production origin, whether sea-caught or farmed, at all points of sale to the consumer.

Difference in price per kg of farmed fish compared to wild fish can provide the consumer with one means of differentiating between the two – wild salmon

can cost up to 10 times the price of its farmed variety. Other visual aspects may help the consumer: a set of identical, plate-sized sea bass on the fishmonger's stall will generally mean farmed, and the subtle differences in colour between wild and farmed salmon can also help a well-trained eye spot which is which.

To ensure that consumers are getting what they pay for, to enforce existing legislation and avoid fraudulently or mis-labelled products, there is a need for objective analytical tools able to confirm the origin of wild and farmed fish. This chapter reviews the tests that have been developed for this purpose, including their use in surveys on market products.

23.2 Methods to confirm the origin of wild and farmed fish

Various methods for the authentication and/or characterisation of fish species have been developed over the last twenty years. The oldest method is based on visual inspection of the fish morphology: body size and shape, fins, jaws and teeth. In some cases farmed fish have been seen to have smaller tails than wild ones because of the lack of space and high density in some farms, and are often fatter due to reduced activity. However, this method of identification can only be applied to the whole fish and not to fish fillets and other processed products, more commonly found in retail outlets.

The most widely and routinely used methods to identify fish species are those using proteins or DNA sequences as species-specific markers (Sotelo *et al.*, 1993), and these have been extensively investigated for both fresh and processed fish products (Dooley *et al.*, 2005). However, these methods are not suitable for distinguishing between wild and farmed samples of the same species, where compositional methods linked primarily to differences in diet are more appropriate.

23.2.1 Chromatographic techniques

Lipid content and fatty acid profiles

Compositional analyses have been used both for characterisation and for authentication purposes. The lipid content and to a greater extent the fatty acid composition of tissue lipids of animals are closely related to diet, making these suitable markers for identifying wild or cultured production methods. Chromatographic techniques are the methods of choice for the analysis of fatty acid mixtures, the old Bligh and Dyer method (Bligh and Dyer, 1959) being the most frequently used to isolate and purify lipids from biological materials. It is based on a liquid-liquid extraction, a mild treatment so as to minimise oxidative decomposition of fish lipids due to their highly unsaturated structure.

Most of the GC techniques developed to study fish lipids require a derivatisation step prior to analysis. Transesterification with methanol is the most commonly used; the fatty acids are then studied as fatty acid methyl esters or FAMEs. Most studies have focused on the liver oil or the fish flesh; however,

other parts of the fish have also been investigated. For example, for Atlantic salmon, total lipid content and composition have been characterised for different anatomical fractions: skin, red and white muscle, belly flap, dorsal fat depot, backbone, head, visceral tissue, liver (Aursand *et al.*, 1994).

The long chain n-3 polyunsaturated fatty acids (PUFA) are the most characteristic components of fish lipids, the main polyenoic acids being eicosapentaenoic acid (EPA or 20:5n-3) and docosahexenoic acid (DHA or 22:6n-3). Levels of linoleic (18:2n-6) and linolenic (18:3n-3) acids, more commonly found in vegetable oils, are generally low in marine lipids. Feed formulations used in aquaculture are made up of fishmeal and fish oil, often derived from species such as capelin, menhaden (*Brevoortia* spp.), sand eel, sprat, Norway pout, blue whiting, horse mackerel, Atlantic herring (*Clupea* spp.), anchovy (*Engraulis* spp.), and pilchard. Since fatty acid profiles can vary from species to species, these differences can be found in the farmed fish fed on specific fishmeal. With declining fish stocks and the corresponding growth in the use of fishmeal and fish oil for aquaculture, there is a move towards incorporating plant-derived products such as soybean meal or vegetable oils in the fish feed. The variations in fatty acid profiles due to these changes also offer a useful means of differentiating between wild and farmed fish.

Various studies have confirmed these differences in fatty acid composition in wild and cultured fish. Alasalvar *et al.* (2002) reported dissimilarities in fatty acid profiles of the flesh lipids of cultured and wild sea bass, with oleic acid (18:1n-9) and linoleic acid (18:2n-6) being significantly higher in the farmed fish. Both these fatty acids can be linked to the use of plant-derived oils in the feed. On the other hand, cultured sea bass contained lower amounts of EPA and DHA, with a higher ratio of n-3 to n-6 in the wild fish.

Interestingly, in an earlier study carried out by Chen *et al.* (1995) on the characterisation of the fatty acid compositions of cultured and wild sturgeon, it was found that wild sturgeon have lower levels of DHA or EPA. The higher amounts of long chain n-3 fatty acids in the cultured fish were attributed to the use of herring or menhaden in the fishmeal.

Potential fraudulent practices do not always entail substituting cheaper farmed fish for its more valuable wild counterpart. In areas where fishing rights are restricted, to protect certain fish populations for instance, there is the potential for endemic fish species to be illegally sold as cultured fish. Tritt *et al.* (2005) used fatty acid composition and chemometric data treatment to discriminate between wild and farmed largemouth bass, black and white crappies, all freshwater fish that are popular with anglers. This study focused on four main fatty acids: linoleic (18:2n-6), linolenic (18:3n-3), arachidonic (20:4n-6) and docosahexaenoic (22:6n-3). The statistical models built up using different data treatments all gave good classification rates were obtained for the wild and cultured species. This work was unusual in that it examined juvenile, age-0, rather than adult fish.

As shown in the examples given above, most studies on the use of lipid content or fatty acid composition to distinguish between wild and cultured fish

have concentrated on the most economically important fish species, such as salmon (Ackman and Takeuchi, 1986; Blanchet *et al.*, 2005), sea bass (Alasalvar *et al.*, 2002; Saglik *et al.*, 2003) and gilthead sea bream (Saglik *et al.*, 2003) or environmentally important species such as the North American red drum (Villareal *et al.*, 1994).

Although it has been clearly demonstrated that fatty acid composition is closely linked to the fish feed, there are other factors that may affect the overall profile, such as age, fatty acid metabolism and so on. Add to this the possible changes in the feed composition to farmed fish to reflect available supplies, and the fact that the diet of wild fish can vary with season, migration patterns and other external factors, it is clear that most discriminant models based on fatty acid profiles may be subject to a number of uncontrolled variables. To provide a more robust solution it is probably best to envisage other complementary methods.

Volatile component profiles

Other chromatographic techniques have been explored to enable a differentiation between wild and cultured fish. For example Alasalvar *et al.* (2005) looked at over 70 volatile compounds, including aldehydes, ketones, alcohols, aromatics, terpenes, and furans by dynamic headspace gas chromatography-mass spectrometry in samples of cultured and wild gilthead sea bream. Although both origins showed very complex volatile profiles, there was a significant difference between them. However, the profiles varied in relation to storage time and conditions, making this analysis only useful on fresh fish and as a back up to other techniques.

Distinction of farmed and wild salmonids from pigment analyses

Consumers will generally choose dark-pink-coloured salmon or trout over paler specimens (Sylvia *et al.*, 1996). The distinct pink pigment in wild salmonids is due principally to the presence of astaxanthin, a carotenoid that occurs naturally in a wide variety of living organisms. It is produced by algae where it protects against ultraviolet light and acts as an antioxidant. From the algae, the carotenoid makes it way up through the food chain to crustaceans such as shrimp, crawfish, crabs and lobster, that are tinted red by accumulated astaxanthin, and on to wild salmon and trout. Farmed salmon or trout, on the other hand, will only display the characteristic pink colour if a similar carotenoid is included in the fish feed. Addition of carotenoids will also play an important role in the growth and survival of the fish.

Astaxanthin is either produced synthetically or from various organisms such as the microscopic algae *Haematococcus pluvialis* or from Phaffia yeast, *Phaffia rodozyma*. Another source is krill meal, which has a high content of astaxanthin bound as esters. Since it is an expensive raw material it is often substituted by, or more commonly used in conjunction with, canthaxanthin, a carotenoid not normally found in Atlantic salmon and at very low levels in Pacific salmon. Both the Food and Drug Administration in the United States (21 CFR 73.35 and

21 CFR 73.75), and the European Union (Commission Directive 2003/7/EC) have set limits on the accepted levels of these carotenoids as pigment enhancers in commercially sold fish.

A number of methods have been developed to analyse the carotenoids in farmed and wild salmonids mostly based on chromatographic techniques, as reviewed by Bjerkeng (1997). Separating canthaxanthin from astaxanthin, and hence farmed from wild salmon, is relatively straightforward and the difficulty arises when differentiating endogenous astaxanthin from its synthetic counterpart or from diverse natural sources. Since natural astaxanthin occurs in different stereoisomeric forms, 3R,3′R, 3S,3′S and 3R,3′S, the distribution of these isomers can provide evidence of a farmed or wild origin. For example, wild salmon contain a low level of the 3R,3′S form, so its detection is a good indicator of a cultured origin. Methods for analysing astaxanthin stereoisomer composition often rely on HPLC with a chiral stationary phase (Weber, 1990), but a direct and rapid liquid chromatography is described by Turujman *et al.* (1997).

The wide range of sources of natural astaxanthin available and the possibility of manufacturing the pigment with a given stereoisomer composition, means that this method alone is not sufficient to unambiguously distinguish between wild and farmed salmonids in all cases.

23.2.2 Stable isotope analyses

Stable isotope analyses have for a long time been the domain of geologists and geochemists, but over the last twenty years an increasing number of applications have been developed for food authenticity testing. The theory behind isotopic techniques is that the major elements making up natural products also exist in their naturally occurring isotopic form: hydrogen, ^{1}H and deuterium, ^{2}H; carbon-^{12}C and carbon-^{13}C (see Table 23.1). This means that the same chemical compound derived from a different source can have exactly the same chemical formula but a quite different isotopic make-up. These subtle differences in isotopic content arise from fractionation accompanying physical, chemical and biochemical processes brought about by the differences in mass between the element and its isotope. These minute deviations can be measured using dedicated instruments.

Table 23.1 Naturally occurring isotopes of common elements and their relative abundance

	Hydrogen			Carbon			Nitrogen		Oxygen		
Atom	^{1}H	^{2}H	^{3}H	^{12}C	^{13}C	^{14}C	^{14}N	^{15}N	^{16}O	^{17}O	^{18}O
Atomic number	1	1	1	6	6	6	7	7	8	8	8
(weight)	(1)	(2)	(3)	(12)	(13)	(14)	(14)	(15)	(16)	(17)	(18)
Proportion (%)	99.985	0.015	(a)	98.904	1.096	(b)	99.63	0.37	99.759	0.037	0.204

Note: (a) and (b) are radioactive isotopes and present at a very low abundance in nature

Stable isotope mass spectrometry

Isotope ratios, relating the heavy isotope to its lighter counterpart ($^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$) are used as the parameters for describing isotopic distribution in a compound or product. These can be determined by Isotope Ratio Mass Spectrometry (IRMS) and are commonly expressed on a relative scale as the deviation δ‰ (parts per thousand or per mil) with respect to the isotope ratio content of an international standard, R_{std} (Kelly, 2003). The standards used are VSMOW (Vienna – Standard Mean Ocean Water) for $\delta^{18}O$‰, VPDB (Vienna – Pee Dee Belemnite, a calcium carbonate) for $\delta^{13}C$‰, and AIR for $\delta^{15}N$‰. These international standards are produced and certified by the International Atomic Energy Agency in Vienna.

$$\delta\ (‰) = (R_{sample}/R_{std} - 1) \times 1000 \qquad 23.1$$

where R is the ratio of the heavy to the light isotope ($^{13}C/^{12}C$, $^{18}O/^{16}O$, or $^{15}N/^{14}N$) in the sample R_{sample} and in the standard R_{std}.

Several correlations exist between isotopic distribution or content and the geo/climatic environment of the product or molecule under consideration. ^{13}C and ^{15}N contents are generally related to diet. In plants, ^{13}C content is principally affected by isotopic fractionation occurring during the photosynthetic assimilation of CO_2 via the C3 (Calvin), C4 (Hatch and Slack) or CAM (Crassulacean acid metabolism) metabolic pathways. This leads to relatively large ranges of isotopic composition in carbohydrates, lipids and other metabolites from the different plant species, with C3 derived metabolites relatively depleted in ^{13}C compared to the richer C4 compounds. ^{15}N content in plants is strongly correlated with local agricultural practices, and in certain circumstances can be used to verify labelling claims such as organic production (Bateman *et al.*, 2005).

In animals, ^{15}N content, and to a lesser extent ^{13}C composition, is linked to the relative trophic level of the organism (DeNiro and Epstein, 1981). Both ^{15}N and ^{13}C increase with each ascending trophic level, from algae to zooplankton to crustaceans and herbivorous fish, to omnivorous and carnivorous fish. The average enrichment in $\delta^{15}N$ from prey to predator is around 3‰, although variations can occur among primary and secondary consumers (Adams and Sterner, 2000).

The hydrosphere is the main source of oxygen and hydrogen, and content is influenced by the isotopic fractionation occurring during the entire hydrological cycle including evaporation, condensation, precipitation, and so on. $\delta^{18}O$ and $\delta^{2}H$ values in plant water are therefore correlated with local climatic conditions.

Taken all together, the overall isotopic fingerprint (^{13}C, ^{2}H, ^{18}O and ^{15}N) provides a useful means of verifying geographical origin of a food product (Kelly *et al.*, 2005; Camin *et al.*, 2007).

Stable isotope analyses have been applied to the discrimination of wild and cultured fish. $\delta^{15}N$ and $\delta^{13}C$ values measured on the fish have been used to differentiate wild and farmed Atlantic salmon (*Salmo salar*) from Newfoundland (Dempson and Power, 2004). Moreno-Rojas *et al.* (2007) used the same

parameters on gilthead sea bream from four origins. The study showed that wild and farmed sea bream could be separated on the basis of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, albeit on a limited database. A similar study was carried out on Atlantic salmon with the aim of identifying organically farmed salmon (Molkentin *et al.*, 2007) but came to the conclusion that isotopic techniques alone were insufficient for this purpose.

Site-specific natural isotope fractionation studied by nuclear magnetic resonance

A second method for measuring isotopic distribution involves high-resolution ^{2}H NMR spectroscopy, a technique called SNIF-NMR®, site-specific natural isotope fractionation studied by nuclear magnetic resonance (Martin *et al.*, 2006), which was first developed to study the origin of sugars in natural products such as wine and fruit juices.

SNIF-NMR has been applied to lipids of plant origin (Royer *et al.*, 1999). Measuring site-specific $^{2}H/^{1}H$ ratios by deuterium NMR for complex mixtures such as lipids is an intricate technique and requires a number of steps. When carried out on the extracted fatty acid methyl esters, it is not possible to identify each fatty acid in the ^{2}H NMR spectrum. Instead it is necessary to work with clusters associated with different moieties of the fatty acid chain (Fig. 23.1). If the molar composition of the mixture in terms of fatty acids is known precisely, it is possible to calculate the isotope ratios of each cluster, using the relation given in equation 23.2.

$$(D/H)_1 - (f_i/F_i)(D/H)_{tot} \qquad 23.2$$

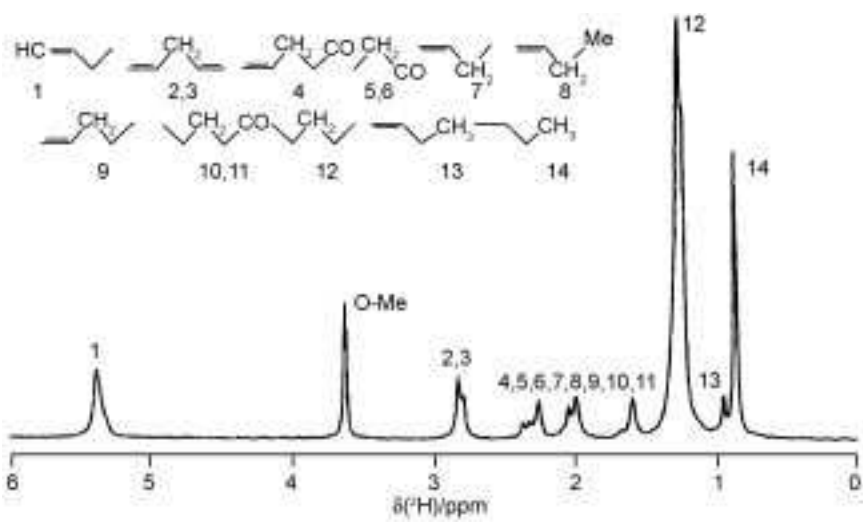

Fig. 23.1 ^{2}H NMR profile of a salmon oil sample showing how the clusters are composed of the equivalent hydrogen irrespective of the fatty acid in which it is contained.

Table 23.2 Mean D/H and $^{13}C/^{12}C$ ratios measured on different individual fatty acids in fish lipids by ^{2}H NMR

	$(D/H)_{tot}$ (ppm)	$^{13}C/^{12}C$ (δ)
14:0	122.9	
16:0	121.0	
18:0	122.2	
16:1n-7	137.2	
18:1n-9	126.9	
18:1n-7	114.0	
20:1n-9	131.3	
22:1n-9	129.3	
18:3n-3	119.3	
20:5n-3 (EPA)	117.2	−23.5
22:6n-3 (DHA)	122.3	−23.8

where f_i are the effective molar fractions of the different isotopomers calculated from the cluster intensities, F_i is the statistical molar fractions deduced from the site populations (number of hydrogen atoms) and the proportions of the fatty acids in the mixture. $(D/H)_{tot}$ is the overall deuterium/hydrogen ratio obtained by IRMS on the mixture of fatty acids.

One of the first studies to use this technique to determine the origin of fish oils combined isotopic analysis using high-resolution ^{2}H NMR spectroscopy with ^{13}C IRMS. This joint approach, carried out by Aursand *et al.* (1997), led to a clear characterisation in the ^{2}H NMR spectrum of all the major fatty acids found in fish lipids, including the internal deuterium distribution in nearly all the chemical sites in EPA and DHA. The mean D/H (deuterium to hydrogen ratio) and the $^{13}C/^{12}C$ ratios have been measured for the different individual fatty acids (Table 23.2), with an overall D/H ratio ranging from 114.0 to 137.2 ppm (vs. VSMOW) and a $^{13}C/^{12}C$ ratio from −23.5 to −33.2‰ (vs. VPDB), typical of ^{13}C content of marine sources.

23.2.3 High resolution ^{13}C NMR spectroscopy

From NMR spectroscopic studies of lipid extracts of muscles and industrial fish oils, it has been shown that quantitative ^{13}C NMR is effective for determining the fatty acid composition of the oil, and in addition can also provide information on the positional distribution of fatty acids in triacylglycerides. ^{13}C NMR spectra of lipids from samples of different products or species show sufficient variation that they can be used to differentiate between fish species, between wild and farmed fish as well as between fish oils treated or processed in different ways.

Preliminary assignment of all or most of the chemical shifts in the ^{13}C NMR spectra of fish oils was carried out by Gunstone (1991). High-resolution ^{13}C NMR spectroscopy has also been used to determine the n-3 fatty acid distribution in lipid extracted from white muscle of Atlantic salmon (Aursand *et al.*, 1993). In addition to total concentration of lipids, and total amount of n-3

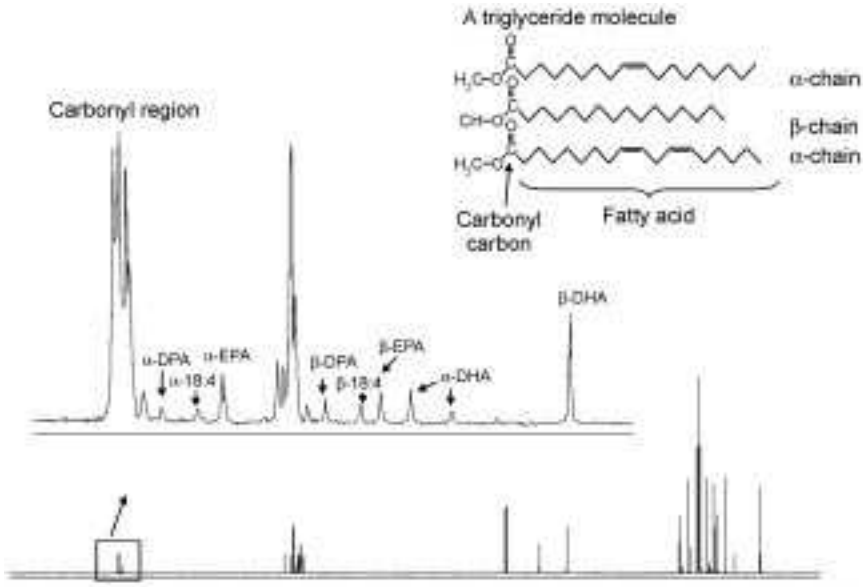

Fig. 23.2 ^{13}C NMR spectrum of extracted salmon lipids (recorded at 600 MHz).

fatty acids, the ^{13}C NMR technique can also provide the positional distribution (α or 1,3- and β or 2-) of the important fatty acids, EPA and DHA, in the triacylglycerol molecules, by analysing both the olefin and carbonyl spectral regions (Aursand *et al.*, 1995). The data obtained using ^{13}C NMR are in good agreement with the results of traditional, and more time-consuming methods.

The spectrum of fish lipids obtained from ^{13}C NMR (Fig. 23.2) can also be considered as a fingerprint, giving an overall profile of the chemical composition of the sample and the composition of the triacylglycerides (positional distribution of the fatty acids in the triacylglyceride molecules). This semi-quantitative approach was chosen in order to investigate the potential of ^{13}C NMR to distinguish between farmed and wild salmon in the COFAWS project (COFAWS, 2001–2004), in order to reduce the longer experimental time required by quantitative measurements. Since the resulting spectra are complex, with small differences in nearly 600 to 700 signals in a ^{13}C NMR spectrum, analysis by visual inspection is difficult and multivariate data-analysis is needed to classify the samples. Various data treatment techniques were applied, including PNN (probabilistic neural networks) and KNN (k-nearest neighbours) giving excellent results for wild versus farmed predictions, but less successful results for country of origin predictions (Aursand, 2004).

23.2.4 Combined methodological approach

As shown in the examples chosen above, most techniques provide a useful method of distinguishing farmed and wild fish, but do not necessarily take into

account all variables. A more robust approach is to combine two or more techniques. In a study to differentiate cultured and wild sea bass (*Dicentrarchus labrax*), Alasalvar *et al.* (2002) used trace mineral composition in addition to fatty acid profile and lipid content. The study found that iron (Fe), aluminium (Al), titanium (Ti) and vanadium (V) were significantly different between the flesh of farmed and wild sea bass, Fe being higher in the wild fish, which was attributed to a greater proportion of dark muscles with higher Fe content in wild compared to farmed sea bass.

One of the first studies to combine compositional and isotopic analyses in order to characterise salmon (*Salmo salar*) was carried out by Aursand *et al.* (2000). This used gas chromatography, IRMS and high-resolution SNIF-NMR to study different types of commercial marine oils (salmon, haddock, cod, tuna, anchovy, tortoise) and lipid extracts from muscle of wild (from Norway) and farmed (from Norway and Scotland) salmon. In addition, lipids extracted from two fish feeds used for farmed salmon were also studied to investigate the dependence of the deuterium composition of the feed on the salmon oil. A statistical analysis of the fatty acid composition, overall ^{2}H and ^{13}C isotope ratios and molar fractions of the isotopomeric deuterium clusters was carried out to select the most efficient variables for distinguishing the different groups of salmon and fish studied. A classification analysis based on four fatty acids compositions, three deuterium molar fractions and overall $(D/H)_{tot}$ (overall deuterium/hydrogen isotope ratio) of fish oils gave a 100% correct classification of all the oils. The data was also able to discriminate between salmon farmed in Norway and in those originating from Scotland.

The promising results from this study led the way to a shared-cost RTD funded by the European Community under the 'Competitive and Sustainable Growth' Programme (1998–2002). The aim of this project COFAWS (2001–2004) was to establish an analytical methodology suitable for distinguishing between farmed and wild salmon, to be provided to the relevant technical committees of the anti-fraud and fisheries directorates of the Commission to detect incorrect labelling or dumping of non-approved fish on the community market. The results are given below.

Results obtained on European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) using the same methodology are also presented in the following sections.

23.3 Application of combined compositional and isotopic methodology to different fish species

23.3.1 Salmon (*Salmo salar*)

The promising potential of stable isotope analyses to distinguish between wild and farmed salmon identified by Aursand *et al.* (2000) was further investigated in the COFAWS (2001–2004) EU project. A consortium was brought together to study different analytical techniques and, in particular, to provide well-characterised samples of authentic wild and farmed salmon.

As described earlier, certain stable isotope ratios can provide information on the origin of a food product. It has also been shown that the potential of these techniques for authenticity testing is vastly improved by investigating inter-and intramolecular isotope correlations in organic compounds (Schmidt *et al.*, 1993). This approach was followed in the COFAWS project by measuring a number of different isotope ratios ($^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$) using IRMS on various components of the salmon: flesh lipids and derived products such as fatty acids, glycerol, phospholipids, salmon flesh, water. Over 300 fish samples were studied: wild and farmed salmon from different geographical origins (Scotland, Ireland, Norway, Iceland, Faroe Islands, Canada, Alaska, Tasmania) and different seasons.

In addition to establishing this isotopic data base of authentic fish, a study was carried out of farmed fish raised on defined feeding programmes in order relate the isotopic data found in the fish muscle triacylglycerides to dietary and environmental influences. In this study, different groups of growing salmon, from the smolt stage in flow-through seawater tanks to a weight of 0.5–1 kg were fed on feed representative of Northern and Southern hemisphere diets.

Preparation of the samples for stable isotope measurement included extraction of the salmon flesh lipids using the Standard NF V03-030. A small amount of the extracted oil was dried and used directly for isotopic analysis. The GC analysis of the fatty acid methyl esters was also carried out on the bulk oil. The glycerol/choline fraction was isolated from the remaining oil after saponification. $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) were measured using an EA-IRMS instrument equipped with a combustion oven, in which samples are burnt at a temperature of at least 1030 ºC in a quartz reactor in an elemental analyser (NA 2100 Proteins, CE Instruments). $\delta^{18}O$ (‰) is measured with an IRMS instrument equipped with a pyrolysis oven, in which samples are pyrolysed at a temperature of at least 1060 ºC in a quartz reactor in an elemental analyser (NA1500 Serie2, CE Instruments).

An analysis of variance (ANOVA) on all the isotopic and fatty acid parameters measured showed three of these variables were the most discriminant (Fig. 23.3): percentage oleic acid (C18:2n-6), $\delta^{15}N$ of the nitrogen in the choline fraction extracted from the salmon lipids, and $\delta^{18}O$ of oxygen in the salmon oil.

A principal components analysis (PCA) of these three parameters (Fig. 23.4) demonstrates conclusive differentiation between wild and farmed salmon (Thomas *et al.*, 2005, 2008).

The information obtained from both the IRMS and GC methods closely reflect the fish diet, which is the principal factor of discrimination. Higher levels of 18:2n-6 found in the farmed salmon can be attributed to the use of greater amounts of plant oils in the fish feed. Nitrogen isotope ratios also reflect dietary input with wild salmon showing high $\delta^{15}N$ values. This isotopic enrichment due to their higher trophic position in the food chain is also demonstrated in the feeding trials carried out. Farmed salmon fed a Southern hemisphere diet which was richer in marine-derived ingredients showed higher

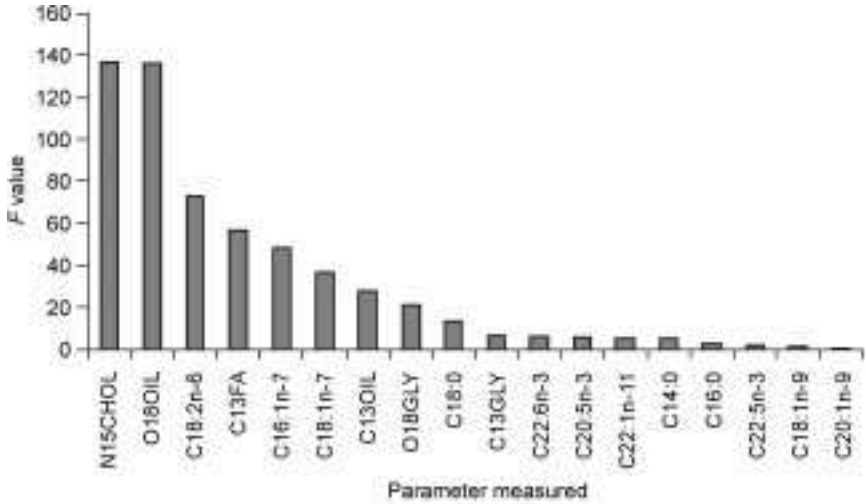

Fig. 23.3 ANOVA on the IRMS and GC data obtained on the authentic salmon samples ($F_{critical}$ = 4 at a 5% confidence interval).

δ^{15}N values than those with a dietary input from a Northern hemisphere diet (Authors, unpublished data). Oxygen isotope data also showed differences between the wild and farmed samples linked to differences in the metabolic fish water. Molkentin *et al.* (2007), on the other hand, found that δ^{18}O of total

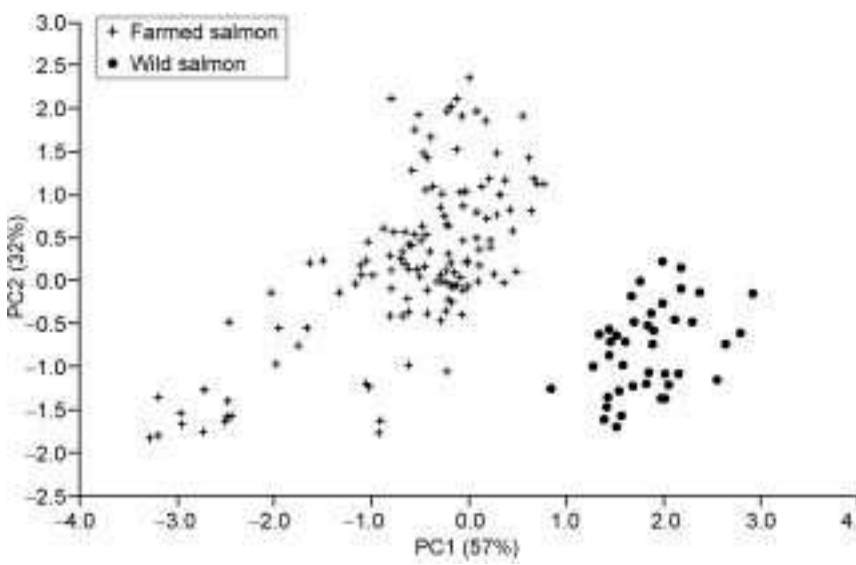

Fig. 23.4 PCA diagram (combination of isotopic and compositional variables) on salmon oil. PC1 and PC2 represent the first and second principal components, with percentage explained variance indicated in brackets.

muscle did not differ between wild and organically farmed salmon. However, these authors only used total muscle flesh, whereas in study carried out by Thomas *et al.* (2005, 2008) the δ^{18}O of the salmon oil was used. It should also be noted that this study uses the correlation between nitrogen and oxygen data and the not the individual values.

Carbon isotopic data, not shown here, can also provide further discrimination of the wild and farmed salmon (COFAWS, 2001–2004). In general, farmed salmon have lower δ^{13}C values, reflecting the use of plant-derived constituents in the fish feed.

In 2005 and 2006 a survey of the UK market was conducted by the Food Standards Agency as part of their food authenticity programme in order to verify 'wild' claims of fish on sale. The methodology described above was used. Of the 'wild' salmon samples collected by local authorities, 15% were found to be farmed (Food Standards Agency, 2007).

23.3.2 Sea bass (*Dicentrarchus labrax*)

European sea bass (*Dicentrarchus labrax*) aquaculture is an increasing activity in Greece, France, Spain, Italy and Turkey, and farmed sea bass is now found in markets and supermarkets in most European cities. With the difference in price between the wild and cultured sea bass, there could be the temptation to fraudulently pass off cheaper cultured sea bass for wild.

The methodology described above, involving a combination of compositional and isotopic analyses, was also applied to authentic cultured and wild sea bass obtained from different European origins.

From the compositional analyses (oil content, GC profile) it was found that cultured sea bass have higher flesh lipid content, which can be used as a preliminary criterion for differentiating between the two origins. The fatty acid profile of the flesh lipids of the farmed bass also showed higher amounts of C18:2n-6 and C20:1n-9, and a lower content of C20:4n-6. This is consistent with the use of commercial fish feed that contains fewer marine-derived and more plant-based constituents.

As expected, additional significant differences between the two origins of sea bass were shown by the results of the isotopic analyses. As in the study on salmon (Thomas *et al.*, 2005, 2008), farmed sea bass showed lower δ^{13}C values for the bulk fish oil due to the lighter carbon-13 content of the feed used. Nitrogen isotopic data, on the other hand, showed the opposite trend to that found in farmed and wild salmon, with wild sea bass having lower δ^{15}N than farmed. In the study by Bell *et al.* (2007), who also noted the same trend, this is attributed to possible effects of maturity, growth rate or seasonal variations.

By combining percentage fatty acid content and δ^{13}C fish oil, δ^{15}N choline and δ^{18}O fish oil, wild and cultured sea bass can be clearly distinguished (Fig. 23.5). These results are also borne out by subsequent work done by Bell *et al.* (2007), in which the methodology described by Thomas *et al.* (2005, 2008) was used to discriminate between wild and cultured European sea bass.

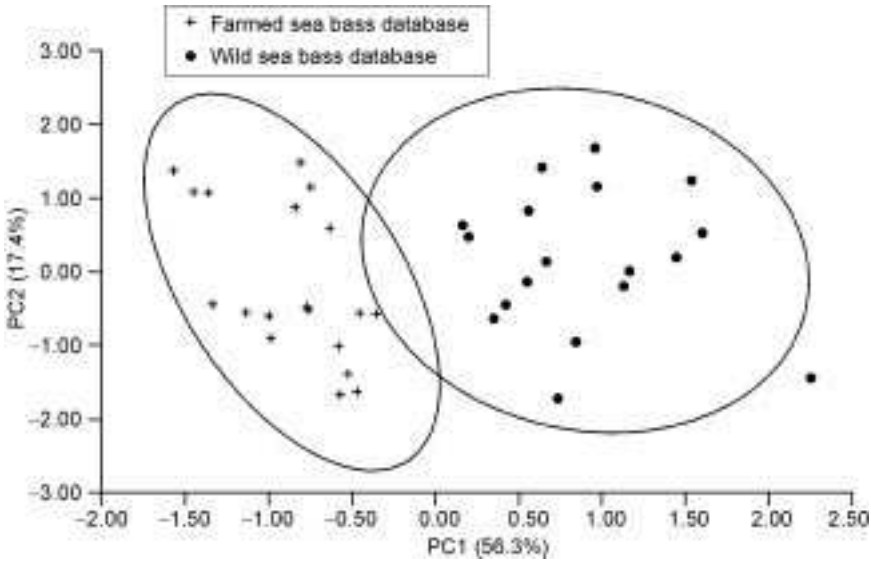

Fig. 23.5 PCA on percentage fatty acid content, δ^{13}C fish oil, δ^{15}N choline and δ^{18}O fish oil to differentiate wild and cultured sea bass (*Dicentrachus labrax*).

Sea bass was also included in the survey of the UK market conducted by the Food Standards Agency in 2005–2006. In this case, 6 of 59 'wild' samples collected by the local authorities were mislabelled.

23.3.3 Sea bream (*Sparus aurata*)

A similar database was established for gilthead sea bream (*Sparus aurata*). As with sea bass, wild sea bream contain significantly higher levels of 20:4n-6 and significantly lower levels of 18:2n-6 compared to their farmed counterparts. δ^{13}C analysis of the total oil fraction and glycerol/choline fraction also gave expected values reflecting the lighter ^{13}C content of farmed fish diets due to the greater content of plant derived oils. ^{15}N isotope values followed the trend seen for salmon rather than sea bream, with higher values for wild compared to farmed sea bream.

Analysis of δ^{18}O from total oil extracted from flesh lipid of sea bream was also different for farmed and wild sea bass. As described previously for salmon, these differences are linked to metabolic water in the fish and may reflect geographical origin.

Principal components analysis was used to examine the multivariate structure of the bream data set. Plots of Factor 1 vs. Factor 2 (Fig. 23.6) demonstrate clear separation of wild and farmed bream.

Sea bream was also covered by the UK survey carried out by the Food Standards Agency. 11% of the 'wild' sea bream samples collected were shown to be incorrectly labelled.

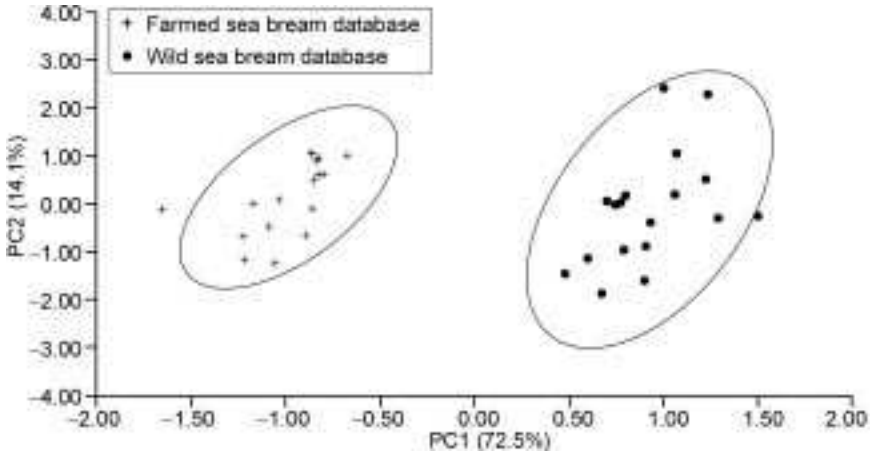

Fig. 23.6 PCA on percentage fatty acid content, δ^{13}C fish oil, δ^{15}N choline and δ^{18}O fish oil to differentiate wild and cultured sea bream (*Sparus aurata*).

23.3.4 Other species

Aquaculture is increasingly relied on as a solution to the problem of dwindling fish stock. Other fish species are now commonly cultured, such as trout, halibut, cod, and all these species are potential targets for the combined compositional and isotopic methodology described above. First results (Authors, unpublished data) are promising. However, as shown in the discussions above, there is no fixed trend for expected fatty acid or isotopic results. Chen *et al.* (1995) showed that wild sturgeon have a lower n-3/n-6 ratio than cultured, whereas the reverse was found for sea bass (Bell *et al.*, 2007). ^{15}N isotope content is higher in wild salmon compared to farmed, but lower in wild sea bass compared to farmed. Based on these assessments it is important to establish a robust database for each of the fish species being studied.

23.4 Future trends

23.4.1 Impact of modified feed in fish diet on established methods

Lipid composition of the fish diet has a considerable influence of the fatty acid composition of the flesh lipids of farmed fish. However, in most cases there is no direct correlation between the two, since a number of other factors intervene such as preferential catabolism of certain fatty acids. The fatty acid composition of flesh lipids from farmed fish have been extensively studied, primarily from the point of view of human nutrition, since the presence of some fatty acids, in particular the long chain n-3 fatty acids, are reported to play an important role in the prevention of cardiovascular disease.

A number of studies have looked at the flesh lipid composition of farmed Atlantic salmon (*Salmo salar*) fed different diets. An early study by Polvi and Ackman (1992) looked at four groups of farmed salmon fed different diets in

which the lipid source was herring oil, rapeseed oil, EPA/DHA ethyl ester concentrate and hen egg fatty acids as ethyl esters. Lipids from the edible muscle from each diet group were analysed and phospholipid and triacylglyceride compositions determined and compared to those of the diets. The results showed that, although the lipid composition of the fish flesh had been modified, there was no evidence of a direct relationship with that of composition of the feedstuff.

A more recent study by Torstensen *et al.* (2005) has investigated the tailoring of salmon flesh lipid composition by replacing the fish oil component in the feed by 100% or 75% vegetable oil. Careful formulation of the latter so that it contained similar proportions of total saturated, monounsaturated and polyunsaturated fatty acid contents to fish oil, and a finishing diet of 100% fish oil, resulted in farmed salmon with a fatty acid profile of suitable composition to fulfil human nutritional requirements.

As further research is conducted on the modification of fish feed in this way, analytical models established on the basis of fatty acid composition and even stable isotope profile will have to evolve to keep pace.

23.4.2 Effect of environmental factors

In addition to human intervention in the modification of farmed fish fatty acid composition, a number of environmental factors, such as nature and availability of food, season, location and year of catch, also contribute to variations in fatty acid composition and contents. For example, in a study of the effects of season and location of catch of the fatty acid compositions of some marine fish species, a correlation between low water temperature and high n-3/n-6 ratios was observed (Armstrong *et al.*, 1994). Therefore databases established to differentiate wild and farmed fish should also take into account variability introduced by such external factors.

23.4.3 Technological progress in analytical methods

In addition to a widening range of applications, considerable developments in IRMS instrumentation have considerably improved both the specificity, precision and ease of use of stable isotope techniques. An example of relevance to this chapter is the possibility of performing the sequential analysis of ^{15}N and ^{13}C in the same analytical run on fish samples (Zebuhr *et al.*, 2000). In this work, various samples of dried fresh pike, of varying size, were analysed and their $\delta^{15}N$ and $\delta^{13}C$ values measured on the whole fish with no preparation step. The isotopic results showed excellent reproducibility ($\delta^{13}C$: $-29.79 \pm 0.20‰$ and $\delta^{15}N$: $13.94 \pm 0.17‰$ with respect to AIR and VPDB standards, respectively) and no obvious dependence of sample weight on delta values.

Further precision can also be obtained over carbon isotope measurements of the bulk fish oil described in the previous sections by determining the $\delta^{13}C$ values of the individual fatty acids in the oil. This is done using a gas

chromatograph coupled to an isotope ratio mass spectrometer via a combustion interface. This approach was used by Bell *et al.* (2007) in the discrimination of wild and cultured sea bass. There are still some analytical problems inherent to the technique, particularly for analytes that are in low amounts or that co-elute.

23.4.4 Rapid screening techniques

Most of the techniques described in the previous sections require fairly lengthy sample treatment and analyses. Some rapid methods have been developed to measure fish lipid composition using various spectroscopic techniques and suitable chemometric treatment of the data obtained.

Near infrared reflectance spectroscopy (NIRS) analysis, for example, provides a rapid means of measuring free fatty acids in fish oils using partial least-squares (PLS) regression to build calibration and validation sets (Cozzolinoa *et al.*, 2005). Xiccato *et al.* (2004) also used NIRS to predict the production system of farmed European sea bass between extensive ponds, semi-intensive ponds, intensive tanks and intensive sea-cages.

Another profiling technique that is becoming increasing used in based on ^{1}H NMR spectroscopy. Gribbestada *et al.* (2005) showed that it is possible to identify single chemical components, such as hypoxanthine, amino acids, anserine, lactate and some fatty acids, in extracts, whole muscle and whole fish from the high-resolution ^{1}H MR spectrum and use this information for the authentication of Atlantic salmon.

23.5 Sources of further information and advice

Further information on fish oil composition and production technology of relevance can be obtained from 'Fish oil, technology, nutrition and marketing' (Hamilton and Rice, 1995), the electronic version of which is now available on-line at http://www.pjbarnes.co.uk

The development of stable isotope analyses is being continued under the EU funded TRACE project (TRACE, 2005–2009). Further information on authenticity and traceability, including access to the regular TRACE newsletter can be obtained from http://www.trace.eu.org.

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24

Farmed fish labelling

P. Najran,* Food Standards Agency, UK

24.1 Introduction: the importance of aquaculture and correct labelling

Global demand for fishery products is increasing as the health benefits of fish consumption become more widely known. At the same time, consumers are increasingly concerned to know how and where their food, including fish products, has been produced, along with any environmental impacts of production. Therefore, labelling rules for fish are becoming increasingly important.

The scale of global demand can be seen in figures compiled by the Food and Agriculture Organisation (FAO), which valued the global fish catch for 2000 at US$81 billion, and international fish trade to be worth US$55 billion. Human consumption of fish increased to 100.7 million tonnes in 2002, up from 93.6 million tonnes in 1998, and 2.6 billion people depend on fish as an important source of dietary protein.[1] Commercial aquaculture has been one of the fastest growing food production sectors globally since the mid-1980s, and provided nearly 50% of the annual world fisheries production of 120 million tonnes in 2004. Half of all aquaculture production is finfish, a quarter is aquatic plants and the remaining quarter is made of crustaceans such as shrimps, prawns and molluscs such as oysters, clams and mussels. The top ten species groups for aquaculture production in 2004 are summarised in Table 24.1.

Figure 24.1 illustrates total wild catch of fish (no shellfish or aquatic animals) compared to total aquaculture production, showing that there has been an increasing trend towards farmed fish production; indeed aquaculture production

* Any views expressed in this chapter are solely those of the author and not of the Food Standards Agency.

Table 24.1 Top 10 species groups for aquaculture production (not including aquatic plants), 2004[2]

Species group	Production (tonnes)	Production % of world total	Value (billion US$)
Carps and other cyprinids	18 303 847	40.3	16.4
Oysters	4 603 717	10.1	2.8
Clams, cockles, arkshells	4 116 839	9.1	3.3
Miscellaneous freshwater fishes	3 739 949	8.3	6.0
Shrimps, prawns	2 476 023	5.5	9.7
Salmons, trout, smelts	1 978 109	4.4	6.6
Mussels	1 860 249	4.1	1.0
Tilapias and other cichlids	1 822 745	4.0	2.2
Scallops, pectens	1 166 756	2.6	1.7
Miscellaneous marine molluscs	1 065 191	2.3	0.6
Other species	4 334 931	9.5	12.9
Total	**45 468 356**	**100.0**	**63.4**

Source: FAO Stats, 2004[2]

has nearly doubled in the period between 1984 and 2004. Asia makes up around 90% of total aquaculture production.[3,4] Table 24.2 shows aquaculture production by the top 20 producers in 2004, with China by far and away the largest producer with 30.6 million tonnes. In contrast (but still not an inconsiderable volume), UK farmed fish accounted for about 207,000 tonnes of the total supply in 2004

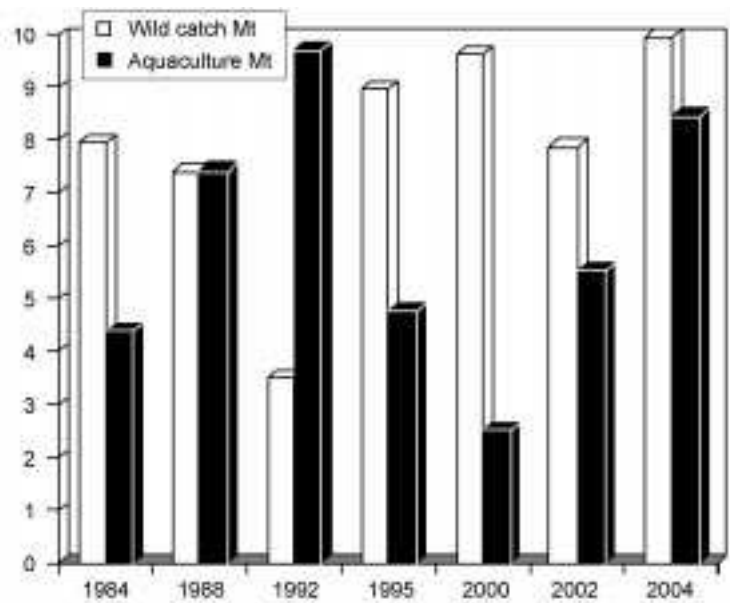

Fig. 24.1 Total wild and farmed fin fish.

Table 24.2 Global aquaculture production by top 20 producers, 2004[2]

Rank	Producer	Production (million tonnes)	% of global production
1	China	30.6	67.2
2	India	2.5	5.5
3	Vietnam	1.2	2.6
4	Thailand	1.2	2.6
5	Indonesia	1.0	2.2
6	Bangladesh	0.9	1.9
7	Japan	0.8	1.7
8	Chile	0.7	1.5
9	Norway	0.6	1.3
10	USA	0.6	1.3
11	Philippines	0.5	1.0
12	Egypt	0.5	1.0
13	Korea Republic	0.4	0.9
14	Myanmar	0.4	0.9
15	Spain	0.4	0.9
16	China, Taiwan	0.3	0.6
17	Brazil	0.3	0.6
18	France	0.2	0.4
19	UK	0.2	0.4
20	Malaysia	0.2	0.4

Source: FAO Stats, 2004[2]

with most of this attributable to production of farmed Atlantic salmon. Indeed, the year-round global supply of salmon and its popularity in consumers' diet has been as a result of the huge increase in its production by aquaculture.

Consumers rely on product labelling to provide them with accurate information, including product name and how it was produced (i.e., wild or farmed), so that they can make informed purchasing decisions that match their dietary preferences, lifestyle choices and cost/quality preferences. Correct labelling is also required to prevent misleading descriptions and food fraud. The majority of food products are generally correctly named and labelled, but the problem of unscrupulous traders substituting cheaper products for more expensive ones is not new. Recently, the fish escolar (also known as snake mackerel) was discovered being described as sea bass, which sells at a premium,[5] and DNA evidence was used to prosecute a fishing vessel which passed off cheap sand sole as expensive Dover sole.[6]

This chapter focuses primarily on labelling rules for fishery products as offered for retail sale to the consumer, rather than labelling rules that exist under European Union (EU) food hygiene legislation. The main focus is on labelling of farmed fish, but labelling requirements for wild fish also need to be considered in order provide the overall picture.

24.2 General food labelling legislation

EU food labelling rules have been developed from various statutes and are designed to ensure the principle of smooth functioning of the internal market, to inform and protect the consumer, and to protect agricultural markets and ensure EU market stability. The rules include laws pertaining to general food labelling applying horizontally across all foodstuffs, labelling for: nutrition, different food groups in marketing regulations, novel foods, certain processed foods in food composition rules, registration of unique characteristics (e.g., protected designation of origin (PDO), protected geographical indications (PGI), traditional recipes) and national labelling rules that are notified to the European Commission to ensure fair trade within the EU.

In the UK, consumer protection rules and food labelling are governed by the Trade Descriptions Act 1968 (which has been recently replaced*),[7] the Food Safety Act 1990 (FSA1990),[8] EC Food Labelling Directive 2000/13/EC (implemented via the Food Labelling Regulations 1996 (as amended))[9,10] and EC General Food Law Regulation 178/2002/EC (and the related parts of FSA 1990 (Amendment) Regulations 2004 and General Food Regulations 2004).[11–13] The overall effect of this legislation is to require that labelling information is a true indication of the nature, substance or quality of the food and to ensure that consumers are properly informed and not misled. These general rules also apply to advertising and presentation of food, which should not mislead consumers.

24.2.1 Trade Descriptions Act 1968

The Trade Descriptions Act 1968 applies throughout the chain of supply and makes it an offence for a trader to: apply a trade description to any goods which is false or misleading to a material degree; and supply, or offer to supply, any goods to which a trade description is applied which is false or misleading to a material degree.

24.2.2 The Food Safety Act 1990

The Food Safety Act 1990 provides the enabling powers under which all food regulations, including those on food labelling, are made. Section 14 of the Act makes it an offence for anyone to sell, to the purchaser's prejudice, any food which is not of the nature, substance or quality demanded. Section 15 of the Act makes it an offence to give or display a label with any food sold, or publish an advertisement, which falsely describes the food or which is likely to mislead as to the nature, substance or quality of the food as well as to sell any food which is misleadingly presented. These provisions apply throughout the chain of supply.

* As the book was going to press, most of the Trade Descriptions Act 1968 was replaced by the Consumer Protection from Unfair Trading Regulations 2008 implementing the Unfair Commercial Practices Directive 2005/29/EC. Similar provisions to the 1968 Act apply in the 2008 Regulations.

24.2.3 Council Directive 2000/13/EC and the Food Labelling Regulations 1996

Food labelling is harmonised in the EU by Council Directive 2000/13/EC relating to the labelling, presentation and advertising of foodstuffs. This is implemented in the UK by The Food Labelling Regulations 1996 (as amended). The regulations apply to most foods which are ready for delivery to the ultimate consumer or to catering establishments.

One of the key principles of the Directive is to inform and protect the consumer and it establishes that there be certain minimum mandatory information labelling of all foods placed on the market (see section on 'pre-packed foods'). Any other information given voluntarily or in accordance with specific EU or national rules must not mislead.

Article 2 of Directive 2000/13/EC requires that the labelling, advertising and presentation of a food must not be such as could mislead a purchaser to a material degree, particularly:

- as to the characteristics of the food and, in particular, as to its nature, identity, properties, composition, quantity, durability, origin or provenance, method of manufacture or production;
- by attributing to the food effects or properties that it does not possess;
- by suggesting that the food possesses special characteristics when in fact all similar foods possess such characteristics.

Pre-packed foods

When sold pre-packed, food must be labelled with the following mandatory requirements:

- the name of the food;
- a list of ingredients;
- the quantity of certain ingredients or categories of ingredients;
- the net quantity;
- an indication of minimum durability;
- any special storage conditions or conditions of use;
- the name and address of the manufacturer or packer, or EC seller;
- place of origin, if omission would mislead to a material degree with regard to its true origin or provenance;
- instructions for use, if appropriate use could not be made of the product without those instructions.

In general for pre-packed foods, the labelling particulars must be shown on the packaging, or on a label attached to the packaging, or on a label clearly visible through the packaging when sold to the ultimate consumer; particulars may be provided in relevant trade documents on or before delivery when the food is sold to a catering establishment. In all cases, the particulars must be easy to understand, clearly legible and indelible, and when the food is sold to the ultimate consumer, they must be in a conspicuous place so as to be easily visible. They must not be hidden, obscured or interrupted by any other written or pictorial

matter. When a datemark and/or the net quantity is required, they must appear in the same field of vision as the name of the food (see Fig. 24.3 on page 601).

Non-prepacked foods

It is open to EU Member States to determine to what extent the requirements of the EC Food Labelling Directive should apply to non-prepacked and similar foods, provided the consumer still receives sufficient information. In the UK, in recognition of the practical difficulties, minimal labelling rules apply. Generally, such food need only be marked or labelled with the name of the food and the category name of certain additives if present in the food as well as labelling as appropriate if these foods or ingredients have been irradiated or derived from genetic modification. That said, it will be seen that the fish labelling rules apply to both pre-packed and loose fishery products sold at retail.

Non-prepacked foods sold at catering premises for immediate consumption are not required to be labelled with any particulars. However, under the general principles of food and trade descriptions law, any descriptions which are applied must be accurate and not misleading.

Name of the food and ingredients list

The name of the food and ingredients list are important information for indicating the nature, substance and quality of food. There are names which either EC or UK law has specified must be used for certain foods. If there is such a name prescribed by law (e.g., milk, butter, fish names, etc.), this must be the name that is used. If no prescribed name exists, a customary name may be used; a customary name is one which has come to be accepted over time by consumers. In other cases, a descriptive name precise enough to indicate the true nature of the product and distinguish it from other foods must be used. The name of the food must include, or be accompanied by, an indication of its physical condition (e.g., powdered) or treatment (e.g., dried, frozen or concentrated) where a purchaser could be misled by the omission of that information. A complete list of ingredients is generally required in descending order by ingoing weight. The name used for an ingredient must generally be a name which can be used if the ingredient were being sold as a food by itself.

Historically, in the UK prescribed names for specific fish species have existed in national food labelling regulations dating back to the 1980s and earlier. These required that the prescribed names be used to describe certain fish species, for example that sole is from the species *Solea solea* or that mackerel must come from any species of *Scomber* only. This national list of designated names was taken forward, being replaced and repealed by the new EU rules (see Section 24.3.2).

24.2.4 General food law regulation, 178/2002/EC

Council Regulation 178/2002/EC provides the basis for public health protection and consumers' interests in relation to food taking due account particularly of

diversity in supply of food. It establishes general principles and requirements of food law and food safety procedures as well as establishing the European Food Safety Authority. The Food Safety Act 1990 (Amendment) Regulations 2004 and the General Food Regulations 2004 align domestic legislation with the general principles and requirements of Regulation 178/2002, and introduce new enforcement provisions.

Articles 8, 14, 16, 18 and 19 of the EC Regulation are of particular interest as they establish consumer protection principles, food safety requirements, food presentation, traceability and responsibilities of food businesses in terms of notifying, withdrawing and/or recalling products not complying with food safety requirements. Article 16 on 'presentation' requires that the labelling, advertising and presentation of food shall not mislead consumers and it applies in addition to the 2008 Regulations replacing the Trade Descriptions Act 1968 and sections 14 and 15 of the Food Safety Act 1990.

The purpose of the traceability provision at Article 18 is to facilitate targeted and accurate withdrawals and recalls of unsafe food. All food businesses must meet the traceability requirements of this Regulation, namely to be able to identify who supplies to them and to whom they have supplied their products (i.e., one step back – one step forward). This information must be made available to the competent authorities on demand. Article 18 traceability provisions apply in addition to any more detailed sector-specific requirements in separate legislation such as in the case of traceability requirements for certain fishery and aquaculture products (see Section 24.3.6).

24.3 Fish labelling legislation

Rules on fish labelling are also covered by two European regulations, Council Regulation (EC) 104/2000[14] and Commission Regulation (EC) 2065/2001.[15]

Council Regulation (EC) 104/2000 relating to the common organisation of the markets in fishery and aquaculture products[14] incorporates specific labelling requirements at Article 4. Like other marketing regulations, the major objectives stated in the 'recitals' (i.e. 'whereas' clauses) are the need to ensure market stability, emphasising the importance of the fishing industry especially to the economy of some coastal EU regions, to promote sustainable fishing through the production and marketing of fishery products, and to apply common marketing standards to ensure product quality and fair competition. The recitals also state that consumers must be provided with 'a minimum amount of information on the main characteristics of products' and that Member States must adopt a list of accepted names for use in their territory for trading in the relevant products (see Section 24.3.2).

Council Regulation (EC) 104/2000 is considered to be the 'parent' regulation, as it provides the basis for labelling fish sold at retail to the final consumer. Since its main purpose is as a marketing standard to protect specific markets, it should be noted that the labelling requirements are linked only to those fishery

products and aquaculture products covered by Chapter 3 of the EU Customs Code Combined Nomenclature (CN codes). This has created some anomalies for fish labelling, since whether or not a product needs to be labelled with consumer information depends on its presentation and if it is caught by the relevant customs codes.

Article 4 of Council Regulation 104/2000 aims to improve transparency and knowledge of fishery products to consumers. Specifically, it requires all fishery and aquaculture products that are marketed within the Community to be labelled at the point of retail sale to the final consumer with *all* of the following information:

(i) the commercial designation of the fish/shellfish (i.e. an agreed commercial name for that species);
(ii) the production method (i.e. whether it is farmed or wild, and if wild, whether caught at sea or inland waters);
(iii) the catch area (i.e. an area of the ocean in the case of sea caught fish, or country of production* in the case of farmed fish or fish caught in inland waters).

For the purposes of Council Regulation 104/2000, 'fishery products' means both products caught at sea or in inland waters, while 'aquaculture' is defined in Council Regulation 2792/1999 (referred to at Article 4(3) of Regulation 2065/2001) as the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants.

Commission Regulation 2065/2001 provides more detailed requirements for the labelling provisions, not only in terms of production method and geographic origin but also for traceability purposes. The Fish Labelling Regulations, 2003, in England, Scotland, Wales and Northern Ireland, enable the EC Regulations to be enforced;[16–19] these regulations make it an offence for certain fish or shellfish to be offered for retail sale without the product labelling providing the required consumer information or the relevant information for traceability and control purposes.

24.3.1 Scope

The consumer information is required for fish and shellfish whether it is sold at retail to the final consumer loose from fish counters or pre-packed, and applies to fishery products sold under the following Chapter 3 EU customs code presentations:

- live fish;
- fresh, chilled and frozen fish (whether whole, gutted, de-tailed, etc.);
- fish fillets and other fish meat (whether minced or not);
- dried, salted or brined fish;
- smoked fish (whether hot or cold smoked fish);

* Country of production means EU Member State or third country of origin, the latter being a country that is not an EU member.

Table 24.3 Fishery and aquaculture categories (in accordance with Council Regulation 104/2000)

CN Code		Description of goods
(a)	0301	Live fish
	0302	Fish, fresh or chilled, excluding fish fillets and other fish meat of heading No 0304
	0303	Fish, frozen excluding fish fillets and other fish meat of heading No 0304
	0304	Fish fillets and other fish meat (whether or not minced), fresh, chilled or frozen
(b)	0305	Fish dried, salted or in brine; smoked fish whether or not cooked before or during the smoking process; flours, meals and pellets of fish, fit for human consumption
(c)	0306	Crustaceans, whether in shell or not, live, fresh, chilled, frozen, dried, salted or in brine; crustaceans in shell, cooked by steaming or by boiling in water, whether or not chilled, frozen, dried, salted or in brine; flours, meals and pellets of fish, fit for human consumption
	0307	Molluscs, whether in shell or not, live, fresh, chilled, frozen, dried, salted or in brine; aquatic invertebrates other than crustaceans and molluscs, live, fresh, chilled, frozen, dried, salted or in brine; flours, meals and pellets of aquatic invertebrates other than crustaceans, fit for human consumption

- crustaceans (except crustaceans which are both cooked and peeled); and,
- molluscs (except cooked molluscs).

The regulations therefore apply to all uncooked fish to which no other ingredients (except salt, smoke) have been added and which have simply undergone physical processing, such as slicing, flaking, cutting, etc. Examples include fish that has been flaked or diced to be sold as such to the consumer to add to recipes or sandwiches, tuna or salmon steaks, sliced fish (e.g., smoked salmon slices), shellfish and frozen fish blocks not treated in any way.

Table 24.3 shows the full list of fishery and aquaculture products to which Regulation 104/2000 applies, as described by Chapter 3 of the Customs Codes.

Fish labelling rules are enshrined in a marketing regulation which defines product scope by customs codes, and some anomalies exist. One example is the labelling of crustaceans. Cooked unpeeled prawns and uncooked peeled prawns must be labelled with the required consumer information, but prawn products which are both cooked and peeled are not subject to the labelling rules because they are not listed as a Chapter 3 product in this presentation. Why would the consumer not be equally interested in this product's place of origin simply because it has been subject to processes of both cooking and peeling? This is an area that perhaps needs to be considered in any future review of the existing rules.

24.3.2 Commercial designation

EC marketing regulations set out names and descriptions which must be used for certain foods when they are marketed to the consumer, and Council Regulation 104/2000 is no exception. It requires Member States to establish and publish a list of commercial designations for fish species accepted within their territory. Fish and shellfish must be named using the accepted commercial designation for the country in which they are to be sold.

The commercial designation is the common name prescribed by law and is determined by the international, scientific (Latin) name of the fish species. For example, only fish of the species *Hippoglossus hippoglossus* (L.) can be described as 'halibut', no other fish species can use this name. Similarly, 'scampi' is one of the accepted commercial designations for the species of scientific name, *Nephrops norvegicus* (L.). This species is also known by other agreed prescribed names of 'Norway lobster', 'Dublin Bay prawn' and 'Langoustine'. Labelling rules require that where there is a name prescribed by law for a food, that is the name that must be used in the labelling of the food whether sold individually or in a composite (e.g., coated) fish product. A product can be called 'scampi' or one of the alternatives if it is from the flesh of species *Nephrops norvegicus* (L.) only. For composite products, general food labelling rules apply which means that the fish ingredient can be simply referred to by the generic name, 'fish', provided the label does not mention the specific fish species name, e.g. 'fish pie' as opposed to 'cod pie'. However, if the fish pie was marketed as 'cod pie', then because the type of fish is given and this is an agreed prescribed name, it must be from one of the fish species agreed for the common name, cod.

The purpose of the commercial designations list is to ensure fish species are correctly described as regards their natural composition, as identified by the scientific name, so that consumers can be confident in what they are buying. In the UK, this list takes the form of a schedule to the national regulations and currently includes over 350 fish species, documenting the commercial names under which each species must be sold. The national schedule of commercial names was reviewed and updated in 2005 to incorporate newly commercialised species, and to add changes to existing commercial names that had arisen in the light of new scientific information. These revisions came into force in 2006 through an amendment to the Fish Labelling Regulations.[20–23] Given that new fish species are being commercialised all the time, it is unlikely that this will be the last such revision.

The commercial designations legislated in the UK are compiled and agreed following consultation with experts in fish taxonomy and by referring to authoritative fish name references, names in internationally recognised global databases of aquatic species. A public consultation is also carried out on any proposed changes to the national legislation, including any revisions to the fish names list.

The commercial designation can be made more precise by qualifying it with additional words, for example *Lutjanus campechanus* must be called 'snapper'

but could also be called 'red snapper'. Also, with the exception of salmon, smoked fish may be called by a customary name. For example, *Clupea harengus* (L.) would ordinarily be described as 'herring', but when smoked is known by its customary name of 'kipper'.

Member States must recognise the commercial designations laid down by other Member States for the same species in the same language. This means that the UK would need to accept any English fish names established by the Republic of Ireland as alternatives to those on the UK lists and vice versa.

It is important to note that there is no mandatory requirement to give the scientific (Latin) name of the fish at point of retail sale to the consumer, but a business can do so if it wishes. However, the scientific name is required in traceability papers at each stage of marketing (see Section 24.3.6).

If a business does choose to use the scientific Latin name, it must be used *in addition* to the commercial designation and not as a substitute for it. For example, *Oncorhynchus mykiss* for 'rainbow trout' as shown in the mocked-up label in Fig. 24.3 on page 601.

The Food Standards Agency (FSA), as the competent authority for the UK, is responsible for establishing and updating the list of agreed commercial designations. Any new species not included on the list may be marketed under a provisional commercial designation agreed and laid down by the competent authority. The provisional name can be used for up to five months, after which, if approved, it must be accepted as a definitive commercial designation to be added to the national list of legal names. Where, for whatever reason, no definitive or provisional commercial designation has been granted, the name used must describe the true nature of the product accurately in accordance with general labelling rules. In such cases, the producer should apply for a provisional designation as soon as is practicable with the ultimate aim of making it definitive.

24.3.3 Production method

The production method relates to the manner in which the fish is 'harvested', that is whether it is produced by aquaculture (i.e. farmed) or caught at sea or in freshwater.

Article 4 of Regulation 2065/2001 stipulates how the production method should be declared to the consumer:

- *for fish/shellfish products of aquaculture,* the terms 'farmed' or 'cultivated' must be used to indicate that they have been farmed;
- *for products caught at sea or in freshwater*, the terms 'caught' or 'caught in freshwater' must be used. However, these terms can be omitted if it is clear from the name that the fish is wild, e.g. North-East Atlantic haddock.

In the UK, it is recommended that the term 'farmed' is used for fish and the term 'cultivated' is used for shellfish production. It is also recommended[24] that the production method be given equal prominence alongside the commercial

designation, so that consumers are provided with meaningful information to help with their purchasing decision, for example 'farmed Scottish trout' (see Section 24.3.7).

24.3.4 Catch area

For fish and shellfish caught at sea, one (or more) of 12 catch areas, based on FAO statistical classifications, must be declared (see Table 24.4). Figure 24.2 shows a map identifying the FAO catch areas. For example, the catch area, 'North-East Atlantic', would need to be indicated on the label for any fish/shellfish caught off the UK (e.g., North Sea, Irish Sea), Norway, Iceland or Denmark (West Coast), while fish caught on the east coast of Denmark or around the Danish islands would be labelled 'Baltic Sea'. Fish caught in Australian waters would be labelled 'Pacific Ocean' or 'Pacific', while fish caught off Canada would cite the catch area 'North-West Atlantic' on the label.

The regulations require that the label shows the name of the catch area (i.e. ocean, sea) rather than the numeric designation. It is considered acceptable to abbreviate catch areas, e.g. North-East Atlantic may be written as N.E. Atlantic. For traceability purposes in commercial documents, the numeric identification of the catch area is considered sufficient.

For products caught in freshwater, the origin must indicate the EU Member State or third country of origin. For example, trout caught in the freshwaters of Norway would need to be labelled as 'Norwegian trout' or 'trout caught in the freshwaters of Norway'.

For farmed and cultivated fish and shellfish products, the origin *must* indicate the country in which the product underwent 'final development'. Although not legally defined, the term 'final development' is understood to mean when the

Table 24.4 FAO catch areas[25]

Catch area	Identification of the area[a]
North-West Atlantic	FAO area 21
North-East Atlantic[b]	FAO area 27
Baltic Sea	FAO area 27.IIId
Central-Western Atlantic	FAO area 31
Central-Eastern Atlantic	FAO area 34
South-West Atlantic	FAO area 41
South-East Atlantic	FAO area 47
Mediterranean Sea	FAO areas 37.1, 37.2 and 37.3
Black Sea	FAO area 37.4
Indian Ocean	FAO areas 51 and 57
Pacific Ocean	FAO areas 61, 67, 71, 77, 81 and 87
Antarctic	FAO areas 48, 58 and 88

[a] FAO Yearbook (2000).[26]
[b] Excluding the Baltic Sea.
Source: reproduced from Annex of Commission Regulation (EC) No 2065/2001.

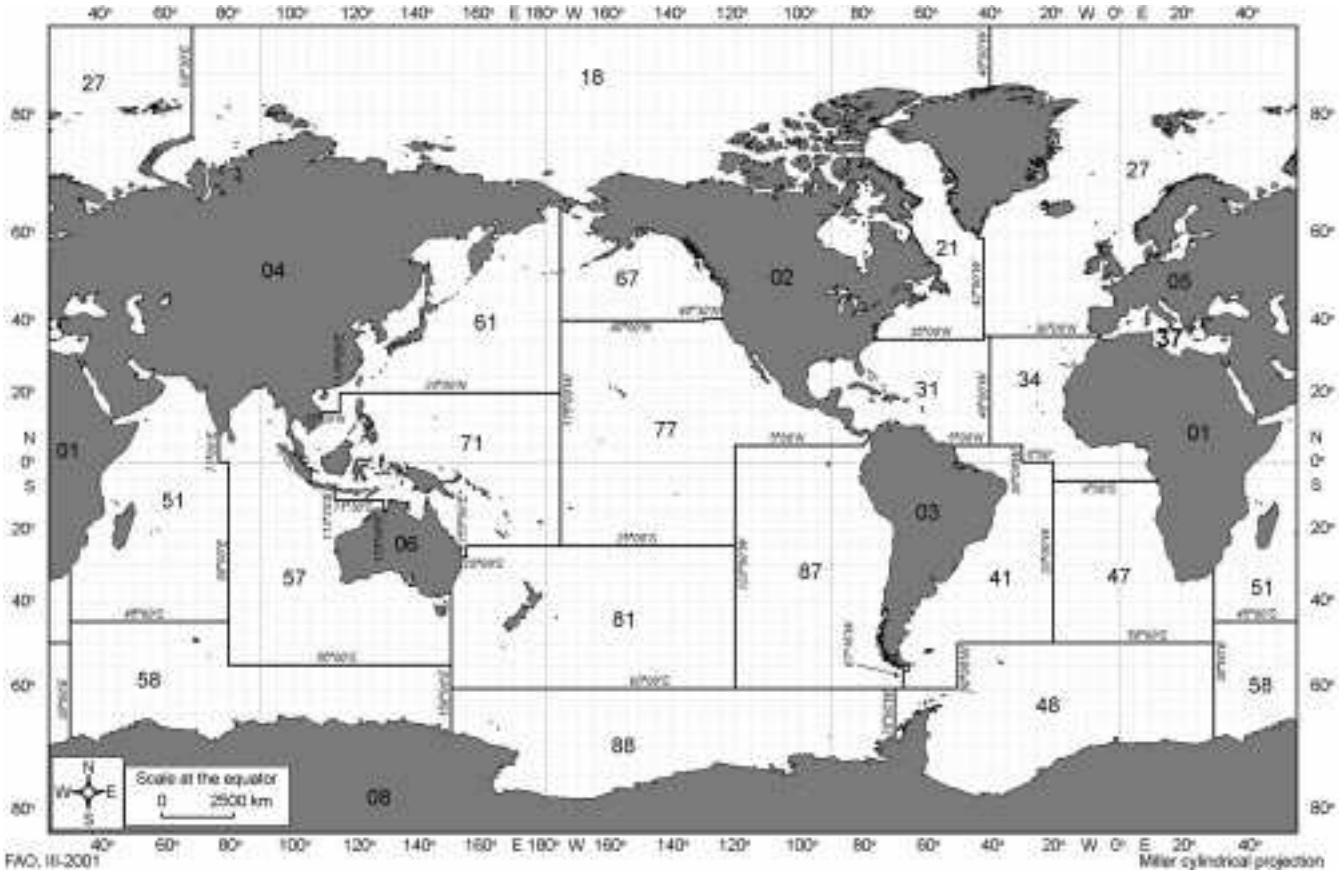

Fig. 24.2 FAO map of major world fishing areas.[26]

fish is 'harvested' from the water upon reaching its final size (that is, full maturity or any earlier stage to which it is being grown for sale to the final consumer). Thus, if sea bass started its life farmed in France and Italy but was 'finally farmed' in Greece, the labelling is required to state, for example, 'Farmed Sea bass' or 'Greek sea bass farm reared in Greece'. That said, separate country of origin labelling guidance[27] advises that labelling should show all countries so that consumers are given accurate and meaningful information on the true origins of the fish. Therefore, in the aforementioned example, it is recommended that the product should be labelled as 'Farmed Greek sea bass reared in France and Italy'.

Furthermore, national legislation has adopted the EC provision that allows a product that has been farmed in more than one EU Member State or third country to indicate these countries on the labelling. This is not a mandatory requirement but the option to list all countries of origin on the label can be taken up.

A more precise geographic origin in terms of catch areas or production area can be given under Article 5(2) of Regulation 2065/2001. However, the FAO marine catch area for fish caught at sea or, in the case of farmed fish, the Member State or third country of production, must be declared even if a more precise area is also given. Therefore, a product could be labelled as 'Farmed Scottish Cod', but as Scotland is not the Member State, the label will still need to give an indication that the product came from the United Kingdom. Similarly, Cornish mackerel would still need to indicate that it has been caught in the N.E. Atlantic.

There is some flexibility in how the two pieces of information are given on the label, in that they do not necessarily have to be located together. For example, 'Farmed Scottish Cod' could be the product name on the front of the pack, with reference to the UK on the back. For loose products, the information could also be given by displaying a notice in the area where the product is sold (see Section 24.3.8).

24.3.5 Labelling of different types of fish batches

For products containing a mixture of different species, the product must be labelled with the commercial names, production methods and catch areas of each and every species included in the product. For example, a seafood mix comprising salmon, haddock and prawn must give the required information for each of these three species.

Mixtures of the same species originating from several production methods must be labelled with each production method in the order in which origin predominate. An example of such labelling may be, 'a mix of farmed Norwegian cod and cod caught in the N.E. Atlantic'.

In contrast, fish mixtures of the same species but originating from different catch areas or fish farming countries must declare the origin that is most representative quantitatively, together with an indication that the product also comes from different areas but without listing all the other catch areas. Businesses

must judge whether their product labelling is accurate and not misleading to the consumer. For example, a batch of 'farmed haddock' originating predominantly from Norway but also from Iceland or Canada could be described as 'farmed haddock originating from Norway, Iceland and Canada'. This would be consistent with separate guidance on country of origin labelling, which suggests that all countries be indicated in product labelling for meaningful consumer information. Clearly, if a single country of origin can be traced throughout the supply chain, then the product should be labelled with that single country of origin.

Further examples of different scenarios for mixed fish batches of the same species and their associated labelling requirements are shown in Table 24.5, reproduced from FSA fish labelling guidance.[24]

24.3.6 Traceability requirements

The EC rules require all businesses to provide traceability information. This means that information on the commercial names, production method, catch area and scientific name of the species must all be available at each stage of marketing. The scientific (Latin) name of the fish species is not mandatory at retail sale but is required in traceability papers at each stage of marketing, when the product changes ownership along the distribution chain.

The term, 'each stage of marketing' is considered to cover all stages of the supply chain from production, from when the fish is first caught, landed or harvested, to the point of retail sale, and includes each point along the distribution chain where ownership changes hands, for instance from producer to processor, to distributor, wholesaler to retailer, caterer, etc. Importation is also regarded as a 'stage of marketing' where labelling information is needed. As retail sales are specifically controlled by the consumer labelling requirements, they are not considered a 'stage of marketing' in this context.

Traceability information can be given in a number of ways and can include:

- labelling or packaging of the product itself;
- commercial documents accompanying products, including the invoice or sales note.

It is important to note that the traceability requirements under Regulation 2065/2001 apply for this specific product sector in addition to the general traceability requirements for all foods under the EC General Food Law Regulation 178/2002 (see Section 24.2.4). The main difference is that, under fish labelling rules, it is the product details that must be passed on at each stage of marketing, while the traceability requirement under 178/2002 requires food businesses to identify their suppliers and those to whom they have supplied products (i.e., one step back – one step forward).

24.3.7 Format for labelling information

Businesses have some flexibility on where information is located because there are no specific provisions as to where and how the name, production method and

Table 24.5 Mixed batches of fish of the same species – labelling requirements[24]

A number of scenarios can be envisaged. Using, for example, a mixture of fish of the same species such as Cod obtained predominantly from the UK either caught (catch area being the North-East Atlantic) or farmed but same species Cod from other areas present also (e.g. Norway, Baltic Sea). The following scenarios with their labelling requirements are outlined:

Species/ Commercial designation (CD)	Production method (PM)	Catch area (CA)	Labelling information requirements
Same species	Same PM	Same CA	Single entity therefore labelling should indicate: – CD, PM, CA e.g. ''North Sea Cod'' NE Atlantic indicated somewhere on the label
Same species	Different PM	Different CA	CD all PMs main CA + others e.g. 'A mix of both caught and farmed cod, mainly from the UK but also from other areas' (but we *recommend* that other CAs or countries are named) NE Atlantic required to be indicated somewhere on the label but we *recommend* also referring to the Baltic Sea
Same species	Same PM	Different CA	CD PM main CA + others e.g. 'A mix of cod from the N. E. Atlantic, but also from other areas' (but we *recommend* that other CAs or countries are named) CA already indicated in the name but we *recommend* also referring to the Baltic Sea
Same species	Different PM	Same CA	CD all PMs CA e.g. 'A mix of NE Atlantic cod and UK farmed cod' or 'NE Atlantic cod' or 'UK farmed cod' depending on whether it is a fish or piece of fish from such a batch. If use Scottish cod, then NE Atlantic or UK indicated somewhere on the label if not already in the product name as above

Source: Reproduced from FSA Fish Labelling Guidance, March 2003.

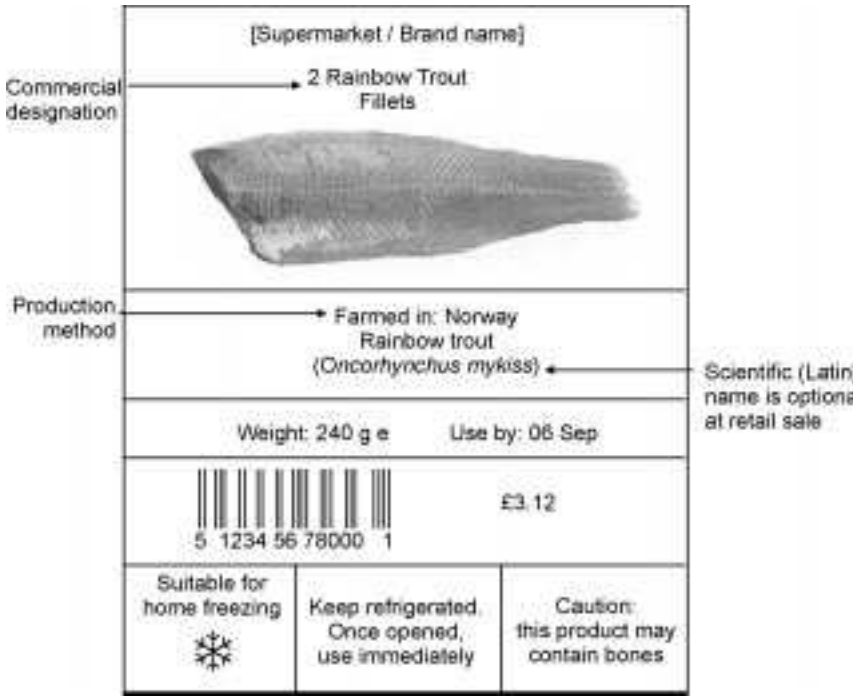

Fig. 24.3 Example of retail label showing relevant consumer information on front of pack.

catch area should be declared on the label. However, in line with general labelling rules, fish labelling information should be presented so that it is easy to understand, clearly legible, indelible and easily visible. Information on production method and catch area should be accurate and meaningful. It is recommended that the information is located where it is most informative and appropriate for both the consumer and producer/retailer and consistent with general labelling requirements. Figure 24.3 illustrates an example of a mock food label illustrating consumer information in accordance with the regulations.

24.3.8 Labelling of products sold loose

Since the rules apply to fishery and aquaculture products offered for retail sale to the final consumer, products not pre-packed but sold loose at supermarket fish counters, fishmongers and market stalls must also give consumers the required information. In line with general food labelling rules, the information, whether it be on a label or ticket attached to the food or on an in-store notice or poster, should be 'readily discernible' to the consumer.[10]

Best practice advice recommends that, where farmed fishery products are being marketed, the production method should be indicated next to the product name to provide meaningful consumer information.[24]

Catering establishments are exempt from consumer information requirements

for products that are ready to eat as they do not constitute a retail sale. The exemption applies only to sales of products that are ready to eat, since there may be instances where catering businesses sell retail products as well as products ready for consumption. In such cases, the business would still need to provide the required labelling for those fish products that were sold at retail. If a fish product is called by its specific name (e.g., 'cod and chips' rather than 'fish and chips') in a catering establishment and there is a name for it prescribed by law, the agreed commercial designation must be used to describe the product (see Section 24.3.2).

24.3.9 Exemptions

The Regulations do not apply to fish that has been further processed, preserved, treated or cooked. Marinated or canned fish, such as canned tuna, sardines, pickled cockles and poached salmon fillets, does not have to meet the consumer information requirements under fish labelling rules.

Also outside the regulations is fish to which other ingredients have been added, such that the fish becomes an intrinsic part of the end product. These are classed as composite products and are not Chapter 3 products. Examples include coated, breaded or battered products, such as fish fingers or coated scampi, fish with colouring, surimi-based preparations such as crabsticks, ready meals, fish pies, sandwiches (e.g., tuna sandwiches) and rollmops.

Other products outside the regulations include smoked fish with ingredients additional to those of the smoking process, such as smoked salmon treated with honey and sugars, and smoked mackerel with added colouring. In all these cases, the products are still subject to the general food labelling rules.

Small quantities of fish sold directly to the final consumer, either by fishermen (e.g., from the quayside) or aquaculture producers (e.g., from lakes, ponds, etc.), are specifically exempt from the information requirements. The term 'small quantity' is taken to mean sales not exceeding 20 Euros.

Finally, it should be noted that fish and shellfish products sold by caterers or catering establishments are also not covered by the labelling requirements. This includes restaurants, canteens, takeaways, etc., where the product is sold ready for consumption without any further preparation (see Section 24.3.8).

24.4 Scottish Farmed Salmon: a special case

Fish labelled as 'Scottish Farmed Salmon' must meet specific requirements under the EU protected food name regulations, which take precedence over EU fish labelling regulations.

Council Regulation (EC) No 510/2006 (which repeals and replaces EC Regulation 2081/92) provides a system to protect food names for agricultural products and foodstuffs where a link exists between the product in question and a defined geographical area.[28–30] Under this system, a named food or drink

(separate arrangements exist for wines and spirits) registered at the European level will be given legal protection against imitation throughout the EU. There are two different types of geographical description namely, 'protected geographical indications' (PGI) and 'protected designations of origin' (PDO). PGI products must have been produced or processed or prepared within the geographical area and have a reputation, features or certain qualities attributable to that area.

The application to register the name, 'Scottish Farmed Salmon', was made by Scottish Quality Salmon (now known as Scottish Salmon Producers Organisation). Following assessment of the application by the Department for Environment, Food and Rural Affairs (Defra) and the European Commission, Scottish Farmed Salmon was registered as a PGI product under Commission Regulation (EC) No 1437/2004.[31] Therefore, since 31 August 2004, only fish products conforming to the registered PGI specification (i.e., salmon from the species *Salmo salar* and, among other criteria, farmed in the defined geographical area of the western coast of mainland Scotland, Western Isles, Orkney and Shetland Isles) can be labelled as Scottish Farmed Salmon. This requirement is independent of the EU fish labelling regulations, given that the European Commission has clarified that the PGI rules take precedence. However, whilst the PGI takes precedence it does not preclude the need to comply with the fish labelling rules with respect to stating the Member State or third country of origin on the product label.

The main purpose of the PGI is to protect the name, 'Scottish Farmed Salmon' against misuse and to provide consumers and others in the food chain (e.g., suppliers, retailers, catering establishments, etc.) with confidence in the product being bought. The PGI protects the name whether it is used as a product name in the title or as text elsewhere on the label. Any salmon farmed in Scotland that does not meet the PGI specification cannot use the name 'Scottish Farmed Salmon' (or any synonyms of it such as 'farmed Scottish salmon', 'salmon farmed in Scotland', etc.).

As already mentioned, fish labelling regulations do require that the Member State or third country of origin be given on a product and, in EU terms, that means 'UK' as Scotland is not a Member State. Accordingly, salmon meeting the PGI specification would still need a declaration somewhere on the label that the product was farmed in the UK. Producers not meeting the PGI specification could still fulfil the requirements of fish labelling rules by using the term, 'farmed in the UK'. Alternatively, producers of salmon farmed in Scotland that does not comply with the PGI specification might use the term, 'produce of Scotland', together with reference to the UK somewhere on the label. However, the term, 'Scottish Farmed Salmon', cannot be used to describe the product.

A key requirement under the protected food name scheme is that producers wishing to use the protected name must be audited to ensure that they are conforming to the registered specification. Food Certification Scotland Ltd is the approved independent inspection body for Scottish Farmed Salmon.

Products covered by the PGI include not only whole or gutted salmon but

also salmon fillet, steak portions, smoked salmon, salmon pate, and ready meals and other dishes where Scottish farmed salmon is the main ingredient and is listed as such on the label. However, if salmon is only a minor ingredient and is not referred to as Scottish farmed salmon, then the PGI does not apply.

The PGI specification acknowledges that, in order to avoid discriminating against Scottish wild salmon interests, the use of the names, 'Scottish smoked wild salmon' and/or any combination of the terms 'Scottish' and 'salmon' in terms of wild salmon can continue to be used.

A UK application to amend the 'Scottish Farmed Salmon' PGI specification to cover organically farmed salmon has been submitted to the European Commission under Article 9 of Council Regulation No 510/2006 and is currently being considered.

24.5 Eco-labelling

Consumers are increasingly keen to make food purchasing decisions based on animal welfare considerations and the environmental impact of the production process. Equally, producers, manufacturers and retailers wish to show that their food has been produced in a sustainable way, and that they have taken due regard of environmental, ethical and social issues. This is exemplified by the growing popularity of 'fair-trade' and 'organic' labels on foods generally.

The increasing demand for products that incorporate environmental and ethical considerations has led to an extensive range of 'eco-labelling' or 'environmental labelling' schemes. Generally, such schemes provide information about the environmental aspects of products or services, such as protection of natural resources, biodiversity and habitats, minimising energy and water in manufacturing, and waste management and recycling. There is no single label which covers all aspects of a product's environmental credentials. Different labels and certifications exist in the marketplace to address different needs and concerns, tailored to specific products.

The International Standard Organisation (ISO) has developed guides (ISO 14020-14025) for environmental labels and declarations.[32–34] Eco-labelling falls under type I – environmental labelling (ISO 14024). These are voluntary schemes aimed at reducing environmental effects and are verified by a third party. The third party, an independent individual or organisation, verifies that the product meets meaningful and consistent standards for environmental protection, and this should be indicated on the label. Type II are self-declared environmental claims (ISO 14021), which are made without independent third party certification, and type III are environmental declarations mainly for use in business-to-business communication (ISO 14025).

Within the fisheries sector, most eco-labelling schemes are private initiatives that make various environmental claims. Examples of marine labelling schemes in the UK include the Tartan Quality Mark (TQM) label, which assures that the salmon is Scottish and that production methods are independently inspected at each stage, the Freedom Food logo, which is currently the only animal welfare

label specifically for farmed Atlantic salmon and requires producers to be approved to RSPCA standards, and the Marine Stewardship Council (MSC), which has developed an international environmental standard for sustainable and well-managed fisheries. These examples and others, together with their corresponding logos and accrediting bodies, are illustrated in Table 24.6.[35]

Given that eco-labelling schemes in the fisheries industry have so far been managed by the private sector, the main question for legislators is how far eco-

Table 24.6 Examples of marine labelling schemes in the UK

Issuing or accrediting body	Assured scheme	Logo	Products
Soil Association Certification Ltd	Soil Association	Code: organic certification UK 5	Farmed fish, dairy foods, meat, etc.
Organic Food Federation	Organic Food Federation		Farmed fish, meat, etc.
Food Certification Scotland (FCS)	Tartan Label (Scottish Salmon Producers Org)		Farmed salmon
Freedom Food Ltd	Freedom Food RSPCA monitored		Meat, poultry, dairy eggs and farmed salmon
Marine Stewardship Council (MSC)	Marine Stewardship Council		Wild-capture marine seafood www.msc.org
Earth Island Institute (EII)	Dolphin Safe	See EII for details	Canned tuna

Source: Fishonline, published by the Marine Conservation Society: www.fishonline.org. Logos reproduced by kind permission of accrediting bodies.

labelling schemes should be controlled and legislated, with a view to protecting consumer interests through a common approach.

24.5.1 International work on eco-labelling

The FAO agreed that potential eco-labelling guidelines should be consistent with the 1995 FAO Code of Conduct for Responsible Fisheries.[36] In 2005, the FAO issued voluntary guidelines on eco-labelling for fish and fishery products.[37] These aim to provide guidance to governments and organisations by outlining general principles so that eco-labelling schemes are based on best scientific evidence, transparent standards, and independent auditing and verification. Minimum requirements and criteria are also specified for assessing whether a fishery can be certified and an eco-label granted. The guidelines define eco-labelling schemes as entitling 'a fishery product to bear a distinctive logo or statement which certifies that the fish has been harvested in compliance with conservation and sustainability standards. The logo or statement is intended to make provision for informed decisions of purchasers whose choice can be relied upon to promote and stimulate the sustainable use of fishery resources.'

The World Trade Organisation's (WTO) Committee on Trade and Environment has also been looking at standards for voluntary environmental labelling schemes. WTO members acknowledge that such labelling schemes can be useful for informing consumers, but they should not create any unnecessary barriers to trade and allow access to markets, particularly for developing countries.[38]

24.5.2 European initiatives on eco-labelling

In 2005, the European Commission launched a consultation on three possible options for a Community approach to eco-labelling,[39] to address questions of competition (mutual recognition of eco-labelled products), trade (unjustified barriers to trade), and consumer protection policies (conflicting or inadequate/misleading schemes). The proposed options were: (i) no action, (ii) creation of a single Community eco-labelling scheme, and (iii) establishment of minimum requirements for voluntary eco-labelling schemes. In its consultation paper, the Commission expressed a preference for the third option, voluntary minimum requirements, as the one that was most proportionate in terms of costs, flexibility and accessibility for small and medium-sized businesses (SMEs) and small fisheries, and consumer protection. The consultation has involved not only Member States and European institutes (i.e. the European Parliament, Council, European Economic and Social Committee), but also a wide range of stakeholders, including the fishery sector, scientific community and consumers.

At the Agriculture and Fisheries Council meeting in April 2007, Ministers confirmed a preference for European legislation on fisheries eco-labelling to consist of the establishment of minimum requirements for voluntary schemes. The Commission will now issue a progress report on the consultation process and publish proposals for a legislative framework in 2008.

Clearly, any eco-labelling scheme needs to make it easy for consumers to readily understand and differentiate between fishery products on sale. Any such scheme needs to make it clear what the eco-label is certifying, in particular whether the label refers to a method of fishing, certain fishing practices, a particular fish stock, or whether the fish originates from a specific fishing area, or a combination of these. Whatever criteria are adopted for eco-labels, harmonised minimum voluntary standards would reassure consumers that eco-labelling schemes follow minimum requirements and principles.

24.6 Future trends

EC labelling requirements, particularly with regard to production method and geographic origin, are still relatively new. A recent FSA survey on retail labels found that the majority (71%) of businesses provided information on whether the fish was 'farmed' or 'wild' and its geographic origin in accordance with legal requirements, but the remainder provided either no information, partial information, or information that did not meet the requirements.[40] These findings suggest that awareness of the legal requirements needs to be raised, particularly among SMEs. In response, the FSA has published a quick reference guide to key fish labelling requirements.[41]

The demand for seafood is expected to grow at 1.5% per year through 2020.[42] Labelling will have to evolve, because wild fish catches will not increase and so the rising demand for fish will have to be met by aquaculture, involving new production techniques and new species being farmed. Added to this is the possibility of commercial production of genetically modified (GM), or transgenic, fish for food use.

These developments will present challenges for product presentation and labelling. Information must be accurate, giving the consumer not only the correct names of new farmed species, but also how these fish have been produced. Information on new aquaculture techniques or novel processes used in their production must be provided. There may also be legal requirements to indicate method of capture, as some in the fishery sector are already lobbying for this to be included in labelling.

With the key food labelling criteria already taken care of in legislation as outlined above, consumers and stakeholder groups are demanding more transparency to address issues such as product provenance, food miles, quality, environmental impacts, production methods and social/ethical issues. For these 'value-based' or 'eco-label' messages, the challenge is to balance what information is essential versus what is desirable, given that space on product labels is already very limited. The choice will be to decide which of the eco-label messages are truly meaningful and understandable, and will help consumers to make informed choices about fish sourced from sustainably managed resources.

For now, environmental labelling schemes are mostly voluntary and are run on a private basis, but future legislation cannot be ruled out if issues of misleading

labels, consumer confusion, barriers to trade, etc., arise from their use. In the final analysis, general food law requires that any information voluntarily provided must not mislead about a product's 'nature, substance or quality'.

24.7 Acknowledgements

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24.8 Sources of further information and advice

- The 2006 updated fish names list: http://www.food.gov.uk/foodindustry/guidancenotes/labelregsguidance/updatednamesfish.
- FSA Guidance Notes on Food Labelling Regulations 1996: http://www.food.gov.uk/foodindustry/guidancenotes/labelregsguidance/foodlabelregsguid
- FSA Guidance Notes for Food Business Operators on Food Safety, Traceability, Product Withdrawal and Recall – A Guide to Compliance with Articles 14, 16, 18 and 19 of General Food Law Regulation (EC) 178/2002 (Issued in July 2007): http://www.food.gov.uk/foodindustry/guidancenotes/foodguid/generalfoodlaw
- FSA Guidance on Clear Food Labelling (October 2002): http://www.food.gov.uk/foodindustry/guidancenotes/labelregsguidance/clearfoodlabelling

Useful website links:

- Fish names database http://www.fishbase.org/search.php
- EU protected food names schemes (i.e. PDO, PGI, etc.): http://www.defra.gov.uk/foodrin/foodname/pfn/intro/index.htm
- Examples of eco-labelling
  www.freedomfood.co.uk
  www.msc.org
  www.orgfoodfed.com
  www.soilassociation.org

All legislation referenced should be available at:

- www.opsi.gov.uk/legislation/index.htm;
- www.europa.eu.int/eur-lex/

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