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Hatchery Manual for Sea Cucumber Aquaculture in the U.S. Affiliated Pacific Islands

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Masahiro Ito

Hatchery-Based Sea Cucumber Farming

It is common knowledge that aquaculture farms will result in employment opportunities for island communities and provide potential source for exports. A hatchery-based sea cucumber production is to make available for stock enhancement program and for aquaculture-based farming enterprises. The author (Masahiro Ito) is an independent sea cucumber hatchery consultant and a former director of aquaculture research and extension of COM. He has propriety technology in possession which can significantly boost the sea cucumber juvenile production in the hatchery. He has agreed to write this manual to contribute to the benefit of the U.S.-Affiliated Pacific Islands and its future industry development. The main objective is to provide the advanced methodologies and to improve the hatchery technology for the holothurian sea cucumbers, particularly the sandfish (*Holothuria scabra*) in the U.S.-affiliated Pacific islands.

Status of World Sea Cucumber Trading

The sandfish sea cucumber business was once prosperous and has been a valuable source of income for decades in the tropical and subtropical coastal communities, but it was based on “boom and bust” business resulting over-fishing to the level of extinction of this high-valued species. Similar phenomenon on almost all sea cucumber fishery have occurred worldwide. Despite of these facts, a sustained demand for bêche-de-mer (processed sea cucumbers) from China and other Asian sea food markets has pushed up the price of this favored *holothurian* sea cucumber species. Most of the sandfish product which has been regarded as one of the most valuable tropical sea cucumber is traded and sold in the dried form in the Asian market mainly in Hong Kong where the products are distributed into mainland China. Dried sea cucumbers are brought from all over the world to be bought and sold in Hong Kong. Traders and wholesalers are located along Nam Pak Hong Street in the Sheung Wan area in the north-west of Hong Kong Island. Hong Kong and Guangzhou in Guangdong province, China, have been tightly connected since the birth of Hong Kong in the 19th century. Through this channel, most of the dried marine products imported into Hong Kong are re-exported to Guangdong, from where they are traded throughout China. Currently, retail prices of the sandfish in Hong Kong are from around US$50 for the low quality with small sized products to US$300 per kg for high quality with larger size and the highest quality sandfish fetches between US$500 and US$800 per kg. The “Australian” or “Australian-made” sandfish have always been regarded as the highest quality and price in Hong Kong wholesale and retail markets.

This hatchery manual includes the following topics; i.e. broodstock management and juvenile production work of the sea cucumber sandfish, notes on microalgae culture, complete larval development as well as descriptions of post-larvae and juveniles of the sandfish and the black teatfish (*Holothuria whitmaei*):

1) quarantine culture of the broodstock, recovering them from spawning stress and conditioning for spawning induction by using down-weller “Habitat Simulator” system;
2) microalgae culture of benthic diatoms and knowledge of heterotrophic algae (micro-organisms) for feeding the settled pentactula and early juvenile stages;
3) spawning induction methods with disinfection of spawners, fertilization, collection and incubation of eggs;
4) larvae rearing including specific knowledge of feeding capability of larval stages and combination of feed, calculating amount of larval feed mix, controlling algal cell suspension, adjusting feeding amount and rearing water volume, and knowledge of optimal larval development by expecting

proportions of larval and post-larval stages between day-1 and day-11;

5) settlement techniques including preparations of settlement plates and tanks, maintenance of benthic diatom culture and water quality, nutrient media preparation and culture techniques of benthic diatoms and/or naturally occurring epiphytes, knowledge on the types of benthic diatoms (*Navicula* sp. & *Cocconeis* sp.) and symbiotic heterotrophic micro-organisms in the mangrove ecosystem for feeding pentactula and early juveniles;
6) culture of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 6 – 15 mm, 0.2 – 1g size), including calculation of feeding mix amount for the juvenile culture and preparation of feed mix;
7) grow-out culture using the down-weller "habitat simulator" tanks from day-56 or 8 weeks (onset of the 2nd phase nursery culture) until 5-6 months old (approximately 20 – 50mm, 5 – 20g size).

Broodstock Management

Elsewhere, the sandfish broodstock are usually held either in FRP (fiber-reinforced plastic) raceways, concrete tanks or earthen ponds for spawning work (Figs. 1a-c). The COM's hatchery in Pohnpei, Federated States of Micronesia, uses freshly caught sandfish broodstock from nearby its hatchery a day prior to the spawning induction work without doing any conditioning work.


Figure 1a. Habitat simulators.


Figure 1b. Concrete tanks.


Figure 1c. Earthen ponds.

The sandfish habitat is characterized by a seagrass bed of the tidal flat along the mangrove-covered shoreline from low-tide line to 10 – 20m deep in subtidal zone with soft muddy or sandy substrate. Seagrass bed is characterized by turtle-grass such as *Thalassia* spp. or by eel-grass such as *Zostera* spp. in the Indo-Pacific region (Fig. 2a-b). It is said that stocking density of the sandfish grow-out in a pond is one or two animals per square meter and the broodstock may be stocked at 3 – 5 per m-2 in a tank for conditioning if they are provided good aeration, water flow (water exchange rate at 400% per day), ample feeding with periodic tank cleaning at least once a fortnightly or renewal of tank with fresh muddy sand substrate (Duy, 2011; Purcell et al., 2012).

A key technological innovation developed by the author is a land-based broodstock culture system with a "down-weller" or "habitat simulator" tank system. The system uses a combination of closed recirculating seawater and partial flow-through method, which enables a long-term holding and domestication of healthy broodstock for selective breeding programs rather than relying on wild-caught parents on each hatchery operation. The tank system holds 5 - 10 broodstock per m-2 by providing with good air, water circulation (100% daily water exchange) and enabled to feed without periodic tank cleaning or renewal of

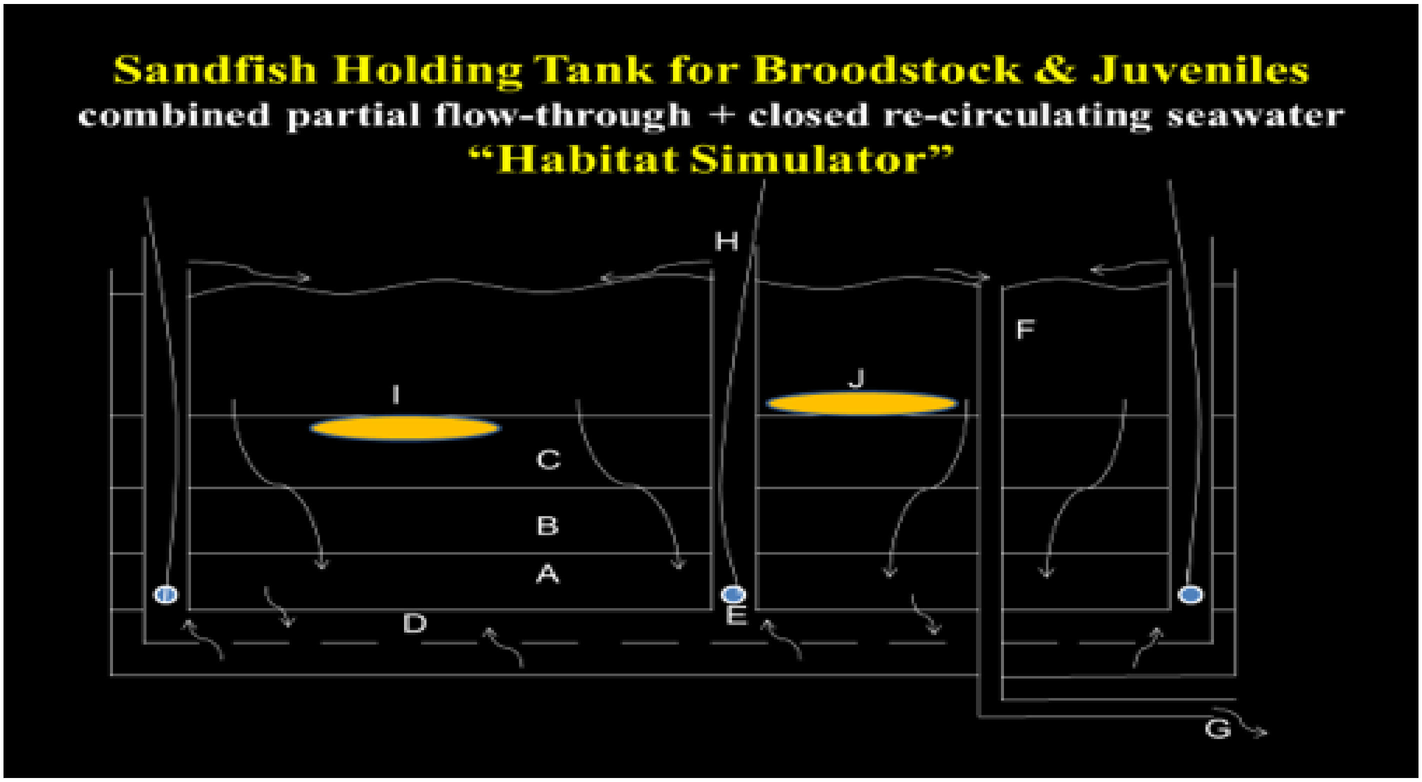


Figure 3. Diagram of down-weller "Habitat Simulator" tank. A: coral rock, B: coral gravel & sand, C: fine sand & silt, D: perforated pipe, E: air stone, F: overflow standpipe, G drain, H: airlift pipe, I & J: sandfish

tank. The down-weller was consisted of two or three layers of substrates to form a false-bottom structure; sand and mud, which are collected from tidal flat areas in the mangrove shore. Water re-circulates through the surface muddy and sand layer by air-lift pump which also maintains aerobic condition of the tank bottom and substrates (Fig. 3).

For quarantine purpose, an Australian private hatchery uses this tank system to prevent spreading potential disease among the wild-caught or domesticated broodstock (Fig. 4). Furthermore, this system has been used for recovering the spawners which had been injured or stressed during the transportation and/or spawning induction work. In Pohnpei of the Federated States of Micronesia, the COM built 2,500L rectangular tanks with down-weller system were made for broodstock recovering (Fig. 5) and juvenile grow-out (Fig. 6). Routine maintenance of the down-weller system for the broodstock is to: 1) avoid the macro-green algae (e.g. *Enteromorpha* spp.) over-grown on the tank surface; and 2) adjust aeration so as not to give strong air to drag too much seawater into the perforated piping system. To prevent green algae over-grown, use shade-screen to cut too much sunlight onto the tank. Continuously strong air-lift causes hardening the sandy substrates from strong downward water movement.

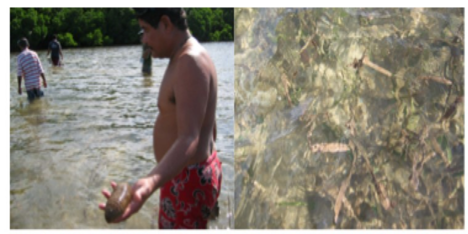


Figure 2a. Seagrass beds of turtlegrass (*Thalassira* species).

Figure 2b. Seagrass beds of eelgrass (*Zostera* species).

Figure 4. Down-wellers for commercial

Figure 5. Quarantine and recovery for broodstock in Pohnpei, Micronesia.

Figure 6. Down-wellers for juvenile grow-out in Pohnpei, Micronesia.

Figure 7a. Transporting in bags.

Figure 7b. Selecting broodstock for spawning.

1-1. Feeding broodstock

The broodstock in the "down-weller" tanks are fed daily with 1~2 % BW (body wet weight) per individual depending on the purposes; i.e. grow-out or quarantine at 1%, fattening or recovery at 1.5~2%. For practical reason, it is recommended to feed them weekly. The feed consists of dried alga Spirulina, fishmeal and seaweed of which ratio varies depending on the sizes and conditions of the animals; approximately 1 vs. 20 vs. 10. Mud/silt collected from tidal flat zone of the mangrove-covered shore is also included as an important food for both broodstock and juveniles. The amount of mud is equivalent to a total weight of other three foods. The amount of food should be adjusted by increasing or decreasing according to their average body weight. Therefore, the body weight needs to be measured at least monthly or bi-monthly. Feeding the broodstock in the Habitat Simulator can be done without a renewal of sandy and/or muddy substrates because this down-weller system itself contains organic matters such as seaweed and detritus. Currently with no mortality has been recorded by using with a combination of dried seaweed, fishmeal and mangrove-silt.

1-2. Transporting broodstock

When transporting broodstock for a long distance (4 hours or more) from wild habitat to the hatchery, the animals should be packed individually in a plastic zip-bag in a polystyrene box or ice-chest (Fig. 7a-b). It is better using ambient seawater the same water collected at the habitat and better inserting ice-gel pack (s) in the box to keep temperature at lower than 25 °C during the transportation. If the animals are found eviscerated (vomited the gut/internal organ), they should be removed from the spawners and held in the recovery-fattening tank for at least six months period for the next spawning work.

2. Microalgae Culture Management

For microalgae culture of benthic diatoms refer to “Trainer's manual for hatchery-based pearl farming” (Ito, 2005), “Development of pearl aquaculture and expertise in Micronesia” (Ito, 2006) or “A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati.” (SPC, 2015). Detailed descriptions of the microalgae culture management and techniques had been written by the author during the COM Land Grant Program’s pearl project in 2001-2013, which offered basic but practical knowledge on the microalgae culture in the tropical conditions.

2-1. Precaution for microalgae culture

- soak in freshwater and wash with detergent, brushing off dirt/wastes. Although it is not always necessary, hydrochloric acid (5 - 10% HCl solution) can be used for cleaning flasks by soaking when the dirt is difficult to clean off. Collect the used hydrochloric acid in a glass bottle for re-use.
- rinse with freshwater 5 - 10 repeats, completely wash off residue of detergent or chemicals.
- dry flasks upside down and avoid air-born dirt inside the flask.
- spray alcohol (isopropyl-alcohol or ethanol 75 % solution), rinse with distilled /filtered rainwater, and wait for dry upside down.
- put the lid on (aluminum foil) or place them in a dust-free cabinet for longer storage.
- rinse with filtered (0.2 µm or 1µm) seawater and, if available, UV-sterilized seawater before use.
- make sure washing your hand, particularly dirty finger nails and oily fingers, with soap and rinse off any residue of soap/chemicals, and then spray alcohol before commencing work.
- spray alcohol on the surface of culture flasks/containers/fittings/ working bench when entering the room.
- keep the floor and bench clean and dry and, if necessary, clean a floor with chlorinated freshwater.
- soak your feet in the chlorine bath before stepping into the room.
- periodically check and clean air filter/air outlet of air pump, air-conditioner and ventilator.

- always keep the room door/windows closed and avoid unnecessary entry into the room.

The hatchery staff tend forget general precautions for the microalgae culture work and how to properly operate the autoclave. Usually, hatchery operation elsewhere uses 121 °C for 45-60 minutes for larger flasks such as 3~5L high density culture, and small 100~250mL flasks for stock culture are sterilized for 10-15 minutes at 121°C. Periodical maintenance of autoclave is also necessary by changing or refreshing water in the chamber.

2-2. Culture methods for the sea cucumber hatchery

Sea cucumber hatchery work involves microalgae culture of planktonic and benthic diatoms. The author also uses mud/silt collected for the tidal flat zone of the mangrove shore for feeding the settled pentactula and early juvenile stages as well as broodstock. This kind of mud contains nutrient rich, particularly Omega-3 (ω3) fatty acids, derived from heterotrophic algae (micro-organisms).

Live microalgae are not required for feeding broodstock (adults) of the sea cucumbers. During the larval and post-larval rearing, however, live and/or dried microalgae are used toward settlement stage (pentactula stage) and after settlement to juveniles. The author simplified feeding methods for the larval rearing to reduce workload of culturing live microalgae. With higher survival rate at 30 - 40 % from day-1 to the settlement stage, the author has been using a single live planktonic diatom species of *Chaetoceros muelleri* together with dried form of microalga, Spirulina sp. For settlement phase and post-settlement rearing of juveniles, the author developed to use two kinds of live benthic diatoms (*Navicula* sp. and *Coconeis* sp.) by combining with dried Spirulina during the pentactula and early juvenile stages and during the juvenile stage by combining fishmeal, seaweed, Spirulina and tidal flat mud. Note that there are eight types of benthic diatoms and *N. ramosissima* (Type-A benthic diatom) and *C. scutellum* (Type-B benthic diatom) are commonly used at abalone hatchery for post-settlement juvenile culture in Japan (Kawamura, 1998). The author has been using two types as live epiphytes on the settlement substrates for the sea cucumbers, such as *N. jeffreyii* for type-A and *Coconeis* sp. for type-B. Master stock culture of these benthic diatoms can be purchased commercially such from Commonwealth Scientific Industrial Organization (CSIRO) in Australia or elsewhere. Culture media of these benthic diatoms or naturally occurring epiphytes are same as planktonic diatom such as *C. muelleri* with nutrient media strength varies from 1/100th to 1/10th. Starter high density (3L - 5L flasks) & mass culture (20Lcarboys - 100L polycarbonate tanks) are used for the above three diatom species. For these benthic diatom culture techniques and work plan, refer to Chapter 4 (Larval Rearing) and 5 (Settlement Techniques).

For specific knowledge of heterotrophic algae, refer to some of many publications such as "*Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean" (Honda et al., 1998), "Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*" (Jiang et al., 2004), "Effects of dried algae *Schizochytrium* sp., a rich source of docosahexaenoic acid, on growth, fatty acid composition, and sensory quality of channel catfish *Ictalurus punctatus*" (Li et al.,2009), and "Heterotrophic cultivation of microalgae as a source of docosahexaenoic acid for aquaculture" (Taberna, 2008).

Figure 8a. Cold water treatment using ice cubes.

Figure 8b. Cold water treatment in algae room.

Figure 9a. 1ppm iodine bath.

Figure 9b. Disinfecting (1 min.)

Figure 9c. Rinse with freshwater.

Figure 10a. Thermal shock.

Figure 10b. Gently stirring.

Figure 10c. Siphoning droppings.

Figure 11a. Sp*irulina* bath (12g/60L seawater)

Figure 11b. Monitoring water temp.

Figure 12a. Spawning.


Figure 13a. Collecting eggs.


Figure 13b. Sampling for counting eggs.


Figure 13c. Cutting broodstock.


Figure 13d. Stripping gonads.


Figure 14. Incubating eggs in 1,000L

3. Spawning Induction

Spawning induction work involves; conditioning and disinfection of spawners; inducing by stimulations or stressing such as exposing to the air, changing water temperature, water pressure and/or salinity, and chemical or food; fertilization and washing of eggs; and collection, sampling, counting and incubation of eggs (see Figs 8-14). When using a 2,500L tank for a small-scale juvenile production work, about 50 - 60 broodstock (spawners) are used for single larval run. Prior to spawning induction work before transferring from cold water treatment to spawning tank, all the spawners are disinfected by iodine, in which the animals were immerged in 1 ppm iodine bath (freshwater) for 1 minute. Spawning induction are usually done by: 1) stress by handling with exposure to the air, 2) thermal shock from cold (20-22 °C) to warm water (32-34 °C), 3) chemical stimulation by dried microalga Spirulina (20g/100L) in seawater for 30 minutes, 4) changing water pressure (decreasing/increasing water level), and/or changing salinity (decreasing salinity to about 30 ppt.

Collection of the spawned eggs are usually two-step approaches; 1) the first batch by scooping the spawned eggs by beakers and 2) the second batch by draining spawning induction tank. For a small-scale work, the former method is better to obtain cleaner with enough number of eggs. This also requires careful and continuous observation of female spawning posture. Noticeable change is observed in gonophore shape by swelling outwardly. Therefore, swift and timely scooping actions to collect eggs are required. If the latter method is used, collection of eggs should be commenced soon after several females spawned before the spawning tank becoming cloudy from too many sperms.

For incubating the fertilized eggs, stocking density should be less than 10 eggs per mL. Seawater is filtered to 1 µm by using filter-bags or cartridge filters, which does not necessarily required sterilization by in-line UV sterilizer unless virus infection or other disease has been reported from the surrounding environment. A combination of plankton screen (50 µm and 80 µm or 90 µm) is essential for collecting eggs.

3-1. Spawning procedures

The following describes timeline of spawning induction work which is based on a combination of 1) physical stress; 2) exposure to air, 3) thermal shock from cold to warm water, 4) chemical (dried algae Spirulina-bath) and/or 5) changing water pressure.

- Start preparing boiled seawater in deep pan (20-40L) using firewood (or use immersion heaters in the spawning tank) to maintain the induction tank water temperature at 33-34 °C.
- Cleaning off dirt from the body surface, measuring body weight (BW) and selecting spawners to be at least 200g, so the smaller ones should be returned to the broodstock holding tank. Before transfer to a cold water, quickly rinse with filtered seawater.
- Prepare cold seawater (1 µm filtered) beforehand, in the preceding day by placing it in the algae room. Transfer spawners to 100L cold treatment tank at about 20-22 °C and keep them for at least 2~3 hours, preferably for overnight.
- Transfer spawners to iodine (freshwater) bath at 1ppm of iodine (or 100ppm of Betadine®*) for 1minute (= 60 seconds). *Betadine® contains 1%W/V iodine. Therefore, 1g Betadine contains 0.01g iodine. To make 1ppm iodine solution (or 0.1g iodine per 100L), add 10g Betadine® per 100L to make 1ppm iodine (freshwater) bath.
- Start spawning induction work immediately after the iodine bath, rinse off iodine with filtered seawater and transfer to the spawning tank (2,500L raceway with approximately 1,000L water volume) at 33-34°C.
- Wait spawning (male and female) for at least an hour and keep cleaning droppings on the tank floor by siphoning. Use a plunger to stir gently spawning tank water and keep mixing the warm water.
- If the spawners dose not respond to the above thermal shock, transfer them to "Spirulina bath" for 30 minutes at 12g of Spirulina in 60L of filtered seawater. Spirulina is dissolved faster and better in freshwater (or rainwater), so prepare 12g Spirulina in about 500mL rainwater before making 60L seawater solution.
- Rinse off the Spirulina with filtered seawater and introduce them again to the spawning tank.
- Wait for the spawning. Males usually spawn before females release eggs.
- Observe spawning posture of female(s) and scoop the eggs with beakers when the female releases the eggs. If excess eggs are needed after confirming the females finished spawning, drain the spawning tank to collect remaining eggs inside the tank. Use a combination of 50 and 80 µm-pore size mesh screen to collect and wash the eggs. If no female responded after two hours, return all the spawners to broodstock holding tank.
- After washing/rinsing off sperms for 10-20 minutes, transfer the eggs into a 20L bucket to make 15L volume of 1 µm filtered seawater.
- Take at least two samples of 2mL volume while stirring the bucket by a plunger.
- Count the eggs under microscope with an aide of Rafter Counting Chamber and estimate total number of eggs obtained. While counting the eggs, check the fertilization by confirming the 1st polar body or more advance embryonic development such as 2-cell stage, 4-cell stage, and so on.
- Stock the eggs in incubator tanks, maximum stocking density of the eggs being 10 eggs per mL.

3-2. In vitro Fertilization (Gonad Stripping Method)

At present, a method using thermal shock with or without Spirulina bath treatment has been effective, but it has not always resulted in 100% success rate of the sea cucumber spawning induction work. Sooner or later, it is inevitable to develop an effective method for spawning both males and females. A Japanese group of scientists (Kato et al., 2009) found that neuronal peptides induced oocyte (ovum) maturation and gamete spawning of the Japanese sea cucumber *Apostichopus japonicus*. They extracted the neuronal peptides and so synthesized it chemically, which was effective to mature 150 µm diameter or larger eggs. They also experimented to inject this synthesized hormone into the body cavity of the sea cucumbers, resulting the male and female spawned 60 minutes and 80 minutes later, respectively. Unfortunately, they did not describe how far the eggs developed as embryos and whether their hatchery work went through to settlement as pentactula stage. Therefore, no information was available from their study for fertilization and hatching rates as well as survival rates during the larval and post-larval rearing works. They also stated that this chemical did not work effectively on immature ova and, thus, they concluded that the maturation mechanisms and process of ova/spermatozoa still needed further studies. Synthesizing and producing such a neuro-hormone commercially could be very expensive and won't be available for the Indo-Pacific region in foreseeable future. The important fact is that developing techniques of artificial maturation of oocytes (ova/spermatozoa) and activation of gametes (sperms) are the keys to success in obtaining the fertilized eggs. In this end, a gonad stripping method* could be an alternative to spawning induction work near future, either using synthesize neuronal hormone or other chemicals such as ammonia-seawater which has been used for commercial pearl oyster hatcheries in Japan, or just use of natural seawater.

**Note that the gonads are removed from the parent animal by cutting a small portion of the body and the gametes are obtained by stripping/squeezing the gonads (Fig. 13c-d). This is called "gonad stripping method".*

Although no one has been successful for in vitro fertilization of the sea cucumbers, the author thought that it was worthwhile for the hatchery technicians to understand principle and procedures of this method. During the hatchery training workshop in May 2015 at the Fijian Government's hatchery in Galoa, the author used filtered seawater (1 µm nominal pore) without using any other chemicals for the gonad stripping method for the sandfish (Ito, 2015). As a result, fertilized eggs subsequently underwent embryonic and larval development to the settlement as pentactula stage. Although the number of eggs and resultant pentactula were very small, several hundred, and low survival rate at less than 10 % to day 11, this method may be economical and could be the first step towards future improvements for a large-scale juvenile production on a regular basis.

4. Larval Rearing

Larval rearing of the sandfish sea cucumber requires knowledge of feeding capability of larval stages, suitable combination of food, calculating amount of feed mix, controlling algal cell (feed mix) suspension, adjusting feeding amount and rearing water volume, water quality control, and knowledge of optimal larval development in changing proportions of larval and post-larval stages; i.e. from hatching as auricularia stage to settlement as pentactula stage. Approximately 18-24 hours after spawning depending on water temperature, larval rearing work commences by draining the incubator to collect gastrula and/or auricularia larvae. For collecting larvae and post-larvae, a combination of 80 µm and 100 µm mesh screen is to be used. Larval rearing tanks should be completely drained every other day, on days 1, 3, 5, 7, 9 and 11. The larval specimens need to be kept alive but immobilized/anesthetized by isopropyl alcohol for counting, measurements and microscopic photographs. For longer term preservation, use formalin (10% seawater formalin). Water temperature in the larval rearing could be better between 27-30 °C.

Hatchery facility should be maintained good conditions in terms of hygiene and efficiency for larval and post-larval rearing work:

- animals such as cats need to be kept away in- and out-side the hatchery, around the indoor and under-cover tanks and indoor storage areas;
- microalgae culture room should not be a sort of storage room with scattered lab supplies and equipment on the culture benches, dusty air-conditioners and air pumps without filter maintenances;
- air supply system needs to be functioning effectively, with sufficient air pressure in the under-cover areas as well as in the microalgae culture room;
- in-use or used tools should not be scattered on the floors, e.g. filter cartridges, filter-bags, hoses, pipes, buckets, air-stones, airlines, pipe-fittings, nets, plumbing machines; air leaks from many outlets with unnecessary accessories and fittings;
- sea water and freshwater supply piping are better to be simple and do not need unnecessary connections, diversions and outlets fittings;
- the hatchery staff understand general hygiene procedure before and during the hatchery operation.

4-1. Precautions before, during and after working at hatchery

For precautions of the onsite hatchery work, refer to “Trainer's manual for hatchery-based pearl farming” (Ito, 2005) or “A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati.” (SPC, 2015). The following were also described in those manuals.

- make sure your hands are clean. Wash your hand with soap, particularly dirty finger nails, before starting work.
- Soak your feet in “chlorine-bath” before entering in the larval and/or microalgae room.
- don’t work with you own shoes. Always ware designated boots or work with barefoot.
- always rinse again the cleaned and dried equipment with filtered rainwater (1 µm) before use.
- for the used equipment/tools, wash first with chlorinated (public) water to wash out the waste/dirt.
- second-wash by using detergent and wash-off the dirt thoroughly with a soft sponge or brush.
- rinse with 1 µm-filtered rainwater and completely wash out residual soap/detergent.
- soak in chlorine-batch (a diluted Sodium Hypo-chloride, NaHClO) for overnight. Don’t mix with soap or this may release Cl2 (chloride gas).
- rinse completely with filtered rainwater (1 µm). Make sure “no residual chlorine”.

Figure 15. Seawater filters and UV sterilizer.

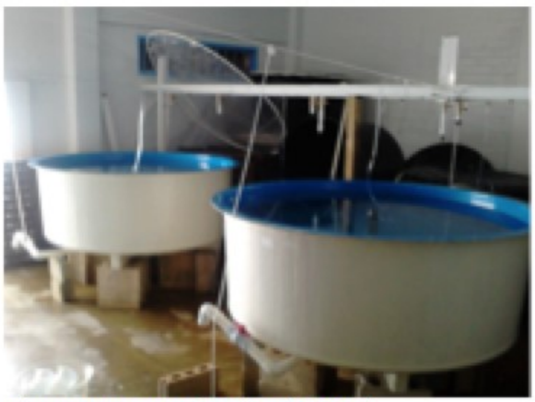


Figure 16a. Larval rearing (2,000L).

Figure 16b. Larval rearing (5,000L).

Figure 17. Sieves for collecting larvae.

- always hang and dry the equipment after being cleaned. Do not leave them on the floor or dirty bench.
- if necessary, use isopropyl-alcohol spray (75 % solution) and wait for it to dry. *Note that the use of methanol (methyl-alcohol) will become a health hazard in a small microalgae culture room.
- don't touch inside of the cleaned surface of equipment and tools such as bucket/ container/tank/tub/flask, etc.
- wash filter bags, cartridges and housings after every use. Wash out the dirt with pressurized freshwater, filtered rainwater (1µm), soak in chlorine-bath, rinse with filtered rainwater (1 µm) and dry them on a designated bench. Keep the filter bags, cartridges in sealable plastic bags each with alcohol-sprayed inside. For the filter housings, spray alcohol inside and store them upside-down on the bench.
- make sure always clean the floor; wash with freshwater (chlorinated town-water or rainwater). It is the best that the floor is a "dry" condition when you start working in the following morning.
- don't disturb animal (larvae/juveniles/broodstock) and minimize giving shock or stress to the animals. Avoid unnecessary entry to the microalgae culture room and larval rearing unit.

4-2. Preparation for the larval rearing

The seawater for the larval rearing should be filtered to 1µm with a bag filter or cartridge filter. In-line UV-sterilizer is not necessarily required (Fig 15). When the day-1 larvae exceeded 0.35 larvae per mL in a rearing tank, the stocking density should be adjusted to make acceptable number of larvae in each tank: e.g. between 0.5 - 0.7 million larvae in a 2,000L tank or 1.25 - 1.75 million larvae in a 5,000L tank (Figs. 16). Gentle aeration is given throughout the larval rearing and the tank must be protected by a lid (tank cover) from debris from the ceiling. If the stocking density is less than 0.25 larvae per mL, the rearing water volume must be adjusted (reduced) to maintain required range of algal cell suspension based on *C. muelleri* culture density and number of larvae in each day. A combination of sieves is usually 80/100 µm throughout larval rearing and each sieve must be deep and wide enough (30 cm deep x 50 cm wide) to do sieving efficiently from a larger diameter drain pipe e.g. 25.4 cm (2 inch) pipe (Fig. 17).

Apart from tools and equipment, preparation of the live microalgae species (*C. muelleri, Navicula sp.* and *Cocconeis* sp.) need to be cultured at least two weeks before commencing spawning and larval run. For continuous culture and feeding the larvae, several subcultures should be made after commencing larval rearing, instead of starting from new stock culture (Figure 18). Larval rearing period with microalga *C. muelleri* feeding would finish within two weeks on day-14 after spawning. When a hatchery operation is planned a single spawning0larval run, therefore, it is not useful to start any new culture of *C. muelleri*. Generally, 7 days needed for a 2 - 5L high density starter cultures to be ready for starting 20 - 100L mass cultures, and these mass culture needs further 4 - 5 days to use for feeding the larvae. The author uses dried microalga Spirulina to mix with live diatom *C. muelleri* for larval and post-larval rearing. *Navicula* sp. and *Cocconeis* sp. are used for feeding settled pentactula and early juvenile stages up to two months old in settlement tanks. If live microalgae are used for sea cucumber hatchery, therefore, it is necessary to culture diatoms.

Figure 18. Microalgae (diatoms) for feeding larvae and post-larvae.

4-3. Feeding methods for larval rearing

Mixture of *C. muelleri* and Spirulina are given for feeding the larvae twice a day. Feeding is maintained by estimating algal cell suspension in each larval tank, starting from approximately 1,000 up to 20,000 cells per mL during the two-weeks larval rearing. Total cell suspension in each day is attained by combining these two feeds and expressed as the number of *C. muelleri* cells, where amount of Spirulina is computed and expressed as the number of *C. muelleri* cells. Feeding ratio of *C. muelleri* and Spirulina is approximately 80 % and 20 %, respectively. Feeding is divided into two; one in the morning between 8 and 10 AM, and the other in the late afternoon between 4 and 6 PM. The author developed daily feeding tables with simplified data-inputting methods, therefore, the hatchery staff are usually trained to use such feeding tables.

A hatchery protocol with daily work schedule for larval rearing was summarized and shown in Table 1. The hatchery staff needs to count the number of living larvae and post-larvae after tank draining every two days and *C. muelleri* to input daily culture density (million cells per mL). The feeding amount of both *C. muelleri* and Spirulina are obtained instantly in those feeding tables. All feeding amount were converted and expressed as *C. muelleri* cells. For the Spirulina, the hatchery staff simply measures the dry-weight according to the feeding table, prepare for AM or PM feeding amount, dissolve in the freshwater (about 500 mL) and wait for an hour before adding to the rearing tanks. It is advised the stocking density of larvae from onset of larval rearing on “day 1” to be between 0.25 - 0.35 larvae per mL.

Table 1. Hatchery protocols for juvenile production of the sandfish sea cucumber.

HATCHERY PROTOCOL **(WORK SCHEDULE)** **days of run (size.; µm)** **Algal cell suspension** **(as *C. muelleri* cells mL^{-1})**	***larval & post-larval development based on the water temperature at 29 ± 1 °C** ***larval rearing tank (LR) = 1 x 5,000L** ***settlement tank (ST) = 4 x 5,000L (bottom area = 5,000 m^2); settlement plates=50cm x 50cm x 800~1,000 plates per 5,000L tank** ***plates = corrugated plastic plates** ***juvenile down-weller tank (JHS) = 4 x 10,000L (bottom area = 10,000 m^2); sandy bottom covered with mangrove mud (dried mud sieved through 100 µm screen)**
-3d before spawning	Start conditioning settlement tanks and plates; add benthic diatoms (i.e. *Navicula sp.* & *Cocconeis sp.* 200L each in 5,000L settlement tank) & nutrient (100% strength) to culture in the settlement tanks
0 (egg: 150-160)	Collecting (sieves 50 & 80 µm mesh screen), washing, counting & incubating eggs (up to 50 million eggs per 5,000L incubator)
1 (420 x 320) 900-1,500 cells/mL	Draining incubator & collecting gastrula & early auricularia (sieve 80 & 100 µm) approx. 20 hours after fertilization; sampling, counting & stocking to larval rearing tanks (1.5 million larvae in 5,000L tank); start feeding larvae with live microalga *Chaetoceros muelleri* and dried microalga *Spirulina* sp. as soon as stocking larvae.
2 - 4,000-7,500 cells/mL	
3 - 7,000-12,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% Water Exchange); early auricularia (20%) + mid-auricularia (80%)
4 - 8,000-14,000 cells/mL	
5 (800-1000) - 8,500-15,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); mid-auricularia (20%) + late-auricularia (8
6 - 9,000-17,000 cells/mL	
7 (800-1200 x50-80) 9,500-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia; remove settlement plates to dry; drain settlement tanks & refill the settlement tanks with new benthic diatoms (200L each in 5,000L tank) & nutrient (1/20th strength)
8 - 10,000-20,000 cells/mL	Spray *Spirulina* on the settlement plates (*Spirulina* 30g per liter freshwater solution) & dry the plates
9 - 8,000-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia + post-late auricularia (= metamorphosing to doliolaria) + doliolaria (~ 5%)
10 - 6,000-16,000 cells/mL	Return the plates to the settlement tanks
11 (400-1200 x 60-80) 4,000-13,000 cells/mL	Larval tank draining (80 & 100 µm), collect larvae & post-larvae & transfer them to the settlement tanks; late-auricularia + fully developed late auricularia + post-late auricularia + doliolaria + pentactula; *survival rate from day-1 = ave. 30% (approx. 0.5 million each in 5,000L settlement tanks)
12 - 2,000-6,000 cells/mL	*feeding only with *C. muelleri* for the swimming stages (no need of using *Spirulina*)
13 - 500-2,000 cells/mL	*feeding only with *C. muelleri* for the swimming stages (no need of using *Spirulina*)
14 - 200-1,000 cells/mL	Finish using *C. muelleri* & *Spirulina* for feeding larvae; fully developed late auricularia + post-late auricularia + doliolaria + pentactula
15 (~ 1mm)	Start flow-through over 24 hrs. 100% WE; (inlet water = 1 µm filter & outlet water = 100 µm screen); post-larvae; Start feeding daily with 3 foods (fishmeal 5g + seaweed 5g + mud 12.5g) per 100,000 post-larvae; *Sieve mud, fishmeal & seaweed with 100 µm screen for feeding; *no need of giving *Spirulina* as the settlement plates still covered with *Spirulina*
21 (1 ~ 2mm)	flow-through 24 hrs.100% WE; (inlet = 1 µm filter & outlet = no screen); continue daily feeding at 0.25% of BW with 3 foods (fishmeal 10g + seaweed 10g + mud 25g) per 100,000 early juveniles; *sieve feed-mix with 100 µm screen for feeding
28 (1-mo) (2~5 mm, 0.01~0.1g)	flow-through 24 hrs.100% WE; start daily feeding at 0.25% of BW with 4 foods (*Spirulina* : fishmeal : seaweed : mud = 1 : 2 : 2 : 5); *start giving *Spirulina*; *settlement success rate from day-11 to 1-mo = 20-50%
30	Start weekly feeding at 0.25% of BW with 4 foods (*Spirulina*, fishmeal, seaweed & mud)
Day **35**	Continue weekly feeding
Day **42**	Continue weekly feeding
Day **49**	Start transferring juveniles (2-mo = ave. 1g BW, 10-20mm BL) from settlement tank (ST) to down-weller "habitat simulator" for juveniles (JHS);
Day **56** (2-mo = J2) (0.2~2g)	*increase proportion of fishmeal and seaweed (Spirulina, Fishmeal & Seaweed = 1 : 2.5 : 2.5); *stocking density of JHS = approx. 10,000~1,500 x J2 per m^2 (= 10,000~15,000 xJ2 in a 10,000L JHS tank);

(Example 1)

If the rearing tank volume is 2,000 L (=2,000,000 mL),
C. muelleri culture density is 3.0 million cells/mL, and
today's total amount of *C. muelleri* required is 2,000 mL

"total *C. muelleri* cells in the rearing tank" =
"culture density of *C. muelleri* " x " today's required amount of *C. muelleri* "
(= 3 million x 2,000 = 6.0 billion cells)

Therefore, today's " *C. muelleri* cell suspension " in the 2,000L tank
= 6 billion cells / 2,000,000 mL = 3,000 cells/mL

(Example 2)

If today's required total feeding amount of larva is 12,000 cells/larva/day,
number of larvae in 2000L tank is 500,000,
today's required algal cell suspension is 3,000 cell/mL, and
today's algal culture density is 3 million cells/mL

"required total algal cells in the 2,000 L rearing tank"
= 12,000 (cells/larva/day) x 500,000 (larvae) = 6 billion cells
*algal cell suspension in this 2000L tank = 6 billion cells / 2 million mL = 3,000 cells/mL

Therefore, today's "total amount of algae" = 6 billion cells / 3 million cells/mL = 2,000 mL

For feeding the larvae, the hatchery staff must take algal samples (= *C. muelleri*) and counts the culture density; input each counting results into the designated cell in the MS Excel spreadsheet ("HIROITO CONSULTING's Sandfish Feeding Tables"), of which table automatically calculates the daily (in the morning-AM and afternoon-PM) feeding amount of both *C. muelleri* and Spirulina. "estimated available food per larva as CM" is based on the past results of larval feeding experiments of the sandfish and other sea cucumber species by the author and others in Japan and elsewhere, which ranges from 3,000 to 60,000 cells/larva/day.

Note that a commercially available dried alga Spirulina contains certain amount of dried form of cells (e.g. 2.6 billion cells per 1 g). The author found about three different sizes (e.g. 10 µm x 15 - 50 µm) and shapes (e.g. rectangular). Average size of *C. muelleri* is about 10 x 5 µm. 1 g of Spirulina cells are approximately to 42.8 billion *C. muelleri* cells. Therefore, it is necessary to estimate approximate volumes of those different sizes of Spirulina cells and to convert to the number of cells to *C. muelleri.* This is because total feeding amount is expressed as the number of *C. muelleri* cells and the feeding ratio (based on the number of cells) of *C. muelleri* vs. Spirulina is 80 % vs. 20 %.

4-4. Example of two spawning-larval runs in September 2017 at COM's Nett Point Hatchery in Pohnpei, FSM.

During the work, 1.4 million eggs (trial 1) and 5.3 million eggs (trial 2) were stocked in the 1,000L incubators. Approximately 20 hours after spawning, each yielded 0.42 million larvae (trial 1) and 2.5 million larvae (trial 2). As the two 2,000L round tanks were needed each with approximately 600,000 larvae for the trial 2. The followings were the results of larval runs between day 1 and day 11:

- trial 1 at survival rate of 10.9 % with 63,750 swimming stages on day 11 including late auricularia, doliolaria and pentactula from 416,250 larvae on day 1;
- trial 2-1 with 67,500 from 620,625 at survival rate of 15.3%;
- trial 2-2 with 221,250 from 620,625 at survival rate of 35.6%

Average survival rate of the three larval runs was 20.6% from day 1 to day 11. Total 353,500 larvae and post-larvae were transferred to three 2,500L settlement tanks.

4-5. Larval Development

Detailed morphological descriptions of the complete larval and post-larval development for both sandfish (*H. scabra*) and black teatfish (*H. whitmaei*) were given in Appendix 1 with notes on larval growth and durations in Appendix 2. Comparisons of morphological features of larval and post-larval stages of these two holothurian sea cucumbers were also given in Appendix 3.

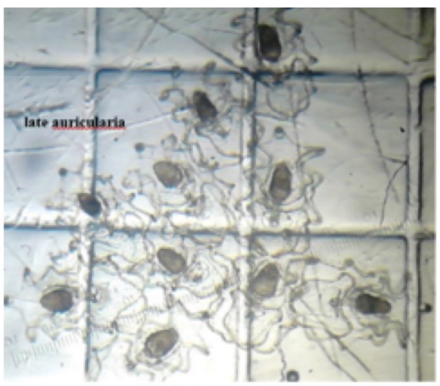


Figure 19. Late auricularia.

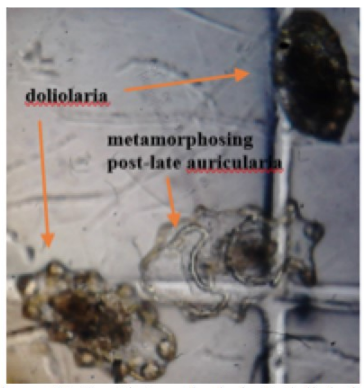


Figure 20. Post-late auricularia & doliolaria.

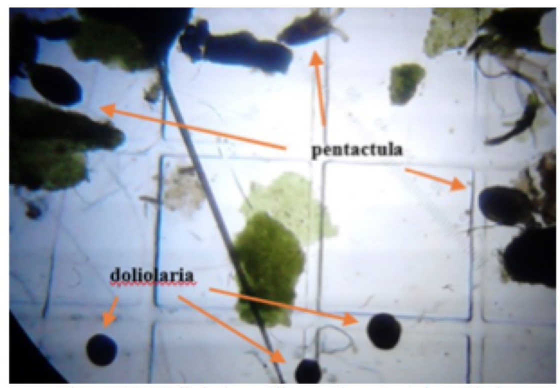


Figure 21. Doliolaria settling and pentactula settled.

5. Settlement Techniques

Settlement techniques involves conditioning of settlement plates and tanks, culturing benthic diatoms. The pentactula generally settles on the sandy sea grass bed such as *Talassira* spp. (for sandfish) in tidal flat zone, coralline gravels and rocks in the intertidal or littoral zone (e.g. black teatfish, greenfish). In the hatchery, the pentactula settle anywhere in the tank and so corrugated plastic plates are usually used as settlement substrates. Surface of settlement substrates such as plastic plates and inside the tank are conditioned with dried alga Spirulina and/or with epiphytes either naturally occurring or cultivated. The author uses two species of benthic live diatoms (*Navicula* sp. & *Cocconeis* sp.) as epiphytes on the settlement substrates. On day 11, larval rearing tank was drained and all swimming stages including fully developed late auricularia, metamorphosing post-late auricularia and doliolaria were collected onto a 100 µm screen (Figs. 19-20). A small percentage of pentactula up around 5% (Fig. 21) might have also settled on this day, so soft brushes were used to collect those settled individuals. Samples were taken for counting to estimate number of living individuals of all stages. Then, they were transferred to settlement tanks.

Table 2. Dimension of settlement plates used at COM's Hatchery in Pohnpei, FSM.

Settlement Plates (type)	Length (m)	Width (m)	Surface Area - both sides (square meters)
Large A (LA) =	0.65	0.44	0.57
Large B (LB) =	0.44	0.44	0.39
Medium (M) =	0.39	0.33	0.26

5-1. Conditioning settlement substrates.

Conditioning settlement plates and tanks should commence at least two weeks prior to settlement. To start culturing benthic diatoms *Cocconeis* sp. and *Navicula* sp. in the settlement tank, 2,500L tank for example, 100L each of mass-cultured those diatoms and a full strength of nutrient media (i.e. medium F2 in Australia and Fiji, OFCF/JICA's medium in Kiribati, MI medium and Kent's F2 media at COM in FSM) together with sodium meta-silicate 15 - 30 g were added to the 2,500L tank. About 10 days after the initial diatoms culture, the settlement plates were removed for drying and the tanks were drained to renew the culture water (1 µm filtered seawater) and to refill with new benthic diatoms (100L mas culture each) and 1/20th strength of nutrient media. After a couple of days for drying, the settlement plates were sprayed (or painted) with Spirulina (30g/L solution) and keep drying for a day or two (Figs. 22-23). On day 10, a day prior to transfer larvae and post-larvae to settlement tanks, return the Spirulina-sprayed plates in the settlement tanks. During the work at COM's hatchery in 2017, three different sizes of corrugated plates were used for settlement (refer to the table 2 of plate's dimensions). In a 2,500 L settlement tank, 300 - 400 plates of the type-M are mainly deployed. The plates were washed by detergent, disinfected by chlorine and dried for a couple of days before growing benthic diatoms on the plates. For larval rearing with a 2,000L tank, two of 2,500 L rectangular tanks (raceways) were used for settlement of doliolaria to pentactula, approximately up to 100,000 pentactula in each settlement tank (Fig. 24). On day 11, about 200,000 swimming stages, which consist of feeding and non-feeding stage of late auricularia, post-late auricularia and doliolaria, are expected in each 2,000L rearing tank out of 600,000 larvae on day 1.

5-2. Transferring swimming and settled stages to settlement tank.

Onset of the settlement (on day 11) is called "the 1st phase nursery culture", which continues for about 2 - 3 months until the juveniles of 1- 2 g body weight (BW in wet weight) in average are transfer further to a down-weller "juvenile habitat simulator" tank or bag net "hapa" for a pre-growout or "the 2nd phase nursery culture".

Living individuals on day 11 are usually consisted of the settled pentactula stage (10 - 15%), transitional doliolaria stage (20 - 25%), metamorphosing late auricularia = post-late auricularia stage (30 - 40%), fully-developed late auricularia (20 - 30%) and late auricularia stage (10 - 20%). It is better to transfer procedure when the proportion of settling or settled animals (50% < post + dolio + penta) occupy more than a half in the larval rearing tank. The larval rearing tank is drained to collect all the animals onto 80 µm and 100 µm sieve. Collected specimens should be poured over the plates in the settlement tanks.

Figure 22. Spraying *Spirulina*.

Figure 23. Drying plates after spray.

Figure 24. Settlement tank.

Toward day 11, all the settlement substrates (plates and tank surface) should be covered with light brown -colored benthic diatoms. If sunlight is too strong, the settlement tanks will be dominated by green alga (e.g. *Enteromorpha* spp.). In that case, the tanks should be covered by shade-cloths (75-80% shading rate). If pink- colored organisms (pathogenic bacteria *Pseudomonas* spp.) began visible after a week of benthic diatom culture in the settlement tank, those plates, tank surface and fittings such as air stones, air tubing, PVC pipes or ropes must be removed from the tank and disinfected by chlorine or discarded all or part of them to avoid further infection of the pathogenic bacteria. Such infection is always caused by inexperienced hatchery technician's careless setting of the settlement tank and plates, poor skill of culturing microalgae and larval rearing.

After day 11, the larval rearing continues in a static water condition because there are still swimming and feeding stages of auricularia larvae (late auricularia, fully developed late auricularia and metamorphosing post-late auricularia). Therefore, it is necessary to provide microalga *C. muelleri* until around day14. From day 15, the rearing water in the settlement tank should be switched from static to flow-through, continuously flowing in from filter bag and flowing out to overflow-drain pipe at 100 % water exchange rate over 24 hours. 100 μm screen is used to cover the drain outlet for minimize loss of swimming stages (Fig. 25).

5-3. Examples of juvenile productions using the settlement plates.

The measurements of the settlement tank (ST), settlement plates (LA, LB, M), sample plates showing surface areas (in square meters) and estimated number of juveniles were given in the following tables 3 and 4: 30 small sample plates, 0.1m x 0.1m corrugated plastic, were also made for estimating settlement on the tank surface sides and bottom. Total of 79,180 one-month-old juveniles (on day 28, J1W1) were estimated to settle in three tanks (Figs. 26-27).

6. Pentactula and Juvenile Rearing

Pentactula and the early juveniles settled on the plates or tank surface are given additional food daily with a mixture of Spirulina, fishmeal, seaweed and mud. The mixtures of these food are homogenized and passed through 100 μm mesh screen before feeding. The juveniles on the plates and tank surface are kept in the settlement tank for two or three months and then the juveniles are transferred to "the 2nd phase nursery culture" either in the down-wellers (or habitat simulators) for juveniles or hapas bag net. The 2nd nursery culture continues further two or three months before being transferred to grow-out farm/ ponds/ enclosure. In Australia, a large-scale private farming enterprise does not conduct "the 2nd phase nursery culture", except for selective breeding programs, but those grown juveniles of average 2 - 5 g BW from the settlement tanks are also transferred to ocean grow-out sites (Fig. 28-29).

Culture techniques of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 10 – 20mm, average 1g size) include selection and collection of food, preparation of food (e.g. drying, sieving and storing) and calculation of amount of food based on the number and weight of juveniles. As the feeding begins with mixture of three food (fish meal, seaweed and mud), rearing method for the 1st nursery phase switches from a static water method to a flow-through method with 100 % water exchange rate over 24 hours. 1 μm filter bag is attached to the inlet of seawater and a 100 μm screen is attached to the drain outlet, the latter is for catching any swimming stages such as doliolaria and auricularia which

Table 3. Surface areas of settlement tank and plates used at COM's hatchery in September - October 2017 in Pohnpei, FSM.

Surface Area (square meters)	bottom	sides	up-down	total surface area
2,500L mark =	5.00	5.00	1.00	11.00
	Surface Area - both sides (square meters)	No. of plates used in Tank #1	No. of plates used in Tank #3	No. of plates used in Tank #2
plate-LA (0.65 x 0.44)	0.57	32	7	0
plate-LB (0.44 x 0.44)	0.39	0	142	0
plate-M (0.39 x0.33)	0.26	0	92	212
	Total surface areas of plates in ST#1-3	18.24	83.29	55.12
	Total surface area ratio (tank vs. plates)	1.66	7.57	5.01

Table 4. The estimated number of the early juveniles on day 28 (1-month after spawning) during the juvenile production work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

	Tank #1	Tank #3	Tank #2
Results of Sampling on Day 28 (J1W1) plate-LA	498 juveniles on 6 out of 30 plates	906 juveniles on 4 out 15 plates	
Estimated number of juvenile on the plates	2,485	3,398	
Results of Sampling on Day 28 (J1W1) plate-LB		5,279 juveniles on 20 out of 215 plates	
Estimated number of juvenile on the plates		53,372	
Results of Sampling on Day 28 (J1W1) plate-M	161 on 2 out of 2 M (side) converted as 1/2 of LA (side)		355 juveniles on 33 out of 212 plates
Estimated number of juvenile on the plates	161		2,503
Results of Sampling on Day 28 (J1W1) sample plates (0.1mx0.1mx30plates)			68 juveniles on 30 out of 30 plates
total number of juveniles (on plates)	**2,646**	**56,770**	**2,503**
total number of juveniles (tank surface)	**3,179**	**11,589**	**2,493**
total number of juveniles (plates + tank)	**5,825**	**68,359**	**4,996**

Table 5. Examples of daily feeding amount after settlement (if total 200,000 settled) for the subsequent two weeks between day 15 and day 27.

	ratio against 100,000 juveniles =	**2.00**	**(If total 200,000 juveniles)**	
	Spirulina (g)	**Fishmeal (g)**	**Seaweed (g)**	**Mud (g)**
d14	**N/A**	**10.00**	**10.00**	**25.0**
weekly	**N/A**	**70.0**	**70.0**	**175.0**
d21	**N/A**	**20.0**	**20.0**	**50.0**
weekly	**N/A**	**140.0**	**140.0**	**350.0**

Table 6. Feeding schedule for the sandfish juvenile grow-out for the COM's hatchery work in September – October 2017 in Pohnpei, FSM.

No. of Juveniles	***feeding amount changes from 0.25% - 0.25% - 0.25% - 0.5% - 0.5% - 1% of the average body weight in each month**							
26,458 (estimated number of pentactula on day 15)	daily amount of feed (g) = **smaller size**				daily amount of feed (g) = **larger size**			
survival rate to 1M = 19%	Spirulina	Fishmeal	Seaweed	**Mud (g) = 3-feed total**	Spirulina	Fishmeal	Seaweed	**Mud (g)=3-feed total**
1-2M (0.01-0.5g) (ratio) 0.25% BW	1	2	2	3-feed total (g)	1	2	2	3-feed daily total (g)
***estimated number on day 28 in ST2 (2,500L tank)**								
4,996	0.02	0.05	0.05	0.12	1.2	2.5	2.5	6.2
4,996	0.14	0.29	0.29	0.72	2.2	4.4	4.4	10.9
4,996	0.26	0.52	0.52	1.31	3.1	6.2	6.2	15.6
4,996	0.38	0.76	0.76	1.90	4.1	8.1	8.1	20.3
***2M transferred from ST#2 to JHS#3: 4 weeks total feed=**	**5.68**	**11.37**	**11.37**	**28.42**	**74.3**	**148.6**	**148.6**	**371.6**
2-3M (0.2-2g) (ratio) 0.25% BW	1	2.5	2.5	3-feed daily total (g)	1	2.5	2.5	3-feed daily total (g)
5,000	0.4	1.0	1.0	2.5	4.2	10.4	10.4	25.0
5,000	0.8	2.1	2.1	5.0	5.7	14.3	14.3	34.4
5,000	1.3	3.1	3.1	7.5	7.3	18.2	18.2	43.8
5,000	1.7	4.2	4.2	10.0	8.9	22.1	22.1	53.1
survival rate to 3M = 100% 4 weeks total feed =	**29.2**	**72.9**	**72.9**	**175.0**	**182.3**	**455.7**	**455.7**	**1225.0**
3-4M (1-5g) (ratio) 0,25% BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
5,000	1.1	5.7	5.7	12.5	5.7	28.4	28.4	63
5,000	2.6	13.0	13.0	28.6	7.1	35.5	35.5	78
5,000	3.1	15.6	15.6	34.4	8.5	42.6	42.6	94
5,000	3.6	18.2	18.2	40.1	9.9	49.7	49.7	109
survival rate to 4M = 100% 4 weeks total feed=	**73.6**	**367.9**	**367.9**	**809.4**	**218.8**	**1093.8**	**1093.8**	**2406.3**
4-5M (2-10g) (ratio) 0.5% BW	1	10	10	3-feed daily total (g)	1	10	10	3-feed daily total (g)
5,000	2.4	23.8	23.8	50.0	11.9	119.0	119.0	250
5,000	3.3	32.7	32.7	68.8	14.9	148.8	148.8	313
5,000	4.2	41.7	41.7	87.5	17.9	178.6	178.6	375
5,000	5.1	50.6	50.6	106.3	20.8	208.3	208.3	438
survival rate 5M = 100% *4M transfer to farm/pond 4 weeks total feed=	**104.2**	**1041.7**	**1041.7**	**2187.5**	**458.3**	**4583.3**	**4583.3**	**6926.0**
5-6M (5-20g) (ratio) 0.5% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
5,000	4.0	80.6	40.3	125.0	16.1	322.6	161.3	500
5,000	5.0	100.8	50.4	156.3	20.2	403.2	201.6	625
5,000	6.0	121.0	60.5	187.5	24.2	483.9	241.9	700
5,000	7.1	141.1	70.6	218.8	28.2	564.5	282.3	875
survival rate to 6M = 100% *5M transfer to farm/pond 4 weeks total feed=	**155.2**	**3104.8**	**1552.4**	**4812.5**	**621.0**	**12419.4**	**6209.7**	**19250.0**

are returned to the settlement tank. Depending on presence of the swimming stages i.e. late auricularia and post-late auricularia, feeding with *C. muelleri* ends on around day 14. The juvenile foods are prepared as follows:

- after collecting from the wild, seaweed (Fig.30) and mud (Fig. 31) are sundried (Fig. 32);
- mud is sieved through 200 or 250 µm screen (Fig 33) and the seaweed is chopped in fine pieces (Fig. 34);
- after weighing, seaweed and fish meal are dipped in 1 µm filtered seawater for a several hours or overnight (Fig. 35)
- Spirulina is dipped in freshwater (rainwater) for several hours or overnight;
- Use blender to make the softened-foods finer pieces;
- sieve through 100 µm before feeding the animals

The protein content is the most important element in feeding pentactula and juveniles. Low fat content (crude fat around 5%) is desirable to maintain better water quality during the larval rearing and the 1st phase nursery culture in the settlement tanks. The author uses dried alga Spirulina instead of Algamac® (*Algamac ProteinPlus®) because Spirulina has much higher protein with low fat contents and the latter has higher fat contents. The author's choice of fishmeal is for milkfish (herbivore fish) farming because of less crude fat contents (about 3 %) compared to other fishmeals (Fig. 36). The author also uses the seaweed Sargassum spp. and Gracilaria sp., which contains about 10% crude protein with other essential nutrients, for free feed from the wild supplementing Spirulina and fishmeal. Mud or silt can be collected from the tidal flat area of the mangrove shore. The author has been using to mix with other feed (i.e. seaweed, fishmeal and Spirulina) for the juvenile grow-out as well as broodstock conditioning. The mud


Figure 24. Settlement tank.

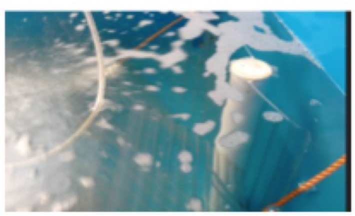


Figure 25. Screen-covered drain outlet.

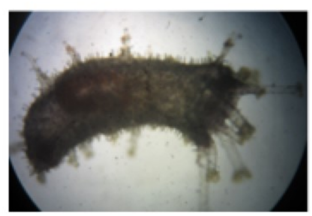


Figure 26. Day 28 juvenile.


Fig 27. Day 28 settled juveniles.


Figure 28. 5 g size 3-mo juveniles


Figure 29. Juveniles 3-mo in ocean grow-out site.


Figure 30. Seaweed *Sargassum* sp.


Figure 31. Mud from mangrove tidal flat.


Figure 32. Drying seaweed and mud.


Figure 33. Dried and sieved mud.


Figure 34. Dried seaweed.


Figure 35. Preparation for feeding.

Figure 36. Fishmeal.

Figure 37. Down-weller “habitat simulators” for juvenile grow-out.

contains detritus with organic substances and symbiotic heterotrophic micro-organisms in the mangrove forest (i.e. Schizochtrium spp.) which are known to have high contents of poly-unsaturated fatty acid (ω3 fatty acid). This heterotrophic (propagating without using sunlight) organisms live in the mangrove shore. *Algamac® and Algamac ProteinPlus® are commercial products from Aquafauna BioMarine, USA.

Figure 38. Estimating settled 2-mo juveniles by detaching from the plates.

From day 28 or one month after spawning to six-month-old juveniles, an example of feeding schedule was given in the following table 6: juveniles from 2-month-old (Settlement Tank #2 = ST2 with 4,996 J2W1) to 3-month-old in the 2,500L down-weller (Juvenile Habitat Simulator #3 = JHS3 with 5,000 J3W1) where the juveniles were produced from the spawning on September 8, 2017.

Juveniles attached on the plates are transferred on around day 56 (two-month-old after spawning) to the down-weller “habitat simulators for juveniles” (Figs. 37-38). Although the larger the animals, it is the easier for counting and estimating the standing crop, the juveniles were large enough (10 - 20 mm, 0.2 - 2g) to estimate the number of individuals by a naked eye.

Between 2 - 3 months old (1 - 5 g) after spawning, down-wellers “habitat simulators for juveniles” are stocked with juveniles either being attached on or being detached from the settlement plates. Stocking density of each tank was approximately 1,500 juveniles per square meter. Those remaining juveniles in the settlement tanks need to be fed weekly but are to be transferred in earlier occasions to hapas or ocean grow-out sites. The juveniles in the habitat simulators will be fed weekly for subsequent two or three months before transferring to grow-out phase (farming) in the ocean sites, earthen ponds or restocking sites.

7. Grow-out Culture

At around four or five months after spawning, the juveniles should reach to average 5 - 10 g. So, they are ready for farming. There is no published information available on the feeding techniques or feeding amount for both settled pentactula, early juveniles and juveniles up to six months after spawning. Also, there is no information about feeding techniques after six months old to harvesting size to export. The

Table 7. The results of counting of two-month-old juveniles attached on the settlement plates and estimate of those attached on the tank surface during the hatchery work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

day 49 (Oct. 27-29, 2017)	**Settlement Tank #1 (from LR2-1)**	**Settlement Tank #2 (from LR1)**	**Settlement Tank #2 (from LR2-2)**
counted total number of juveniles (on plates)	**2,154**	**2,975**	**17,067**
estimated total number of juveniles (tank surface)	**3,670**	**2,021**	**51,304**
estimated total number of juveniles (plates + tank)	**5,824**	**4,996**	**68,371**

Table 8. Restocking JHS tanks with 2-mo juveniles.

2.5t Habitat Simulator Tank for Juveniles (with plates)	**No. of Juveniles Counted**
JHS#1 =	**5,985**
JHS#2 =	**4,642**
JHS#3 =	**5,129**
JHS$4 =	**4,569**
JHS#5 =	**1,871**
total =	**22,196**

Table 9. Number of 2-mo juveniles in the settlement tanks.

2.5t Settlement Tank after Transfer (without plate)	**No. of Juveniles Estimated**
ST#1 =	**3,670**
ST#2 =	**2,021**
ST#3 =	**51,304**
total =	**56,995**

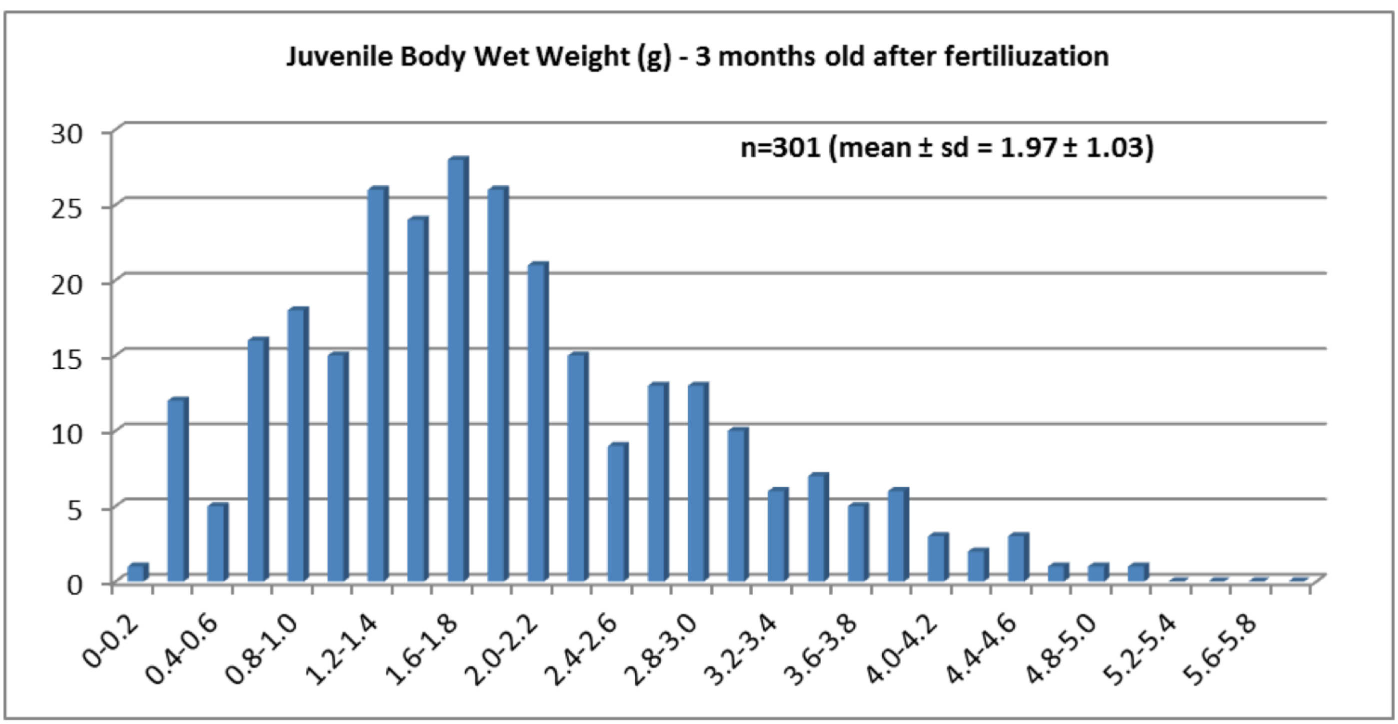
Table 10. Feeding table for 6-month-old juveniles in the downweller habitat simulator for juveniles.

	daily amount of feed (g) = **smaller size**				daily amount of feed (g) = **larger size**			
	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total
6M (10-40g) (ratio) 1% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
4,750	NA	306.5	153.2	475.0	NA	1225.8	612.9	1900
4,750	NA	536.3	268.1	831.3	NA	1379.0	689.5	2138
4,750	NA	766.1	383.1	1187.5	NA	1532.3	766.1	2375
4,750	NA	990.0	498.0	1543.0	NA	1685.5	842.7	2613
survival rate to 12M = 95% - 4 weeks total feed=	NA	18233.9	9116.9	28262.5	NA	40758.1	20379.0	63175.0

feeding table (Table 10) was developed by the author for private farming enterprise in Australia. Note that the feeding tables should be used only if actual or estimate of number of juveniles are not available. Feeding the juveniles and/or young adults are generally based on and calculated by the average body weight (wet weight). When they reach preferably to 10 - 20 g (average 5 - 10g) size after 3 - 5 months from spawning, they are ready for grow-out in the farm (ocean enclosures and/or ponds). Stocking density for the grow-out farming could be at 2 - 3 individuals per square meter (m2). In a hapa method elsewhere, 1- 2 g size about 2 or 3-month-old juveniles are stocked at 200 individuals per m2. On the other hand in a down-weller juvenile habitat simulator (e.g. 10,000L in Australia) with tank floor area of approximately 10 m2, initial stocking density is about 1,000 juveniles per m2). It is recommended to reduce the stocking density to at least half or preferably to 1/4 on around 5-month-old.

The following pages describe how to estimate feeding amount for the juveniles of 3-month-old. Also, explaining how accurate these feeding tables and how to use these tables developed by the author. For rearing early juveniles, it is recommended to feed the animals with 1/4 of adult grow-out amount = (1% body weight) x ¼ = 0.25% daily, and then increase the amount gradually to 0.5% and later around 5-month-old to 1%. The hatchery and farmhands must monitor the animals daily or at least weekly, such as measuring body weight by sampling monthly to adjust (increase) amount of weekly feeding amount. The following is an example of 300 juveniles of 3-month-old collected from a hapa elsewhere and measured body weight (mean ± sd = 1.97 ± 1.03 g). The histogram of these 301 (say 300) showed that they were smaller and closer to minimum weight group (1g group), indicating that they had not been fed well.

The following is how to estimate the weekly feeding amount of the 300 juveniles of 3-month-old, when they are required daily with 1% of body weight of food. Also, how-to estimate the feeding amount by using the juvenile feeding table when actual body-weight measurements were not done onsite.



(EXAMPLE) Histogram of the 3-month-old juvenile body weight obtained from hapa in a pond.

"How-to-Compute" weekly feeding amount (1% Body Weight) for 3-month-old "300" juveniles from hapa.

1.97g x 1% BW per day = 1.97g x 0.01 = 0.0197g per day per juvenile
0.0197 x 300 juveniles per day = 5.91g per day = 5.91 x 7days = 41.37g per week in total
*Spirulina vs. Fishmeal vs. Seaweed = 1 : 5 : 5 for the 3-month-old juveniles
therefore,

Spirulina = 41.37 x 1/(1+5+5) = 3.76g per week (0.54g per day)
Fishmeal = 41.37 x 5/(1+5+5) = 18.8g per week (2.69g per day)
Seaweed = 41.37 x 5/(1+5+5) = 18.8g per week (2.69g per day)

* If number of juvenile is 300, then the feeding amount is 300/10,000 of the figures given in the following Feeding Table.

daily amount of 3-feed total = 25 x 300/10,000 = 0.75g when given 0.25% BW

If given 1% BW,
daily amount = 0.75g x 1/0.25 = 3g and weekly amount = 3g x7days = 21g
Spirulina = 21 x 1/(1+5+5) = 1.91g
Fishmeal = 21 x 5/(1+5+5) = 9.55g
Seaweed = 21 x 5/(1+5+5) = 9.55g
However, the amount is based on the Smaller Group (1g).

If the juvenile's average body wet weight is 1.97g (= 2g), the feeding amount should be increased

and then, re-calculated for the "300" juveniles.

i.e. Each 1 g increment is (875-1,75)/4 = 1,75 per week

1g weight group = 175; 2g weight group = 175 + 175 = 3,50g; 3g weight group = 350 + 175 = 525g

Feeding Table for the 10,000 Juveniles (3 M): Smaller Group (1g) and Larger Group (5g)								
	Smaller Group (1g)			Larger Group (5g)				
10,000 3M juveniles	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
3-4M (ratio) 0,25%BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
wk-1 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-2 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-3 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-4 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
Weekly Total (g)	15.9	79.5	79.5	175.0	79.5	397.7	397.7	875.0

4g weight group = 525 + 175 = 700g; 5g weight group = 700 + 175 = 875g

therefore,

weekly amount of the 2g weight group could be 350g per "10,000" juveniles

weekly amount of the 2g weight group of the "300" juveniles = 350g x 300/10,000 = 10.5g when given 0.25% BW

therefore,

Spirulina = 10.5 x 1/11 = 0.95g

Fishmeal = 10.5 x 5/11 = 4.77g

Seaweed = 10.5 x 5/11 = 4.77g

If given 1% BW, weekly feeding amount of 2g weight group = 10.5 x 4 = 42.0g

therefore,

Spirulina = 0.95 x 1/0.25 = 3.8g

Fishmeal = 4.77 x 1/0.25 = 19.1g

Seaweed = 4.77 x 1/0.25 = 19.1g

The estimated weekly feeding amount from BW measurements (1.97g ± 1.03g) was total 41.37g = Spirulina 3.76g + Fishmeal 18.8g + Seaweed 18.8g

Thus,

the above “Feeding Table” is very accurate (+0.04g < > +0.67g), almost the same feeding amount obtained from actual BW measurements.

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