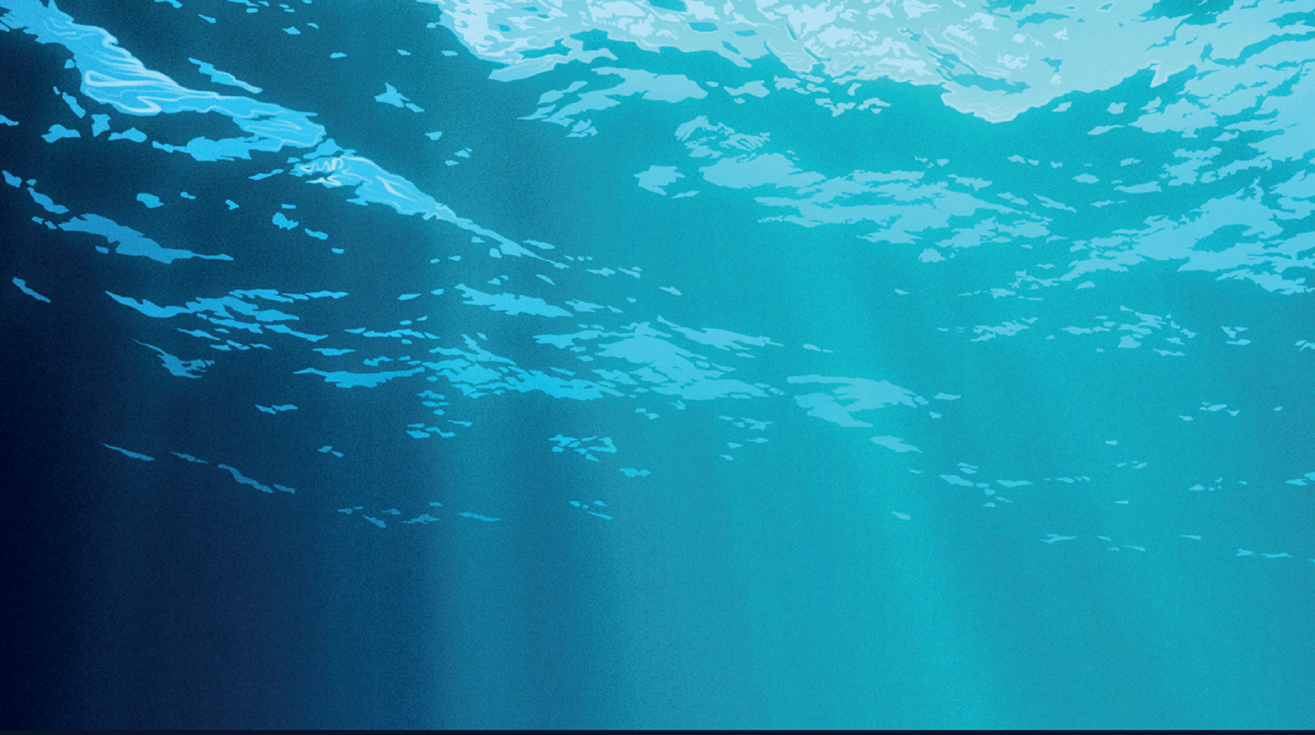

VANNAMEI SHRIMP FARMING

Edited by

S. Felix, Tzachi Samocha and M. Menaga

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VANNAMEI SHRIMP FARMING

This book arose from an attempt to find a new way to approach the shrimp aquaculture's future, facing up to the central insight that a global, technology-driven blue revolution will require new forms of governance to match the technological and social changes brought by innovative aquaculture practices. Each chapter contains evidence-based background information emphasizing core science, intended for the professional who already possesses a basic understanding of the principles of shrimp aquaculture and layout of each chapter includes a table of contents, materials and methodologies and a concluding set of objectives of the experimental study for the better understanding of the subject matter to the readers. The aim of this book is to provide a basic understanding of the modern culture techniques currently used in shrimp aquaculture research, primarily for vannamei, such that readers can develop an understanding of both the power and limitations of Intensive systems.Recently, in the scientific literature, there has been a profusion of information pertaining to many advanced culture systems such as raceways, reciruclatory aquaculture systems and many advanced culture practices such as biofloc technology and probiotics based culture practices.The material covered in the chapters of this book provides background to newcomers interested in Intensive shrimp culture techniques and a description of the current state of research and scientific understanding of advanced systems and standard management practices in regards toenvironmental sustainability of shrimp aquaculture would be much more helpful for the farmers and the industrial stakeholders. For researchers currently working in the field on specific culture systems and practices this text provides invaluable information that relates innovative intensive culture systems.

Prof. S. Felix, presently the Vice-Chancellor of Tamil Nadu Dr. J. Jayalalithaa Fisheries University & the President of the World Aquaculture Society (Asia Pacific Chapter). He is having 34 years of teaching experience. He is specialized in Aquaculture and Aquaculture Biotechnology and Systems development and had mobilized more than 150 Crores for the University in the form of Research & Development grants.

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Preface

The potential for increased production of *Penaeus vannamei* throughout the globe through advanced culture practices holds out hope for improved nutrition and a better livelihood for millions of people. The concept for this book arose with this in mind. The purposes envisioned early in its development quickly proved too lofty, however, and as this work took shape it became apparent that, rather than trying to change the world with a single volume, more practical editorial approaches would probably be required. Aquaculture has increased tremendously in the last decades and is predicted to continue to grow incredibly with higher growth rate. The aim of this book is to provide a scientific forecast of the development with a focus on the environmental, technological, social and economic constraints that need to be resolved to ensure sustainable development of the industry and allow the industry to be able to feed healthy aquaculture products to the future generations. We made this book with a degree of simplification to upkeep the readers' interest and attention. As a result, this text would present exhaustive methodology for adopting vannamei shrimp culture practices with latest technologies to balance between practical application and more technical considerations. Citations have been identified not only for their illustrative value, but also to serve as starting points for those wishing to pursue particular topics in greater depth. The works cited throughout this book reflect the dedicated efforts of many scientists over the years who share their interest in the topic. Its our sincere hope as we finalize this work that it will capture the interest of a new generation of aquaculturists who, collectively, may realize our vision of taking up the shrimp aquaculture to its next level!

Authors

CHAPTER

1

VANNAMEI FARMING IN INDIA

S. Felix

Shrimp farming sector in India is highly remunerative and a fast growing sector with 50240.77 ha area under the culture which yields an export production of 353413.1 MT with an enormous scope for increasing the foreign exchange. Although higher production levels are needed, increased aquaculture production is limited globally by the availability of suitable land and water.

Shrimp farming in India, till 2009, was synonymous with the mono culture of tiger shrimp, *Penaeus monodon*. About 1,90,000 ha brackishwater area have been developed for shrimp culture in the country spread over all the coastal states. Since 1995 culture of *P. monodon* is affected by White Spot Syndrome Virus (WSSV) and the development of shrimp farming has become stagnant.

Most of the Southeast Asian countries like Thailand, Vietnam, Indonesia were also culturing *P.monodon* and since 2001-02 onwards most of them have shifted to culture of exotic Whiteleg shrimp, *Penaeus vannamei* because of the availability of Specific Pathogen Free (SPF) and Specific Pathogen Resistant (SPR) broodstock. In India, Pilot-scale introduction of *P. vannamei* was initiated in 2003 and after a risk analysis study large-scale introduction has been permitted in 2009. The commercial farming of Pacific white shrimp production started from the year 2009-10 is the largest cultured shrimp in terms of production and productivity. Andhra Pradesh tops in the area under culture and production followed by Tamil Nadu and Gujarat

P.vannamei is a suitable species for semi-intensive culture with the availability of pathogen free seed. The major issues to be considered are bio-security and maintenance of water quality through constant monitoring. It also requires higher technical knowledge to achieve better production in sustainable manner.

BIOLOGY

P. vannamei is native of Pacific coast of Mexico and Central and South America as far south as Peru. It is mainly found on mud bottoms, down to a depth of 75 m. It is commonly known as white legged shrimp or Mexican white shrimp. It is greyish-white in colour. The maximum weight of females in the wild is about 120 g. The males are smaller at 60-80g. It lives in the column and prefers clayey loam soil.

The growth at 30°C is much higher than at 25°C. The optimal range of temperature for the species is between 30 and 34°C. At 20°C growth virtually stops. It can tolerate salinity levels of 0 to 50 ppt. Growth is uniform within 10-40 ppt. They can grow in freshwater also but the growth is slow below 10 ppt. The pH range of 7 to 9 is tolerated with optimal growth at pH 8.0. Dissolved oxygen levels above 4.5 ppm are required for optimal growth. Turbid water with flocculated particles of more than 0.5 micron resulted in better growth than clean water mainly because of the presence of algae and bacteria. Ammonia -N and Nitrite - N levels should be less than 0.1 ppm and 1 ppm respectively.

P. vannamei is an omnivorous scavenger and is less aggressive and less carnivorous than P. monodon. Food intake is more during evening and night. Retention time of food in the gut is 2.2 to 5 hours. Food is digested at modest acidities of pH 5.5-7.0. Growth of *P. vannamei*, under confined culture conditions was similar to *P. monodon* till they attain 20g size. Beyond that the growth rate was poor. The shrimps attained the size of 20g within a period of 100-120 days depending on the stocking density.

Advantages of *P. vannamei*

Culture of *P. vannamei*, is being taken up in many countries because of the following characteristics:

- It grows as fast as *P. monodon* upto 20 g.
- It is easier to culture in very high stocking densities of upto 150/sq. m due to their less aggressive nature.
- It is tolerant to wide range of salinities of 0.5 to 45 ppt.
- It is very tolerant to low temperatures of upto 15 degree centigrade.
- It requires low protein feed (20-35%).
- It is an easy species to breed and hence domestication of the species is very successful with the production of SPF stock. Commercial availability of SPF and high-health stock is an added advantage.
- Selective breeding work for the production of SPR broodstock is easier because of the short generation period and easier captive breeding.

- Higher survival rates in hatchery (50-60%).
- Has a very good market in the USA, as the most preferred species with higher meat yield (66-68%).

Limitations of *P. vannamei*

Though the above advantages make *P. vannamei* a very important cultivable species, but the following disadvantages create some apprehension for their introduction in the country:

- *P. vannamei* is highly susceptible and a carrier of Early Mortality Syndrome (EMS), Enterocytozoon hepatopenaei (EHP), Taura Syndrome Virus (TSV), White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) and Lympoid Organ Vacuolization Virus (LOVV). Though SPF stocks are available for these viruses, the performance of these in the virus ladden environment is doubtful. WSSV is prevalent in the country and its infectivity and pathogenicity for *P.vannamei* is similar to that of *P. monodon.*
- *P. vannamei* is being cultured in very high densities under intensive management, which might lead to environment related issues like nutrient loading.
- *P. vannamei* is highly susceptible to hypoxic conditions and hence there is a need for continuous aeration during high density cultures.
- Handling, processing and transport are relatively more difficult in *P.vannamei* compared to *Penaeus monodon*.
- There is high competition in the International market with world -wide production.

SPECIFIC PATHOGEN FREE STOCK

P. vannamei is highly susceptible to a number of viral pathogens. White Spot Syndrome Virus (WSSV), Early Mortality Syndrome (EMS), Enterocytozoon hepatopenaei (EHP) , Taura Syndrome Virus (TSV), Yellow Head Virus (YHV), Infectious Hypodermal Haematopoietic Necrosis Virus (IHHNV), Lymphoid Organ Vacuolization Virus (LOVV), Reo like Viruses (REO) are some of the virus reported in the species.

In order to eliminate the presence of the virus in the seed, Specific Pathogen Free (SPF) stock has been developed by producing a number of generations in highly bio-secure facility with continued surveillance of pathogen presence. Although SPF shrimp are, by definition, free of all specifically listed pathogens, SPF shrimp may be infected with a known pathogen that is not included on the SPF list of the shrimp supplier, or with an un-known pathogen that has not yet been described.

Offspring of SPF shrimp are not considered SPF unless they are produced and maintained at an SPF facility. SPF status changes with the pathogen condition of the

shrimp, as well as the type of environment within which they are cultured (level of biosecurity). One of the main advantages of culturing *P. vannamei* is commercially available as high health animals from Specific Pathogen Free (SPF) stocks while *P. monodon* have very limited availability from SPF stocks.

Vulnerability of *P. vannamei* to the diseases

Stocking of pathogen free post larvae alone will not guarantee a disease free culture since the pathogens could still enter the culture environment horizontally and infect shrimps during the culture. Viral pathogens still enter the culture through:

- By persisting in the soil
- Intake water
- Aquatic vectors introduced through intake water, by crabs and other animals
- Contaminated land animals and birds
- Contaminated farm inputs
- Contaminated farm implements

Crabs are one of the carriers of viral pathogens and providing crab fencing in shrimp farms is considered as one of the important bio-secured measure. Carriers like crabs could also move from pond to pond over land barriers. Birds such as crow/ water crow pick up the dead and moribund shrimps affected with viral disease from ponds and may drop in unaffected ponds, there by transmitting the virus mechanically. Feed ingredients of aquatic origin and wet/ moist feeds could be potential source of pathogens. Pond to pond transmission could occur through the use of farm implements and farm workers.

PREPAREDNESS FOR THE FUTURE

In the last few decades, the shrimp industry experienced the viral disease outbreak that led to the collapse, or near collapse of the flourishing shrimp industries in many countries. A disease named firstly as early mortality syndrome (EMS), later defined as acute hepatopancreatic necrosis disease (AHPND), was found in China, 2009. And later spread to other countries such as Vietnam, Thailand and Malaysia then spreading to the south and Central America. Though this disease has not been pronounced in India, an equivalent threatening disease known as *Enterocytozoon hepatopenaei*(EHP),a microsporidian infects the postlarvae of *Penaeus vannamei* has handicapped the Indian Shrimp aquaculture Industry which led to the decline in production coupled with a decline in global market prices for shrimp is of serious concern to the farmers.

The environmental impacts of shrimp aquaculture often causes a potential effect, and this convention leads to the outbreak of diseases. The Impacts are potential because effects depend on characteristics of the environment in which aquaculture production systems are embedded. Contrasting to broad issues related to resource use, many of the problems commonly attributed to shrimp aquaculture such as the adverse effects of waste discharge, consumptive water use, and release of chemicals and antibiotics are related to facility operations and can be addressed on shorter time scales through technology-based approaches (standard management practices, or SMPs). Farmers are not following the required standard management practices (SMPs) especially with regard to pond preparation resulting in high mortality such as through RMS (Running Mortality Syndrome) and EHP (*Enterocytozoon hepatopenaei*).This needs to be corrected but government extension services alone (State Fisheries and Central Institutions) cannot change farmers mindset. As farmers are mainly dependent on advice from feed technicians, consultants and fellow operators, the latter group could play a larger role in the dissemination of information.

CHAPTER

2

SHRIMP NURSERY TRIALS WITH NO WATER EXCHANGE

Tzachi M. Samocha, David I. Prangnell, Leandro F. Castro and Thomas R. Zeigler

INTRODUCTION

Postlarvae (PL) nutrition and adequate oxygenation and mixing of the culture medium are all factors that have a major impact on shrimp performance in any culture systems and more so in intensive zero-exchange, biofloc-dominated production systems.

Because of its nutritive value, to ensure production of high quality PLs, commercial shrimp hatcheries have been using newly hatched, live or frozen, *Artemia* nauplii to feed early larval and PL stages. Nevertheless, shortage and high prices of good quality *Artemia* cysts have been forcing these hatcheries to use *Artemia* substitutions for partial replacement. Furthermore, when young PLs are being raised at high densities nursery systems, their feed has to be nutritious with adequate level of attractants to stimulate consumption in order to avoid negative impact on PL performance.

Feeding of properly formulated high quality liquid or dry feed can be more convenient and cost-effective than live *Artemia*-based diets. However, offering the PL with the correct feed quality, quantity, and the particle-size for their body size is a prerequisite for PL performance especially in intensive nursery systems where availability of natural food is limited. Other factors such as adequate dissolve oxygen levels and mixing of the water column are equally important to ensure even distribution of the feed and preventing the development of anoxic areas especially on the tanks' bottom during the first few week of the nursery phase.

Previous studies at Texas A&M AgriLife Research Mariculture Lab at Flour Bluff, Corpus Christi, Texas have been conducted in shallow raceways (0.45 m average water depth) using airlift pumps, air diffusers, and a pump-driven Venturi injector supplemented

with pure oxygen to maintain adequate dissolved oxygen (DO) and mixing of the culture water in intensive nursery and grow-out tanks of the Pacific White Shrimp with good results. To support high yields of >7 kg/m^3 and 9.5 kg/m^3, respectively, the Venturi injector had to be supplied with pure oxygen.

Recent trials at this facility showed that high pressure, pump-driven, a^3 injectors were suitable for maintaining DO and water mixing in high-density biofloc-dominated tanks operated with no water exchange for production of marketable shrimp with yields greater than 9 kg $m^3$with no need for oxygen supplementation. Nevertheless, their performance in nursery systems during the early nursery stage when PL are very small and fragile has not been adequately studied.

The present trials were design to study the performance of *Litopenaeus vannamei* young PL in the nursery phase under two growing conditions; a) when offered two dietary regimes; with and without EZ *Artemia,* in airlift pump operated 40 m^3, zero-exchange, biofloc-dominated, raceways (RWs), and b) when fed the EZ *Artemia* and the dry feed in 100 m^3, zero-exchange, biofloc-dominated, RWs operated with high-pressure a^3 injectors.

METHODS

Two 62 days nursery trials were conducted with five to ten-day old *P. vannamei* PLs (av. wt. 0.94±0.56 mg) produced by commercial hatchery (Shrimp Improvement System, Islamorada, FL, USA) from a cross between Taura-Resistant and Fast-Growth, Specific Pathogen-Free, breeding populations.

Stocking densities in the six 40 m^3 and the two 100 m^3 RWs were 675 and 540 PL/m^3, respectively. RWs were filled, two days prior to stocking, with 5 ppm chlorinated natural seawater at reduced salinity of 30 ppt and with nitrifying bacteria-rich water (up to 10% of the RWs water working volume). No water exchange was conducted in either system throughout the trials. Chlorinated municipal freshwater was used to compensate for evaporative and water losses during biofloc cropping aiming at total suspended solid concentration between 250 and 350 mg/L range. To avoid development of anoxic patches on the RWs bottom, water was mixed manually everyother day for the first three weeks. Water DO levels were increased over time based on the system requirement using the built-in oxygenation tools in each RWs. Similarly, water mixing and turbulence was increased as the PLs increased in size. Each RW was equipped with foam fractionator and settling tank to maintain biofloc concentration at the desired range. Although each RW had cyclone filter, these devices were not used throughout the nursery phase. Small amount of commercial nitrifying bacteria, *KI-Nitrifier™, (Keeton Industries,* Wellington, CO, USA) and controlled white sugar applications were used during the first half of the trial to expedite the establishment of healthy nitrifying bacteria in the culture media. No sugar

was added once the nitrifying bacteria were established. A brewed commercial probiotic, *Ecopro (EcoMicrobials™,* Miami, FL, USA), was added every three days at a rate of 400 mg/m^3 with sporadic increase in concentration up to 300 mg/m^3 based on *Vibrio* counts. Culture medium green forming and yellow forming *Vibrio* colonies concentrations were monitored twice-weekly on TCBS agar plates. Each RW had a YSI 5500 in-line dissolved oxygen (DO) monitoring system. Each of the 40 m^3 RW was equipped with one optical DO probe while each of the large 100 m^3 RW was provided with two sensors for continuously monitor DO and temperature.

Trial 1

The study was conducted in six EPDM-lined 40 m^3 (0.45 m average water depth) RWs (Figure 1) each aerated, circulated, and mixed with eighteen 50 mm airlift pumps, six 0.9 m air diffusers *(Aero-Tube™, Tekni-Plex, Inc.,* Wayne, PA, USA), a 2 HP pump-driven 5 cm Venturi injector, and bottom pipe with spray nozzles. The original experimental design involved feeding PLs in three RWs a combination of EZ *Artemia (Zeigler Bros. Inc.,* Gardners, PA, USA) and dry-feed *(Zeigler Raceway Plus* <400 um) for the first 8 days post-stocking, while those in the other three RWs were to be fed only the dry-feed.

Shrimp size variation at stocking was extremely high (0.94±0.56 mg) which necessitated abandoning the dry-feed only treatment as a large number of the small PL had empty guts. On days 3-5 after stocking, 42% of the feed, in the dry-feed only treatment, was replaced with EZ *Artemia* to stimulate feed intake. Identical feed and rations were fed to the shrimp in both treatments after the first 8 days: *Zeigler Raceway Plus* (<400 um, 400-600 um, 600-850 um), and *Zeigler Shrimp 40-9* with V-pak™ (1 mm, 1.5 mm, and 2 mm). From the second day on, feed was added continuously by belt feeders. Feed particle size and rates were adjusted on an ongoing basis according to shrimp growth sampling (twice a week), shrimp size variation (once every two weeks), and assumed growth, FCR, and survival.

Trial 2

This trial was conducted in two 100 m^3 (1.1 m average water depth) RWs (Figure 4). Each RW was equipped with fourteen high-pressure a^3 injectors *(All Aqua Aeration,* Orlando, FL, USA) driven by up to two 2 HP pumps for all aeration, circulation, and mixing needs. Shrimp were fed the same dietary regime as those in the EZ *Artemia* and dry-feed treatment in Trial 1. The pump intakes were protected by three screens (500 u, 800 u and 1 mm) to prevent PLs losses.

Fig. 1. General view of the biofloc-dominated 40 m^3 raceway operated with air-lift pumps, air diffusers, Venturi injector, and bottom pipe with spray nozzles used in the 62-d nursery trial with *Litopenaeus vannamei.*

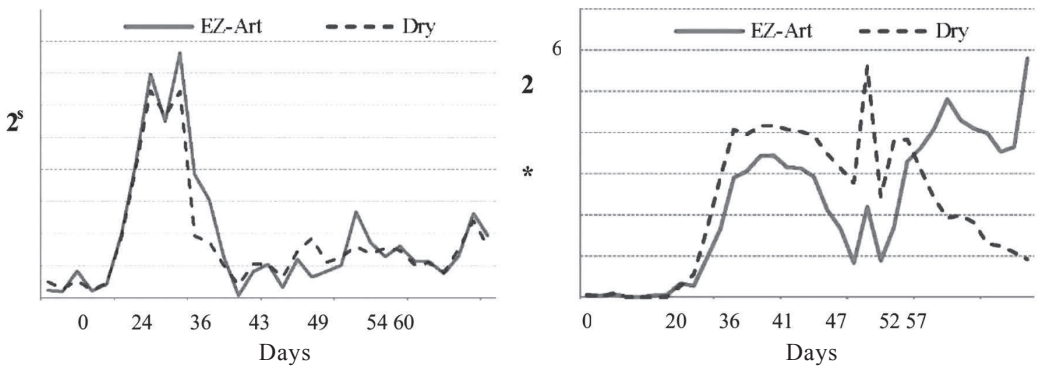


Fig. 2. Changes in TAN and nitrite-N in 62-d nursery trial with *Litopenaeus vannamei* in 40 m^3 raceways operated with no water exchange.

RESULTS

Trial 1

Following the adjustments made in the feeding regimes, there were no significant differences in final survival, weight, growth rate, yield or FCR between RWs at the conclusion of the 62-d trial. No significant differences (>0.05) were found in any water quality indicators between RWs. Mean temperature, salinity, DO, and pH were 26.6ºC (Range: 20.8-30.2ºC), 30.4 ppt (Range: 29.4-31.5 ppt), 6.47 mg/L (Range: 4.43-8.52 mg/L), and 8.2 (Range: 7.6-8.5), respectively. Mean TAN and NO2-N were 0.79-1.17 mg/L (max 4.95

mg/L) and 1.44-3.17 (max 10.93 mg/L), respectively, and had no observed negative impact on PLs. Unlike observations from previous nursery trials, TAN and nitrite concentrations stayed lows throughout the trial (Figure 2). Non-sucrose fermenting (green colony forming units- GCFU) *Vibrio* remained below 100 CFU/mL and less than 28% of the sucrose fermenters (yellow colony forming units- YCFU) concentration and were only observed on 14% of plates (Figure 3).

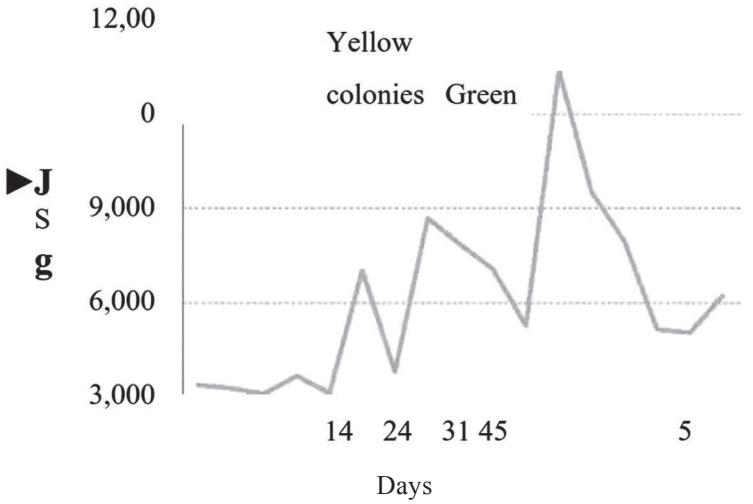


Fig. 3. Changes in yellow and green *Vibrio* forming colonies in 62-d nursery trial with *Litopenaeus vannamei* in 40 m^3 raceways operated with no water exchange.

Trial 2

At the conclusion of the 62-d trial, survival was very high, and FCR was low (Table 1). The a^3 injectors operated with only one 2 HP pump from day one were capable of maintaining adequate mixing and dissolved oxygen (>4.5 mg/L) in the RWs throughout the trial, with no visible damage to the shrimp (Figure 7). Use of 500 u 800 u, and the 1 mm filter screens on the pump intakes enabled adequate supply of water to operate the injectors without damaging the shrimp. The average weight of the shrimp at harvest was higher (6.46 vs. 5.57 g) and the CV of the individual weight samples lower than the shrimp in the 40 m^3 RWs system (33.3 vs. 44.3%). Water quality variables were all within the range suitable for *P. vannamei* culture. Mean temperature, salinity, DO, and pH were 26.6ºC (Range: 22.2-30.2ºC), 30.4 ppt (Range: 29.7-31.1 ppt), 6.67 mg/L (Range: 4.41-8.46 mg/L), and 8.1 (Range: 7.6-8.58), respectively. Mean TAN and NO2-N were 0.76-0.80 mg/L (max 2.72 mg/L), and 1.60-2.27 (max 5.46 mg/L), respectively (Figure 5). TSS was kept below 511 mg/L for the duration of the trial. GCFU *Vibrio* concentrations remained below 50 CFU/mL and less than 2% of the YCFU concentrations throughout the trial (Figure 6).

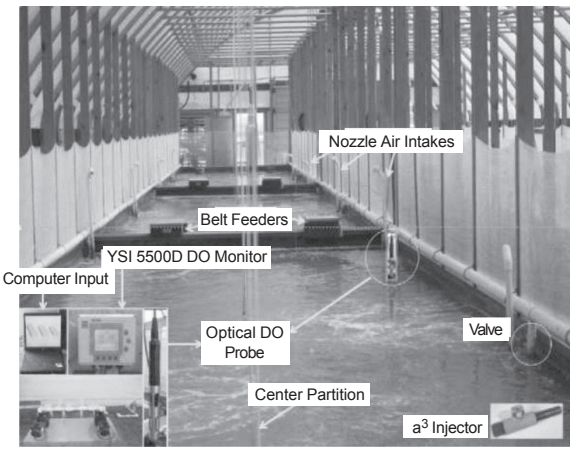


Fig. 4. 100 m^3 biofloc-dominated raceway operated with a^3 injectors and used in the 62-d nursery trial with *Litopenaeus vannamei.*

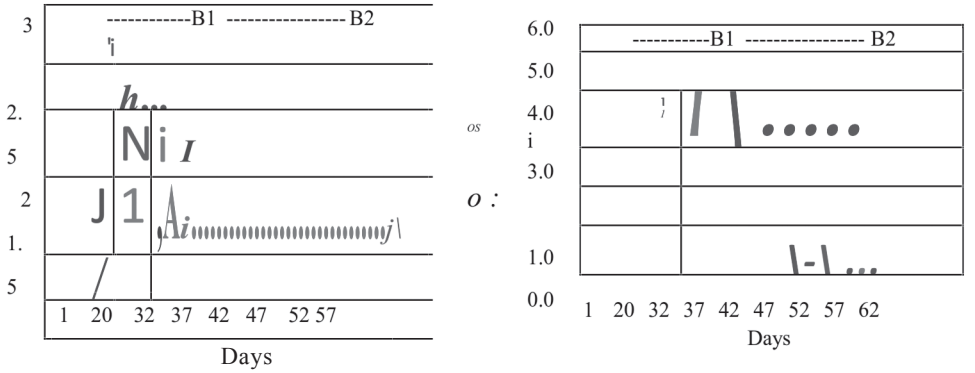


Fig. 5. Changes in TAN and nitrite-N in 62-d nursery trial with *Penaeus vannamei* in 100 m^3 raceways operated with no water exchange

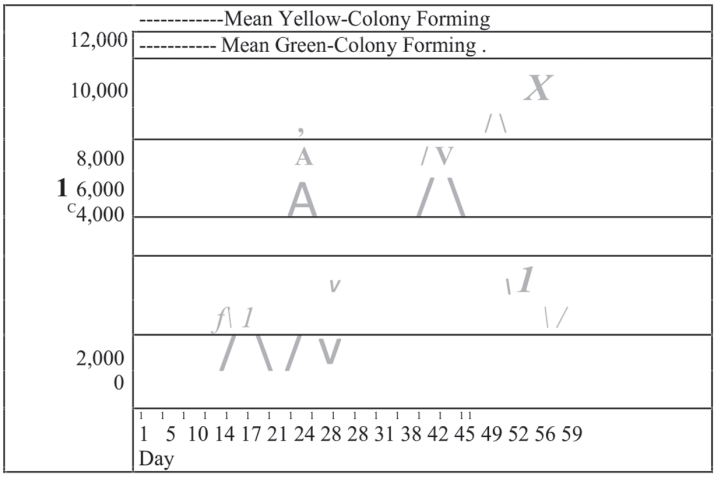


Fig. 6. Changes in yellow and green *Vibrio* forming colonies in 62-d nursery trial with *Litopenaeus vannamei* in 100 m^3 raceways operated with no water exchange.

Table 1: Summary of a 62-d nursery trial with young postlarvae (0.93±0.56 mg) *Litopenaeus vannamei* stocked (540 PL/m^3) in two 100 m^3 RWs when fed EZ Artemia + Dry-feed in biofloc-dominated water under with no water exchange conditions.

Parameters	Raceway B1	Raceway B2
Survival (%)	97.80	94.60
Final weight (g)	6.49	6.43
Growth (g/wk)	0.73	0.73
Yield (kg/m^3)	3.43	3.28
FCR	0.81	0.81
Sugar added (kg/RW)	33.36	33.09
Bicarbonate added (kg/RW)	26.00	25.00

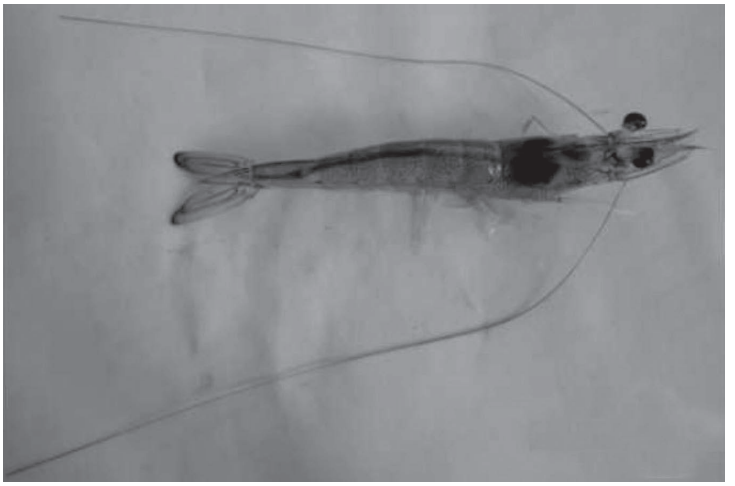


Fig. 7. A juvenile harvested from a 100 m^3 raceway showing the excellent condition of shrimp harvested from this system.

DISCUSSION

Because of low water temperatures during the first four weeks (20.8-26.7^{o}C) it took 62 days for the PL to obtain an average weight above 5.4 g in the 40 m^3 RW system. It is possible that the high shrimp size variation may have contributed to the extended duration of the trials. The problems with the small PL fed dry-feed only illustrate the importance of observing the shrimp very closely, and being prepared to respond to unexpected events such as the arrival of small or variably sized PLs. Use of EZ *Artemia* was a key to providing proper nutrition to the shrimp in the earliest phases, contributing to the final harvest success. Proactive management was critical for controlling FCR, and related water quality.

The results from the 100 m^3 RWs demonstrate that the high-pressure a^3 injectors can be used successfully for all oxygenation and mixing needs in nursery tanks stocked with very small PL (<1 mg). Furthermore, only one 2 HP pump was needed to support a yield > 3.3 kg/m of juvenile shrimp (> 6.4 g) with high survival and low FCR under zero-exchange, biofloc-dominated conditions. Manual water flow control for each a^3 injector, via ball valve, was a key to maintaining adequate water DO and to prevent damage to the young PL from strong mixing action. Future studies will evaluate potential use of a programmable variable speed pump to streamline the control of water flow needed for oxygenation and mixing.

The data from the 100 m^3 RWs suggest that the culture medium was more evenly mixed, with biofloc which developed sooner, with alkalinity which declined at a faster rate, and nitrifying bacteria that established sooner than in the 40 m^3 RW system. The mean am/pm DO over the nursery period in these RWs (6.55-6.79 mg/L) was also slightly higher than in the 40 m^3 RWs (6.36-6.57 mg/L). This suggests that the design of the 100 m^3 RWs, with a^3 injectors, provided a superior environment for the development of nitrifying bacteria, through enhanced mixing and higher DO as demonstrated by the higher amount of bicarbonate required to maintain water alkalinity compared to the airlift pump operated RWs. Furthermore, the results suggest that the100 m^3 RWs provided a superior environment for shrimp to grow compared to the 40 m^3 RWs as indicated by higher shrimp survival, growth rate, and yield.

The fact that under the conditions of this trial it was feasible to get juvenile shrimp with an average weight >6.4 g with FCR of 0.81 suggest that with additional fine-tuning of management we may be able to produce marketable size shrimp with similar low FCR values.

Although differences were found between the two raceway systems in shrimp performance, more studies are needed to determine if these difference are due to differences in stocking densities, water quality, system design or mixing dynamics.

Finally, the high shrimp performance, low GCFU *Vibrio* concentrations, and comparatively lower ammonia, and nitrite concentrations observed in these trials may demonstrate the benefits from using inoculum of nitrifying-rich water, probiotic, and nitrifying bacteria during the nursery phase of this species.

CHAPTER

3

DESIGN AND OPERATION OF HIGH-DENSITY, BIOFLOC-DOMINATED PRODUCTION SYSTEMS OF PACIFIC WHITE SHRIMP, *PENAEUS VANNAMEI*

Tzachi M. Samocha[7], David I. Prangnell, Terrill R. Hanson, Granvil D. Treece, Timothy C. Morris, Leandro F. Castro and Nick Staresinic

ABSTRACT

Seafood supply from the ocean has reached its carrying capacity. Aquaculture thus must meet future demand. Like all farming activities, aquaculture impacts the environment. Informed consumers increasingly drive adoption of sustainable practices that reduce discharge of waste, feed ingredients derived from over-harvested fish stocks, antibiotics, excessive use of water, and escape of cultured stock to wild gene pools. This requires a shift from traditional flow-through to recirculating aquaculture systems.

This presentation describes a sustainable alternative: high-density, indoor, biofloc-dominated shrimp production with no water exchange based on *in situ* microbial floc that removes harmful metabolites and supplements nutrition.

INTRODUCTION

The demand for protein from increasing world population — together with decreased fishery landings - has resulted in rapid growth of aquaculture. The world shrimp farming industry's annual growth over the last decade has been estimated at 10 percent. This rapid expansion has stimulated the intensification of production systems, which has resulted in the release of nutrients and organic waste, and sometimes the spread of diseases to receiving streams.

Reducing aquaculture's environmental impact is a widely accepted goal of seafood producers, retailers, and consumers. Consumers drive this trend by demanding seafood that meets sustainable production practices that reduce aquaculture's environmental footprint to include: Discharge of waste and pathogens to receiving streams, finite supply of feed ingredients from over-stressed fish stocks, use of antibiotics, excess use of diminishing water resources, escape of cultured stock to wild-stock gene pools, growing preference for locally-raised products with adequate traceability.

Increasing concerns over negative environmental impacts from shrimp farm effluent waters along with wide spread outbreaks of diseases have led to the development of culture systems with minimal or zero water exchange (Hopkins *et al.*, 1993). With little or no water exchange, properly managed Recirculating Aquaculture Systems (RAS) can reduce or eliminate the amount of nutrients and pathogens released into receiving streams. Biofloc systems are a unique type of RAS that maintain a community of suspended microalgae, autotrophic and heterotrophic bacteria which develop in limited-exchange systems (Ray *et al.*, 2010). Operating high-density shrimp culture systems with limited or no water exchange results in production of large volume of suspended flocculated organic particles referred to as biofloc. The promoted biofloc in these systems have been reported to have beneficial effects on shrimp culture to include: (1) improved water quality (WQ) through removal of toxic nitrogen species, (2) improved feed utilization and shrimp performance from natural productivity (Xu *et al.*, 2012; Xu and Pan, 2014); and (3) enhanced shrimp health through possible probiotic effect (Kim *et al.*, 2014).

BIOFLOC COMPOSITION, STRUCTURE AND DEVELOPMENT

"Biofloc" is a general term that describes an assemblage of living (bacteria, cyanobacteria, algae, fungi, protozoans) and non-living (detritus, uneaten feed, waste products) components that form suspended aggregates in many aquatic ecosystems, including aquaculture production systems. The aggregates (Figure 1) are composed can vary in size from the microscopic to > 1 mm with wet-weight density of slightly > 1 g/ml (Sears *et al.*, 2006).

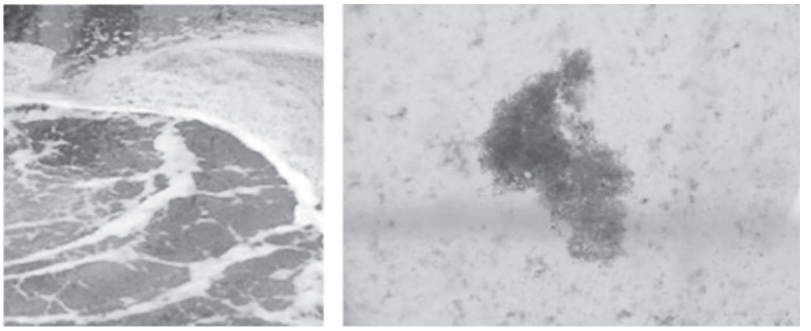


Fig. 1. Appearance of the water surface (left) and a microscopic view of an individual biofloc aggregate (right).

Biofloc microorganisms vary among systems and also within the same system over time (Leffler and Brunson, 2014). Bacteria typically dominate biofloc aquaculture systems. Not only are they abundant (up to 100 million bacteria/ml), but they exhibit high diversity.

Emerenciano *et al.* (2013) provides a thorough discussion of the many factors that determine floc composition, among which are temperature, salinity, pH, photoperiod, the intensity of vertical mixing, and the type of organic carbon available for bacterial metabolism.

Biofloc develops in newly filled systems soon after a suitable source of organic matter -uneaten feed, shrimp waste, or an added organic compound - has accumulated to a sufficiently high level. The rate of floc development can be advanced by 'boosting' or adding an organic carbon source to stimulate floc formation (De Schryver *et al.*, 2008).

Maintaining a dense floc concentration throughout a crop cycle can reduces the need for formulated feed (Avnimelech, 2015), especially in semi-intensive outdoor systems, which typically accounts for half or more of production costs in traditional aquaculture.

The nutritional quality of biofloc is related to the carbon-to-nitrogen ratio of culture water, dietary protein level, and light intensity. These and other factors are discussed in detail by Crab *et al.* (2012), Emerenciano *et al.* (2013), Ekasari *et al.* (2014), and Martins *et al.* (2014).

The quantity and quality of organic matter stored by bacteria ultimately determine the nutritional value of floc. This stored organic matter depends on the amount and type of organic carbon available for bacterial metabolism (Crab *et al.*, 2012). If the proper organic substrates are provided, then biofloc will store high-quality organic compounds that contribute to the nutritional needs of the shrimp.

On a dry-weight basis, the proximate analysis of biofloc breaks down as follows: protein: 12-50%, lipids: 0.5-41%, carbohydrates: 14-59%, and ash: 3-61.4%. The wide variation in these figures is due to differences in composition between young and mature floc aggregates and the culture conditions.

Beyond its proximate analysis, marine biofloc typically is rich in the amino acids valine, lysine, leucine, phenylalanine, and threonine, but it can be deficient in the essential amino acids arginine, methionine, and cysteine, as well as deficient in Vitamin C (Crab *et al.*, 2012; Taw, 2012; Ekasari *et al.*, 2014).

Biofloc alone, therefore, is insufficient to guarantee the level of growth and survival required by high-density shrimp culture. Thus, supplementation of formulated feed is required to satisfy the nutritional requirements of the shrimp in these systems.

BIOFLOC AND WATER QUALITY

Beyond its nutritional value, biofloc also can be managed to improve WQ in culture tanks. There are two types: autotrophs and heterotrophs.

Autotrophs are organisms that "nourish themselves" by producing the organic compounds from inorganic compounds. They obtain carbon from inorganic carbon sources such as carbon dioxide and bicarbonate. They are further classified as *photo*autotrophs, that derive energy from sunlight, and *chemo*autotrophs that derive energy from inorganic chemical compounds. In aquatic environments, the former are the algae, and the latter include bacteria such as the nitrifiers that obtain energy from the oxidation of ammonia to nitrate. The heterotrophs on the other hand obtain carbon from organic carbon sources.

Both autotrophs and heterotrophs found in biofloc improve WQ by assimilating or transforming dissolved inorganic nitrogen (ammonia, nitrite, and nitrate) that are harmful to shrimp. To this end, a biofloc-dominated system can be managed to favor autotrophic bacteria, heterotrophic bacteria, or some combination of the two in a mixed system.

BIOFLOC AND IMMUNE RESPONSE

Shrimp have a non-specific, labile immune system, which means that they have no specific (antibody-antigen) immune mechanism to respond to new pathogens entering production units (Roch, 1999). However, the dense microbial population in biofloc systems may play a role in activating the non-specific shrimp immune system, resulting in a type of defense that may permit a quick response against bacterial diseases (Kim *et al.*, 2014). In addition, biofloc has a probiotic effect, in which short-chain fatty acids (lipopolysaccharides, peptidoglycans, and P -1, 3-glucans) in bacterial and fungal cell walls play a role (Crab *et al.*, 2012). Biofloc microorganisms also suppress pathogen growth by competing for space, substrate, and nutrients, and by excreting inhibiting compounds (Emerenciano *et al.*, 2013).

Biofloc systems favor development of heterotrophic bacteria when the C:N ratio is high. These bacteria can rapidly remove ammonia from culture water. The biomass production per unit nitrogen of heterotrophs is about 40 times greater, with greater O_2 consumption and CO_2 production per unit nitrogen than nitrifiers. Feeding shrimp high-protein feed with no supplemental organic carbon results in low C:N ratio which favors development of chemoautotrophic bacteria, including nitrifying bacteria, which oxidize ammonia to nitrate and cause reduction in alkalinity.

Biofloc-dominated systems, such as the one developed at the Texas A&M AgriLife Research Mariculture Lab (ARML), rely on floc aggregates that contain heterotrophic bacteria *and* nitrifying bacteria. This "mixotrophic" system provides an efficient way to improve WQ and supplement shrimp diet.

In no exchange systems, continued use of the same culture water leads to accumulation of total nitrogen, usually as nitrate.

The correct response to high nitrate in closed-system aquaculture is to incorporate a denitrification step in system design. The conventional approach is to operate an anaerobic digester in which denitrifying bacteria reduce nitrate to nitrogen gas (Van Rijn *et al.*, 2006).

AUTOTROPHIC VERSUS HETEROTROPHIC SYSTEMS

Biofloc generally consists of a mix of chemoautotrophic and heterotrophic bacteria and photoautotrophic algae. Each type of microorganism plays a role in managing the concentrations of dissolved nitrogen compounds in culture water.

Among the chemoautotrophs, one group of nitrifiers oxidizes ammonium to nitrite (NH_4+ NO_2^-) and another oxidizes nitrite to nitrate ($NO_2^-NO_3^-$). The nitrate end-product is less harmful to shrimp than either ammonium or nitrite, so it can accumulate to higher levels before requiring removal.

Under no water exchange, sufficient organic carbon is available from the feed and shrimp waste for heterotrophic bacteria to metabolize approximately 1/3 of the ammonia. The remaining 2/3 is available for nitrification by chemoautotrophs (Ebeling *et al.*, 2006). Compared to a purely heterotrophic biofloc system, the mixotrophic system demands less oxygen, requires fewer carbohydrate additions, generates less CO_2, and produces lower microbial biomass. If supplemental organic carbon is added to the culture the system would shift toward a more heterotrophic regime.

Evolution of Recirculating Aquaculture Systems

The expansion of the shrimp farming industry has stimulated the intensification of the production systems. Recently, these systems started to incorporate Biofloc Technology (BFT), which can reduce nutrient releases, escape of non-native culture species, and spread of pathogens to the environment. The incorporation of BFT facilitated high density shrimp production using limited or no exchange practices with a much smaller footprint than outdoor ponds (Samocha *et al.*, 2010; 2012).

Advantages of Indoor Biofloc Systems

Few of the advantages associated with the use of these systems are: water conservation, stable WQ, reduced fertilizer use, small footprint, year-round production, faster growth, lower disease susceptibility with greater biosecurity, more efficient use of protein in feed, lower feed requirements, higher yields, and sustainability.

Disadvantages of Indoor Biofloc Systems

Few of the disadvantages associated with the use of these systems are: high capital investment per unit area, high energy input, power failure is critical, technical operating complexity, potential exposure to toxins, and disease risk.

Major Water Quality Indicators in Recirculating Aquaculture Systems

Different WQ parameters play significant role in RAS among them are: dissolved oxygen, temperature, nitrogen species (TAN, NO_2, and NO_3), pH, alkalinity, salinity, solids, ionic composition, and heavy metal concentrations. Thus, to maximize output, a successful operation should have in place the tools to monitor and maintain the WQ within the optimum range. Any deviations from the required range can result in sub-optimal performance that can also result in poor survival.

Key Factors to Consider in the Design and Operation of Biofloc Production System

In addition to WQ there other components that have significant impact on the economic viability of biofloc systems which include: site location, water availability and required pre-treatment, the type of building, the size and type of the culture tanks, availability of DO monitoring and control systems, methods used to maintain DO and to keep biofloc in suspension, cost and availability of power and adequate power backup, availability and cost of temperature control, availability and type of feed storage, feed quality and feeding practices, theft and predator control, biosecurity protocols, solid control and waste disposal methods, water storage, post-harvest water treatment methods, equipment storage space, workshop, office and staff accommodation, harvest and product handling procedures, quality control, availability of cold storage, and marketing program.

Careful examination and selection of the components listed above together with the use of well-trained workforce are critical for building and operating an economically viable super-intensive, no exchange, biofloc dominated shrimp production system.

The Texas A&M-ARML Super-intensive, Biofloc-dominated, No Water Exchange, Shrimp Production System

Shrimp production trials were performed in two systems both located inside greenhouse structures with no active water temperature control. Exhaust fans and roof shade cloth served to lower above optimal water temperatures during the summer months. Passive heat retention by the greenhouse structures enabled extension of the production trials from early spring to late fall in this temperate climate location.

The first experimental system had six raceways (RWs - Figure 2) lined with 1 mm ethylene propylene diene monomer membrane (EPDM, Firestone Speciality Products, Indianapolis, IN, USA.

All but one RW, which had a concrete bottom, had sand under the liner with 0.5% slope. Each has a surface area of 103.7 m^2 and working water volume of 40 m^3 with 0.45 m average water depth.

Every RW had five boardwalks and anti-jump netting around it to prevent escapement. Water circulation, mixing and oxygenation was provided by one 2 hp pump, six 0.9 m long air diffusers, and eighteen 5 cm airlift pumps. Every RW was equipped with a center partition positioned over a 5 cm PVC pipe with spray nozzles for homogenous distribution of oxygenated water from a 5 cm pump-driven Venturi injector (see Figure 4). All six RWs were equipped with online DO monitoring system (5500D, YSI Inc., Yellow Springs, OH, US -see Figure 3). Each RW had a settling tank (Figure 5), a foam fractionator (Figure 6), and a multi-cyclone filter (Figure 7) for solid control. The system was also provided with separation tanks to help drying the cropped-bioflocs to facilitate disposal and to conserve water (Figure 8). Six spring-loaded belt feeders were used to deliver feed 24/7 in each RW.

Biofloc systems operated with air blowers alone are generally capable of producing shrimp yields between 2 kg/m^3 and 4 kg/m^3. As mentioned above, each RW was equipped with a pump-driven Venturi injector which enabled yield shrimp production of > 9.5 kg per cubic meter of water.

The second system had two 100 m^3 RWs with 100 m^2 surface area and were lined with the same membrane used in the other system. Unlike the other system, these RWs were built half buried above the natural soil and had an average water depth of 1 m. In addition, the two RWs had a common concrete harvest basin outside the greenhouse which enabled the use of fish pump for harvest.

Similar to the other system, each RW had anti-jump netting, boardwalks, belt feeders, and center partition (Figure 9). Unlike the other system, aeration and water mixing in each RW were generated solely by two pumps of 2 hp forcing high-pressure water (45 psi) at a flow rate of 28.4 lpm through each of the 14 injectors *(a^3® All-Aqua Aeration,* Orlando, FL, US -see Figure 10).

Biofloc concentrations were maintained with help of ST (Figure 11) and a FF (Figure 12) using a similar setup used in the other system for drying and disposal of the bioflocs (Figure 8).

Raceways were equipped with the same online DO monitoring system used in the small RW system.

Figure 10.Water and air flow of a^3 injector used for water aeration and mixing in the 100 m^3 RW: One of two 5 cm PVC distribution pipe (a), A valve controlling water supply to the a^3 injector (b), A barrel union adapter (c), A water supply pipe (d), An air suction pipe (e), An a^3 injector (f), A mixture of air bubble and water streaming out of the injector (g), A boardwalk (h), and A 5 cm PVC ball valve for quick fill of the raceway (i). Blue arrows: high pressure water supply; Red arrows: atmospheric air suction.

The use of two 2 hp pump along with the 14 a^3 injectors in each RW supported marketable size shrimp yield of more than 9 kg/m^3 while using atmospheric air only to satisfy the shrimp and the biofloc oxygen demand.

Figure 11. (1) A 2 m^3 outdoor fiberglass ST used in each RW; (2) A top view of the ST; (3) A view of the piping system at the shallow end of the RW; (4) A view of the 5 cm PVC pipe returning water from the ST back to the RW: (a) a sleeve preventing the mixing of water entering and leaving the ST, (b) A hose delivering water from the RW into the ST, (c) A valve controlling water flow into the ST, (d) Water distribution pipe to the a^3 injectors, (e) The pipe returning water from the ST back to the RW, (f) A valve feeding the a^3 injector, and (g) A valve for quick fill of the RW.

Figure 12. (1) A photo of the homemade FF, and (2) A schematic drawing of the FF: (a) 30 cm PVC pipe, (b) An acrylic pipe, (c) foam delivery pipe, (d) temporary foam storage tank, (e) A valve controlling flow to the FF, (f) a^3 injector, (g) An air intake pipe, (h) A valve controlling return flow to the RW.

NURSERY TRIALS

Raceway System I [40 m^3]

All but one nursery trial were conducted in the small RW system. Nursery tank stocking densities varied from few hundred to several thousand post-larvae (PLs) per m^3 of culture water. Trials initiated with virgin water, showed that PLs could tolerate high concentrations of ammonia (>26 mg/L of TAN) and nitrite (35 mg/L NO2-N Figure 13) without negative impact on shrimp performance.

The objectives of the nursery trials were to evaluate the impact of select WQ management and control tools in order to establish optimal working ranges for several key parameters affecting the system. The results suggested the following operational ranges for these parameters: TSS (250-350 mg/L), SS (10-14 ml/L), alkalinity (140-160 mg/L $CaCO_3$). Continued monitoring of DO in the nursery RWs using the online DO monitoring system helped optimize feed delivery (e.g., use of automatic feeders for 24/7 feed delivery). Other studies determined the preferable feed and feed particle size for PLs of different sizes. Additional trials help define the role of different commercial feeds and feed management on shrimp performance.

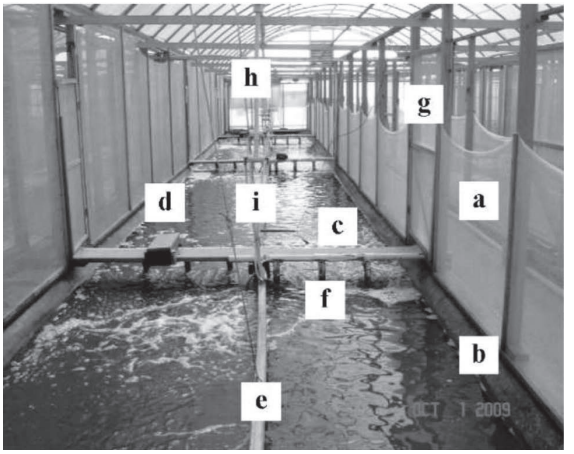


Fig. 2. Photos of a 40 m^3 RW with some of its support systems: (a) Anti-jump netting, (b) Freeboard, (c) a boardwalk, (d) Belt Feeder, (e) Center partition, (f) A bank of three 5 cm airlift pumps, (g) Access door, (h) 2.5 cm PVC air distribution pipe, (i) Ropes for positioning the center partition in place.

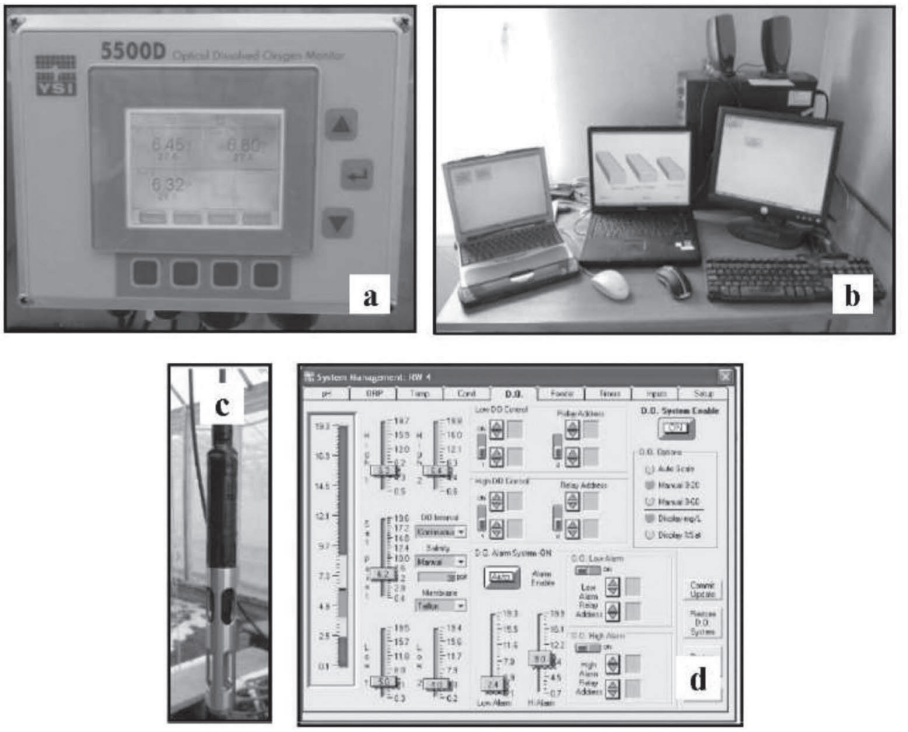


Fig. 3. *YSI 5500D,* an online dissolved oxygen monitoring system: (a) on-site display, (b) computer display with audio, (c) an optical dissolved oxygen probe, (d) programming and a screenshot of alarm-setting software.

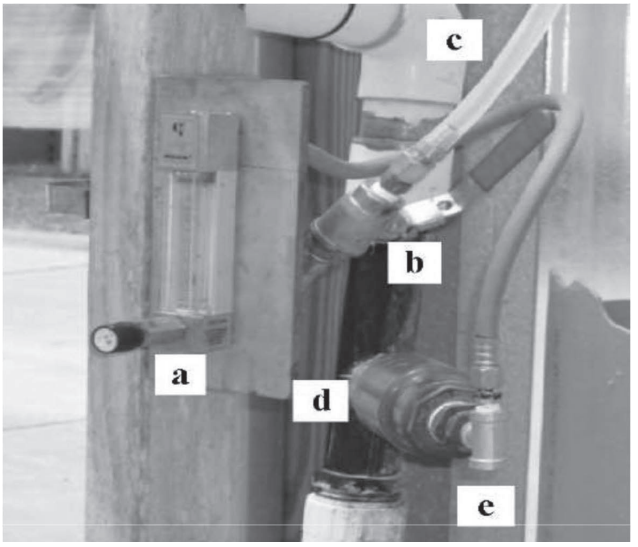


Fig. 4. A Venturi injector assembly: (a) oxygen flow meter, (b) oxygen supply valve, (c) oxygen supply hoses, (d) a check valve, and (e) atmospheric air intake.

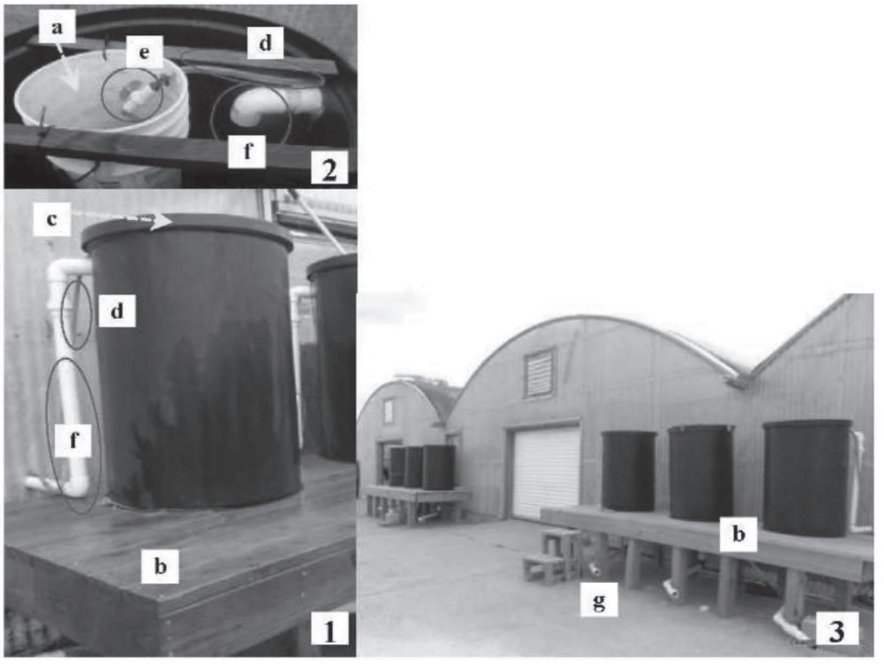


Fig. 5. STs used in the 40 m^3 raceway system: (1) a side view of tank, (2) top view of a tank, (3) a view of all six STs: (a) sleeve preventing mixing of water entering and leaving the tank, (b) STs wooden structure support, (c) a lid of ST, (d) 1.6 cm water supply hose from the raceway, (e) 1.6 cm PVC water supply control valve, (f) 5 cm PVC water return pipe.

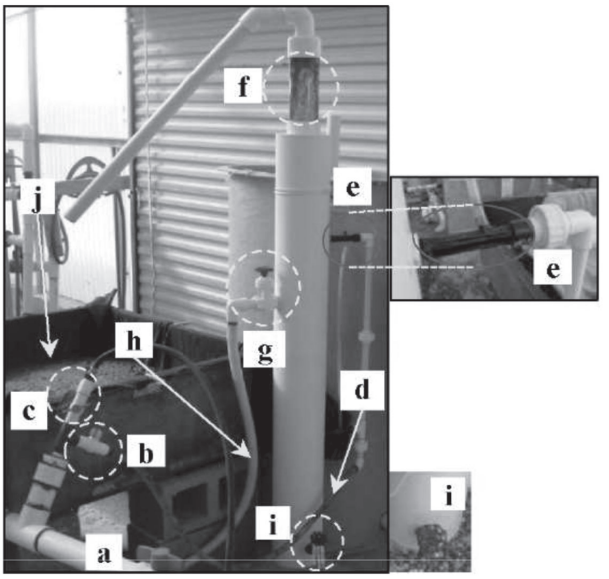


Fig. 6. FF in the 40 m^3 raceway; (a) 5 cm PVC valve mounted on the pump discharge pipe, (b) 1.6 cm PVC valve controlling water supply into the FF; (c) 1.6 cm PVC valve controlling water supply into the ST, (d) 1.6 cm hose connecting the valve and the FF; (e) one of the two 2 cm Venturi injectors; (f) clear acrylic tube; (g) 2.5 cm PVC gate valve controlling water flow from FF to raceway via 2.5 cm flexible hose (h), (i) FF drain valve, and (j) foam collection tank.

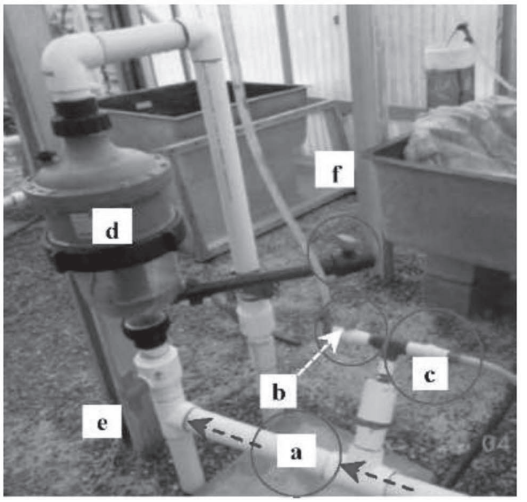


Fig. 7. Multi-cyclone mounting and valve arrangement in the 40 m^3 raceway: (a) 5 cm PVC pump discharge pipe, (b) 1.25 cm PVC valve controlling water supply to the FF, (c) 1.25 cm PVC valve controlling water supply flow to the ST, (d) multi-cyclone filter, (e) 5 cm PVC valve controlling water supply to the multi-cyclone filter, and (f) waste drain valve of the multi-cyclone filter.

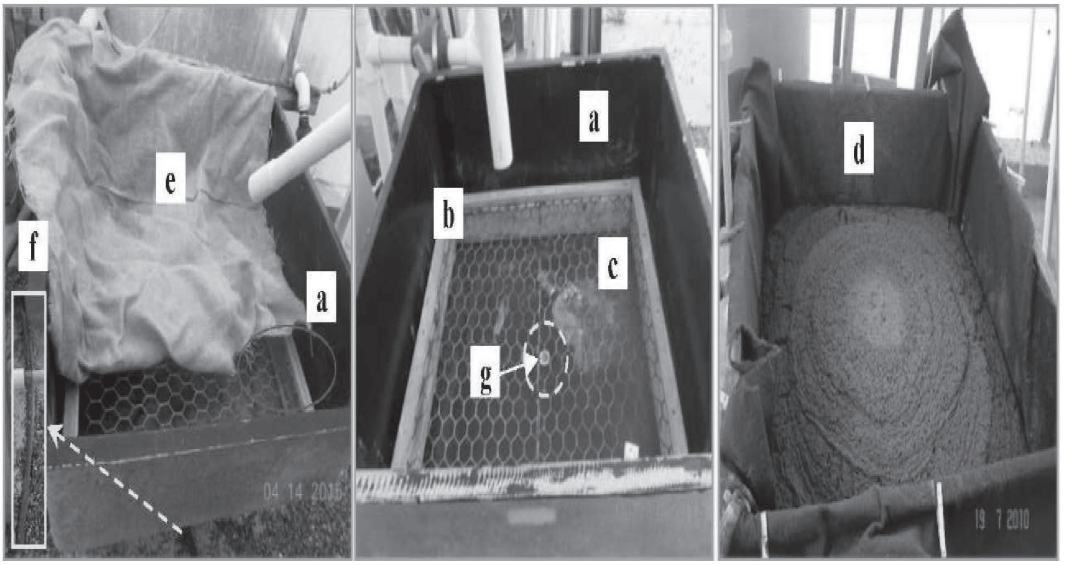


Fig. 8. Bioflocs separation tanks with drying bioflocs (a), a false-bottom is created by placing a wooden frame (b), covered with chicken wire (c), and covered by a geotextile membrane (d), or burlap cloth (e) for water separation, with hose returning water back to the raceway (f) via an outlet at the bottom of the tank (g).

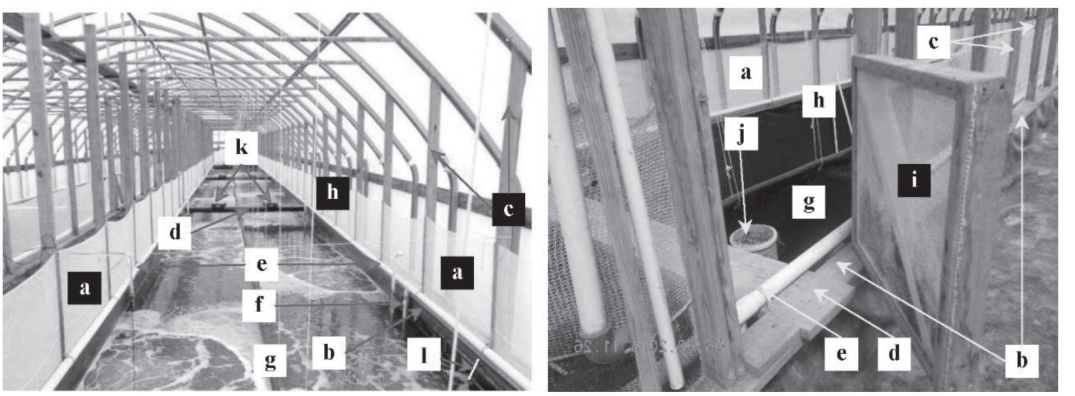
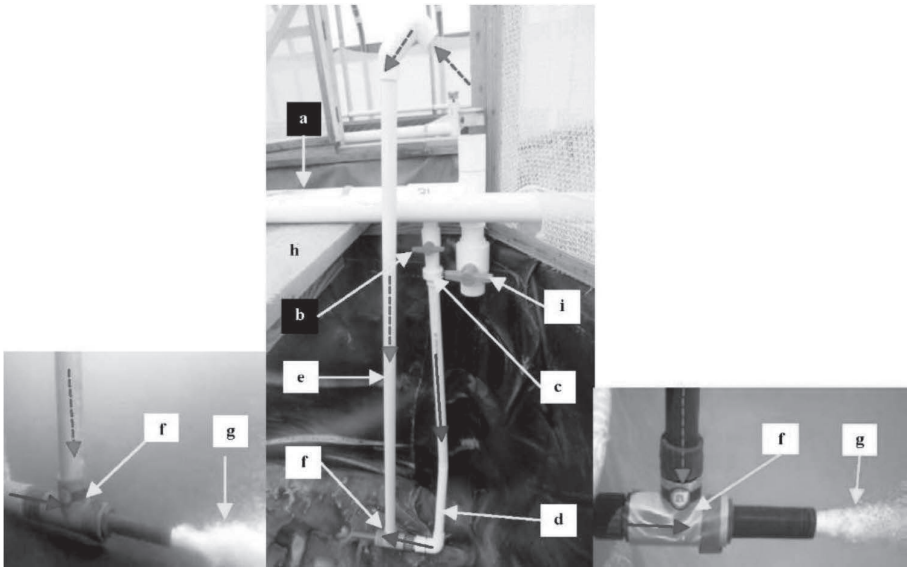


Fig. 9. Photos of the 100 m^3 RWs: Anti-jump netting (a), Footing made of planks mounted on top of the RW walls (b), Vertical planks attached to the RW footing and the greenhouse structure (c), Boardwalk (d), 5 cm PVC distribution pipes (e), 2.5 cm PVC a^3 water supply pipe (f), Center partition (g), Partition positioning rope (h), Access door (i), Screened pump intake (j), Belt feeders (k), and Freeboard (l).



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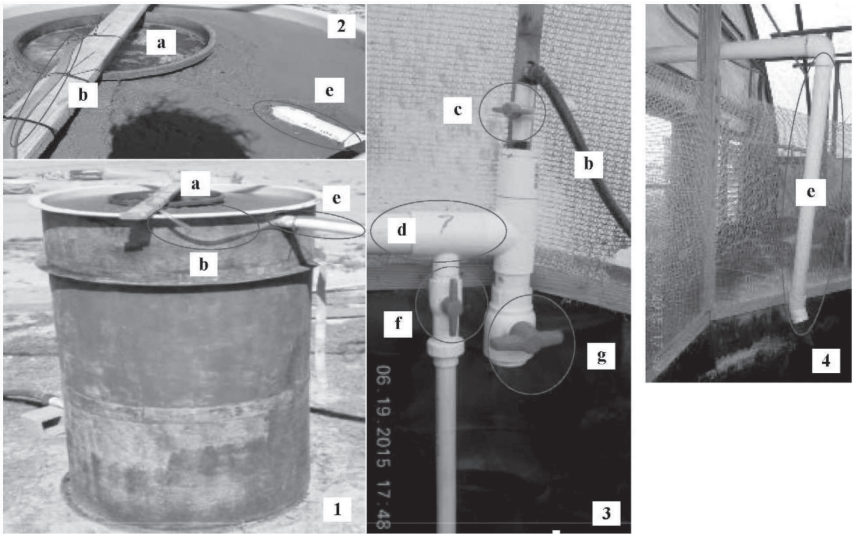

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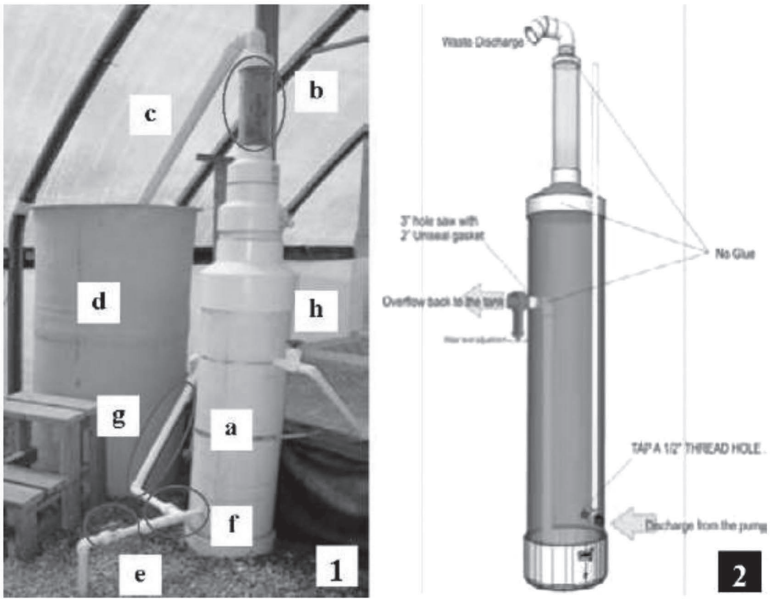

Fig. 12. (1) A photo of the homemade FF, and (2) A schematic drawing of the FF: (a) 30 cm PVC pipe, (b) An acrylic pipe, (c) foam delivery pipe, (d) temporary foam storage tank, (e) A valve controlling flow to the FF, (f) a^3 injector, (g) An air intake pipe, (h) A valve controlling return flow to the RW.

Routine applications of probiotic into the culture water demonstrated the ability of the probiotics to suppress pathogenic *Vibrio* development in the culture medium. Furthermore, studies showed that exposures of PL to high TAN and NO_2 levels can be avoided by inoculating virgin water with nitrifying bacteria.

Finally, the studies showed that the small RW system was capable of producing juvenile shrimp of about 2 g in size with high survival, low FCR, and high yields with no water exchange (Table 1).

Table 1: Results from a 71-d nursery trial in four 40 m^3 RWs stocked with *P. vannamei* at a density of 4,000 PL/m^3 using two solid control methods; water exchange (WE) or a combination of pressurized sand filter and FF.

Treatment	Size at harvest (g)	Yield (kg/m^3)	Survival (%)	FCR
FF-1 [1]	1.9^a	7.6^a	100^a	0.97^a
FF-2 [1]	2.0^a	6.9^a	92^a	1.08^a
WE-1 [2]	1.7^b	3.9^b	56*	1.64^a
WE-2 [2]	1.4^b	4.7^b	82^a	1.36^a

[1] RW operated with FF and 3.35% daily water exchange.

[2] RW operated with no FF and 9.37% daily water exchange.

Values with similar superscripts are not significantly different (a=0.05).

Raceway System II [100 m^3]

The nursery trial conducted in the two RWs showed that the injectors can be used from the day of stocking without damaging the young PLs. Table 2 shows the excellent results obtained in a 62-day nursery trial in the two RWs when stocked with the *P. vannamei* at a density of 540 PL/m^3. The results document extremely low FCR, with excellent survival and yield greater than 3.4 kg/m^3 which required operation of only one of the two pumps to maintain suitable water DO and mixing.

Table 2: Summary of a 62-d nursery trial with *P. vannamei* stocked at 540 PL/m^3 in two 100 m^3 RWs and fed *EZ-Artemia* and dry feed in biofloc-dominated water with no water exchange.

Parameters	RW B1	RW B2
Survival (%)	98	95
Final weight (g)	6.5	6.4
Yield (kg/m^3)	3.4	3.3
FCR	0.81	0.81
Water use (L/kg)	420	447
Sugar added (kg/m^3)	0.33	0.33
Bicarbonate added (kg/m^3)	0.26	0.25

GROW-OUT TRIALS

RW System [40 m^3]

Numerous studies were conducted over the years in this systems using different portions (20% to 90%) of the water used in previous nursery trials. The nitrifying-rich bacteria in this water kept TAN and NO2-N concentrations low (usually between 1 mg/L to 6 mg/L Figure 13) while maintaining high shrimp yields (>9.5 kg/m^3) requiring no organic carbon supplementation. As expected under these conditions, nitrate levels showed increase trend throughout the production trials (from about 40 mg/L to about 450 mg/L NO_3-N Figure 13).

Although shrimp are capable of tolerating nitrate concentrations higher than 450 mg/L NO_3-N, under water salinity of 30 ppt, if the culture water is to be reused in subsequence productions, this water had to be treated to reduce nitrate levels as concentrations above that levels can be harmful for the shrimp. Trials conducted at the facility with high nitrate water showed that the concentration can be easily reduce to below 50 mg/L as NO_3-N using digester operated under anaerobic conditions. Furthermore, acceleration of the process were achieved by supplementing the digester with organic carbon.

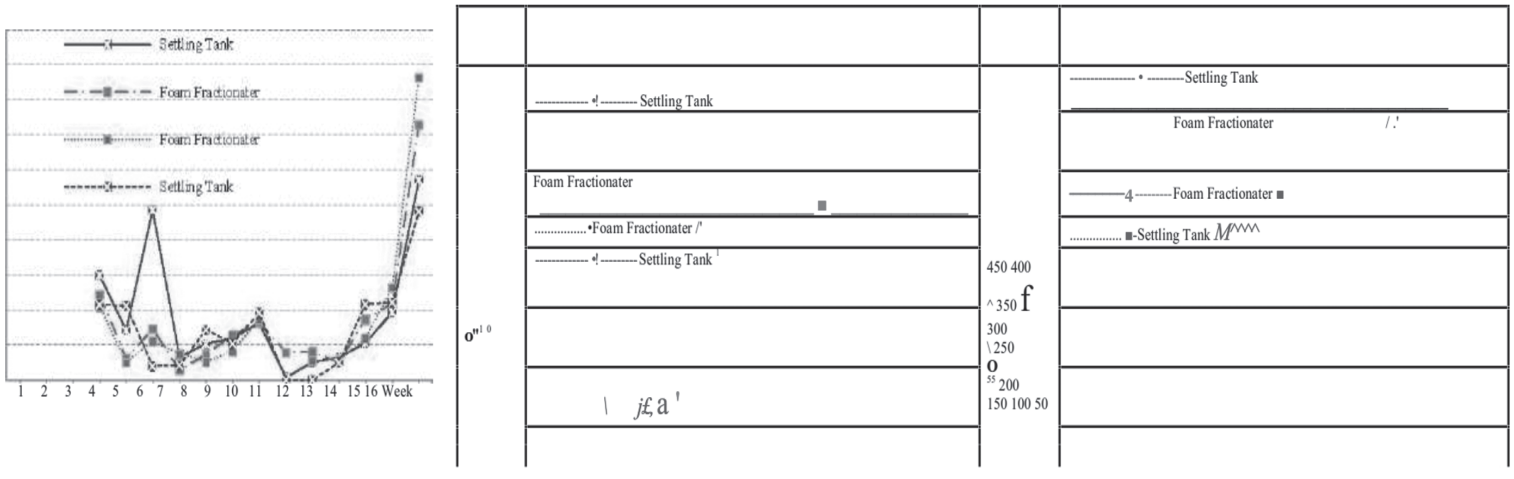

Fig. 13. Typical changes in TAN, NO_2-N and NO_3-N over one grow-out cycle in the 40 m^3 RW system.

As expected, under strong nitrification activities, alkalinity and pH are reduced and required constant adjusted to minimize stress on nitrifying bacteria and shrimp. Alkalinity adjustments were made using sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), or calcium carbonate ($CaCO_3$). Although shrimp can tolerate alkalinities above 400 mg/L as $CaCO_3$, efforts were made to keep alkalinity concentrations between 120 and 160 mg/L. As pH in these systems tends to drop from about 7.8 to below 7 during the production cycle, the alkalinity supplementations, in most cases helped maintain the pH at about 7.4. Nevertheless, when supplementations with different carbonate species were not sufficient to increase pH under high stocking densities (250 - 500 juveniles/m^3) and no water exchange, when shrimp are fed 24/7 specially formulated high-quality feed, TSS and SS increase. The concentrations of these indicators can reach levels that can negatively affect shrimp performance. Trials performed in our facility evaluated and selected the most cost-effective tools to maintain optimal concentrations for the shrimp (e.g., use of pressurized sand filters, FFs, STs, multi-cyclone filters, etc.). These trials showed that although shrimp were growing well at relatively high concentrations of particulate load (e.g., > 500 mg/L and 30 ml/L for TSS and SS, respectively), targeted levels were set between 250 and 350 mg/L and between 10 ml/L and 14 ml/L, respectively. These lower concentrations were recommended to provide system operators enough time to make any adjustments to bring the particulate load to the desired levels. Production trials in this system were done at stocking densities between few hundred and five hundred shrimp/m^3.

Other trials with feed of different quality and prices showed that better shrimp performance can be expected when shrimp were fed specially formulated high-quality feed designed compare to feed formulated for a semi-intensive outdoor production ponds (Table 3).

Table 3: ***Penaeus vannamei*** **performance in a 67-d grow-out trial in 2012 with 2.7 g juveniles stocked at 500/m^3 in six 40 m^3 RWs when fed two commercial feeds under no water exchange.**

Feed	Av. Wt. (g)	Growth (g/wk)	Yield (kg/m^3)	Survival (%)	FCR	Water use (L/kg shrimp)	Operation FF	(h) ST
HI-35[1]	22.1	2.0	9.7	87	1.25	125	812	87
SI-35[2]	19.7	1.8	8.7	88	1.43	138	1,253	391
Diff	2.4	0.3	1.0	(1.0)	0.18	14	441	304

HI-35, Zeigler Bros. Inc., Gardners, PA, US. *SI-35, Zeigler Bros. Inc.,* Gardners, PA, US.

Information from online DO monitoring system showed significant DO reductions when feed was delivered few times a day which was corrected by switching to 24/7 feed

delivery. This practice together with the use of high quality feed and careful monitoring of feed consumption enabled production of marketable size shrimp with good growth (1.8-2.0 g/wk), survival (79%-88%), yields (9.4-9.9 kg/m^3), and low FCR (1.39-1.45).

Table 4 below summarizes the information obtained from a 2011 trial in the small RW system. It is important to note that the system had to be supplemented with pure oxygen using the Venturi injector to support the high oxygen demand of the shrimp and the bacteria in the culture medium.

Table 4: Summary of a 2011 grow-out trial with *P. vannamei* juveniles in five 40 m^3 RWs stocked at 500/m^3, operated with no water exchange, and fed a high quality feed with 35% protein.

RW	Average stockin	Weight Harvest	Days	Growth (g/wk)	Survival (%)	Yield (kg/m^3)	FCR	Water use (L/kg shrimp)	Salinity (ppt)
1	1.9	22 .2	81	1.8	88	9.7	1.39	147	18
2	1.9	23.6	82	1.9	82	9.6	1.44	139	18
3	1.9	23.4	82	1.8	82	9.4	1.45	126	18
4	1.9	23.8	83	1.9	79	9.4	1.45	138	18
5	1.4	25.1	85	2.0	79	9.9	1.44	127	30
Av.		23.6		1.9	82	9.6	1.43	135	
SD		0.9		0.1	0.3	0.2	0.02	9	

The trials showed that excellent results can be achieved when shrimp are exposed to optimal growing conditions. However, this system requires constant monitoring and adjustments to avoid stressing the shrimp. Furthermore, our studies documented that exposing the shrimp to unfavorable conditions for few days can stress them and lead to pathogenic bacteria outbreaks with poor results. Thus, great efforts are required to make sure shrimp are maintained in the comfort zone.

RW System100 m^3

Monitoring and control of key WQ indicators in this system were similar to those used for the small system. However, only limited grow-out trials were conducted in this system since the construction of the two RWs was completed only in 2010. The grow-out trials in this system were designed to evaluate the effect of different stocking densities, different particulate matter control tools, and feed management on shrimp performance. These trials showed that the homemade FFs and STs were capable of keeping particulate matter concentrations under control.

As was the case for the small system, when nitrifying bacteria rich water was used for the grow-out trials under no water exchange and with adequate adjustment of alkalinity and pH, concentrations of TAN and NO_2 stayed very low while NO_3 levels continued to increase throughout the production trials.

Since aeration and mixing in these RWs was generated by the pump-driven a^3 injectors, fine-tuning of the water flow was needed to improve shrimp performance (minimize energy expenditure stemming from constant swimming against the current generated by the injectors). This together with 24/7 feed delivery and the delivery of the feed away from the pumps' intakes helped reduce the FCR from as high as 2.56 to 1.43.

Other trials also showed that shrimp growth rates can be greatly improved (an increase from 1.38 to 2.31 g/wk) when RWs were stocked with juveniles produced by breeding populations of two genetic strains (Fast-Growth x Taura Resistant) compare with those produced by Taura Resistant strain. One additional significant finding from these trials was the high yields (> 9 kg/m^3) achieved without the need to use pure oxygen (e.g., the system oxygen demand was met by operating the a^3 injector using atmospheric air). Table 5 summarizes the results from a trial conducted in this system in 2012.

Table 5. Summary of a 63-d trial in 2012 in two 100 m^3 RWs at 500/m^3 with *P. vannamei* juveniles (3.6 g), a^3 injectors, high quality commercial feed, and no water exchange.

RW	(Juveniles/m^3)	Stocking	Harvest (g)	Growth (g/wk)	Survival (%)	Yield (kg/m^3)	FCR L/Kg	Water Use
1	500	3.6	22.8	2.1	81	9.2	1.43	112
2	500	3.6	22.7	2.1	78	8.9	1.53	121
Average			22.7	2.1	80	9.0	1.48	117

ECONOMICS OF RAS AND BIOFLOC SYSTEMS

Table 6 compares production costs in earthen ponds and RAS using data from the USDA-funded US Marine Shrimp Farming Program. Pond data are from an intensive farm in Arroyo City, Texas and RAS trials conducted at the Oceanic Institute, Hawaii over four years (2005, 2006,2007, and 2009).

Production costs per unit shrimp were less in RAS than in earthen ponds. Even at higher stocking densities, survival and growth in the RAS trials were better than in ponds. At harvest, shrimp produced in RAS were just as large and in some cases even larger than shrimp from ponds.

Closed, indoor super-intensive RAS can be operated for less than earthen ponds (Moss and Leung, 2006). See in Table 6 shows cost comparison between a farm and a

closed, indoor super-intensive RAS system. The cumulative distribution of total cost for ponds and RAS indicates that RAS has a lower cost per unit weight than ponds.

Table 6. Grow-out trial comparison (USMSFP at USDA review panel, Shaun Moss, personal communication).

	Texas farm	Hypothetical USMSFP	USMSFP	USMSFP	USMSFP
	2001-2002	1999	2005	2006	2007	2009
Stocking (shrimp/m^2)	50	140	705	401	828	450
System size (m^2)	20,234	n/a	58.4	75	337	40
Survival (%)	50.0	80.0	70.3	90.6	67.9	96.3
Harvest weight (g)	18.0	23.0	17.9	21.0	18.3	23.1
Growth (g/wk)	1.00	1.50	1.37	1.49	1.50	1.39
Production (kg/m^2)	0.45	2.60	8.90	7.60	10.30	9.75
Cost ($/kg)	**6.72**	**13.05**	**4.96**	**4.85**	**3.66**	**5.51**

Economic projections suggest that biofloc systems can be profitable when they target niche markets for live or fresh (never frozen) shrimp (Hanson and Posadas, 2004; Hanson *et al.*, 2013). The Texas A&M-ARML has reduced indoor biofloc operating costs from $11.00/kg, the US average for super-intensive systems, to about $4.53/kg. That work also suggests the feasibility of extending the number of crops from 3.5 to 5.5 per year.

CURRENT ISSUES WITH INDOOR BIOFLOC SHRIMP CULTURE

Advances in indoor biofloc systems have been impressive, but current knowledge is not complete. For example, the failure of some indoor biofloc projects can be traced to the complex inter relationships that characterize the diverse and difficult-to-control microbial biofloc community. This assemblage can be unstable in small tanks stocked at high densities and driven by the large input of feed required for good shrimp growth. If the microbial community of the biofloc system is not balanced properly, inimical chemicals can accumulate, particularly TAN, NO_2 and NO_3. WQ changes are exacerbated when water is reused over multiple crop cycles.

Biofloc systems also are susceptible to outbreaks of noxious organisms, such as *Fusarium solani* (responsible for closure of a commercial facility in Kentucky) and *Vibrio* sp. (which caused a commercial operation in Texas to abandon biofloc). The Waddell Mariculture Center research facility has experienced outbreaks of the cyanobacterium *Synechococcus* sp. and the dinoflagellates *Gymnodinium* sp. and *Pfiesteria piscicida,* each with an unpredictable and decidedly negative impact on production. The Texas A&M-ARML indoor biofloc systems also have experienced crop-threatening outbreaks of *Vibrio.*

The systems and the results described in this paper suggest the need for the refinement of production management of super-intensive, biofloc-dominated operated with no water exchange to make these system more economically viable.

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CHAPTER

4

ISOLATION AND IDENTIFICATION OF BACTERIAL ISOLATES FROM AMF DRIVEN NURSERY RAISING OF *PENAEUS VANNAMEI* IN SUPER INTENSIVE SYSTEMS

M.Menaga, S. Felix and A. Gopalakannan

INTRODUCTION

The expansion of the shrimp farming industry has stimulated the intensification of the production systems (FAO, 2012). The hyper-intensive shrimp culture system was developed in the 70's with stocking density between 200 and 500 shrimp m^{-2}, 300% daily water exchange, use of small tanks, and more balanced feeds (Ortega-Salas and Rendón, 2013). Recently, these systems started to incorporate biofloc technology (BFT) (Venero *et al.*, 2009), which has the potential to reduce environmental impacts associated with the intensification process (McIntosh, 2000 and Schryver *et al.*, 2008). The incorporation of BFT facilitated high density shrimp production using limited or no exchange practices with a much smaller area than outdoor ponds (DuRant *et al.*, 2011,Samocha *et al.*, 2010 and Samocha *et al.*, 2012).Application of bio-floc technology (BFT) in aquaculture offers a solution to avoid environmental impact of high nutrient discharges and to reduce the use of artificial feed. In BFT, excess of nutrients in aquaculture systems are converted into microbial biomass, which can be consumed by the cultured animals as a food source.

The microorganisms play important role in nutrient cycling such as methane production (Chan *et al.*,2005), sulphate reduction (Li *et al.*,1999) and ammonia oxidation (Hastings *et al.*, 1998) in natural aquatic system. Microbiological aspects particularly bacterial growth patterns,characterization of biofloc and possible manipulation of microbial community

is necessary for successful design and operation of biofloc technology (Azim *et al.*, 2008). The most abundant microbes in the floc particles are chlorophytes (green algae) diatoms, dinoflagellates, nematodes,rotifers, and cyanobacteria (DeVantier *et al.*, 1998; Ray *et al.*, 2010). Heterotrophic ammonia-assimiliative and chemoautotrophic nitrifying bacteria are primarily responsible for maintaining water quality and lead to minimal –exchange in intensive systems (Ebeling *et al.*, 2006; Hargreaves, 2006).

The most easiest and common method to determine the presence and type of microorganisms is a simple microscopy.Since the method is based on visual microscopy it is generally not possible to identify all of them.It can be used to gain a value of the proportion of filamentous and zoological flocs within a water sample. 16s rDNA PCR is being increasingly used for the identification of bacterial isolates that could not be identified by conventional methods as in the case of noncultivable bacterial species . It is also highly reliable as it provides unambiguous data even for rare isolates, which are difficult to identify with the phenotypic identification schemes. One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles.

Although bacterial pathogens are conventionally identified by their cultivation and biochemical characterization, there are many bacterial species that are not cultivable by standard method. Biochemical identification methods also involve laborious, time-consuming processes which are at times misleading due to the presence of variants among bacterial species. Molecular methods like PCR have been proved to be very useful in the identification of bacteria. PCR amplification of 16s rDNA gene which is conserved in bacteria and sequencing enables the identification of bacterial species. This technique has been proved to be helpful to identify non-cultivable bacteria and other bacterial species in shorter time with relatively higher accuracy. Rapid and accurate identification of the bacterial pathogens would help to undertake suitable management measures to avoid disease outbreaks and production losses in aquariculture. The present study was carried out with an objective to identify bacterial isolates from AMF based aquaculture systems by 16s rDNA gene amplification systems and sequencing.

MATERIALS AND METHODS

Experimental System and Setup

PL_{14} (±0.002 g) of SPF vannamei were stocked in the nursery raceways(50 m^3) at a stocking density of 6000PL/m^3 in indoor raceways and 2300PL/ m^3 in outdoor lined ponds (100m^3) at Advanced Research Farm Facility, Madhavaram, Chennai. Commercial shrimp feeds (Growel manufactured crumble feeds (0.5-1mm)) were used throughout the experiment. The MF-CEED (Microbial Floc-Carbohydrate Enriched Distillery Effluent)

formulated by enriching the locally available rice flour to the distillery spent wash (26% w/w carbon and specific gravity of 1.5) was used as an organic carbon source to achieve the required C/N ratio of 15:1. Input C/N ratios were calculated based on the carbon-nitrogen contents of the feed and the carbon content of the MF-CEED.

Microbiological analysis of water

For microbiological study,the samples were collected from the respective systems for every ten days during morning hours of the day between 8.00 to 10.00 a.m.The microbial floc suspensions were taken in the Imhoff cone and allowed to settle for 15-20 minutes.The settled biofloc suspension were transferred aseptically into sterile Uricol (Hi-media) bottles and stored in refrigerated condition.

Enumeration of total heterotrophic bacteria

Total heterotrophic bacteria were enumerated on nutrient agar medium from both systems.10 ml of the biofloc water sample was transferred aseptically to the 90 ml physiological saline which gives 10 times dilution of the sample, i.e 10^{-1} dilution for further dilution,1 ml from 10^{-1} was mixed with 9 ml saline (10^{-2}) and so on to get the appropriate dilution for plating. Three dilutions were selected for plating in triplicate.0.1 ml of each of the dilutions was inoculated on the surface of the respective pre-set agar plates. By using glass spreader the inoculums was uniformly speeded well on the surface of the agar medium. After 30 minutes, the plates were incubated at 37°C for 48 hrs.After 48 hours of incubation, the colonies were enumerated using a colony counter. The average colony counts of triplicate plates were taken for the total heterotrophic bacterial count and expressed as colony forming unit per ml(CFU ml^{-1}). Similarly vibrio count and fungi count from raceways and lined pond water samples were determined by the spread-plate technique Thiosulfate citrate bile salts sucrose (TCBS) agar for Vibrio count,Sabouraud dextrose agar for total fungal count were used respectively. A total of 10 isolates of bacteria from aerobic microbial floc in both systems were subjected to biochemical tests for finding the relative difference to categorize the bacterial groups, out of which five isolates were given for 16s r DNA amplification.

DNA extraction from AMF

Microbial suspension (1ml) was kept in Hi-Bead tubes (Hi-Media Laboratories Limited, Mumbai) and the lysozyme-SDS based phenol chloroform method of extraction was followed with slight modifications. 500 µl of lysis 1 solution (0.15 M NaCl, 0.1 M EDTA; pH 8;15mg lysozyme/l) was added to the bead tube and mixed by horizontal vortexing for 2 minutes.It was then incubated at 37°C for 1 hour in a water bath. After that 500 µl of

lysis 2 (0.1 M NaCl, 0.5 MTris-HCl; pH8 :12 % SDS) solution was added to it and incubated again for 1-2 hours at 60°C in a water bath.The suspension was then centrifuged at 8000xg for 10 minutes in a Spinwin (Tarson,India) centrifuge and 1 ml of supernatant was recovered in separate vials.To this supernatant,1 ml of saturated phenol (pH 8)was added,mixed and centrifuged at 8000xg for 8 minutes. The aqueous layer was recovered and an equal volume of chloroform was added, mixed and centrifuged for 30 minutes at 4°C at 8000xg in a microprocessor –based high speed research refrigerated centrifuge (Eltek, India). The supernatant was decanted and pellets were washed with 500 µl of 70 % ethanol and centrifuged at 8000xg for 15 minutes using the centrifuge. Finally, the vials were decanted again and kept in a dry bath (SLM-DB-120,Genei, India) at 60°C for some time to evaporate the remaining ethanol. The DNA was then re-suspended in 50µl of nuclease-free water and stored at -20°C.

Genomic DNA confirmation by Agarose Gel Electrophoresis

The elute was checked for the presence of genomic DNA using 1.5 % agarose gel pre-stained with ethidium bromide (0.5 µg ml-l),1.0 µl of 6x loading dye was mixed with 10.0 µl of genomic DNA and loaded into the wells of the gel. Loading dye1.0µl was mixed with 5.0 µl 1Kb DNA molecular weight marker and loaded into the wells. Electrophoresis was carried out in 1 x TAE buffer at 70 volts for about 60 minutes. The gel was visualized and photographed using Bio-Rad Universal Hood 11 Gel Imager (Bio –Rad Laboratories,USA).

SEQUENCES ANALYSES

Sequences were analyzed for similarity with sequences deposited in public databases using the Basic Local Alignment Search Tool (BLAST) (McGinnis & Madden 2004) at the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST). Alignment and further phylogenetic analysis was performed by means of **Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0** (Tamura, Dudley, Nei & Kumar 2007). The resulting alignment were manually checked and corrected when necessary and unambiguously aligned nucleotide position were used for construction of a 16S rRNA sequence based phylogenetic tree, using the neighbor-joining (NJ) method (Saitou & Nei 1987) because of its known accuracy. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei & Kumar 2004) and are in the units of the number of base substitutions per site.

Table 1: Primer sequences used

S.No	Primer Sequence (5"!3')
1	27F5'-AGAGTTTGATCMTGGCTCAG-3'
2	1492R5'-TACGGYTACCTTGTTACGACTT-3'
3	518F 5'-CCAGCAGCCGCGGTAATACG-3',
4	800R 5'-TACCAGGGTATCTAATCC-3')

Table 2: Components of PCR reaction

Sl.No	Reagents	Volume(50 µl)
1.	Master mix	25
2.	Forward primer (100 picomole)	0.4
3.	Reverse primer (100 picomole)	0.4
4.	Template	3
5.	Nuclease free water	21.2

The PCR consisted of 30 cycles each of initial denaturation at 95°C for 5 minutes,denaturation at 95°C for 30 seconds,annealing at 61.3°C for 30 seconds and extension at 72°C for 1 minute,and a final extension for 15 minutes.The PCR products were examined by electrophoresis on 1 % agarose gel prestained with ethedium bromide and visualized in Bio-Rad Universal Hood 11 Gel Imager (Bio-Rad Laboratories,USA).

The PCR products were tested for the various biochemical tests such Gram staining, Observation of bacterial motility by hanging drop technique, 0/129 sensitivity (2,4 diamino 6,7 di-isopropyl pteridine),Lysine test, Ornithine test, Urease test, Phenyl analine test, Nitrate reduction test, H_2S test, Citrate test, Vokes Proskaeur (VP) test, Methyl red test, Indole test, Melanote test and Indole test as per APHA 2004.

The closely related sequences were stored at -80°C and directly send for sequencing to Chennai, Genei, India. The partial bacterial 16SrDNA gene sequences were subjected to BLAST (www.ncbi.nlm.nih.gov/BLAST/) search on the Genbank nucleotide database NCBI (National Centre for Biotechnology Information) to identify the sequences with higher similarity.

Collection and Identification of plankton and microbial organism in Aerobic Microbial Floc

One ml of biofloc sample was taken by micropipette for plankton identification using Leica LCD projection microscope (10x and 40x). All the samples were analysed immediately in live condition and were identified according to Edmondson,1959.

Confocal Microscopy

The fluorescent images of bacterial floc were taken using a confocal microscope (Leica TCS 4PI) on a biweekly basis. Water samples were collected from one tank in each treatment and processed immediately for microscopy.Each sample was diluted with pre-filtered water of the same tank, stained with fluorescence 42 -6-Diamidino-2-phenylindole (DAPI), and observed and photographed at magnification 10.

Statistical Analysis

One way ANOVA followed by Tukey's test (Zar,1984) was used to compare treatments at a significant level of 0.05.

RESULTS

Bacterial count in water

The total heterotrophic count and total fungal count were significantly higher in indoor raceways.The total vibrio count was significantly higher in lined ponds than compared to raceways.The minimal and maximal fungal count encountered in raceways were $1.5\pm0.14x10^3$ and $2.1\pm0.11x10^4$ respectively .The outdoor lined ponds shows the highest vibrio count of $1.95\pm0.25x10^3$ and the lowest of $7\pm0.33x10^2$.The total heterotrophic count increased gradually from $3.0\pm0.22x10^3$ on 10^{th} day to $2.1\pm0.27x10^7$ on 50^{th} day in raceways.

Biochemical test performance

Gram staining process followed by a series of biochemical testing was conducted on all 10 isolates for further identification purposes. A series of biochemical tests were conducted in order to identify each type of bacterial isolates.All the isolates exhibits a negative results to ONPG,Lysine, Ornithine,Urease, Pheny alanine, VP test,Indole and Melanote test. The results obtained are presented in Table no 4.

The overall relative abundance of each bacterial group throughout the study in indoor raceways and outdoor lined ponds.Data are presented as mean ± standard error.Superscripts within a row indicate significant differences ($P<0.05$) between the indoor raceways and outdoor lined ponds.

Identification of Bacteria from AMF

The bacteria from five isolates were identified using molecular technique based on the 16S rDNA gene amplification using polymerase chain reaction (PCR) followed by sequencing of the amplified gene. The primers binds to respective base positions of the 16S rDNA genes of *Streptococcus* sp. with 99 % similarity,*Bacillus* sp with 85 % similarity and *Brevundimonas vesicularis* with 99 % similarity.

Table 3: Relative abundance of bacterial group

Sampling Interval	Total Heterotrophic Count (THC)		Total Fungal Count (TFC)		Total Vibrio Count (TVC)	
Days	Indoor Raceways Mean (10^{-3}CFU /ml)	Lined Ponds Mean (10^{-3}CFU/ml)	Indoor Raceways Mean (10^{-2} CFU/ml)	Lined Ponds Mean (10^{-2} CFU/ml)	Indoor Raceways Mean (10^{-2} CFU/ml)	Lined Ponds Mean (10^{-2} CFU/ml)
10	$3.0\pm0.22x10^{3a}$	$1\pm0.2x10^{3b}$	$1.5\pm0.14x10^{3a}$	$3\pm0.15x10^{2b}$	$4.5\pm0.22x10^{2a}$	$7\pm0.33x10^{2b}$
20	$1.3\pm0.25x10^{4a}$	$9\pm0.32x10^{3b}$	$2\pm0.22x10^{3b}$	$9\pm0.25x10^{2a}$	$1\pm0.13x10^{3b}$	$1.1\pm0.16x10^{3a}$
30	$6.0\pm0.26x10^{5a}$	$4\pm0.24x10^{5b}$	$4\pm0.22x10^{3b}$	$2\pm0.11x10^{3a}$	$1.8\pm0.22x10^{3a}$	$2.35\pm0.16x10^{3b}$
40	$1.3\pm0.14x10^{7b}$	$1.1\pm0.38x10^{6a}$	$1.2\pm0.24x10^{4a}$	$8\pm0.32x10^{3b}$	$8\pm0.22x10^{2a}$	$1.7\pm0.32x10^{3b}$
50	$2.1\pm0.27x10^{7b}$	$1.5\pm0.26x10^{7a}$	$2.1\pm0.11x10^{4b}$	$1.1\pm0.36x10^{4a}$	$6.5\pm0.22x10^{2b}$	$1.95\pm0.25x10^{3a}$

Table 4: Observation of Biochemical test of bacterial isolates from AMF based P*enaeus vannamei* nursery culture

Sl. No.	Characterization	R1	R2	R3	R4	R5	P1	P2	P3	P4	P5
1	Motility	+	+	+	+	+	-	+	+	+	-
2	Gram staining	+	+/-	+/-	-	+/-	+	+	-	-	+
3	ONPG	-	-	-	-	-	-	-	-	-	-
4	Lysine	-	-	-	-	-	-	-	-	-	-
5	Ornithine	-	-	-	-	-	-	-	-	-	-
6	Urease	-	-	-	-	-	-	-	-	-	-
7	Phenyl alanine	-	-	-	-	-	-	-	-	-	-
8	Nitrate reduction	+	+	+	+	+	-	+	+	-	+
9	H_2S	-	+	+	-	+	-	+	-	-	-
10	Citrate	-	-	-	+	-	-	-	+	-	-
11	VP test	-	-	-	-	-	-	-	-	-	-
12	Methyl red	+	-	-	+	-	+	-	+	-	+
13	Indole	-	-	-	-	-	-	-	-	-	-
14	Melanote	-	-	-	-	-	-	-	-		

Table 5: Sequence length and closest phylogenetic affiliation of 16s r DNA band

Sample code	Sequence Length(bases)	Phylogenetic Relationship Species	Similarity
R1	CSRKWTRRAATTTGYTTWATCRAGGCAGTCGTA ACAAGWATAGCGTWCAGTA	Sequencing failure	85
R2	TTTCYTTGAYRMGCWTCTTCRAWWRCARTYST AACARRAMTMGTCTCWAGARTTAGTGGCGGAC GGGTGAGTAACACGTGGGAACGTGCCTTTAGGT TCGGAATAACTCAGGGAAACTTGTGCTAATACC GAATGTGCCCTTCGGGGGAAAGATTTATCGCCT TTAGAGCGGCCCGCGTCTGATTAGCTAGTTGGT GAGGTAATGGCTCACCAAGGCGACGATCAGTA GCTGGTCTGAGAGGATGATCAGCCACATTGGGA CTGAGACACGGCCCAAACTCCTACGGGAGGCA GCAGTGGGGAATCTTGCGCAATGGGCGAAAGC CTGACGCAGCCATGCCGCGTGAATGATGAAGGT CTTAGGATTGTAAAATTCTTTCACCGGGGACGA TAATGACGGTACCCGGAGAAGAAGCCCCGGCT AACTTCGTGCCAGCAGCCGCGGTAATACGAAG GGGGCTAGCGTTGCTCGGAATTACTGGGCGTAA AGGGAGCGTAGGCGGACATTTAAGTCAGGGGT GAAATCCCGGGGCTCAACCTCGGAATTGCCTTT GATACTGGGTGTCTTGAGTATGAGAGAGGTGTG TGGAACTCCGAGTGTAGAGGTGAAATTCGTAGA TATTCGGAAGAACACCAGTGGCGAAGGCGACA CACTGGCTCATTACTGACGCTGAGGCTCGAAAG CGTGGGGAGCAAACAGGATTAGATACCCTGGT AGTCCACGCCGTAAACGATGATTGCTAGTTGTC GGGATGCATGCATTTCGGTGACGCAGCTAACGC ATTAAGCAATCCGCCTGGGGGAGTACGGTCGCA AGATTAAAACTCAAAGGAATTGACGGGGGGCC CGCACAAGCGGTGGAGCATGTGGATTTAATTCG TAGCCACCGCGCAGCAATCCTTACCACCCAGTA TG	*Bacillus sp.*	
R5	TCCGGGGGGTAGTAAACACAACTCTCCATGGGG GCCCACTGGGCGGGGTGTACAAGACCCGGGAA CGTATTCACCGTAGCATGCTGATCTACGATTAC TAGCGATTCCAACTTCATGTAGTCGAGTTGCAG ACTACAATCCGAACTGAGAACAACTTTATGGGA TTTGCTTGACCTCGCGGTTTGGCTACCCTTTGTA TTGACCATTGTAGCACGTGTGTAGCCCAAATCA TAAGGGGCATGATGATTTGACGTCATCCCCACC TTCCTCCGGTTTGTCACCGGCAGTCAACTTAAA GTGCCCAACTTAATGATGGGGACTAAAATGAA GGGGTGTGCTCGTTGCGGGGCTTAACCCAACAT CTCACAACACAAGCTGAGAACACCCATGCACC ACCTGTGACTCTGTCCCCCGAAGGGGAAAACTC TATCTCTGGAGGGGTCACAGGATGTCAAGATTT GGTAAGGTTCTTCGCGTTGCTTCAAATTAAACC	*Staphylococus captis*	90%

[Table Contd.

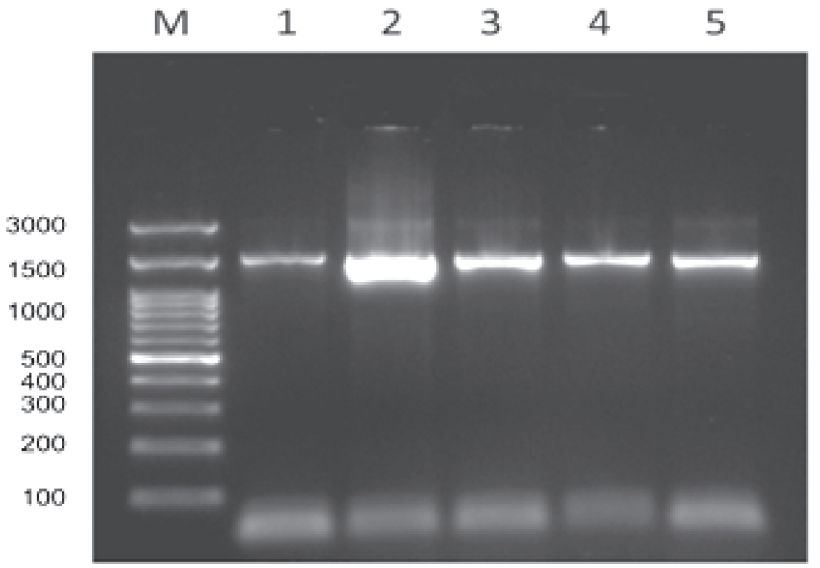
Contd. Table]

Sample code	Sequence Length(bases)	Phylogenetic Relationship Species	Similarity
	ACATGCTCCACCGCTTGTGCGGGCCCCCGTCAA TTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCC CAGGCGGAGGGCTTAATGCGTTAGCTGCACCAC TAAGGGGCGGAAACTCCCTAACACTTAGCACTC ATCGTTTACGCGGTGGACAACCAGTGAATCTAA CCCTGTTGTACCCCCCCGTTTTTCCCCATCAGGT TCAGTTACAGACAAGAAGAACCCCTTCACCAGT GGGTGTGCCTCCATAACTCTGCGCATTTCCCCG TTCACCAAGGAAGTTTCACCTCTCCTCTTCTGCA CTAAATTTTCCAGGTGTCGAAGGACCCTCCAAG AGTTGAACCGCGGCGGTTTCTCCATCCAAACTT AAAGAAACCGCCTACCGCCGCGCTTTCGCGCCC ATTGAATTCCCGAATAGACACTAGCCACCCTAC ATA		99
P2	GAATAAGATTGCTATCRAGGGAGTCGTACAGGT AGCGWCACTACTGT	Sequencing failure	
P4.	AATAATTTTKGCTTA		
	TCGAAGCARTCGTAACAAGAATAGCKAYAGTA MTTGCTCCCAAGATTAGCGGCGGACGGGKGAG AAACACGKGGGCAACCTGCCTGTAAGACTGGG ATAACTTCSGGAAACCGGAGCTAATACCGGATA ATCCCTTTCCTCACATGAGGAAAGGCTGAAAGA CGGSGTCTASYTGTCMYTTMCRAATGGGCCCCC GGCSCATTASCTAKTTGGGGAGGTAACGGCTCA CCAAGGCCACRATGCKTASCCAACCTGAAAGGG TGATCGGCCACMCTGGGACTGAAACMCGGCCC AAACTCCTACGGGAGGCASCAKTAGGGAATCTT CCSCAWTGGACRAAAGTCKGACGGASCAMCSC CSCGGGAKTGATGAAGGTTTTCGGATCGWAAA ACTCTGTTGTTAGGGAAGAAMAAKTATCGAAK TAMCTGCCGGTMCCTTGACGGWMCCTAACCAA AAAGCCMCGGYTAACTACKTGCCASCASCCSCG GWAATACKTAGGTGGMARGCGTTGTCCGGAAT TATTGGGSGTAAAGSGCGCGCRGGKGGTTCCTT AAKTCTGATGTGAAASCCCACGSYTCAMCCGTG RAGGGTCWTTGKAAACTGGGGAAYTTGAGTGC GAARAGGAAAAGTGWAWTTCCAAGTGTAGCG GTGAAATGCGTAGAGATTTGKAGGAACACCAST GGCGAAGGCGACTTTCTGSTCTKGTAAYTGACA CTGAGGCGCGAAAGCGTGGGAGCRAACAGGAT TAKATACCCTGTRGTCYACGCGYAAACGATGAG TGCTGAGTGTRKAGGTTTCTGCCCTYTAGTGCT GCAGCAAATCGCATTATGCACTCGCTGTGAGTA ACGACGCACGATGCAWACCTCATAGGAAATTG AG	*Brevundimonas vesicularis*	

- Accession nos. are to be allotted by NCBI.

PLANKTON IDENTIFICATION

A 3-D picture of individual bacterial flocs using a confocal microscope is shown in Fig. 1. The blue channel dominating the picture indicates the clusters of bacterial nuclei.There are trace amounts of mucopolysaccharides,typically secreted by bacteria (green channel). In AMF,the planktons such as *Chlorella* sp., *Oscilatoria* sp., *Stephanodiscus* sp., *Coscinodiscus* sp., *Navicula* sp. (Pennate diatoms), *Scenedesmus*, *Vorticella*, *Gloenkinia*, *Chlorogonium*, dinoflagellates, *Amphiprora* sp., *Nitzschia* sp. (Pennate diatoms), *Chaetoceros* sp., *Cyclotella* sp., *Triceratium* sp., *Cymbella* sp., *Stentor* sp., *Paramecium* sp., *Cyclidium* sp., *Peranema* sp., *Petalomonas* sp., *Rotifer*, Nematode or round worm, *Chaetonotus* sp.*Cyclops* sp were identified from both systems.However, predominantly the ciliates,protozoans and rotifer population was grazing within the AMF particles in indoor raceways than compared to outdoor ponds.The structure of AMF was seen in anomalous flocculation with bacteria and zooplankton especially nematodes and rotifers.



M –Marker ;**1-**R1 ;**2-**R2 ;**3-**R5 ;**4-**P2 ;**5-**P4

Fig. 1. Agarose gel electrophoresis picture obtained after 16SrRNA amplification

Discussion

Shrimp, post larvae and juveniles are able to consume a little micro algae,cyanobacteria and other microorganisms directly from the water column (Kent *et al.*, 2011).An important aspect to emphasize is the seasonal variation in the microbial communities mainly affected by ecological succession (predation-competition phenomenon) especially when the cultures are performed outdoor (Vezzulli *et al.*, 2002). In outdoor cultures with enough light and natural photoperiod,the diatoms were the dominant community (Godoy *et al.*, 2011), while

under indoor conditions with low light or lack of light, the heterotrophic bacteria may dominate (Becerra-Dorame *et al.*, 2011). The ciliates,a protozoan community was dominated at low salinities in the outdoor culture (Maica *et al.*, 2012). MF-CEED shows high 2.1×10^7 heterotrophic bacterial count in indoor culture and clearly indicates that MF-CEED supports the growth of fungi, yeast and bacteria. The range of bacteria 10^7 to 10^8 cells mL^1 from the present study in indoor raceways and outdoor ponds were in compliance with the findings of Avnimelech (2012), Otoshi *et al.*,(2006) and Bauman and Pearson (2003). In 'young' biofloc for instance,heterotrophic bacteria are mainly abundant,whereas in 'old' biofloc,fungi took its prominence (Kuhn & Lawrence, 2010). The diatom population was found highly in both the systems at the later part of the culture (Ju *et al.*, 2008). It has a major role in maintaining water quality through the process of photosynthesis (Hargreaves, 2006) The heterotrophic count in indoor raceway was far higher than by the earlier findings (Becerra-Dorame *et al.*, (2011) in *P.vannamei* nursery. Relatively low abundance of dinoflagellates throughout the study in the indoor and outdoor systems was encountered.

High survival recorded in indoor raceways was due to the dominance of heterotrophic bacteria under lower light level and intensity can affect bacterial community structure, which has a significant effect on the survival and production of the cultured species (Lopez-Tarin 2011). The presence of *Bacillus* predominantly in the indoor raceways had the lower vibrio load when compared to the outdoor pond culture and inhibited the proliferation of pathogens (Crab, 2010). The *Bacillus* sp identified from this study has the tendency to grow efficiently with low-cost carbon and nitrogen sources, because its enzymes are very efficient breaking down a great variety of proteins, carbohydrates and lipids from animal and vegetable origin, into their constituent units (Sonnenschein *et al.*, 1993, Arellano & Olmos, 2002). *Brevundimonas vesicularis*, isolated and sequenced from outdoor culture can accumulate the poly-beta hydroxybutylate from effective carbon sources (Jang *et al.*, 2010). *S. capitis* should be considered as opportunistic bacteria with potential to become pathogenic for fish under stressful conditions. It is not possible to eliminate the bacteria from the fish or from the environment (Varvarigos, 2001).We encountered the sequence failures due to the reduced DNA quantity obtained from the PCR amplification and necessitates the importance of quantitative and qualitative DNA extraction.

CONCLUSION

The results of this study shows that 16s rDNA amplification helps in the rapid and accurate identification of bacterial pathogens .The AMF production and management of the microbes under indoor raceways yields the productive microbial protein for the assimilation of *Penaeus vannamei* in the nursery culture. Information on the bacterial species that inhabit the aquaculture systems will be helpful in the effective management in AMF technology so as to prevent the disease outbreak or spread of infections the humans involved in the handling activities.

CHAPTER

5

COMPARISON OF WATER EXCHANGE RATE, FEED ECONOMICS AND NUTRITIONAL COMPOSITION OF AEROBIC MICROBIAL FLOC IN INDOOR AND OUTDOOR CULTURE OF *PENAEUS VANNAMEI*

M. Menaga and S. Felix

ABSTRACT

The present study evaluated the nursery culture of *P. vannamei* in a high density Aerobic Microbial Floc (AMF)-dominated system in 50m^3 Indoor Raceways (RW) and 100 m^3 Outdoor Lined Ponds (LP) for 50 days. Each RW was stocked (6000PL/m^3) and LP with 2300PL/m^3 at 7 ppt. The commercial feed contained 30% crude protein (CP), 7% lipid and 4% fiber were fed to the animals. Total ammonium nitrogen and nitrite nitrogen were maintained in culture tanks with minimum water replacement throughout the experiment. Microbial Floc-Carbohydrate Enriched Distillery Effluent (MF-CEED) was used as the carbon source to maintain the C:N ratio of 15:1 in both the systems. Specific growth rate,Feed Conversion ratio and survival were not significant whereas,Final mean body weight,Protein Efficiency Ratio, yield and Average weight gain were significantly higher in RWs.The crude lipid and total ash were significantly higher in indoor raceways whereas crude protein was significantly higher in outdoor lined ponds.The AMF culture in indoor raceways saved about nearly 3times of the water needed to maintain nontoxic conditions during vannamei nursery production in outdoor culture.The feed economic analysis indicates that 8 % gain in terms of feed costs kg^{-1} shrimp live weight produced in indoor raceway culture compared to outdoor ponds. This study demonstrates that MF-CEED has influenced positively in the biochemical composition of flocs and can be used as a cost effective carbon source for nursery shrimp culture.

Keywords: *Penaeus vannamei*, Aerobic Microbial Floc, Raceways,MF-CEED.

INTRODUCTION

Increasing feed prices, wild fish over-exploitation and environmental pollution have obstructed the development of sustainable aquaculture. Production of bioflocs from aquaculture systems for use as an alternative aquatic feed material has been attracting worldwide interest (Crab *et al.*, 2007). Bioflocs, microbial community growth taking up dissolved nitrogen with biofloc technology (BFT), contain 20–60% crude protein and 1–5% crude lipid (Azim and Little, 2008, Crab *et al.*, 2010, De Schryver and Verstraete, 2009, Kuhn *et al.*, 2009, Liang *et al.*, 2014, Lu *et al.*, 2012 and Luo *et al.*, 2006). Bioflocs are good sources of vitamins and minerals; however, their content of n-3 fatty acids is low (Crab *et al.*, 2010 and Ju *et al.*, 2008). Several factors affect the nutritional quality of bioflocs. The content of protein is increased when bioflocs grow on glycerol and acetate, and the lipid content is much higher for glucose feeds (Crab *et al.*, 2010). Bioflocs production rates, protein and poly-β-hydroxybutyrate (PHB) contents increase with higher carbon supplementation levels (De Schryver and Verstraete, 2009, Ruan *et al.*, 2011 and Schneider *et al.*, 200a).

Pioneer work such as Reid and Arnold (1992) and Williams *et al.* (1996) demonstrated that it is possible to produce shrimp at high density in raceway systems that use water recirculation. The recent progress made in the area of super-dense cultures, also known as 'suspended-growth systems' (Hargreaves, 2006), 'Biofloc Technology' (Avnimelech, 1999),Aerobic Microbial Floc Technology (Felix.S.,2014) reinforces the idea that it is possible to produce aquatic organisms in an intensive and especially bio-safe way.

Application of bio-flocs technology (BFT) in aquaculture offers a solution to avoid environmental impact of high nutrient discharges and to reduce the use of artificial feed. In BFT, excess of nutrients in aquaculture systems are converted into microbial biomass, which can be consumed by the cultured animals as a food source.

High density cultures in intensive systems require high amounts of feed to be added to the systems. This will cause water quality deterioration due to the high concentrations of organic compounds (Avnimelech,2007). Because only 20-30% of feed are assimilated in fish biomass, and the remaining for about 70-80% of the feed will be accumulated in the water body as uneaten feed and excretion products (Avnimelech & Ritvo, 2003; Gross & Boyd, 2000). Similarly, bacteria mineralize organic nitrogen in uneaten feed and feces to ammonia (Gross & Boyd, 2000).

The high protein and lipid content of the flocs of > 100 μm might be attributed to the concentration of extracellular polymeric substances (EPS) which account for 80–95% of the organic matter in the flocs (Wilen *et al.*, 2008). Most of the aquaculture species require protein at a range of 20–50% in their diet (Tacon, 1987a).

Since the biofloc not only can provide supplemental nutrition, like protein, lipid, mineral and vitamin for the growth of cultured shrimp (Izquierdo *et al.*, 2006, Ju *et al.*, 2008b,Moss *et al.*, 2006 and Xu *et al.*, 2012), but also is a source of abundant natural microbes and bioactive compounds that could exert a positive effect on the physiological health of the shrimp in biofloc-based culture systems (Ju *et al.*, 2008a and Xu and Pan, 2013), there is a great potential in reducing crude protein level of feeds while maximizing the contribution of biofloc to the growth and health of the shrimp.

Several studies noted that application of BFT has improved the production performance of several aquaculture species such as shrimp and tilapia (Avnimelech, 2007; Kuhn *et al.*, 2008, 2009). This may be related to the nutritional content of bio-flocs, therefore information concerning these parameters of bio-flocs and their influencing factors are becoming one of the most important key in the development of BFT.The objective of our experiment was to study the effect of Microbial Floc-Carbohydrate Enriched Distillery Effluent (MF-CEED) as a organic carbon source in the nursery rearing of *Litopenaeus vannamei* in indoor raceways and outdoor lined ponds on the primary nutritional parameters of the aerobic microbial flocs.

MATERIALS AND METHODS

Experimental Design

The aim of the study was to evaluate the AMF efficiency between the two systems such as greenhouse-enclosed indoor raceways(50 m^3) and outdoor lined ponds (100m^3)at Advanced Research Farm Facility,Madhavaram, Chennai, India.The raceways were provided with eighteen 5.1 cm airlift pumps arranged in three equidistance banks on each side of the partition and six 1m long air diffusers.Aeration in each raceways were generated by 10 hp and 3 hp blowers.The aeration in lined ponds were provided with two aspirators (2 HP each) to ensure sufficient aeration and water circulation.

The metahaline seawater collected from nearby creek was mixed with freshwater and chlorinated with a commercial grade calcium hypochlorite (35% active chlorine).The dechlorination was done with sufficient aeration ,the salinity was adjusted for 7 ppt with freshwater.The diluted water was directed to the sand filter for filtration and it was filled in the raceways and lined ponds.

Stocking and Feed Management of Shrimps

After one week of fertilization,raceways were stocked (6000PL m^{-3}) with PL_{14}(±0.002g). During the first week, post-larvae were fed six times a day with 30% crude protein crumble feed (Growel Manufactures,Crumble Feed). Feed was provided manually for the

first 10 days a week at 2 % of the body weight to the total biomass. After 10 days, the amount was adjusted according to feed consumption, gut fullness, shrimp mean weights and estimated survival. Shrimp samples were collected randomly and weighed every 10days to determine the mean body weight in both system.From first stocking until the end of the experiment (50 days), each system received daily carbon additions in the form of MF-CEED to convert the total ammonia nitrogen (TAN) generated from feed into bacterial biomass. The amount of MF-CEED added was calculated assuming that 20 g of carbohydrates are needed to convert 1 g of TAN into bacterial biomass (Avnimelech, 1999).

The fertilizers were added by adopting Taw., (2006) protocol with slight modifications for the development of Aerobic Microbial Floc.The MF-CEED (Microbial Floc-Carbohydrate Enriched Distillery Effluent) formulated by enriching the locally available riceflour to the distillery spentwash (26% w/w carbon and specific gravity of 1.5) was used as an organic carbon source to achieve the required C/N ratio of 15:1. Input C/N ratios were calculated based on the carbon-nitrogen contents of the feed and the carbon content of the MF-CEED.

The PL_{14} (±0.002g) were stocked in the nursery raceways at a stocking density of 6000PL/m^3 in indoor raceways and 2300PL/ m^3 in outdoor lined ponds.Commercial Shrimp feeds (Growel manufactured crumble feeds (0.5-1mm)) were used throughout the experiment.Proximate composition of the experimental diet are given in Table 1. Feeding rates were based on observation of feeding behaviour of shrimp in the treatments during the first few days and fixed at 2 % of the total stocked biomass and adjusted weekly after every sampling.Daily feed rations were split into four equal amounts given at 06:00, 10:00, 18:00 and 20:00 hrs in both raceways and lined ponds.The culture animals were sampled for every ten days throughout the trial for the assessment of zootechnical parameters.

Table 1: Proximate composition of experimental feed on dry weight basis (g/100g)

Proximate analysis of the shrimp feed and MF-CEED utilized as organic carbon source in the experiment. Carbohydrates estimates by difference :dw :-dry weight.

Sl.No	Constituents	% on Dry matter
1	Crude protein	30.25
2	Crude lipid	6.00
3	Crude fiber	2.73
4	Carbohydrate	10.65
5	Ash	41.42

Biochemical Analysis of AMF

Nutritional composition reported for microbial flocs (Table 1) was analyzed by A&L Eastern Laboratories, Inc. (Richmond, Virginia, US). Two independent sampling events (14 days between events) were performed to determine microbial floc nutritional consistency. Microbial flocs were harvested as a dietary ingredient for the shrimp feeding trial during the first sampling event. Settled microbial flocs were harvested from SBRs by siphoning and were air dried in 5 cm layers to solids levels greater than 86%. Microbial flocs were subsequently ground into fine material using a stand mixer with grain mill attachment (KitchenAid® Professional 600 Series, Saint Joseph, Michigan, US).

Physico Chemical Analysis

At the end of the experimental trial, the microbial flocs were harvested from each raceways and lined ponds and the same were used for analysis of proximate composition.Proximate composition analyses of the floc were performed as per standard methods (AOAC,1995) at Chennai.

Proximate composition analyses of crude protein, crude lipid and ash content of the experimental diets were performed by the standard methods of AOAC (1995). Protein was determined by measuring nitrogen using the Kjeldahl method and multiplying by 6.25; lipid by ether extraction using Soxhlet; and ash by combustion at 550°C. Gross energy of the experimental diets was determined by an Adiabatic bomb calorimeter (PARR1281, PARR, Moline, Illinois, USA).

Analysis of Fatty Acid Profile

Extraction of lipid from the Biofloc sample was done by Folch method (Folch et. al.,1957) with slight modifications.The extracted lipid undergone FAME preparation and analyzed in GC-MS for fatty acid profile.

Extraction of Lipids

5 g of sample was finely ground with a mixture of 20 ml chloroform and 10 ml methanol using homogenizer.The mixed solution is then filtered through whatman No.1 filter paper into a separating funnel.Then 0.85 % NaCl is added to this and mixed properly and kept for separation into two layers.The lower chloroform layer with lipid is collected in a pre-weighted flat bottom flask and the solvent was evaporated using a rotary evaporator.Weight of the flask with lipid was taken and weight of lipid was calculated from the difference in the weight of the flask with lipid and without lipid. For fatty acid analysis, fatty acid methyl esters were prepared according to the method of Morrison and Smith (1964) and

identified using a Gas Chromatograph (GC, Agilent 6890, Agilent Technologies Co., Ltd. USA) fitted with an Omegawax 320 fused silica capillary column (Supelco, Billefonte, PA, USA). Fatty acid contents were expressed as a percentage of a particular fatty acid to the total fatty acids (%).

Preparation of fatty Acid Methyl Esters (FAME)

The AOAC (1995) method was followed to esterify the lipid extract .FAME was prepared from the lipids extracted from samples by heating with the methanoic NaOH first and then with BF_3 Methanol for esterification. 5 ml n-heptane was added to recover the methyl esters in organic phase. Saturated NaCl solution was added to the mixture and the aqueous and organic layers were separated uing a separating funnel. The upper n-heptane phase was pipet out and stored in 10ml glass vials in refrigerator until further analysis.

Gas Chromatography-Mass Spectrometry

Fatty acids were separated using a Shimadzu Qp2010 Quadrupole Gas Chromatography Mass spectrometer (GC-MS) instrument equipped with a carbowax (30 m x 0.25 mm ID;0.25µm film thickness) capillary column (cromlab S.A). Helium was used as the carrier gas,injector and detector temperatures were set at 250°C. Injection was performed in split mode (1:15). The column temperature was programmed initially at 50°C for 2 minutes and then to increase at a rate of 10°C per min to a final temperature of 230°C. FAME esters were separated at constant pressure (23.1 kPa) and peaks were identified by comparing the mass spectra with the mass spectral database.

Details of characteristic ions used in the study for identification of fatty acids.

Class of fatty acid	Characteristic ion (m/z)
SAFA	74
MUFA	M-74
PUFA	91
n-6	150
n-3	108

Statistical Analysis

Data were analyzed using SPSS statistical software (V. 16 for Windows). The ONE way ANOVA followed by Duncan's test was used to determine the significant differences in raceways and lined ponds in biochemical composition and fatty acid profile of Aerobic Microbial Floc. All differences were analyzed at significance level of $\alpha = 0.05$

RESULTS

Calculation of water exchange rate

One of the major advantages of AMF systems is reducing the volume of water required for maintaining good water quality.The AMF system only recycles the water along with the nutrients.It does not replace water with fresh water.Only losses from evaporation need replacement.According to Avnimelech,1999 the water replacement was done 10 % in raceways and 20% in Outdoor lined ponds (adapted values are adjusted according to field environmental conditions) on every third day.

Specifications	Raceways	Lined ponds
Total volume	50	100
Initial fill(m^3)	40	80
Water replacement volume (m^3)	4 (@ 10%)	16 (@ 20%)
16 top offs (m^3)	68	272
Total	108	352

Additional water to maintain biofloc water quality in indoor raceways was 68m^3, while water to maintain AMF culture was 272 m^3. The AMF system in indoor raceways saved about nearly 3times of the water needed to maintain nontoxic conditions during vannamei production in outdoor culture.An additional large saving in electrical expenses was achieved,estimated at about 75 % during production time.

The feed economics of shrimp nursery culture by Aerobic Microbial Floc (AMF) Technology

The potential savings on feed that can be obtained by AMF has been calculated according to Avnimelech,(2015).Shrimp juveniles produced with commercial feed at a 30% protein content and at food conversion ratio of 1:0.9 in indoor raceways and 1:1.1 in outdoor lined ponds for 50 days trial are as follows:-

Calculation of the cost saving by the application of AMF

The cost for the production of 1 kg shrimp live weight with AMF

→ 1.2 x ₹ 80 kg -1 feed (in India) = ₹ 96 kg^{-1} fish live weight produced in raceways

→ 0.9 x ₹ 80 kg -1 feed (in India) = ₹ 72 kg^{-1} fish live weight produced in lined ponds

Calculation of the amount of organic carbon needed to grow the flocs

The flocs have a C:N ratio of 4 (Avnimelech,1999)

→ 4 x 0.04 =0.16 kg C in floc biomass is produced kg^{-1} fish live weight produced since all the excess nitrogen should be assimilated in the bioflocs

The yield of bacterial biomass can be added in the water for the flocs to be able to assimilate the excess nitrogen kg^{-1} fish live weight produced

In case MF-CEED (26% C) is used as organic carbon source

→ 0.32/0.26 =1.23 kg acetate needs to be dosed to the water kg^{-1} shrimp live weight produced.

The costs for the production of 1 kg shrimp live weight produced

→ (1.2 kg feed kg-1 fish live weight produced x ₹ 80 kg -1 feed) + (1.2 kg MF-CEED kg^{-1} fish live weight produced x ₹ 10 kg-1 MF-CEED) = ₹ 106 kg^{-1} fish live weight produced in Indoor raceways

→ (0.9 kg feed kg-1 fish live weight produced x ¹ 80 kg^{-1} feed) + (1.2 kg MF-CEED kg-1 fish live weight produced x ₹ 10 kg^{-1} MF-CEED) = ₹ 92 kg^{-1} fish live weight produced in outdoor lined ponds

There was no significant gain appears to be only in the order of 8 % in terms of feed costs kg^{-1} shrimp live weight produced in outdoor ponds compared to indoor raceway culture at higher stocking density .

RESULTS

Proximate Composition of AMF

Nutrients	Raceways	Lined Ponds
Crude protein	29.82 ± 0.60^{b}	37.22 ± 1.80^{a}
Crude lipid	6.5 ± 0.8^{a}	4.4 ± 0.1^{b}
Crude Fibre	4.71±0.62	4.6±0.2
Total Ash	45.5 ± 0.5^{b}	28.95 ± 3.01^{a}
Ether Extract	1.215±0.02	1.85±0.02
Carbohydrate	23.12±1.31	25.01±0.7
Gross Energy KJ g^{-1}	229.5±1.5	287.7±2.2

There were significant difference in Crude protein(29.82 ± 0.60^{b} vs 37.22 ± 1.80^{a}) and Total ash (28.95 ± 3.01^{a} vs 45.5 ± 0.5^{b}) between raceways and lined ponds. The crude fat (6.5±0.8 vs 4.4±0.1) was significantly higher in raceways than lined ponds. The crude fibre(4.71±0.62 vs 4.6±0.2) remains the same in both the culture systems. The Ether Extract (1.215±0.06 vs 1.85±0.02) was relatively higher in lined ponds. The carbohydrate (23.12±1.31 vs 27.01±0.7) was comparatively higher in lined ponds than raceways.Similarly Gross energy (287.7±2.2 vs 229.5±1.5) content of Aerobic Miocrobial Floc in lined ponds was higher.

Fatty acid profile of AMF

Fatty acids (%)	Raceways	Lined Ponds
C14:0 –Myristic Acid	2.92±0.81	3.68±0.04
C16:0-Palmitic acid	29.69±1.18	24.995±0.575
C18:0-Stearic Acid	10.84±0.1	5.155±0.175
C18:2 –Oleic Acid	31.91 ± 0.12^{b}	21.165 ± 0.985^{a}
C18:3 n-6 –Linoleic Acid	13.235±3.005	14.325±1.295
C18:2 n-3–Linolenic Acid	1.5 ± 0.2^{a}	13.05 ± 0.35^{b}
C20:0 –Arachidic Acid	0.375 ± 0.075^{a}	1.81 ± 0.29^{b}
C22:0 –Behenic Acid	1.24±0.34	1.445±0.305
C20:5 n-3(EPA)	0.665±0.075	0.78±0.09
C22:6 n-3(DHA)	0.79±0.05	1.035±0.165
C16:1-Palmitoleic Acid	6.995 ± 0.605^{a}	9.465 ± 0.515^{b}
Others	0.84±0.07	0.6±0.04

The relative contents of C18:2n6 (linoleic acid), C20:5n3 (eicosapentaenoic acid), C22:6n3 (docosahexaenoic acid), *n*-3 PUFA, and *n*-6 PUFA had no significant differences between the systems. Only C18:2 (Oleic acid),C18:2 n-3 (Linolenic acid) were significantly higher in indoor raceways than outdoor lined ponds.The C20:0 (arachidic acid), C16:1 (palmitoleic acid) were significantly higher in outdoor lined ponds than indoor raceways.This study showed that Aerobic Microbial Flocs (AMFs) were good sources of *n*-6 PUFA, which was consistent with the previous studies (Azim and Little, 2008 and Crab *et al.*, 2010).

DISCUSSION

The survival rates recorded in the study indicate that the rearing conditions were favorable for the development of post-larvae, and results were similar to those found in other studies (Cohen *et al.*, 2005 and Wasielesky *et al.*, 2006). The final biomass of shrimp and performance of the post-larvae in RWs and LPs were influenced by the carbon source.The MF-CEED (enriched with rice flour) was better and therefore it can be speculated that the AMF produced from MF-CEED provided better nutrition, contributing to shrimp performance.

Proximate analysis of the biofloc in the present study is in agreement with the findings of Ballester *et al.* (2010) who reported 30.4% crude protein (CP) with wheat flour and molasses as carbohydrate sources. The crude protein content of biofloc collected from BFT treatments was within the range of what have been previously studied (Azim & Little 2008; De Schryver & Verstraete 2009; Crab *et al.* 2010; Ekasari *et al.* 2010). The

lower crude protein level noticed in the harvested biofloc in indoor raceways compared to outdoor lined ponds from the earlier findings (Crab *et al.*, 2010 and Ekasari *et al.*, 2010) may be due to differences in bacteria taking part in floc formation (Rittmann and McCarty, 2001). Ju *et al.* (2008a) reported that chlorophyll-dominated biofloc contained higher crude protein content (42%) than flocs dominated by diatoms (26–34%) and bacteria (38%). This further suggests that the microbiota that constitutes the floc is likely to affect the protein content of the bioflocs (Ju *et al.* (2008). The crude lipid was significantly higher in indoor raceway culture than outdoor lined ponds and this may be attributed by the presence of high diatom density as it contains the lipid upto 25% (Shifrin & Chisholm 1981). High level of ash in floc (45%) was closely similar to the findings of De Schryver and Verstraete (2009) and this may be due to the increased solid concentration.The higher level of gross energy has been recorded by using MF-CEED as carbon source,the reason for this is not clearly known but this may be directly related with the utilization of floc by the shrimps in insitu culture as reported by Hargreaves, (2006) and Azim *et al.* (2008).

Out of the 11 fatty acids detected in Aerobic Microbial Floc, palmitic acid, linoleic acid and oleic acid were the dominant fatty acids, and omega 3 fatty acids were detected in trace level in both the systems . This is in consonance with the findings of Crab *et al.* (2010) who reported that biofloc contain palmitic acid, palmitoleic acid and linoleic acid in the highest and omega 3 fatty acids in trace levels. The lower level of PUFA in biofloc collected from raceways may be due to the dominance of heterotrophic bacteria compared to PUFA rich microalgal community in outdoor culture(Meyers and Latscha, 1997).The unsaturated fatty acids were relatively higher in outdoor ponds as it contains the ciliates and live flagellates predominantly than indoor raceways (Lim *et al.* (1997b). The biochemical composition of floc depends on the microbial composition and this is greatly influenced by the light and the type of culture systems.Hence,lower dominance of autotrophic community in the the present study might be due to lack of direct sunlight in the biofloc indoor raceway facility (Ju *et al.*, (2008a) and dominance of algal communities over bacterial biomass in flocs collected from outdoor lined shrimp culture pond.

Conclusion

Microbial Floc-Carbohydrate Enriched Distillery Effluent (MF-CEED) can be used as a carbon source in biofloc systems, increasing shrimp yield and decreasing the feed conversion rate. Current method of biofloc production using nitrogen fertilizers and MF-CEED as carbon source is cheaper and easier compared to bioreactors. These findings may encourage feed manufacturers to consider biofloc as a viable alternative dietary supplement. Future studies are required to determine the amino acid profile, mineral profile and non-protein content of the biofloc with respect to nutritional requirement of shrimp.

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CHAPTER

6

EFFECT OF CARBOHYDRATE ENRICHED DISTILLERY EFFLUENT AS A CARBON SOURCE IN THE NURSERY CULTURE OF *PENAEUS VANNAMEI* IN SUPER INTENSIVE SYSTEMS UNDER AEROBIC MICROBIAL FLOC TECHNOLOGY

M. Menaga and S. Felix

ABSTRACT

A 50 days nursery trial was conducted in *Penaeus vannamei*, stocked at 6000PLm^{-3} in 50 m^3 raceways and 2300PL m^{-3} in 100 m^3 lined ponds in duplicates. Microbial Floc-Carbohydrate Enriched Distillery Effluent (MF-CEED), obtained as distillery spentwash from the distilleries was used as the carbon source to maintain a C:N ratio at 15:1. Nitrate-N was significantly greater in the outdoor lined ponds, accumulating to a peak mean concentration of 216 mg NO_3-N L^{-1}whereas it was below 24 mg NO_3-N L^{-1} in the Indoor raceways. The raceways showed significantly higher 5-Day Biochemical oxygen demand (BOD_5) and Total suspended solids concentration compared to lined ponds due to increased bacterial biomass. The Protein Efficiency Ratio/survival and yield were significantly higher in indoor raceways. These results indicate that differences in the culture environment on the application of MF-CEED can lead to substantial disparity in system function and shrimp production. Thus, the nursery phase of *Penaeus vannamei* at higher stocking density in Indoor raceways systems ensures the predominant growth of heterotrophic bacteria with effective water quality than compared to the outdoor line ponds in the Aerobic Microbial Floc (AMF) technology.

Keywords: Distillery Effluent, Shrimp, Heterotrophic, Raceways

INTRODUCTION

As the present demand of the fish protein necessitates the intensification in aquaculture,the stocking and rearing density of the culture animals are increased to increase the productivity in per unit area. In aquaculture intensification process, due to limited control over the pathogens,the major challenges were found to be health management and biosecurity (Kautsky*et al.*, 2000 and Moss *et al.*, 2012).

Use of nursery systems can also prevent the spread of diseases as post larvae can be kept under quarantine during the nursery. It was first introduced in shrimp production system (Parker *et al.*,1974). Sturmer and Lawrence(1987), Seidman and Issar (1998) and Samochaand Lawrence (1992) evaluated the nursery phase in raceways and ponds in late 1980's. Nursery phase in shrimp farming have advantages over direct stocking, which increases control over stock inventories, water quality and feed management (Samocha and Benner, 2001). Nursery systems can help to produce larger and hardier shrimp for stocking in the grow-out ponds. Furthermore, stocking these larger juveniles can also result in shorter production cycles and a higher number of crops per year (Clifford, 1985; Briggs and Brown, 1991; Fast, 1991b, Samocha *et al.*, 2001, S.Felix and Samayakannan, 2005). Finally, in viral infected areas, the use of two phase rearing which involves nursery can result in increased shrimp stress and mortality as observed by Stern and Letellier (1992).

The application of biofloc technology in shrimp nurseries bestowed the rapid growth in the cultured animals (Wasieleski*et al.*, 2013). BFT improve production and nutrients retention by manipulating the carbon/nitrogen ratio (C/N ratio) as the potential management measure in intensive shrimp farming systems (Avnimelech, 1999). The inorganic nitrogen components in pond can be converted into bacterial biomass by maintaining the well-balanced carbon nitrogen ratio.This can be done by either the use of lower protein diet or supplying additional simple carbon sources,e.g.glucose,sucrose or complex carbon sources such as cellulose,starch, *etc.* to the pond (Avnimelech, 1999, Hargreaves, 2006 and Crab *et al*., 2007). The nutrients from excreted waste and excess feed will be recycled into bacterial biomass and aggregate to form biofloc which can be taken as supplemental feed for culture animals (Avnimelech, 2006). Intensive nursery rearing of *Penaeus vannamei* in Aerobic Microbial Floc (AMF) driven raceways offers the tremendous production potential in the low saline raceway system (Felix. S., 2015).

The aerobic microbial population includes the heterotrophic bacteria that utilize carbon as an energy source for nitrogen assimilation. The cellular proteins formed in the form of microbial biomass assimilate ammonia rapidly (De Schryver*et al.*, 2008).

Heterotrophic bacteria utilize organic carbon as an energy source and assimilate nitrogen to build cellular proteins. By adding carbohydrates to the water, heterotrophic-

bacteria can rapidly assimilate ammonia (De Schryver*et al.*, 2008). However, the rapid assimilate consumes higher rate of dissolved oxygen (DO) compared to nitrification leading to an accumulation of solids in the water column.The better shrimp growth and feed conversion efficiency in BFT has been documented by Xu*et al.*, 2012 and Gao*et al.*, 2012.

The efficacy of aquaculture as a tool for the treatment of wastes has been demonstrated at Madras,where the distillery spentwash,an effluent after undergoing the biomethanation process, is fed into fish ponds.With production rates of 50 tonnes per hectare per year about 6 hectares of land area has been shown to be adequate for treating $100m^3$ of effluents (Rangaswami,1987).

The purpose of this study was to evaluate the differences in system function and shrimp production between indoor raceway system and outdoor lined pond in the application of Microbial Floc-Carbohydrate Enriched Distillery Effluent (MF-CEED) as a carbon source in the nursery culture of *Penaeus vannamei.*

MATERIALS AND METHODS

Site and Experimental system

The study was carried out in two 50 m^3 greenhouse-enclosed raceways (RWs) and in two 100 m^3 lined ponds in Advanced Research Farm Facility at Madhavaram, Chennai, India. The two raceways were operated with high pressure rapid sand filters. Each raceway had a central longitudinal partition placed over a 5.1 cm (2 inch) schedule 40 PVC pipe grid across the bottom of the raceway to provide oxygen-rich water. Water oxygenation in each raceway was generated by a 10 hp and 3 hp blowers. This setup increased dissolved oxygen levels into the raceway by mixing it with air or pure oxygen demand of the system. In addition, each raceway was provided with eighteen 5.1 cm airlift pumps arranged in three equidistance banks on each side of the partition and with six 1m long air diffusers.

Metahaline seawater (90-95 ppt) collected from the nearby lagoon was stored in 5000L reservoir pond.The seawater was allowed to settle down for a week. At the same time, the water has been allowed to be aged atleast 78 hours within the reservoirs.This aging process eliminates the viral particles in the water. The water has been chlorinated with a commercial grade calcium hypochlorite (Bleaching powder of 35 % of active chlorine) to achieve an initial chlorine concentration of 20ppm with targeted residual chorine of 1ppm after 38 hrs. The dechlorination was done with sufficient aeration,the salinity was adjusted for 7 ppt with freshwater.The diluted water was directed to the sand filter for filtration and it was filled in the raceways and lined ponds. The most important

factors such as screening,chemical treatment and aging process were efficiently monitored before stocking with shrimp.The treated water was added with the fertilizers such as Urea,Triple super phosphate,Dolomite and Feed for the development of Aerobic Microbial Floc(AMF) by following Nyan Taw *et al.*,(2006) protocol with slight modifications to promote heterotrophic bacteria for the culture.In lined ponds the fertilization was done and the aeration was provided by two aspirators.These aspirators were positioned diagonally opposite in direction to ensure the proper circulation of water in the pond.

Stocking and culture management

The PL_{14} (±0.002) were stocked in the nursery raceways at a stocking density of 6000PL/m^3 in a AMF developed water with floc volume of 5 ml and TSS was <80 mg L-1.The blowers were operated continuously to maintain the dissolved oxygen content in the saturation level. And in the case of lined ponds the PL_{14} (±0.002g) were stocked at a density of 2300PL/ m^3 in the biofloc water of 3 ml of floc volume and TSS was <60 mg L^{-1}.The two aspirators were allowed to run alternatively throughout the experiment in the lined ponds.

Commercial shrimp feeds (Growel manufactured crumble feeds (0.5-1mm)) were used in the experiment.Proximate composition of the experimental diet are given in Table 1. Feeding rates were based on observation of feeding behaviour of shrimp in the treatments during the first few days and fixed at 2 % of the total stocked biomass and adjusted weekly after every sampling. Daily feed rations were split into four equal amounts given at 06:00,10:00,18:00 and 20:00 hrs in both raceways and lined ponds.The MF-CEED (Microbial Floc-Carbohydrate Enriched Distillery Effluent) formulated by enriching the locally available riceflour to the distillery spentwash (26% w/w carbon and specific gravity of 1.5). It was used as an organic carbon source to achieve the required C/N ratio of 15:1.

Table 1: Proximate composition of experimental feed on dry weight basis (g/100g)

Constituents	% on Dry matter
Crude protein	30.25
Crude lipid	6.00
Crude fiber	2.73
Carbohydrate	10.65
Ash	41.42

Shrimp Growth parameters Analysis

The culture animals were sampled for every ten days throughout the trial for the assessment of zootechnical parameters.At each sampling, 100 animals were randomly selected and weighed. Survival, growth rate (expressed as weekly weight gain), yield (expressed as yield per unit of water volume), PER and FCR were calculated using the following equations: Survival (%) = (100 × final live shrimp count) / initial stocking shrimp count, Specific growth rate (% day^{-1}) = 100 × [Ln(final body weight) – Ln(initial body weight)] experimental duration (days), Protein Efficiency Ratio =body weight gain/protein fed ,yield (kg m^{-3}) = total weight of shrimp harvested divided by water volume, FCR = total weight of feed offered divided by total shrimp weight gained.

Water quality analysis

Dissolved oxygen (DO) was examined for six times (04:00,09:00,13:00,16:00,21:00,01:00) in a day. Temperature,pH and Ammonium ion were monitored 3 times at 04:00, 12:00, 18:00 daily. The Nitrite-Nitrogen (NO_2-N), Nitrate-Nitrogen(NO_3-N), Phosphate(PO_4), Total Alkalinity, Calcium Hardness, Magnesium Hardness, Turbidity and Imhoff cone volume were tested at 11.00 am periodically. The Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Total Dissolved Solids (TDS),Turbidity were measured for every three days. The five-day biochemical oxygen demand (BOD_5) was recorded throughout the experiment.Besides these, sodium and potassium were determined weekly once during the experiment.TheFloc Volume (ml L^{-1}) was monitored by using Imhoff cones daily.

Table 3: Selected water quality indicators and monitoring procedures used in 50 days nursery trial evaluating the performance of *Penaeus vannamei* in Indoor raceways and outdoor lined ponds.

Parameters	Methods
Temperature(°C)	Thermometer
pH	pH meter
Alkalinity(mg $CaCO_3$ L^{-1})	APHA ,1995
Magnesium Hardness (mg CaCO3 L^{-1})	APHA,1995
Total Hardness (mg CaCO3 L^{-1})	APHA,1995
Dissolved Oxygen (mg L^{-1})	APHA,1995
CO_2 (mg L^{-1})	APHA,1995
TAN (mg L^{-1})	APHA,1995
Nitrite-N (mg L^{-1})	APHA,1995
Nitrate-N (mg L^{-1})	APHA,1995
Phosphate (mg L^{-1})	APHA,1995

[Table Contd.

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Parameters	Methods
Sodium (mg L^{-1})	APHA,1995
Potassium (mg L^{-1})	APHA,1995
TSS (mg L^{-1})	APHA,1995
TDS(mg L^{-1})	APHA,1995
VSS(mg L^{-1})	APHA,1995
Turbidity (NTU)	Nephalometer
BOD(mg L^{-1})	APHA,1995
Floc Volume (ml L^{-1})	ImhoffCone,APHA,1995

STATISTICAL ANALYSIS

Data were analyzed using SPSS statistical software (V. 16 for Windows). Two way ANOVA was used to determine significant differences between raceways and lined ponds in water quality indicators with time and systems as the fixed factors. The shrimp mean final weight, specific growth rate (SGR), Feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER), survival and yield were analyzed using one-way ANOVA after conducting a homogeneity of variance test. Differences were considered significant at $P<0.05$. When significant differences were found Duncan's multiple range test was used to identify differences between the systems according toBiradar (2002) and Zar (2014).

RESULTS

Zootechnical Parameters

(Table-4 Performance of *Penaeus vannamei* post larvae in 50-d high-density nursery trial (6000 PL m^3) in Indoor raceways and (2300 PL m^{-3}) in Outdoor lined ponds in a AMF-dominated system (mean ± SE)).

Parameters	Raceways (R)	Lined Ponds(P)
Initial mean weight (g)	0.024 ± 0.01^a	0.035 ± 0.02^b
Final mean weight (g)	0.907 ± 0.33^a	1.198 ± 0.26^b
Specific Growth Rate (SGR)	7.22±0.04	7.03±0.13
Feed Conversion Ratio (FCR)	1.17±0.06	0.89±0.03
Protein Efficiency Ratio (PER)	2.94 ± 0.12^a	3.87 ± 0.09^b
Yield (kg/m^3)	4.92 ± 0.26^a	2.36 ± 0.02^a
Survival (%)	91.24±1.6	86.1±1.4
Average weight gain (g)	0.88 ± 0.03^a	1.16 ± 0.02^b

Table 4 depicts the mean values of the growth parameters in both the systems.There was a significant difference ($p<0.05$) in final mean body weight (0.907 ± 0.33^{a}vs 1.198 ± 0.26^{b}),Protein Efficiency Ratio (PER) (2.94 ± 0.12^{a}vs3.87 ± 0.09^{b}),Yield (4.92 ± 0.26^{a}vs 2.36 ± 0.02^{a}) and Average Weight gain (g) (0.88 ± 0.03^{a}vs1.16 ± 0.02^{b}) of the vannamei between raceways and lined ponds.There was no significant difference between raceways and lined ponds in Specific Growth Rate (7.59±0.30vs7.87±2.52),Feed Conversion Ratio (1.2±0.02vs0.90±0.01) and Survival (91.24±1.6 vs86.1±1.4)in indoor raceways and outdoor lined ponds.

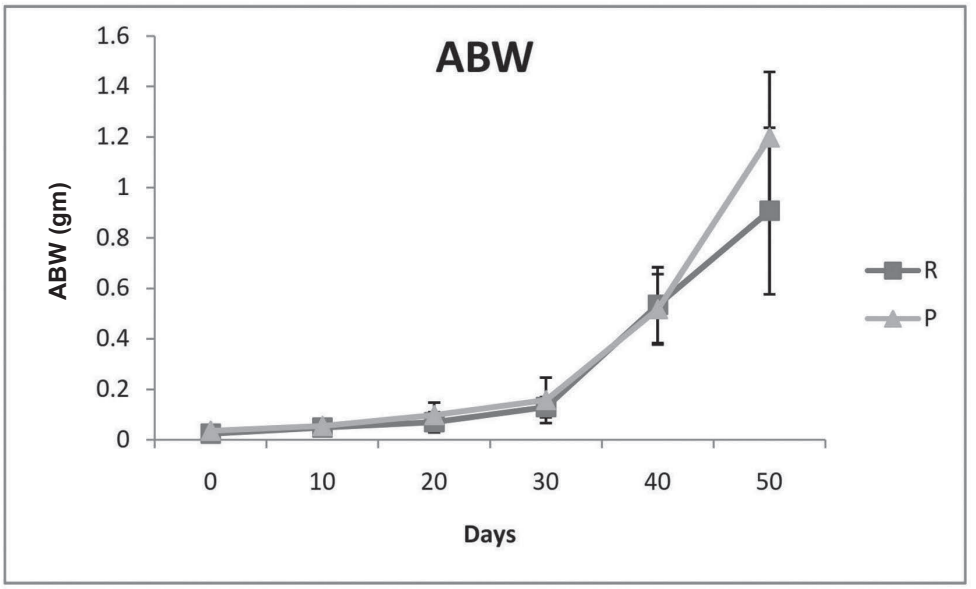

Fig. 1. Weekly Average body weight of shrimp in indoor raceway(R) and outdoor lined ponds(P) during the 50-d nursery study. Values are means (± S.E.) of duplicates per sampling time in each treatment.

***ABW-Average Body Weight**

Water quality parameters

Table 5. summarizes the means and ranges of the daily water quality indicators analyzed in this study. Overall mean (±SD) of water temperature at 06:00,12:00 and 18:00 were (27.25±0.64°Cvs26.71±1.01°C);(30.32±0.61°Cvs33.14±1.43°C); (28.78± 0.41°Cvs31.07±0.76°C); dissolved oxygen (5.52±1.61vs4.83±0.59mg L^{-1}); (5.0±1.52vs4.2±0.54 mg L^{-1});(6.4±1.61vs5.57±0.57 mg L^{-1});(6.2±0.74vs6.01±0.77 mg L^{-1}); (5.9±1.81vs6.1±0.58 mg L^{-1});(5.9±1.75vs5.01±0.47 mg L^{-1}) at 01:00, 04:00.09:00, 13:00,16:00 and 19:00 hrs. pH (7.5±0.14vs7.5±0.19);(7.4±0.14vs7.5±0.21); (7.4±0.13 vs 7.6±0.19) at 06:00,12:00 & 18:00 , Carbondioxide (2.53±2.01vs2.16±0.71 mg L^{-1}), Nitrite-N (0.09±0.30vs0.68±0.07mg L^{-1}) , Sodium (160.4±4.50vs142.4±6.80mg L^{-1}),

Table 5: Summary of water quality indicators for Indoor raceways(R) and Lined ponds (P) operated in a AMF system with *Litopenaeus vannamei* over a 50-d nursery trial). Mean values (± S.D) of duplicates in same row with a different superscript differ significantly (P < 0.05).

Parameters	Time (Hrs)	Raceways(R)	Lined Ponds(P)
Temperature(°C)	06:00	27.25±0.64(26-29)	26.71±1.01(25-29)
	12:00	30.32±0.61(29-32)	33.14±1.43(31-36)
	18:00	28.78±0.41(28-29)	31.07±0.76(30-33)
pH	06:00	7.5±0.14(7.2-7.6)	7.5±0.19(7.2-7.9)
	12:00	7.4±0.14(7.2-7.8)	7.5±0.21(7.0-7.9)
	18:00	7.4±0.13(7.2-7.8)	7.6±0.19(7.3-7.9)
Alkalinity (mg CaCO3 L^{-1})	11.00	122.14±15.69(98-178)	149.25±15.99(110-172)
Calcium Hardness (mg CaCO3 L^{-1})	11.00	156.60±42.79(117-280)	134.71±10.98(102-155)
Magnesium Hardness(mg CaCO3 L^{-1})	11.00	167.96±55.74(106-315)	144.53±32.88(100-185)
Total Hardness(mg CaCO3 L^{-1})	11.00	1906±229.22(1120-2220)	1178±221.68(840-1650)
Dissolved Oxygen (mg L^{-1})	01:00	5.52±1.61(3.8-5.6)	4.83±0.59(3.5-5.9)
	04:00	5.0±1.52(4.0-6.8)	4.2±0.54(3.8-5.5)
	09:00	6.4±1.61(3.8-8.4)	5.57±0.57(4.0-8.0)
	13:00	6.2±1.74(5.5-8.4)	6.01±0.77 (5.2-7.2)
	16:00	5.9±1.81(5.0-8.2)	6.1±0.58(4.8-6.8)
	19:00	5.9±1.75(4.0-8.2)	5.01±0.47(4.5-6.8)

[Table Contd.

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Parameters	Time (Hrs)	Raceways(R)	Lined Ponds(P)
CO_2 (mg L^{-1})	11.00	2.53±2.01(0.5-5.12)	2.16±0.71(0.9-3.10)
Ammonia (NH_3-Nmg L^{-1})	04:00	0.06 ± 0.12^{a}(0.03-0.48)	0.20 ± 0.18^{b}(0.01-0.9)
	14:00	0.05 ± 0.14^{b}(0.04-0.68)	0.16 ± 0.16^{b}(0.01-1.2)
	23:00	0.46 ± 1.67^{a}(0.02-0.43)	0.11 ± 0.13^{b}(0.01-1.3)
Nitrite-NO_2-N(mg L^{-1})	11.00	0.09±0.30(0.01-0.15)	0.68±0.07(0.01-1.71)
Nitrate-NO_3-N(mg L^{-1})	11.00	24.78 ± 5.59^{b}(3-45)	216.28 ± 5.17^{a}(12-257)
Phosphate(mg L^{-1})	11.00	5.61 ± 3.71^{b}(1.65-12.40)	8.14 ± 4.27^{a}(1.59-32.40)
Sodium (mg L^{-1})	11.00	160.4±4.50(147-165)	142.4±6.80(123-150)
Potassium(mg L^{-1})	11.00	147.96±55.74(77-228)	144.53±32.88(100-195)
TSS (mg L^{-1})	11.00	155.87 ± 20.26^{b}(124-237)	116.75 ± 14.63^{a}(85-139)
TDS(mg L^{-1})	11.00	12.01 ± 1.50^{a} (6.40-15.6)	4.25 ± 0.95^{b}(2.25-8.50)
VSS(mg L^{-1})	11.00	107.62 ± 8.92^{a}(94-131)	62.75 ± 11^{b}(47-87)
Turbidity (NTU)	11.00	44.59 ± 13.50^{a}(26.6-79.2)	35.23 ± 3.08^{a}(28.4-41.3)
BOD(mg L^{-1})	11.00	92.28 ± 39.72^{a}(33.21-145.2)	81.85 ± 22.91^{b}(51.44-121.40)
Floc Volume (ml L^{-1})	11.00	12.3 ± 2.083^{a}(8.4-12.30)	6.08 ± 2.13^{b}(2.6-8.00)
Salinity (‰)	11.00	6.9 ± 0.8 (6.5–7.7)	6.8 ± 0.8(6.4-7.2)

Potassium(147.96±55.74vs144.53±32.88mg L^{-1}) and Salinity (6.9 ± 0.8 vs6.8 ± 0.8‰) at 11:00 hrs daily in Indoor raceways and outdoor lined ponds.These parameters were not significantly different ($p>0.05$) between the two systems.

The Floc Volume Concentrations in the culture medium were low during most of the trial, levels were >15 ml L^{-1} and TDS were >250 mg L^{-1}. The significant differences ($P > 0.05$) were found in daily water quality indicators such as TAN,Nitrate-N,Phosphate,TSS, TDS, VSS, BOD, Turbidity, Floc Volume between raceways(R) and lined ponds(P).There was no significant difference between the water quality parameters such as Temperature,pH,Alkalinity,Calcium hardness, Magnesium hardness,Total hardness,Dissolved Oxygen,Carbondioxide,Sodium and Potassium(Van Wyk and Scarpa,1999,Lin and Chen,2001 and Furtado *et al.*, 2015) .

Discussion

The growth of vannamei did not differ significantly between indoor raceways and outdoor lined ponds and this results supports the findings of Krummenauer *et al.* (2011) as the growth of shrimp does not affect even at the higher stocking density (Arnold *et al.*, 2006b,Wasielesky *et al.*, 2013). The survival of the vannamei in ponds was relatively higher in raceways and this may be due to the effective aeration system and the overall effective control of all parameters.As the aspirators in lined ponds could not offer the sufficient aeration and dead ends were formed in the corners of the pond.The oxygen requirement in AMF technology is high to maintain the correct balance between microbial flocs and culture animal.The SGR values in our study for 50 days(7.0-7.2%increase in weight/day)was closely similar to the values reported for stocking densities of 3000-6000 animals/m^3 (8.6-9.2 % weight increase/day (Esparza-Leal *et al.*,2015) for 42 days in BFT systems.The adequate water quality concentrations allowed shedding and proper formation of the exoskeleton,promoting growth and survival in the raceways even at the higher stocking density(McGraw and Scarpa,2003)In this study, the FCR was not significantly different between treatments ($P>0.05$) and the addition of carbon sources (MF-CEED) increases shrimp growth and reduce FCR(Arnold *et al.*, 2006, Arnold *et al.*, 2009 and Audelo-Naranjo*et al.*, 2010). The Protein Efficiency Ratio(PER) was significantly higher in outdoor lined ponds as autotrophic dominated system are rich in protein microbial mass compared to bacteria dominated heterotrophic system and this leads to the assimilation of microbial protein by the cultured animals(Avnimelech,2015). There was a significant difference in the yield between the culture system and this is attributed due to the increased stocking density in raceways.

The highest TAN concentration recorded in our study was 0.68 mg TAN L^{-1} (at pH 7.3, temp. 29.2 °C, and salinity7 ppt) in indoor raceways(R) and 1.3 mg TAN L^{-1} (at pH

7.5, temp. 32.2 °C, and salinity7 ppt) in outdoor lined ponds(P).It was significantly higher in outdoor ponds due to decreased bacterial biomass in the system.Furthermore, we did not observe any dead PL during periods of high ammonia concentration it is probable that TAN did not affect shrimp survival in our trial. It is interesting to note that the patterns of nitrite concentration were different for both the system. Although the increase in nitrite concentrations started after the third week in outdoor lined ponds(P) ,the maximum nitrite-N levels in the outdoor lined ponds were higher than the indoor raceways(R) (1.71 vs. 0.15 mg NO_2-N L^{-1}). This higher concentration was expected since the development of nitrifiers as well as the heterotrophs was slow in the outdoor lined ponds(P) than indoor raceways(R). The low concentrations of nitrite in the indoor raceways reveals the complete oxidation of ammonia to nitrate (Cohen *et al.*, 2005).Nitrate concentrations averaged 24.78 mg L^{-1} and 98.28mg L^{-1} for the raceways and lined ponds, respectively, with final concentrations increasing to 45 mg L^{-1} and 198 mg L^{-1} at the end of trial (Table 5).In our study exposure to a maximum NO_3 concentration of 257 mg L^{-1} or mean of 216.28 mg NO_3-N L^{-1} had no adverse effect on shrimp and did not affect the growth and survival in indoor raceways but affected the survival in outdoor lined ponds at lower salinities(Kuhn *et al.*(2010) as it was relatively lower. In the present study, low TAN and NO_2-N concentrations were observed in both systems below the safe level as cited by Kuhn *et al.*, 2010 and their dynamics didn't seem to be affected by the carbon supplementation (Xu and Pan, 2012).The rapid assimilation of ammonia after the application of MF-CEED as a carbon source was experienced at faster pace in indoor raceways than lined ponds due to increased heterotrophic population.And this study confirms the influence of carbon source in the succession of heterotrophic bacteria over microalgae(Gonzalez-Felix *et al.*, 2007, Ju *et al.*, 2008).

TSS,TDS,VSS and turbidity changes followed the same increasing trend as nitrate, reaching levels higher than 237 mg L^{-1}, 15.6 mg L^{-1}, and 131 mg L^{-1},79.2 NTU respectively at the end of the study in indoor culture.These values in the raceways were significantly higher than the lined ponds (Table 5). The less floc volume and TDS in ponds indicate the reduced population of bacteria,cyanobacteria,rotifers and nematodes (Ray *et al.* (2010b) in lined ponds.The higher turbidity in raceways may be due to the presence of total suspended and dissolved solids (Andrew J. Ray *et al.*, 2014). However, the levels of TSS and TDS during the experiment showed direct correlation with regard to the carbon supplementation and disagrees with the findings of Wu-JieXua *et al.* (2016). The phosphate levels in the outdoor lined pond treatment was significantly higher than the indoor raceways. The slight decrease of phosphate concentration in the raceways dominated by heterotrophic bacteria may have been due to assimilation of phosphate by heterotrophic bacteria (Longnecker *et al.*, 2010 and Zubkov *et al.*, 2007). The five-day carbonaceous Biochemical Oxygen Demand (BOD) ranged from minima of 33.21 and 51.44 to maxima

of 145.20 mg L^{-1} and 121.40 mg L^{-1} in the indoor raceways and outdoor lined ponds respectively. Higher BOD_5 indicates that microbial respiration and oxidative processes consumed a greater amount of oxygen in the heterotrophic dominant systems (Azim and Little,2008,Browdy*et al.*, 2012).The average dissolved oxygen levels in the indoor raceways were above 5.6 mg L^{-1}. The AM and PM maxima and minima DO concentrations reported in Table 5 suggest that DO never dropped below 3.6 mg L^{-1}. The concentration of CO_2 was relatively higher in raceways (0.5-7.10 ppm) than lined ponds (0.9-3.10ppm) and this may be due to the regeneration of ammonia by the dominant population of heterotrophic bacteria in the raceways (Avnimelech, 2015). In both system the pH, alkalinity, temperature were within the acceptable range for the species (Van Wyk and Scarpa, 1999). All these water quality results suggests the heterotrophic bacteria have a maximum growth rate significantly higher than nitrifiers, replicates 5 times per day compared to one replication per day, and are thus able to outcompete autotrophs in indoor conditions (Ebeling *et al.*, 2006). This increased trend towards heterotrophy with increasing carbon supplementation was also noticed highly in the indoor raceways.

CONCLUSIONS

The application of Aerobic Microbial Floc (AMF) technology allows minimal or zero-water exchange practice during the culture period, in the super intensive systems thereby can improve sustainability,biosecurity and production in nursery culture of shrimps (Felix S., 2015). The driving force of AMF systems is the development of floc, which is responsible for water quality control, waste assimilation, and nutrient recycling, which contribute to improved performance of cultured shrimp (Avnimelech, 2015). The results of the present study showed that the carbon source had a significant influence on the development and characteristics of Aerobic Microbial Floc affects the inorganic nitrogen and floc dynamics to some extent, in the outdoor culture of shrimps than indoor culture at higher stocking densities. Three significant conclusions can be drawn as follows: (i)The heterotrophic bacterial dominant floc can be developed rapidly in indoor conditions and can be maintained easily at the C:N ratio of 15:1 (ii) The MF-CEED as a carbon source can rapidly control the TAN and NO_2-N concentrations effectively by heterotrophic assimilation in indoor raceways than autotrophic nitrification in the outdoor ponds (iii) The MF-CEED can be used as a potential carbon source in the indoor raceway culture for the maintenance of good and stable water quality to improve the shrimp performance in the zero water exchange system.

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CHAPTER

7

INVITRO PROBIOTIC PROPERTIES OF *BACILLUS SP* ISOLATED FROM BIOFLOC SYSTEMS

M. Menaga and S.Felix

ABSTRACT

A study was conducted to investigate the probiotic potential of the bacterial species from biofloc systems using *invitro* quantitative assays. Based on the morphological, biochemical and 16S rRNA sequencing analysis, isolated bacterial species were identified as *Bacillus infantis, Exiguobacterium profundum*, *Bacillus subtilis* and *Bacillus megaterium*. The *invitro* probiotic properties such as bile tolerance, growth at different bile concentrations, bile salt hydrolase activity, antibiotic susceptibility test, antimicrobial activity, auto aggregation test, bacterial adhesion to hydrocarbons and resistance to gastric acidity were performed. All the isolates showed higher resistant to bile tolerance test and growth of cultures were observed from 0.5 to 8% bile salt concentrations. The distinct zone of hydrolysis was marked in the tested isolates in bile salt hydrolysis activity. Except *B. infantis* all the other three isolates were predominantly resistant to the tested antibiotics. Antimicrobial activity against three pathogens viz. *Vibrio parahaemolyticus, Vibrio harveyi* and *Aeromonas hydrophila* was observed in all the isolates. *E. profundum* and *B. subtilis* showed improved auto aggregation. Enhanced resistant to bile salt adhesion to hydrocarbon and *invitro* gastric acidity (pH 3) was seen in *B. megaterium*. It is one of such unique studies confirming the probiotic effect of *Bacillus sp.* isolated mainly from biofloc culture. *B.subtilis* and *B. megaterium* exhibited remarkable *invitro* probiotic properties and thus can be recommended as a successful probiotic strain for fish farming.

Key words: Biofloc, GIFT Tilapia, Probiotics, *invitro* properties, Antimicrobial activity

INTRODUCTION

Aquaculture as a food-producing sector is recognized for its ability to contribute to the global fish supply. Aquaculture production is projected to rise to 82 million tonnes in 2050 (FAO, 2016). The potential use of bioflocs as an anti-inflammatory strategy in aquaculture for disease management on a long term basis in contrast to conventional approaches such as antibiotic, probiotic and prebiotic applications was reviewed by Sinha *et al.*, (2008). Probiotics are viable microbial cells that have a beneficial effect on the health of a host by improving its intestinal equilibrium through improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic actions, growth-promoting factors, and an increased immune response (Verschuere *et al.*, 2000).The heterotrophic bacterial dominance established by steering C/N ratio in the water with the interventions in using different external carbohydrate sources or by using low protein feeds helps the bacteria to assimilate the ammonia for converting to microbial biomass(Avnimelech, 1999).The heterotrophic bacteria in biofloc (Halet *et al.*, 2007) produce some natural substances (Dinh *et al.*, 2010; Iyapparaj *et al.*, 2013) and suppress the growth of other pathogenic species like *Vibrio harveyi* (Defoirdt *et al.*, 2007). The main objective of the present study was to isolate, identify and characterize the various probiotic properties of isolates derived from the biofloc based culture.

MATERIALS AND METHODS

Isolation and characterisation of dominant bacteria

Animals reared in biofloc ponds were collected and examined for infection using external morphological analysis [Johansen *et al.*, 2006]. Sampled fishes were killed and dissected under sterile environment. Homogenates of intestinal tracts (oesophagus to rectum) were prepared and preserved in physiological solution (0.85% NaCl). Three pathogenic bacteria such as *Vibrio parahaemolyticus* ATCC-17803, *Vibrio harveyi* ATCC- BAA-2752 and *Aeromonas hydrophila* ATCC- 35654 were obtained from American Type Culture Collections (ATCC, Chroma chemie Laboratory Pvt. Ltd, Karnataka, India) and used as target pathogenic bacteria. All strains were stored in their respective growth medium with 20 % glycerol at -20°C to provide a stable inoculum throughout the study. The intestinal homogenate was serially diluted and 10^{-2}, 10^{-4}, 10^{-6} & 10^{-8} were plated on de Man-Rogosa-Sharpe (MRS) agar as per standard microbiological methods. The plates were incubated for 24 hours at 37°C, morphologically different colonies were picked and further streaked to isolate pure colonies. The four different isolates obtained were characterised morphologically and biochemically as per the method of Bergey's Manual of Determinative Bacteriology.

DNA isolation, PCR amplification and Phylogenetic tree construction

The DNA was isolated from 16hrs old culture using Phenol-Chloroform method. The isolated DNA was amplified for its 16srRNA region using the Forward primer (27F-5'AGA GTT TGA TCM TGG CTC AG 3') & Reverse primer (1492R-5' CGG TTA CCT TGT TAC GAC TT 3'). The amplified DNA was further purified with QIAquick PCR purification kit (Qiagen), and sequenced using Sanger's method. The aligned sequences were submitted to the GenBank database using the BLAST program of NCBI (http://www.ncbi.nlm.nih.gov) and accession numbers were obtained. The phylogenetic tree was constructed to find the relationship between the four isolates using Mega 6.0 software.

Invitro Evaluation of Probiotic Properties

Bile tolerance

MRS supplemented with 0.3% (w/v) bile as well as without bile (control) was inoculated with four bacterial isolates (incubated for 16 ± 2 h at 37ºC). The growth of four isolates in bile tolerance test was monitored by measuring the OD at 600 nm using spectrophotometer. Time to reach log phase at 0.3% bile concentration was determined to find bile tolerance or sensitivity of the cultures (Chateau *et al.* 1994).

Growth at different bile concentrations

Different concentration of Ox-bile [0.5, 1.0, 2.0, 4.0 and 8.0% (w/v)] were added to the MRS broth and inoculated with freshly grown (incubated for 16 ± 2 h at 37°C) bacterial cultures. The growth of the test cultures at different bile concentrations was monitored by measuring the optical density (OD) at 650 nm after 24 h of incubation at 37°C. MRS broth without Ox-bile was used as negative control (Nithya & Halami, 2013).

Bile salt hydrolase activity

Four isolates were grown overnight in MRS broth and spotted using sterile cotton buds on to MRS agar plates containing 0.5% (w/v) ox-bile and 0.37 g/l $CaCl_2$. Plates were incubated at 37°C for 72 hours and were observed for the appearance of precipitation zones as per Sieladie *et al.*, 2011.

Antibiotic susceptibility test

Antibiotic susceptibility of isolated bacteria was determined using disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2016). Ampicillin (10mcg) Amoxicillin (10mcg), Cefixime (10mcg), Cephalexin (10mcg), Chloramphenicol (25mcg),

Ciprofloxacin (10mcg), Erythromycin (15 mcg), Tetracycline (25mcg), Levofloxacin (10 mcg), Norfloxacin (10 mcg) discs were used. Freshly grown culture (16 ± 2 h) were suspended to a density of McFarland 0.5 turbidity standard and diluted to 1:100. The diluted suspension were streaked on Muller Hinton agar plates and kept for incubation at 37°C for 24 hr. The diameters of inhibition zones were measured and results were recorded as sensitive (S) and resistance (R).

Antimicrobial activity

Three pathogenic strains *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Aeromonas hydrophila* were grown in 5 ml sterile nutrient broth for 3 hours. The freshly grown bacterial isolates were spread on to nutrient agar plates using sterile cotton buds. Wells were created using gel puncture and 30 µl of the selected pathogens were seeded in the respective wells. For the diffusion of culture the seeded plates were kept in a refrigerator for 20 minutes and incubated at 37°C for 48 hours. After incubation, plates were observed for presence or absence of zone of inhibition as per Tambekar *et al.*, 2010)

Auto aggregation tests

Auto aggregation ability of the bacterial isolates were determined by Canzi *et al.* (2005). 10 ml of pure bacterial culture was kept in static condition for incubation (3 h at 15°C). After incubation, 1 ml of the broth from the upper suspension was transferred to another test tube after incubation. The transferred suspension OD was measured at 600nm. Auto aggregation was calculated by using the equation, Auto aggregation % = 1-[OD value of upper suspension/OD value of total culture] x 100

Bacterial adhesion to hydrocarbons (BATH)

Adherence ability of the isolates to xylene as a hydrocarbon was performed as per Canzi *et al.* (2005).The freshly grown culture (16 ± 2 h) was centrifuged to harvest the cell pellet for 15 min at 10,000xg at 4ºC. The cell pellet was washed and resuspended in 0.1 M PBS to an absorbance (600 nm) of 0.5. Equal volume of xylene was added to this suspension and the two phase system was vortexed for 3 min. After 1 hour of incubation at 27 ± 2ºC, the aqueous phase was removed and its absorbance was measured at 600nm. Formula: Adhesion percentage = [(A0-A)/A0] 100 was used to estimate the BATH (where, A0 and A were absorbance at 600nm before and after extraction with organic solvents respectively.)

Resistance to gastric acidity

Four isolates were grown overnight in MRS broth at 37°C. An aliquot of 0.5 ml of the overnight culture was inoculated into 50 ml MRS broth adjusted to pH 3 and 7. Bacterial growth was monitored by measuring the absorbance at 620 nm after 6 and 24 h of incubation at 37°C. The surviving index of the isolates was calculated as the percent difference between the variation of optical density (OD) at pH 7.0 (ΔODpH7) and pH 3 (ΔODpH3) as per Shruthy *et al.*, 2011. Formula: Surviving (%) = ΔODpH7 - ΔODpH3 / ΔODpH7 × 100, was used to estimate the Resistance.

RESULTS AND DISCUSSION

Biochemical Characterization of isolated colonies

The isolated bacterial species from biofloc reared GIFT gut were identified as gram positive rods and the biochemical characterization are given in Table 1.

Table 1: Biochemical characterization of *Bacillus sp.*

Biochemical characteristics	*Bacillus infantis*	*Exiguobacterium Profundum*	*Bacillus subtilis*	*Bacillus megaterium*
Indole	-ve	-ve	-ve	-ve
Methyl red	-ve	-ve	-ve	-ve
VogesProskauer's	+ve	-ve	+ve	-ve
Citrate utilization	+ve	+ve	+ve	+ve
Glucose	+ve	+ve	+ve	+ve
Adonitol	-ve	+ve	+ve	-ve
Arabinose	-ve	+ve	+ve	-ve
Lactose	+ve	+ve	+ve	-ve
Sorbitol	-ve	+ve	+ve	-ve
Mannitol	+ve	+ve	+ve	-ve
Rhamnose	+ve	+ve	+ve	-ve
Sucrose	+ve	+ve	+ve	-ve

DNA isolation, PCR amplification and phylogenetic tree construction

In the present study based on 16S rRNA gene sequence analysis the isolates HI 12 (568 bp), HI29 (577 bp), H24 (1001 bp) and H4 (577 bp) were found to be *Bacillus subtilis*-MH424900,*Bacillus megaterium*- MH424904, *Exiguobacterium profundum*-MH424898 and *Bacillus infantis*- MH424755. The phylogenetic tree was also constructed and shown in the figure 1.

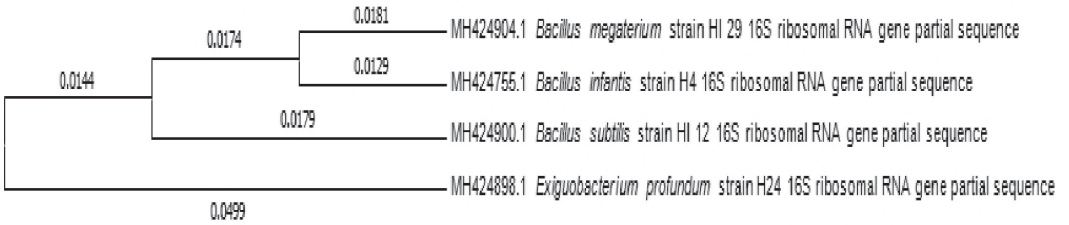

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Bacillus* isolates.

Invitro Evaluation of Probiotic Properties

Bile tolerance

The bile tolerant concentration in the selection of probiotic species for humans and fishes are found to be 0.3% (w/v) (Brashears *et al.*, 2003). The three isolates *B. infantis, B.subtilis* and *B.megaterium* showed a delay in growth below 15 minutes and thus categorized as bile resistant. However, *Exiguobacterium profundum* recorded 60min delay in growth and grouped under weakly tolerant. Bacillus species tested in the present study were categorized in both 'resistant' and 'weakly tolerant' groups as shown in Table-2. All the four isolates showed no effect to the bile tolerance though *Exiguobacterium profundum* bacteria exhibited delayed growth for 1 hour which did not impact in its probiotic potential. The findings of the current study confirmed that these isolates can be used as gut probiotics to thrive in fish intestine.

Table 2: Bile Tolerance of *Bacillus* cultures

Isolates	Delay in growth(min)	Tolerance
Bacillus infantis	10	Resistant
Bacillus subtilis	15	Resistant
Exiguobacterium profundum	60	Weakly tolerant
Bacillus megaterium	15	Resistant

Growth at different bile concentrations

The cultures revealed difference in growth at tested concentrations of Ox-bile (0.5-8% w/v) (Fig. 2). The bacterial culture expressed growth from 0.5-8% of bile concentrations, however decrease in the growth of bacteria with increase in bile concentration was recorded. The findings of the present study is similar to the studies of Nithya & Halami (2013). The *Exiguobacterium profundum* exhibited more stable growth with high bile tolerant potency when compared to other three bacterial species. *Bacillus subtilis* was found to be the low bile tolerant and showed poor growth.

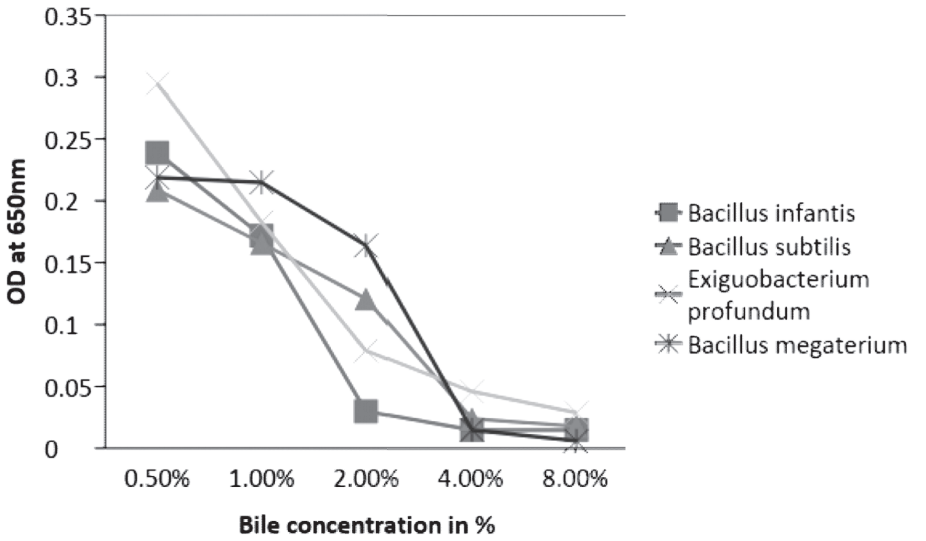

Fig. 2. Growth profile of Bacillus cultures in MRS broths supplemented with different concentrations of Ox-bile

***The cultures were categorized into four groups according to the observed delay of growth (d) in presence of Ox-bile: resistant strains (d ≤ 15 min), tolerant strains (15 < d ≤ 40 min), weakly tolerant strains (40 < d < 60 min) and sensitive strains (d ≥ 60 min) (Chateau *et al.* 1994.**

Bile salt hydrolase activity

Out of the four isolates screened for the bile salt hydrolase activity, all the isolates showed positive results with the formation of distinct zone of hydrolysis (Fig. 3). The white precipitates around the colonies confirm their enzymatic digestion ability of bile salts to

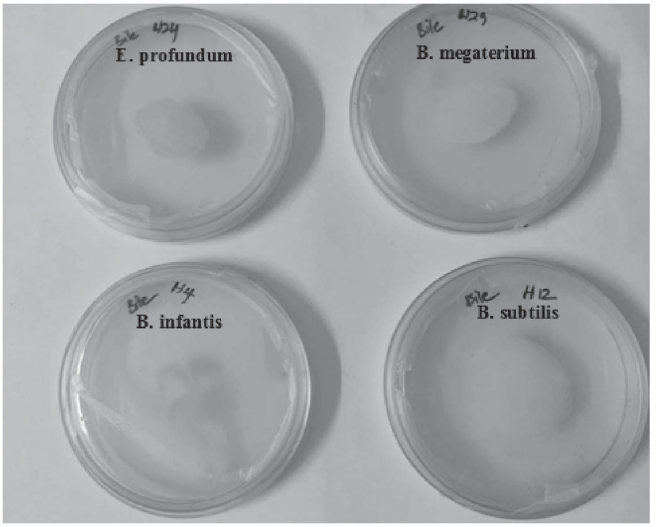

Fig. 3. Bile Salt Hydrolase activity showing the zone of hydrolysis of ox-bile

primary bile salts thereby reducing the levels of serum cholesterol as suggested by Begley *et al.* (2006).Thus these bacterial isolates can exert a probiotic effect on culture animals by reducing their serum cholesterol level.

Antibiotic Susceptibility Test

Out of four bacterial isolates*, Bacillus subtilis, Exiguobacterium profundum* and *Bacillus megaterium* showed a higher resistivity pattern. The lowest antibiotic susceptibility was found in *Bacillus infantis* with higher sensitivity to the tested antibiotics as mentioned in Table-3. These results confirmed the absence of transferable antibiotic resistant genes and they can be categorized as safe probiotic strains as per the breakpoint levels of EFSA, 2008. Among the four isolates, Bacillus subtilis showed intrinsic resistance to antibiotics and can be considered as putative probiotic strain. Apparently, *Bacillus subtilis* 2335 showed invitro activity against *H.pylori* by producing antibiotic Amicoumacin (Pinchuk *et al.*, 2001). Thus this bacterial strain can replace antibiotic treatment effectively to fight against the pathogens (Sorokulova *et al.* (2008).

Table 3: Antibiotic susceptibility test using four isolates

ANTIBIOTICS	*Bacillus infantis*	*Bacillus subtilis*	*Exiguobacterium profundum*	*Bacillus megaterium*
Ampicillin (10mcg)	S^+	R	R	R
Amoxicillin (10mcg)	S^+	R	R	R
Cefixime (10mcg)	R	R	R	R
Cephalexin (10mcg)	S^+	R	R	R
Chloramphenicol (25mcg)	S^+	S^+	S^+	S^+
Ciprofloxacin (10mcg)	S^+	R	S^+	S^+
Erythromycin (15 mcg)	R	R	R	R
Tetracycline (25mcg)	S^+	R	S^+	S^+
Levofloxacin (10 mcg)	S+	S+	S+	R
Norfloxacin (10 mcg)	S+	R	R	R

Zone size: 0 to 5 mm- Resistance(R) and 6 to 15 mm- Sensitive (S+)

Antimicrobial activity

The antimicrobial activity of bacterial isolates against the pathogenic bacteria is one of the determining factors of probiotic potential. In the present study, four isolates tested against three pathogens *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Aeromonas hydrophila* showed different zones of inhibition(Fig.4). *B.subtilis* and *B.megaterium* showed higher antimicrobial activity against the tested pathogens. The results of the antimicrobial activity

were shown in Table-4.This may be due to the spore forming ability of bacillus species which in turn trigger the immune response thereby exerting a probiotic effect. The results were concurrent with the findings of Liu *et al.* (2014) and Irasema *et al.* (2011) against pathogenic Vibrios and *Aeromonas hydrophila* (Aly *et al.*, 2008). This might be due to the production of antimicrobial compounds produced by the *Bacillus sp.* (Chaurasia *et al.*, 2005; Drablos *et al.*, 1999; Gullian *et al.*, 2004; Morikawa *et al.*, 1992; Perez *et al.*, 1993; Yilmaz *et al.*, 2006).

Table 4: Zone of Inhibition against the three pathogens formed during the experiment

Pathogen	*B. infantis*	*B. subtilis*	*E. profundum*	*B. megaterium*
Vibrio parahaemolyticus	✓	X	✓	X
Vibrio harveyi	✓	X	X	X
Aeromonas hydrophila	✓	X	X	X

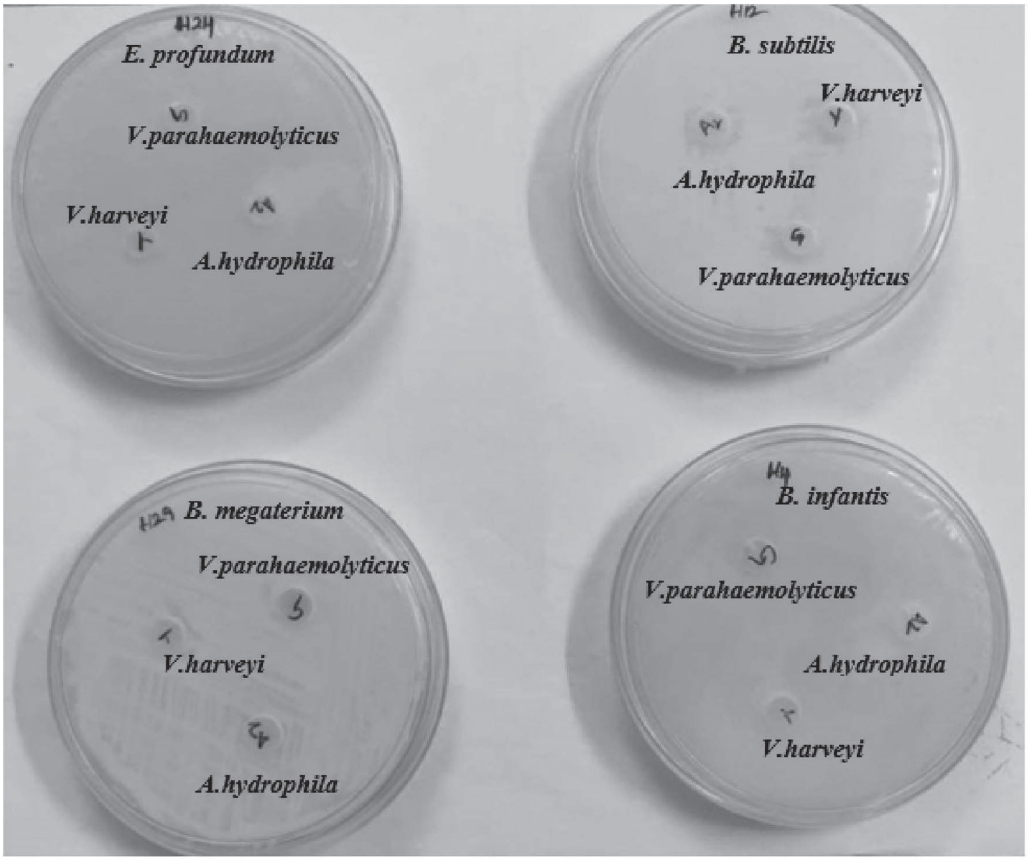

Fig. 4. Antimicrobial activity of four isolates against three pathogens, *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Aeromonas hydrophila*

Auto aggregation test, Bacterial adhesion to hydrocarbons (BATH) and Resistance to gastric acidity

Auto aggregation activity was found to be higher in *Exiguobacterium profundum* (98%) followed by *Bacillus subtilis* (96%), *B. infantis* (93%) and low aggregation was observed in *Bacillus megaterium* (43%).The highest aggregation property of three bacterial isolates (*Bacillus infantis, Exiguobacterium profundum and Bacillus subtilis*) confirmed their therapeutic properties in preventing the animals from disease outbreak (Nithya & Halami.,2013).Summary of the results of these three parameters were shown in Table 5.

The higher bacterial adhesion to hydrocarbon activity was reported to be 56.09% in *Bacillus infantis,* 29.89% in *Bacillus megaterium* and very low activity was expressed in *Exiguobacterium profundum* (10.41%) and *Bacillus subtilis* (15.25%). The culture *Bacillus infantis* exhibited better adhesion to hydrocarbons (>50%) supporting that this strain might have higher level of adherence and colonization ability. This was concurrent with the findings of Otero *et al.*, (2004) suggesting that increased level of adhesion and colonization ability in *B. flexus* Hk1 and *B. licheniformis* Me1strains.

Table 5: Summary of bacterial adhesion to hydrocarbons (BATH), Auto aggregation tests and Resistance to gastric acidity

Probiotic Property	*Bacillus infantis*	*Exiguobacterium profundum*	*Bacillus subtilis*	*Bacillus megaterium*
Auto aggregation test (%)	93	98.86	96.6	43.46
Bacterial adhesion to hydrocarbons (%)	56.09	10.41	15.25	29.89
Resistance to gastric acidity (%)	41.66	86.59	85.7	90.39

Resistance to gastric acidity is one of the key indicators for the good probiotics. *Bacillus megaterium (90.39%)* showed good resistance against gastric acidity followed by *Exiguobacterium profundum (98.86)* and *Bacillus subtilis (85.7).*Therefore, these bacteria can survive in intestine of the culture animal under acidic pH confirming its probiotic potential. The presence of similar heterogeneity within the bacillus species in acidic environments has been reported by Hyronimus *et al.* (2000)

CONCLUSION

All the tested cultures survived in the high bile salt concentration and low pH which confirms its absorbance and presence in fish gut tissue. The experimented cultures showed resistant to bile salt hydrolase activity, Bile tolerance and hydrophobicity towards hydrocarbons. In addition the results of auto aggregation test, antibiotic susceptibility and antimicrobial activity of isolates revealed the bacterial performance in the order of *Bacillus megaterium>Bacillus subtilis>Exiguobacterium profundum>Bacillus infantis*. From

the present study, we observed that *Bacillus megaterium* and *Bacillus subtilis* showed the highest probiotic effect based on the *invitro* properties of probiotic.

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CHAPTER

8

SUPERINTENSIVE PRODUCTION OF JUVENILE PACIFIC WHITE SHRIMP, *PENAEUS VANNAMEI*, IN BIOFLOC-DOMINATED SYSTEMS- LIMITING FACTORS

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INTRODUCTION

Shrimp aquaculture has expanded rapidly over the last three decades with more than half of global shrimp production now farmed (FAO 2014). However, conventional shrimp farming practices in outdoor ponds with high water exchange have caused environmental degradation and significant crop losses from disease outbreaks (Cohen *et al.,* 2005; Balcazar *et al.,* 2007; Samocha *et al.,* 2007; Ekasari *et al.,* 2014). This has led to the development of limited-exchange, biofloc-dominated shrimp production systems that allow for lower environmental impact, improved biosecurity, and water conservation (Ray *et al.,* 2010b). The Pacific white shrimp, *Penaeus vannamei,* has been successfully cultured in such systems at superintensive densities (Cohen *et al.,* 2005; Samocha *et al.,* 2007; Mishra *et al.,* 2008; Correia *et al.,* 2014; Samocha *et al.,* 2015a).

Despite the inherent advantages, several limiting factors in superintensive, limited-exchange, biofloc-dominated shrimp culture are apparent. These include feed optimization, *Vibrio* out-breaks, and accumulative changes in culture water quality and composition.

The microbial community that develops in limited-exchange systems helps to control nitrogenous waste and provides supplemental nutrition to shrimp in the form of microbial pro-tein from biofloc (Avnimelech 1999; Browdy *et al.,* 2001; De Schryver *et al.,* 2008).

Several authors have suggested that this *in-situ* nutrition source may allow some feed components, particularly the protein content, to be reduced without compromising shrimp performance (Wasielesky *et al.,* 2006; Crab *et al.,* 2012; Xu and Pan 2014a, 2014b). Reducing this protein content could reduce feed cost and the nitrogen input into the system. However, other studies have indicated improved shrimp performance in biofloc-dominated systems with higher protein feeds (Mcintosh *et al.,* 2001; Correia *et al.,* 2014; Yun *et al.,* 2015). A large range of dietary protein requirement estimates have been reported for *P.vannamei,* ranging from 30 to 48% (Hu *et al.,* 2008). Determining the optimum dietary protein content will improve the efficiency and sustainability of shrimp production in these systems (Correia *et al.,* 2014; Yun *et al.,* 2015).

Bioflocs are thought to enhance shrimp immu-nity when they are consumed as an additional feed source (De Schryver *et al.,* 2008; Crab *et al.,* 2012; Kim *et al.,* 2014). However, Vibrio out-breaks still frequently occur in intensive shrimp production systems and are one of the most common shrimp disease agents (Saulnier *et al.,* 2000; Huervana *et al.,* 2006; Samocha *et al.,* 2015b). Shifts in Vibrio populations in shrimp culture water and subsequent Vibrio outbreaks typically follow exposure to single or multiple stressors, such as viral infection, poor water quality, or excessive handling (Sung *et al.,* 1999, 2001; Ekasari *et al.,* 2014). Further understanding of Vibrio dynamics and determination of effective Vibrio control measures in limited-exchange, biofloc-dominated shrimp culture systems is needed.

The composition of water in recirculating aquaculture systems operated with limited water exchange is known to change over time without remediation. Typical changes include nitrate accumulation and alkalinity decline as a result of nitrification (Loyless and Malone 1997; Ebeling *et al.,* 2006; Kuhn *et al.,* 2010), phosphate accumulation (Correia *et al.,* 2014), solids accumulation (Ray *et al.,* 2010a; Ray *et al.,* 2011), shifts in phytoplankton and microbial density (Hargreaves 2006, 2013), and depletion of trace elements (Watson and Hill 2006). These changes can generally be managed with appropriate treatment to maximize shrimp production. However, heavy metals may also accumulate in culture water, biofloc, and shrimp tissue in closed shrimp production systems (Colt 2006; Leffler and Brunson 2014), and the degree of accumulation is largely unknown. Environmental contamination with heavy metals has resulted in accumulation in shrimp tissue, though rarely to concentrations harmful to human health(Marsden and Rainbow 2004; Tu *et al.,* 2008; Metian *et al.,* 2010; Mitra *et al.,* 2012). Heavy metal accumulation and element depletion may reduce shrimp and bacterial performance and compromise marketability. These ionic changes and their interactions with diet and water composition in limited-exchange, biofloc-dominated shrimp culture systems need to be established.

The present 49-d study was conducted to investigate the effect of two commercial feeds of differing protein content and an indoor limited-exchange, biofloc-dominated culture

environment on *P. vannamei* performance and body composition, water quality and ionic composition, and Vibrio dynamics.

EXPERIMENTAL SYSTEMS AND DESIGN

The study was conducted at the Texas A&M AgriLife Research Mariculture Laboratory at Flour Bluff, Corpus Christi, Texas in four 40 m^3 Ethylene-Propylene-Diene-Monomer (EPDM)-lined raceways (RWs) enclosed in a greenhouse. A central fiberglass partition ran the length of each RW (25.4 x 2.7 m, 0.45 m average depth). A 3 HP regenerative air blower (Rotron, DR404, Area Inc., Homestead, FL, USA) and 7.5 HP positive displacement air blower (Model 4007-21L2, Tuthill Vacuum & Blower System, Houston, TX, USA) continuously supplied air to six rows of three 50-mm slotted-type airlift pumps on a 45° angle and six 92-cm-long hose diffusers (Aero-TubeTM, Tekni-plex Aeration, Austin, TX, USA) evenly distributed in each RW. Each RW had a 2-HP centrifugal pump (Hydrostorm, Waterco Inc., Augusta, GA, USA) that continuously circulated water through a Venturi injector (Model MIC-1583 A, Mazzei Injector Co., Bakersfield, CA, USA), 50-mm polyvinyl chloride (PVC) pipe with spray nozzles spaced every 0.9 m and positioned under the center partition. The Venturi injector could draw ambient air, pure oxygen or a mixture of both. Each RW had a foam fractionator (Model VL 65, Aquatic Eco Systems, Apopka, FL, USA) and 450-L conical-base settling tank that were operated intermittently to regulate levels of particulate matter.

Each RW was initially filled with 35 m^3 of mature culture water and biofloc with established nitrifying bacterial populations used for a prior 62-d nursery trial and 5 m^3 natural seawater (drawn from Upper Laguna Madre, disinfected with 10 ppm active chlorine after 30 min, and neutralized with sodium thiosulfate). The salinity was reduced to 30 ppt with municipal freshwater.

RWs were stocked at 457 shrimp/m^3 with hybrid Taura Resistant/Fast-Growth juvenile *P. vannamei* (5.3 g ± 1.05) from postlarvae (PL) (Shrimp Improvement Systems, Islamorada, FL, USA) reared in the earlier nursery trial. Over the course of the 49-d study shrimp were sampled twice a week with a cast net to monitor condition and weight gain, and daily feed rations were adjusted accordingly. Observed mortalities were immediately removed.

RWs were operated with no water exchange, other than addition of municipal freshwater twice weekly to maintain salinity at about 30 ppt, compensating for losses due to evaporation and particulate matter control. A commercial probiotic, EcoPro (EcoMicrobials LLC., Miami, FL, USA), was added to the culture water on Days 5-10, 12-23, 29-30, 32-34, 37, 39-45, and 47-48 at 0.2 mg/L as a Vibrio-control measure. This probiotic contained stabilized spores of Paenibacillus polymyxa, Bacillus mega-terium,

Bacillus licheniformis (two strains), and Bacillus subtilis (three strains), at a minimum concentration of 5.5 x 108 colony forming units (CFU)/g. For preparation, 100 g of Ecopro powder was mixed into 10 L of disinfected (10ppm Cl-/1h) water (30 ppt salinity) and aerated for 18 h; 0.8 L of this mixture was then distributed around each RW. Liquid oxygen (LOX) was supplemented through the RWs Venturi injectors as required from Day 14 onwards to maintain dissolved oxygen (DO) above 4 mg/L. Alkalinity was increased to 160 mg/L as $CaCO_3$ using sodium bicarbonate every second day. NaOH was used to increase pH above 7 on Days 33 -40. No supplemental organic carbon was added throughout the study. Targeted total suspended solids (TSS) and settleable solids (SS) ranges were 200-300 mg/L and 10-14 mL/L, respectively.

At the study termination, RWs were drained, shrimp were collected with dip nets, transferred to harvest baskets, and weighed, and survival, final mean weight, total biomass, and yield were calculated based on samples collected from each harvest basket.

Table 1: Proximate composition (%) of the two commercial feeds used as experimental diets.

	HI-35	EXP
Crude protein	36.94	40.98
Crude fat	9.88	10.87
Phosphorus	1.01	1.45
Moisture	6.06	5.50
Ash	9.41	10.74

Feeds and Feeding

Shrimp in two of the RWs were fed the HI-35 feed (Zeigler Shrimp GR Hyper-Intensive 35, 2.4 mm, 35% protein, Zeigler Bros., Inc., Gardners, PA, USA), while shrimp in the other two RWs were fed the Experimental (EXP) feed (Zeigler Shrimp, 2.4 mm, 40% protein) (Table 1).

Feed was offered continuously by six evenly spaced 24-h automatic belt feeders (Zeigler Bros., Inc.). RWs were inspected for uneaten feed daily with a dip net. Daily feed rations were adjusted between growth samplings based on feed consumption, measured and projected shrimp biomass increase, and feed conversion ratio (FCR).

Water Quality Analysis

Temperature, salinity, DO, and pH were monitored twice daily (0800 and 1600 h) using a multiparameter probe (Model 650, YSI Inc., Yel-low Springs, OH, USA). SS was measured daily using Imhoff cones (Method # 2540 F; Eaton *et al.,* 1995), TSS every

second day using the standard gravimetric method (Method # 2540 D; Eaton *et al.,* 1995), and volatile suspended solids(VSS) weekly (Method # 2540 E; Eaton *et al.,*1995). Turbidity was measured weekly using a turbidimeter (Model 2100Q, Hach Company, Loveland, CO, USA). Alkalinity was measured every second day according to Method #2320 B (Eaton *et al.,* 1995). Total ammonia nitrogen (TAN) and NO_2-N were measured twice weekly, and NO_3-N and PO4 weekly using a multichannel flow injection analyzer (FIAlab 2600, Bellevue, WA, USA) following US EPA methods 350.1, 353.2, 353.2, and 365.1, respectively and photometer (Model 9300, YSI Inc.).

Vibrio Monitoring

Vibrio concentrations were monitored twice weekly in duplicate in all RWs by placing collected water samples on thiosulfate citrate bile salts sucrose agar (TCBS) plates (Becton, Dickinson, and Company, Sparks, MD, USA), and at the conclusion of the trial on CHROMagar Vibrio (CVA; CHROMagar, Paris, France). Water samples were individually blended for 20 sec to release Vibrio cells from particulate solids. Agar plates were inoculated with a 10 µL sample using a micropipette and spread with a sterile wire loop according to standard plating methods (Method 9215 C; Eaton *et al.* 1995). Agar plates were incubated for 24 h at 32°C, after which the number of yellow colony forming units (YCFU, sucrose fermenting Vibrio) and green colony forming units (GCFU, non-sucrose fermenting Vibrio) were counted on TCBS; and the number of blue colony forming units (BCFU, Vibrio vulnificus), mauve colony forming units (MCFU, Vibrio parahaemolyticus), and white/colorless colony forming units (WCFU, Vibrio alginolyticus) were counted on CHROMagar.

Samples of hemolymph and hepatopancreas were taken from moribund shrimp and plated onto TCBS and CHROMagar without quantification. In addition, API and 16S rRNA sequencing, and biochemical profiling with Biolog, polymerase chain reaction (PCR) and gel electrophoresis were performed on select isolated colonies by Nicholls State University, Department of Biological Science, Thibodaux, Louisiana, USA to identify specific species present, the presumptive etiological agent(s), and the presence of human toxin genes (tdh and trh).

Pigment Analysis

Once a week 20-50 mL of water from each RW was filtered through a 4.7 cm glass microfiber filter 696 (VWR International, Radnor, PA, USA) to sample suspended solids (biofloc). The filter was then wrapped in aluminium foil and stored at -80°C for later analysis. Algal pigment composition was assessed by means of an HP1100 high-performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) as previously described

in Zimba *et al.,* (1999). Identification of specific divisions of algae is possible using taxon-specific pigment biomarkers. A pigment library was used to identify samples; unknown samples were quantified by linear regression of known commercial standards.

Composition

Water samples (500 mL) were collected from each RW at stocking, on Days 5, 29, and at harvest, and stored at 4°C for later ionic composition analysis. Shrimp were sampled at stocking (20 shrimp), mid-trial (Day 29) (6 shrimp/RW), and at harvest (10 shrimp/RW) and were kept at -20°C for later proximate and ionic-composition analyses. For tissue analyses, shrimp were defrosted, blotted dry, and weighed. The tail muscles from half of the initial and harvest shrimp were dissected (digestive tract removed), blotted dry, and weighed. All samples were then dried in an oven at 65°C for 144 h to obtain dry weight and calculate moisture content. They were then ground to a powder with a coffee grinder (Magic Bullet, Homeland Housewares®, Los Angeles, CA, USA). Ionic analysis was conducted on these samples, feed and culture media at the analytical chemistry lab, University of New Mexico, New Mexico. About 1 -2 g of the sample was digested using 68% HNO_3, a method comparable to US EPA 200.2 for acid digestion, in a Digi-block with temperature controller. The temperature was ramped sequentially every 15min up to 95°C. After acid digestion was complete, digested samples were brought to a specified volume, filtered, diluted, and analysed for cations (salts and metals). An Optima 4300DV Inductively Coupled Plasma - Optical Emission Spectrometer (ICP-OES) (PerkinElmer, Waltham, MA, USA) was used to analyze cations (salts and metals). This method is comparable with US EPA 200.7 for the ICP-OES analysis. The system was calibrated using a blank and three calibration standards. A set of quality control (QC) samples were also analyzed to verify calibration standards and instrument stability at specified frequency. Data were verified and validated using QC measures via check solutions. Results then were converted into mg/kg dry weight based on the sample weight and digestion volume and reported. The proximate composition of the dried samples was analyzed by New Jersey Feed Laboratory, Inc., Trenton, NJ, USA.

Statistical Analysis

Data were analyzed with SPSS statistical software (IBM SPSS Statistics for Windows, Version 22.0, IBM Corp., Armonk, NY, USA). Percentage data were arcsine transformed prior to analysis. Independent samples t tests were used to determine significant differences in survival, mean final weight, growth, biomass, yield, FCR, protein efficiency ratio (PER), and ionic and pigment concentrations between treatments. Linear mixed models were used to determine significant differences in water quality parameters and Vibrio concentrations between treatments. One-way ANOVA, Tukey post hoc test (parametric),

and Games-Howell post hoc test (nonparametric) were used to determine significant differences in ionic concentrations over time. Pearson correlation coefficients between *Vibrio* counts, algal pigment concentrations and water quality variables were determined. Differences were considered significant when $P < 0.05$.

RESULTS OBTAINED

Shrimp Performance

At the conclusion of the trial there was no significant difference ($P > 0.05$) in mean survival, weight, growth, specific growth rate (SGR), total biomass, yield, FCR, or PER between feed types (Table 2). Shrimp fed EXP had higher growth and final weight, while those fed HI-35 had higher survival, resulting in similar final total biomass and yield between feed types. Increasing numbers of mortalities were recovered toward the end of the trial (Day 39 onwards) in all RWs due to confirmed *Vibrio* infections.

Table 2: *Litopenaeus vannamei* performance in the 49-d trial when fed two commercial feeds (mean ± SD). There were no significant differences in any variable at P > 0.05. n = 2.

	HI-35	EXP
Survival (%)	79.86 ± 4.78	75.57 ± 13.07
Final weight (g)	19.82 ±0.38	21.46 ± 1.69
Growth (g/wk)	2.10 ± 0.02	2.33 ± 0.21
SGR[1] (%/d)	2.72 ± 0.00	2.88 ± 0.12
Total biomass (kg)	289.5 ± 22.9	294.4 ± 27.9
Yield (kg/m^3)	7.24 ± 0.57	7.36 ± 0.70
FCR[2]	1.68 ± 0.22	1.63 ± 0.22
per[3]	1.72 ± 0.23	1.55 ± 0.21

[1]SGR (specific growth rate) = 100× (In final weight – In initial weight/days)

[2]FCR (feed conversion ratio) = Total feed intake (g)/Total biomass gain (g)

[3]PER (protein efficiency ratio) = Biomass gain (g)/protein intake (g)

Water Quality

There was no significant difference ($P > 0.05$) in mean temperature (29.9± 0.8^{0}C), salinity (30.3 ±0.4 ppt), DO (5.3 ± 0.8 mg/L), or pH (7.5 ± 0.3) between treatments (Table 3). There was no significant difference between ($P > 0.05$) in mean TAN, NO_2-N, NO_3-N, TSS, VSS, SS, or turbidity between treatments (Table 4). Mean alkalinity was significantly lower ($P > 0.05$) in EXP (142.6 ± 1.1 mg/L of $CaCO_3$) than in HI-35 (158.3±2.8 mg/L) (Table 4). More bicarbonate was added to EXP (40.8 kg/RW [1.02 kg/m^3]) than HI-35 (27.5 kg/RW [0.69 kg/m^3]) to maintain adequate alkalinity. Mean phosphate was significantly

lower ($P > 0.05$) in HI-35 (25.6±2.6 mg/L) than in EXP (31.6 ± 0.2 mg/L) (Table 4). Nitrate and phosphate both accumulated in the culture water over time

Table 3: Mean values of daily water quality indicators during the 49-d trial period using two diets in 40 m3 raceways. Sampling times were 0800 and 1600 h. There were no significant differences in any variable at P > 0.05. n (days) = 48 (a.m.) and 37 (p.m.). DO= dissolved oxygen

Concentrations (mg/L)	Mean ± SD	Min-Max	Mean ± SD	Min-Max
Tan	1.47 ± 0.36	0.24-5.1	1.30 ± 0.25	0.21 -6.0
NO_2-N	0.21 ± 0.06	0-2.25	0.27 ± 0.11	0-1.57
NO_3-N	115.13 ± 8.61	45.73-189.08	135.08 ± 8.61	47.18-231.47
Alkalinity ($CaCO_3$)	158.3 ± 2.8a	102-199	143.1 ± 1.0b	109-189
PO_4	25.6 ± 2.6a	14.4-39.0	31.56 ± 0.17b	14.21-56.96
TSS	348.3 ± 53.3	150-533	363.8 ± 12.6	175-550
VSS	253.2 ± 21.0	142 – 367	221.9 ± 33.9	117-288
SS (mL/L)	26.7 ± 20.2	8-90	11.2 ± 1.1	3.5-31
Turbidity (NTU)	146.6 ± 24.8	94-202	161.2± 45.0	102-241

NTU = nephelometric turbidity unit; SS = settleable solids; TSS = targeted total suspended solids; VSS = volatile suspended solids.

Table 4: Mean values of water quality indicators during the 49-d trial period using two diets in 40 m^3 raceways. Values in any one row not followed by the same superscript letters are significantly different at P < 0.05. n (days) = 17 (TAN), 15 (NO2-N), 6(NO3-N, VSS, and turbidity), 7 (PO4), 27 (alkalinity), 18 (TSS), and37(SS).

		HI-35		EXP	
		Mean ± SD	Min-Max	Mean ± SD	Min-Max
Temperature (°C)	a.m.	29.5 ± 0.6	27.8-30.9	29.4 ± 0.6	27.8-30.7
	p.m.	30.5 ± 0.7	28.9-31.8	30.4 ± 0.7	28.8-31.9
DO (mg/L)	a.m.	5.3 ± 0.7	3.5-6.9	5.3 ± 0.6	3.8-6.8
	p.m.	5.2± 0.7	3.5-6.5	5.2±1.0	4.0-6.4
pH	a.m.	7.5 ± 0.3	6.9-7.9	7.5 ± 0.3	6.9-7.9
	p.m.	7.6± 0.3	6.8-8.0	7.5 ± 0.3	6.7-8.0
Salinity (ppt)	a.m.	30.3 ± 0.3	29.8-31.1	30.4 ± 0.4	29.7-31.3
	p.m.	30.3 ± 0.3	29.6-31.1	30.3 ± 0.4	29.7-31.2

NO_3-N increased from 45 mg/L at study initiation to a maximum of 232 mg/L in EXP and 189 mg/L in HI-35 at study termination (Fig. 1). Phosphate increased from 14 mg/L at study initiation to a maximum of 57 mg/L in EXP and 39 mg/L in HI-35 at study termination (Fig. 2). There was no significant difference ($P > 0.05$) in mean final

NO_3-N (HI-35: 179.4 ± 13.7 mg/L, EXP: 222.2 ± 13.1 mg/L, P = 0.086) or PO_4 (HI-35: 35.1 ± 5.6 mg/L, EXP: 52.4 ± 6.5 mg/L, P = 0.103) concentrations between treatments.

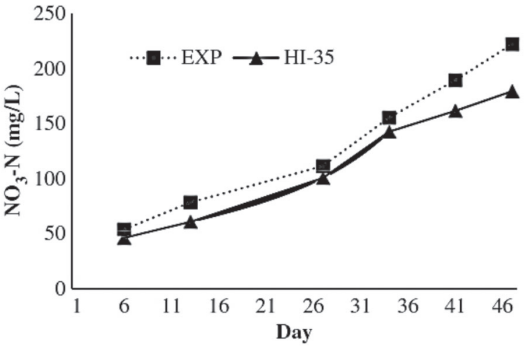

Fig. 1. Mean NO_3-N concentration in culture water during the 49-d trial period using two diets.

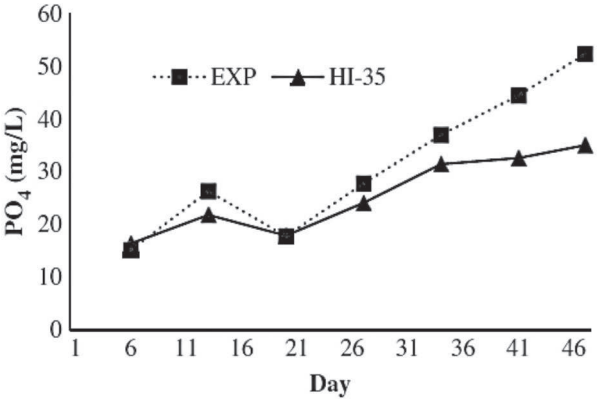

Fig. 2. Mean PO_4 concentration in culture water during the 49-d trial period using two diets

Vibrio

There were no significant differences (P > 0.05) in Vibrio counts between treatments (Table 5). Total Vibrio counts increased over time, particularly in the final week, up to 35,500 CFU/mL. The increased shrimp mortality toward the conclusion of the trial corresponded with an increase in sucrose-fermenting (YCFU) Vibrio concentrations (Fig. 3). CHROMagar plating and API suggested the presence of *V. parahaemolyticus*, *V. vulnificus*, and *V. algi-nolyticus* in the culture water, moribund shrimp hemolymph, and hepatopancreas. 16S rRNA sequencing confirmed the presence of *V. parahaemolyticus, V. vulnificus, V. alginolyticus*, *Vibrio harveyi*, and *Vibrio mytili* in moribund shrimp hemolymph. Biochemical profiling with Biolog and PCR (culture water, hemolymph and hepatopancreas) suggested *V. parahaemolyticus* was the dominant species associated with mortalities. This strain tested negative for humantoxin genes (tdh and trh) by PCR

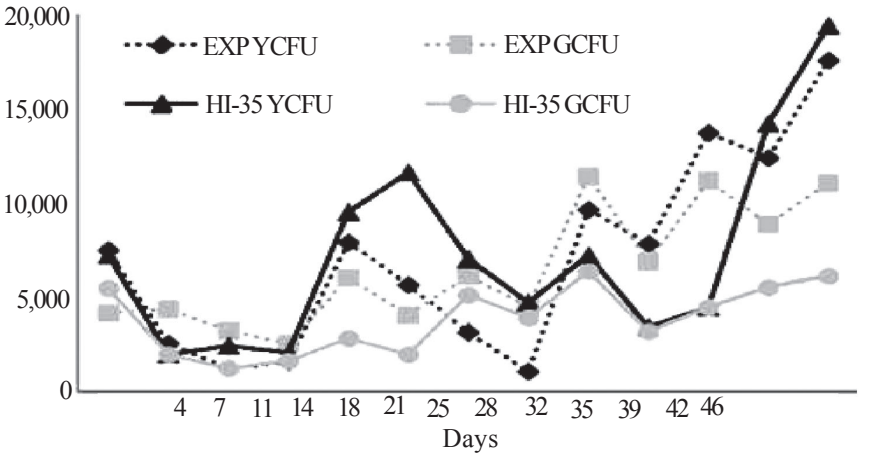

Fig 3. Mean sucrose-fermenting (yellow colony form-ing units [YCFU]) and non-sucrose fermenting (green colony forming units [GCFU]) Vibrio concentrations in culture water during the 49-d trial period using two diets, as expressed on thiosulfate citrate bile salts sucrose agar (TCBS).

Table 5: Mean Vibrio colony counts on thiosulphate citrate bile salts sucrose agar (TCBS) using two diets. There were no significant differences in any variable at P < 0.05. n = 13 (days).

Vibrio colonies (CFU/mL)	HI-35		EXP	
	Mean ± SD	Min- Max	Mean ± SD	Min-Max
Total	11,221 ±1199	2700-30.150	13,652±3636	3600-35,550
YCFU	7364 ±3022	1600-25,050	6960±2744	700-20.900
GCFU	3858 ±1822	600-10,600	6692±892	1850-15.900
% GCFU	39.3 ±8.1	2.9-69.7	55.2 ±17.9	7.7-86.7

There were weak to moderate negative correlations **(P** <0.05) between alkalinity and total **Vibrio**/YCFU/GCFU (—0.4 to —0.57), and between % GCFU and TSS/VSS (—0.43 to —0.56). There were weak to moderate positive correlations **(P** <0.05) between TSS and total **Vibrio**/YCFU (0.34-0.49), and between VSS and YCFU (0.63).

IONIC COMPOSITION

Changes in the concentrations of most ions in the culture water were minimal or below detectible limits (Table 6). K^+ and Mg^{2+} increased significantly ($P < 0.05$) over time, by 13.9-17.4% and 2.1-3.1%, respectively. K^+ increased at a faster rate in HI-35 and was significantly higher ($P < 0.05$) in HI-35 than in EXP at 29 d. Sr^{2+} decreased significantly ($P < 0.05$) over time, by 10.0-11.4%. Br- decreased significantly ($P < 0.05$) in EXP between Days 5 and 49 and was significantly lower ($P < 0.05$) in EXP than in HI-35 at the conclusion of the trial. Cl^- decreased significantly ($P < 0.05$) between Days 29 and

Table 6: Ionic composition of culture water during the 49-d trial period using two diets (mean ± SD). Only ions measured above detectible limits are listed. Values in any one row not preceded by the same subscript letters are significantly different at P < 0.05. Values in any one column (for the same element) not followed by the same superscript letters are significantly different at P< 0.05. n=2.

Element (mg/L)	Treatment	Day 0	Day 5	Day 29	Day 49
B	HI-35	38.58 ± 2.04	38.57 ± 2.20	41.61 ± 1.04	40.28 ± 1.49
	EXP		36.92 ±0.05	38.83 ±1.75	41.38 ± 3.99
Ca	HI-35	$_{A}$389.83 ± 4.66	389.63 ± 0.46	377.71 ± 3.85	387.70 ± 18.16
	EXP		$_{AB}$390.41 ± 6.12	$_{B}$376.34 ± 4.39	$_{AB}$392.49 ± 6.13
K	HI-35	$_{A}$350.33 ± 7.75	$_{A}$353.39 ±3.00	$_{B}$390.40 ± 3.20[a]	$_{C}$411.32 ± 1.17
	EXP		$_{AB}$350.06 ± 5.74	$_{B}$368.48 ± 0.02[b]	$_{C}$395.46 ± 9.67
Mg	HI-35	$_{A}$1126.36 ± 7.75	$_{AB}$1130.95 ± 7.72	$_{B}$1158.92 ± 10.52	$_{B}$1161.40 ± 18.93
	EXP		$_{A}$1124.86 ± 1.07	$_{AB}$1139.26 ± 12.96	$_{B}$1149.79 ± 1.68
Na	HI-35	9213.02 ± 71.92	9166.26 ± 83.45	9357.07 ± 69.39	9176.36 ± 135.77
	EXP		9138.44 ± 160.87	9230.35 ± 52.59	9232.10 ± 9.66
Si	HI-35	23.02 ± 2.70	22.38 ± 2.44	29.07 ± 6.94	26.46 ± 2.43
	EXP		24.14 ± 2.37	25.39 ± 3.08	22.24
Sr	HI-35	$_{A}$6.22 ± 0.14	$_{A}$6.16 ± 0.00	$_{B}$5.87 ± 0.00	$_{B}$5.51 ± 0.15
	EXP		$_{AB}$6.20 ± 0.13	$_{BC}$5.85 ± 0.09	$_{C}$5.60 ± 0.05
Br	HI-35	$_{A}$58.61 ± 1.87	57.13 ± 1.14	56.75 ± 2.08	57.55 ± 0.52[a]
	EXP		$_{A}$59.50 ± 1.02	$_{AB}$57.85 ± 0.79	$_{B}$53.19 ± 0.90[b]
Cl	HI-35	$_{A}$24.090.95 ± 201.70	$_{A}$24,070.64 ± 96.43	$_{A}$24,376.38 ± 421.03	$_{B}$23,027.32 ± 181.93
	EXP		$_{A}$23,909.82 ± 182.77	$_{A}$23,750.44 ± 111.48	$_{B}$22,863.44 ± 263.32
F	HI-35	$_{A}$5.04 ± 2.76	2.32 ± 3.29	6.12 ± 1.32	0.56 ± 0.79

[Table Contd.

Contd. Table]

Element (mg/L)	Treatment	Day 0	Day 5	Day 29	Day 49
	EXP		$_{AB}$1.91 ± 0.15	$_{AB}$4.67 ± 1.99	$_{B}$ND
NO_2	HI-35	3.26 ± 7.99	ND	ND	ND
	EXP		ND	ND	ND
NO_3	HI-35	$_{A}$98.33 ± 43.52	$_{A}$153.24 ± 14.88	$_{B}$511.97 ± 51.33	$_{C}$851.16 ± 79.64
	EXP		$_{A}$196.94 ± 65.19	$_{B}$546.94 ± 53.90	$_{C}$1016.52 ± 7.48
PO_4	HI-35	$_{A}$ND	$_{A}$ND	$_{A}$ND	$_{B}$32.19 ± 3.77
	EXP		$_{A}$ND	$_{AB}$15.87 ± 22.45	$_{B}$43.70 ± 5.36
SO_4	HI-35	$_{A}$2563.20 ± 26.70	$_{AB}$2606.11 ± 65.73	$_{B}$2669.63 ± 46.05	$_{A}$2518.27 ± 18.92
	EXP		2595.85 ± 74.05	2587.60 ± 40.57	2535.28 ± 3.29
Salinity (ppt)	HI-35	30.41 ± 0.23	30.27 ± 0.22	30.21 ± 0.07	30.69 ± 0.69
	EXP		30.19 ± 0.16	30.11 ± 0.13	31.19 ± 1.50

49 in both treatments. NO_3 and PO_4 accumulated in both treatments over time. Concentrations of Al^{3+}, As^{3+}, Ba^{2+}, Be^{2+},Cd^{2+}, Co^{2+},Cr^{3+}, Cu^{2+} , Fe^{2+}, Li^{+}, Mn^{2+}, Mo^{6+}, Ni^{2+}, Pb^{2+}, Se^{4+}, V^{5+}, and Zn^{2+} were Not detected or below detectible limits in culture water.

Changes in the ionic composition of shrimp tissue (whole shrimp and tail muscle) over the course of the trial are summarized in Table 7. In general, B^{3+} (tail muscle), Ca^{2+} (tail muscle), Na^{+} (whole shrimp), Si^{4+} (tail muscle [HI-35]),and Sr^{2+} (whole shrimp [EXP] and tail muscle decreased in shrimp tissue over time; while K^{+} (whole shrimp [HI-35] and tail muscle), and Zn^{2+} (whole shrimp [EXP] and tail muscle) increased in shrimp tissue over time. Cu^{2+} increased significantly ($P < 0.05$) between Days 0 and 29 in whole shrimp in EXP, and decreased significantly ($P < 0.05$) between Days 0 and 49 in tail muscle in HI-35. Cu^{2+} was significantly higher ($P < 0.05$) in EXP than HI-35 in both whole shrimp and tail muscle. K^{+} and Mg^{2+} were significantly higher ($P < 0.05$) in HI-35 than EXP in whole shrimp at Day 49. B^{3+} and Si^{4+} were significantly higher ($P < 0.05$) in EXP than HI-35 in whole shrimp and tail muscle, and tail muscle only, respectively at Day 49. Concentrations of Al^{3+}, As^{3+}, Be^{2+} , Cd^{2+}, Co^{2+}, Cr^{3+}, Li^{+}, Mn^{2+}, Mo^{6+}, Ni^{2+}, Pb^{2+}, Se^{4+}, and V^{5+} were not detected or below detectible limits in shrimp tissue.

The ionic composition of the two feeds is shown in Table 8. HI-35 had higher Fe^{2+}, K^{+}, Mg^{2+}, Na^{+}, and Zn^{2+}, while EXP had higher Al^{3+} , B^{3+}, Ba^{2+}, Ca^{2+},Cu^{2+}, Mn^{2+}, Si^{4+} and Sr^{2+}. Concentrations of As^{3+}, Be^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Li^{+}, Mo^{6+}, Ni^{2+}, Pb^{2+}, Se^{4+}, and V^{5+} were not detected or below detectible limits in feed.

ALGAL PIGMENTS

All algal pigments decreased over the course of the trial (Table 9). No blue-green algal pigments (canthaxanthin, myxoxanthophyll, and zeaxanthin) were recorded. Lutein, violaxanthin, chlorophyll b, and chlorophyll a were significantly higher ($P < 0.05$) in EXP than HI-35 on Day 23. Mean violaxanthin and chlorophyll b were significantly higher ($P < 0.05$) in EXP than HI-35. Similarly, diatom pigments (chlorophyll c2, diadinoxanthin, and fucoxan-thin) were generally higher in EXP than HI-35, though these differences were not significant ($P > 0.05$).

There were moderate positive correlations between chlorophyll b and total Vibrio/ GCFU/ NO3-N/PO4 (0.50-0.76), diadinoxanthin/vio-laxanthin and TAN(0.59-0.77), chlorophyllc2/diadinoxanthin/fucoxanthin/p-carotene andTSS/turbidity (0.56-0.78), and chlorophylla and turbidity(0.77). There were moderate negative correlations between chlorophyllc2/diadinoxanthin/fucoxanthin/chlorophyll oc/p-carotene and NO_2-N (-0.62 to -0.69), and chlorophyllc2/diadinoxanthin and NO_3-N (-0.59 to -0.65).

Table 7: Ionic composition of dried shrimp tissue during the 49-d trial period using two diets (mean ± SD). Only ions measured above detectible limits are listed. Values in any one row not followed by the same superscript letters are significantly different at P< 0.05. n = 10 (0, 49-d), n = 6 (29-d).

Element (mg/kg)	Whole shrimp (W)/tail muscle (TM)	Initial (Day 0)	HI-35Day 29	ESPDay 29	HI-35Day 49	EXPDay 49
B	W	63.68 ± 17.28^{ac}	49.02 ± 2.38^{b}	47.91 ± 2.14^{b}	51.25 ± 3.38^{ab}	69.51 ± 17.08^{c}
	TM	97.09 ± 26.98^{a}			51.00 ± 1.70^{b}	72.44 ± 21.05^{c}
Ba	W	7.05 ±0.72	8.29 ± 1.02	8.04 ± 1.17	7.21 ± 1.37	7.37 ± 0.93
	TM	ND			ND	BDL
Ca	W	27,010.29 ± 1959.08	24,465 ± 3846.59	24,314.52 ± 4582.40	27,372.20 ± 3606.93	23,617.41 ± 3221.18
	TM	2036.77 ± 496.42^{a}			1078.55 ± 236.53^{b}	1192.02 ± 246.86^{b}
Cu	W	78.38 ± 13.00^{a}	73.48 ± 7.68^{a}	116.22 ± 9.50^{b}	67.69 ± 5.64^{a}	100.88 ± 11.25^{b}
	TM	27.52 ± 4.42^{a}			22.16 ± 3.81^{b}	30.60 ± 4.27^{a}
Fe	W	26.98 ± 7.56^{a}	37.22 ± 22.75^{ab}	31.68 ± 4.59^{a}	15.36 ± 6.09^{b}	27.01 ±12.01^{ab}
	TM	9.50 ± 7.25			8.25 ± 7.79	16.12 ± 11.85
K	W	11,694.89 ± 1186.49^{a}	12,852.79 ± 1249.82^{ab}	13,147.79 ± 748.39^{ab}	13,398.15 ± 892.18^{b}	12,040.59 ± 1140.88^{a}
	TM	13,283.36 ± 1792.31^{a}			15,002.81 ± 1236.00^{b}	15,823.70 ± 912.43^{b}
Mg	W	2898.02 ± 334.83^{ab}	3051.92 ± 276.63^{ab}	3069.88 ± 466.56^{ab}	3199.30 ± 230.06^{a}	2759.73 ± 279.66^{b}
	TM	1592.40 ± 206.98			1588.40 ± 113.28	1674.75 ± 81.21
Na	W	8820.33 ± 1228.21^{ab}	10,161.89 ± 1882.22^{a}	9601.63 ± 755.43^{a}	7462.16 ± 819.54^{bc}	6858.75 ± 594.17^{c}
	TM	5630.96 ± 585.23			5210.77 ± 603.74	5558.22 ± 460.45
Si	W	50.07 ± 18.83	42.85 ± 9.68	37.46 ± 4.89	37.69 ± 5.27	47.87 ± 16.24
	TM	65.67 ± 23.60^{a}			37.69 ± 7.65^{b}	57.51 ± 11.68^{a}
Sr	W	344.05 ± 25.56^{a}	318.69 ± 46.55^{ab}	316.12 ± 57.12^{ab}	317.19 ± 37.20^{ab}	267.80 ± 39.72^{b}
	TM	21.34 ± 6.37^{a}			7.97 ± 2.66^{b}	9.16 ± 2.74^{b}
Zn	W	46.92 ± 4.77^{a}	53.45 ± 3.46^{ab}	57.04 ± 3.22^{b}	52.93 ± 5.14^{ab}	52.33 ± 6.88^{ab}
	TM	27.59 ± 6.58^{a}			39.42 ± 3.36^{b}	45.53 ± 5.77^{b}

Table 8: Ionic composition of test feeds (mg/kg). Only ions measured above detectible limits are listed.

Content (mg/kg)	HI-35	EXP
Al	83.32	128.46
B	46.81	53.47
Ba	4.61	8.89
Ca	2199.61	23,482.91
Cu	18.87	38.37
Fe	376.95	359.34
K	15,055.64	10.508.19
Mg	8299.00	2471.85
Mn	51.10	78.84
Na	2004.20	1596.54
Si	44.70	66.91
Sr	15.38	24.88
Zn	193.38	177.65

INTERPRETATION

Shrimp Performance

These results indicate that, under the conditions of this study, increasing the dietary protein content from 35 to 40% did not significantly ($P > 0.05$) improve shrimp performance in terms of growth, survival, FCR, and PER. This concurs with Yun *et al.* (2015) who reported significant increase in growth of *P.*vannamei in biofloc systems as the dietary protein level increased from 25 to 30 to 35%, but no growth difference between 35 and 40% protein diets. Browdy *et al.* (2001) and Gomez-Jimenez *et al.* (2005) reported no difference in *P.* vannamei PL performance when fed diets of differing protein levels, 30 and 45% in ponds and 25, 30, 35,and 40% in zero exchange aquaria, respectively.

Similarly, Xu and Pan (2014a, 2014b) reported no significant difference ($P > 0.05$) in growth, survival, and immune condition of juvenile *P. vannamei* fed between 25 and 35% protein diets in zero-exchange biofloc tanks. In contrast other authors have reported improved *P. vannamei* performance at higher dietary protein content when juveniles were fed 31 vs. 21% protein (Mcintosh *et al.,* 2001) and when PL were fed 40 vs. 30% protein (Correia *et al.,* 2014) in limited exchange systems. McIntosh *et al.* (2001) tested a lower and wider protein range than the present study (21 and 31% vs. 35 and 40%). Correia *et al.* (2014) tested PL in the nursery phase, when shrimp have a higher dietary protein requirement than at later stages (Colvin and Brand 1977; Chen *et al.,* 1985; Velasco *et al.,* 2000). Even though no significant differences in shrimp performance were apparent

Table 9: Algal lipophilic pigments identified from raceway water during the 49-d trial period using two diets. Values in any one row not preceded by the same subscript letters are significantly different at P < 0.05. Values in any one column (for each pigment) not followed by the same superscript letters are significantly different at P< 0.05. n = 2.

Category	Pigment (ng/mL ± SD)	Feed	Day 14	Day 21	Day 23	Day 28	Day 44	Mean
Diatoms	Chlorophyll *c2*	EXP	$_{A}$61.07 ± 16.51	$_{AB}$55.86 ± 43.69	$_{B}$0.00 ± 0.00	$_{AB}$9.05 ± 12.79	$_{B}$4.68 ± 6.62	26.13 ± 13.27
		HI-35	$_{A}$42.01 ± 7.43	$_{A}$30.77 ± 0.75	$_{B}$0.00 ± 0.00	$_{BC}$4.71 ± 6.67	$_{C}$14.71 ± 4.68	18.44 ± 0.63
	Diadinoxanthin	EXP	$_{A}$15.50 ± 4.49	$_{AB}$7.30 ± 4.13	$_{AB}$2.85 ± 0.23	$_{B}$0.92 ± 0.73	$_{B}$1.18 ± 0.95	5.55 ± 2.10
		HI-35	$_{A}$12.79 ± 0.85	$_{B}$7.06 ± 1.52	$_{C}$1.08 ± 0.54	$_{C}$0.39 ± 0.06	$_{C}$0.81 ± 0.22	4.43 ± 0.61
	Fucoxanthin	EXP	23.61 ± 12.48	15.19 ± 9.41	14.68 ± 4.58	6.12 ± 4.60	5.74 ± 4.94	13.07 ± 5.37
		HI-35	$_{AC}$16.53 ± 3.61	$_{A}$10.84 ± 0.88	$_{AB}$5.27 ± 2.98	$_{B}$2.99 ± 0.86	$_{BC}$5.81 ± 0.65	8.29 ± 0.84
Green algae	Lutein	EXP	-	-	$_{A}$16.65 ± 1.68^{a}	$_{B}$2.27 ± 1.16	$_{B}$1.34 ± 0.39	6.75 ±1.08
		HI-35	-	-	$_{A}$5.51 ± 1.30^{b}	$_{A}$1.78 ± 0.85	$_{A}$2.46 ± 1.36	3.25 ± 0.60
	Violaxanthin	EXP	-	-	$_{A}$0.86 ± 0.11^{a}	$_{B}$0.08 ± 0.05	$_{B}$0.04 ± 0.01	0.33 ± 0.06^{a}
		HI-35	-	-	0.16 ± 0.05^{b}	0.04 ± 0.00	0.05 ± 0.02	0.08 ± 0.02^{b}
	Chlorophyll *b*	EXP	$_{A}$0.00 ± 0.00	$_{A}$0.00 ± 0.00	$_{B}$10.61 ± 0.84^{a}	$_{A}$1.89 ± 0.69	$_{A}$1.76 ± 0.61	2.85 ± 0.09^{a}
		HI-35	$_{A}$0.00 ± 0.00	$_{A}$0.00 ± 0.00	$_{B}$2.92 ± 0.23^{b}	$_{C}$1.34 ± 0.05	$_{ABC}$1.77 ± 0.60	1.21 ± 0.18^{b}
Total algae	Chlorophyll *a*	EXP	73.15 ± 40.48	54.65 ± 26.23	83.75 ± 16.77^{a}	26.92 ± 20.99	20.99 ± 17.69	51.89 ± 17.73
		HI-35	$_{A}$43.36 ± 3.71	$_{AC}$39.96 ± 5.37	$_{AB}$25.94 ± 7.87^{b}	$_{B}$13.91 ± 4.53	$_{BC}$27.18 ± 2.04	30.07 ± 0.74
	β-carotene	EXP	5.79 ± 2.16	3.57 ± 1.45	3.60 ± 0.88	1.17 ± 0.57	0.92 ± 0.28	3.01 ± 1.07
		HI-35	$_{A}$4.15 ± 0.10	$_{A}$3.42 ± 0.45	$_{BC}$1.52 ± 0.32	$_{B}$0.63 ± 0.03	$_{C}$1.76 ± 0.20	2.30 ± 0.09

in this study, reduced growth and Vibrio infections, as evidenced by mortality and increasing systemic Vibrio concentrations, encountered toward the end of the trial may have limited growth and survival and prevented the detection of significant differences in shrimp performance. The feeds used in this study supported very high growth rates (>2.1 g/wk), despite high densities and stressful culture conditions.

Although, under the conditions of this study, the small increase in total protein from 35 to 40% did not result in significant differences in final weight at harvest, sample data and trends toward overall increase in final weight suggest that, under optimal conditions, more significant improvements in performance may have been achieved.

Water Quality

All water quality parameters remained within safe limits for juvenile shrimp throughout this trial (Wickins 1976; Tsai and Chen 2002; Cohen *et al.,* 2005; Mishra*et al.,* 2008; Kuhn *et al.,* 2010). Nitrogen input was higher in the RWs receiving the higher protein feed (EXP), which would have resulted in higher ammonia production (Samocha *et al.,* 1998; Yun *et al.*, 2015). This did not translate into significantly different measured TAN and NO_2-N concentrations between treatments, indicating that the heterotrophic and nitrifying bacterial populations were able to efficiently assimilate and oxidize the available nitrogenous waste. However, the higher NO_3-N, significantly lower ($P < 0.05$) alkalinity, and higher bicarbonate requirement in EXP were the consequence of the higher nitrification activities in that treatment to nitrify the available ammonia (Loyless and Malone 1997; Ebeling *et al.,* 2006). This positive relationship between dietary protein content and nitrate accumulation has also been reported by Gomez-Jimenez *et al.* (2005), Correia *et al.* (2014), and Yun *et al.* (2015). The higher PO_4 concentration in EXP was likely due to the higher phosphorus content in the EXP feed (1.45 vs. 1.01%). NO_3-N and PO_4 accumulated in both treatments over the trial as the end product of nitrification and input from feed, respectively, as has been observed by other authors in zero-exchange biofloc systems (Mcintosh *et al.,* 2001; Cohen *et al.,* 2005; Ray *et al.,* 2011; Correia *et al.,* 2014). Some nitrate and phosphate may have been removed through denitrification processes in each RW's settling tank (Ray *et al.,* 2010a; Ray *et al.*, 2011).

Vibrio

Protein content had no significant ($P > 0.05$) effect on culture water *Vibrio* counts, although the number and proportion of GCFU was higher in EXP throughout the trial. Dietary protein content has been shown to affect biofloc composition in shrimp culture systems (Xu and Pan 2014a) and may also have affected *Vibrio* population dynamics between treatments in this trial, either directly or through differing NO_3-N and PO_4 concentrations.

The *Vibrio* species identified in moribund shrimp in this study, particularly V. parahaemolyticus, are common disease-agents in shrimp culture systems, causing substantial economic losses to the industry (Sung *et al.,* 2001; Aguirre-Guzman *et al.,* 2010). Even though biofloc is thought to have a probiotic effect on shrimp and stimulate their immune system (De Schryver *et al.,* 2008; Crab *et al.,* 2012; Ekasari *et al.,* 2014; Kim *et al.,* 2014), Vibrio outbreaks, such as in this trial, are still common in these systems. These outbreaks usually occur in conjunction with a stressor (Sung *et al.,* 1999; Ekasari *et al.,* 2014). Non-sucrose-fermenting (GCFU) Vibrio, which includes V. para-haemolyticus, were much more abundant in this grow-out study (600-15,900 CFU/mL) than in the prior nursery phase (< 100 CFU/mL), with the shift likely caused by harvest-related stress at the end of the nursery phase (Samocha *et al.,* 2015b). In addition, although mean water quality values (i.e., TAN, DO, etc.) were within acceptable levels for shrimp in this study, ranges included high (TAN, nitrite, and nitrate) and low levels (DO and pH) that could potentially be viewed as stressful, potentially compromising shrimp health and increasing susceptibility to Vibrio infections. Cheng *et al.* (2003) reported that a TAN concentration of 0.55 mg/L and above in freshwater suppressed the immune system of Macrobrachium rosenbergii exposed to the pathogen Lactococcus garvieae. Chen *et al.* (2015) reported that long-term exposure to low pH (6.8) reduces juvenile *P*. vannamei immune response and resistance to *V. alginolyticus.*

A commercial probiotic was added to the RWs in an effort to prevent the proliferation of pathogenic Vibrio. Although the probiotic may have delayed Vibrio-related mortalities, it did not prevent them under the conditions of this study. Many probiotics have proven effective at improving shrimp performance and preventing Vibrio infections in juvenile *P.* vannamei in biofloc systems (Moriarty 1998; Balcazar *et al.,* 2007; Krummenauer *et al.,* 2014), while others have had limited effect (McIntosh *et al.,* 2000).

The increase in the population of culture water Vibrio toward the end of the trial corresponded with physical observations of Vibriosis on shrimp and increased mortality. A close relationship can exist between the Vibrio population in the culture water and disease incidence (Amaro *et al.,* 1995; Sung *et al.,* 1999,2001; Pang *et al.,* 2006). This demonstrates that monitoring Vibrio in superintensive shrimp culture systems can be a useful indicator of disease outbreaks, allowing for timely management (Sung *et al.,* 2001). This study demonstrates the need for further research into Vibrio control in superintensive, biofloc-dominated shrimp culture systems.

Ionic Composition

Concern exists over ionic changes - the potential accumulation of heavy metals and depletion of important ions - in limited-exchange biofloc-dominated shrimp production systems. Colt (2006) suggested potential for build-up of toxic concentrations of heavy

metals, particularly copper and zinc in water reuse systems. Leffler and Brunson (2014) reported accumulation of several elements, including heavy metals, in culture water, biofloc, and shrimp tissue when *P.*vannamei were reared in one such system for 128 d. In contrast, changes in most ionic concentrations in culture water over the 49-d present trial were minimal or below detectable limits. The small increase in Mg^{2+} (2.1-3.1%) corresponded to the difference in salinity between the initial and final samples (0.9-2.6%). The increase in K^{+} may have been due to accumulation from the feed, with the higher K^{+} concentration in HI-35 culture water corresponding to the higher K^{+} concentration in HI-35 feed (15,056 vs. 10,508 mg/L). Trace elements such as strontium are often depleted in recirculating systems through uptake by the culture animals (Watson and Hill 2006). In addition, the positively charged strontium, and other cations that were below detectable limits, may have accumulated in the negatively charged biofloc (Wilen *et al.,* 2003; De Schryver *et al.,* 2008), which was periodically removed for solids control. Strontium plays a role in crustacean exoskeleton mineralization and supplementation can increase M. rosenbergii larval productivity and shorten the culture cycle (de Araujo *et al.,* 2002). This suggests that depleted strontium may need to be supplemented into water used for successive limited-exchange shrimp culture cycles. A threshold strontium requirement for shrimp culture has yet to be determined.

The significant differences in the ionic composition of shrimp tissue between feed types all reflected differences in the two feeds. B^{3+}, Cu^{2+}, and Si^{4+} were higher in the EXP feed and in the tissue of shrimp fed this diet, while K^{+} and Mg^{2+} were higher in the HI-35 feed and in the tissue of shrimp fed this diet. A small portion of this difference in whole shrimp could be explained by the presence of any residual feed in the hepatopancreas and digestive tract of shrimp. However, the digestive tract was removed from shrimp tail muscle prior to drying for analysis, indicating that shrimp were bioaccumulating these ions. Lee and Shiau (2002) reported that the whole body Cu^{2+} concentration of juvenile Penaeus monodon increased as dietary Cu^{2+} content increased. Benthic crustaceans accumulate ions from feed and the surrounding water (Marsden and Rainbow 2004). Feed is likely a more important source of Cu^{2+} to shrimp than water (Mitra *et al.,* 2012). Changes in shrimp tissue ionic profile over the trial, both depletion (B^{3+}, Ca^{2+}, Na^{+}, Si^{4+}, and Sr^{2+}) and accumulation (Cu^{2+}, K^{+}, and Zn^{2+}) may be due to normal changes in physiology and metabolic requirements as the shrimp grew (Marsden and Rainbow 2004; Tu *et al.,* 2008)or responses to culture conditions. Farmed and wild shrimp may have different concentrations of heavy metals due to differing metal bioavailability (Paez-Osuna and Tron-Mayen 1996). Other authors have reported that Cu^{2+} and Zn^{2+} concentration in shrimp tissue is positively correlated with shrimp size (Paez-Osuna and Ruiz-Fernandez 1995a, 1995b), as was observed in this trial. The increase in K^{+} and decrease in Sr^{2+} in shrimp tissue over the trial corresponded with equivalent changes in the culture water. The degree of increase in mean K^{+} over the whole trial in HI-35 whole

shrimp (14.6%) and tail muscle in both treatments (12.9-19.1%) was similar to the K^+ increase in the culture water (13.9-17.4%). The lower degree of increase in whole shrimp mean K+ in EXP (2.96%) may be due to the lower K+ content of the EXP feed. Li *et al.,* (2014) reported that the concentrations of Ba^{2+}, Co^{2+}, Cu^{2+}, K^+, Mg^{2+}, Na^+, and P^{3+} in shrimp tissue were correlated with the water concentration. In contrast, while the Sr^{2+} content in shrimp tissue and culture water both decreased over the trial, the degree of mean Sr^{2+} decline was much greater in EXP whole shrimp (22.16%) and tail muscle in both treatments (57.1 -62.6%) than in the culture water (10.0-11.4%). This could be explained by ecdysis-related loss as Sr^{2+} is concentrated in the exoskeleton of shrimp (de Araujo *et al.,*2002; Tu *et al.,* 2008) or the Sr^{2+} content, and that of the other depleted ions, may have been diluted by increasing shrimp size (Mendez *et al.,* 1997; Tu *et al.,* 2008).

In this trial the measured heavy metal concentrations in shrimp tail muscle were all within acceptable limits for human consumption (CEFAS1998; FDA 2000,2011), and only Cu^{2+} and Zn^{2+} increased to a small degree. Shrimp predominantly bioaccumulate, detoxify, and store metals in the hepatopancreas and intestine (Tu *et al.,* 2008) rather than the abdominal muscle (i.e., the main edible part) so that, even with minor heavy metal accumulation, shrimp may still be safe to consume (Metian *et al.,* 2010). This was also indicated in this trial, with all metals above detectible limits (Cu^{2+}, Fe^{2+}, Sr^{2+}, and Zn^{2+}) being at much higher concentrations in the whole body than tail muscle. Further studies should monitor ionic changes in culture water, biofloc, and shrimp over longer culture periods and through reuse of culture water through successive culture cycles.

Algal Pigments

The decrease in algal pigments over the present trial is typical of biofloc-dominated systems. These systems usually transition from phytoplankton dominated to bacterial dominated as feed input, biomass, and suspended solids increase (Hargreaves 2006,2013). These factors, along with increasing shrimp biomass, significantly increase the system's oxygen demand, which is why supplemental pure oxygen was supplied to RWs from Day 14 in this trial. The correlation results suggest that phytoplankton were contributing to overall TSS and turbidity. Even though there were few significant correlations between algal pigment and Vibrio concentrations in the culture water in this trial, the general trend was for phytoplankton to decrease and Vibrio to increase over the course of the trial. The lowest phytoplankton concentrations were observed in the second half of the trial (Days 28-44), which corresponded with an increase in Vibrio concentrations and related shrimp mortality. Other studies have shown that some microalgae species such as *Chaetoceros* sp., *Nitzchia* sp., *Leptolyngbia* sp., and *Chorella* sp. can inhibit pathogenic luminous Vibrio spp. growth in culture systems through secretion of anti-luminous Vibrio substances

(Tenden-cia and de la Pefia 2003; Lio-Po *et al.,* 2005; Huervana *et al.,* 2006). This effect may explain the observed changes in Vibrio populations in this trial. Thus, manipulating the phytoplankton population to control Vibrio populations in shrimp culture systems is a promising possibility worthy of further research.

CONCLUSIONS

Under the conditions of this study, increasing the protein content of feed from 35 to 40% did not significantly ($P < 0.05$) affect *P*.vannamei performance. Both of these nutrient dense diets provided for high growth rates exceeding 2 g/wk, yields of over 7 kg/m^3, and reasonable FCRs despite Vibrio-associated mortality of 20-25%. Use of well-formulated nutrient dense feeds can play a key role in supporting shrimp performance in these high-density zero-exchange systems. Higher feed protein content did increase nitrate accumulation, alkalinity consumption, and bicarbonate use. The presence of Vibrio resulted in cases of Vibriosis and reduced shrimp survival. Periodic monitoring of Vibrio populations in superintensive shrimp culture systems can be a useful indicator of potential disease outbreaks. While Cu^{2+} and Zn^{2+} did increase in shrimp tissue, no heavy metals accumulated to problematic levels in culture water or shrimp tissue. Feed ionic composition and normal physiological changes appeared to be the main factors associated with changes in shrimp ionic composition. The depletion of Sr^{2+} in culture water suggests this ion may need to be supplemented if water is to be reused for successive culture cycles. Further research into overcoming limiting factors in superintensive limited exchange biofloc dominated shrimp culture systems Vibrio control and changes in culture water and shrimp composition is urgently needed.

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CHAPTER

9

SEMI-INTENSIVE SYSTEMS FOR *PENAEUS VANNAMEI* PRODUCTION IN A HYPER-INTENSIVE BIOFLOC-DOMINATED SYSTEM WITH FORMULATED COMMERCIAL FEED

Tzachi M. Samocha

INTRODUCTION

The expansion of the shrimp farming industry has stimulated the intensification of the production systems (FAO, 2012). The hyper-intensive shrimp culture system was developed in the 70's with stocking density between 200 and 500 shrimp m^{-2}, 300% daily water exchange, use of small tanks, and more balanced feeds (Ortega-Salas and Rendon, 2013). Recently, these systems started to incorporate Biofloc Technology (BFT) (Venero *et al.,* 2009), which has the potential to reduce environmental impacts associated with the intensification process (McIntosh, 2000; Schryver *et al.,* 2008). The incorporation of BFT facilitated high density shrimp production using limited or no exchange practices with a much smaller area than outdoor ponds (DuRant *et al.,* 2011; Samocha *et al.,* 2010, 2012)

Feed and feeding practices are important factors affecting any aquaculture operation. Because it represents one of the major costs in shrimp production, accounting for over 50% of the total production costs (Hanson *et al.,* 2009; Son *et al.,* 2011), it can significantly affect profitability. In addition, the effects of feed on water quality and shrimp growth are important factors to be considered (Tacon *et al.,* 2002); more so when dealing with hyper-intensive, biofloc-dominated systems. In a hyper-intensive, biofloc-dominated system, feed can directly affect the suspended solids, pH, alkalinity and concentrations of different nitrogen species, requiring careful monitoring and refinement to maximize production

(Cohen *et al.,* 2005; Furtado *et al.,* 2011; Ray *et al.,* 2010; Samocha *et al.,* 2007). The interactions between feed, water quality and productivity have been evaluated in relation to the characteristics of each kind of culture system resulting in the development of specially designed feeds to enhance shrimp performance in each system (Hasan, 2001; Tacon, 1993).

The feed manufacturers have followed the trend to formulate feed for each kind of system and have available a specially formulated feed to be used in hyper-intensive biofloc-dominated systems(HI-35, Zeigler Bros., Gardners, PA). This feed is much more expensive than any other commercial feeds available on the market for intensive shrimp production system, e.g., the one formulated for semi-intensive systems (SI-35, Zeigler Bros., Gardners, PA). However, this trend to use specially formulated feed for each kind of system has been poorly investigated. For example, the effects of feeding a cheaper commercial feed formulated for semi-intensive system on *Penaeus vannamei* production in a hyper-intensive biofloc-dominated system has not been evaluated. Thus, the aim of the present study was to evaluate the effect of feeding commercial diet formulated for semi-intensive systems on selected water quality indicators, performance, and profitability of a system used for production of *P. vannamei* under high density, no water exchange, and biofloc-rich conditions.

ANIMALS AND EXPERIMENTAL DESIGN

A 67-day study was conducted at the Texas AgriLife Research Mariculture Laboratory at Flour Bluff, Corpus Christi, TX, USA. The trial was composed of two feed treatments with three replicates each. According with the manufacturer, the first feed (SI-35,US$0.99 kg-1) was formulated to have 35% crude protein (CP), 7%lipid and 4% fiber and was designed for semi-intensive shrimp culture systems. The other feed (HI-35, US$1.75 kg-1) was formulated to have 35% CP, 7% lipid and 2% fiber, and was intended for hyper-intensive biofloc shrimp production systems. Both feeds were produced by Zeigler Bros., Gardners, PA, USA. Since the formulation of the two feeds is proprietary, the ingredients composition of the tested feeds cannot be shown in the present study. However, the ingredients contained in each feed are shown in Table 1.

The juvenile shrimp (2.66 g) for the study were raised for 49 days at the facility from ten-day-old postlarvae (PL10). Postlarvae were produced by Shrimp Improvement Systems (Islamorada, FL, USA) from Taura-Resistant and Fast-Growth breeding lines. Shrimp were stocked (500 juveniles m^{-3}) in six 40 m^3 raceways (RW). Each RW (25.4 m x 2.7 m) was lined with Ethylene Propylene Diene Monomer membrane (Firestone Specialty Products, Indianapolis, IN, USA) and was equipped with a center longitudinal partition positioned over a 5.1 cm PVC pipe with sprayer nozzles. Every RW had six banks of three 5.1 cm airlift pumps positioned equidistance on both sides of the partition. In addition, each RW had six 0.91 cm long air diffusers (1.9 cm OD, Aero-TubeTM, Tekni-

plex Aeration, Austin, TX, USA), a 2 hp centrifugal pump, and a Venturi injector capable of introducing atmospheric air or a mixture of oxygen and air. Raceways were filled with 18 m^3 of water used in the preceding 49-day nursery period and another 22 m^3 of natural seawater and municipal freshwater in order to maintain the salinity around 30.

Shrimp were fed by hand for the first three days and from Day 4-11 using a combination of hand-feeding and automatic belt-feeders. From Day 12-47 two thirds of the daily ration was fed by hand during the day while the rest was delivered at night using belt-feeders. Beginning on Day 48, shrimp were fed solely by belt-feeders. Initial daily rations were based on an assumed weekly growth of 1.5 g week-1, feed conversion ratio (FCR) of 1.4, and mortality of 0.5% week-1. Rations were later adjusted weekly based on observed feed consumption and results from shrimp sampling. The ration adjustments were conducted using the following equation:

$$AF = (N \times G \times FCR \times S)/7$$

in which, AF = amount of daily ration; N = number of shrimp stocked in each RW at the beginning of the study; G = expected weekly growth (gweek-1); FCR = expected FCR for the week; and S = expected survival (%) for the week.

Table 1: Ingredients contained and proximate composition (%) of the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi-intensive systems (SI-35) fed to juvenile Penaeus vannamei in a 67-day grow-out trial in hyper-intensive, biofloc-dominated, zero-exchange raceways.

Ingredients	HI-35	SI-35
Grain products	+	+
Marine protein products	+	+
Plant protein products	+	+
Processed grain by-products	+	+
Grain distillers dried yeast	+	
Hydrolyzed feather meal	+	
Soy Lecithin	+	+
Poultry by-product meal	+	+
Limestone	+	+
Fish oil	+	+
Magnesium oxide	+	
Potassium chloride	+	
Dicalcium phosphate	+	+
Yeast extract	+	
Butanoic acid	+	
Calcium propionate	+	+
L-ascorbyl-2-polyphosphate	+	+

[Table Contd.

Contd. Table]

Ingredients	HI-35	SI-35
Vitamin A acetate	+	+
Vitamin D3 supplement	+	+
di-Alpha tocophery acetate	+	+
Vitamin B12 supplement	+	+
Riboflavin	+	+
Niacin	+	+
Calcium pantothenate	+	+
Menadione sodium bisulfite complex	+	+
Folic acid	+	+
Thiamine mononitrate	+	+
Pyridoxine hydrochloride	+	+
Biotin	+	+
Astaxanthin	+	
L-Lysine	+	
Cholesterol	+	
Magnese proteinate	+	+
Zinc proteinate	+	+
Copper proteinate	+	+
Calcium iodate	+	+
Iron proteinate	+	+
Cobalt proteinate	+	+
Calcium carbonate	+	+
Sodium selenite	+	+
Zinc sulfate	+	
Zinc oxide	+	
di-Methionine	+	-
Proximate analysis		
Protein	36.10	35.80
Lipids	7.30	9.86
Carbohydrate	45.44	40.54
Fiber	1.61	2.69
Ash	9.55	11.11
Gross energy (kJg-1)[b]	16.35	16.44

\+ Ingredient present in the feed

[a] Since the formulation of the feeds used in the present study is proprietary, the ingredients composition in terms of g/100g cannot be shown. The list of ingredients shown in this Table were obtained from the label attached to bags of each feed.

[b] Energy was calculated assuming the physiological fuel values of 16.7 16.7 and 37.4kJg^{-1} for protein, carbohydrate and lipids, respectively (FAO, 2003).

PROXIMATE COMPOSITION OF FEED

Samples of each feed were analyzed by the New Jersey Feed Laboratory Inc. (Trenton, NJ, USA) for proximate composition. All values were expressed on a dry weight basis.

Table 2: Mean (± standard deviation; SD), minimum (Min) and maximum (Max) values of daily water quality parameters for the treatments composed of the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi-intensive systems (SI-35) in a 67-day grow-out trial with Litopenaeus vannamei in hyper-intensive, biofloc-dominated. zero-exchange raceways.

	HI-35		SI-35	
	Mean(±SD)	**Min-Max**	**Mean(±SD)**	**Min-Max**
Temperature (°C)	30.04 ± 0.67	27.46-31.59	29.91 ± 0.55	28.06-31.54
Dissolved oxygen (mgL^{-1})	5.75 ± 1.11	4.56-6.96	5.77 ± 1.08	4.49-7.58
pH	7.12 ± 0.22	6.24-7.57	7.12 ± 0.24	6.25-7.51
Salinity	28.43 ± 3.01	24.44-36.51	28.34 ± 2.86	24.56-36.69

Water quality

Water temperature, salinity, dissolved oxygen (DO), and pH, were monitored twice daily using a YSI 650 Series multi-probe meter (YSI Inc. Yellow Springs, OH, USA). Alkalinity was measured twice a week, total suspended solids (TSS) and settleable solids (SS) were monitored three times a week, while turbidity, volatile suspended solids (VSS), 5-day carbonaceous biochemical oxygen demand (cBOD5), total ammonia nitrogen (TAN), nitrite-nitrogen (NO_2–N), nitrate-nitrogen (NO_3–N), and phosphate (PO_4) were monitored weekly. Each RWwas equipped with a YSI 5500D multi-parameter monitoring and alarm system with an optical DO probe (YSI Inc.). Real-time DO data from all six RWs was recorded by the monitoring system and uploaded to a computer which could be accessed from remote locations. Whenever the monitoring system recorded DO levels below 4.5 mgL^{-1}, ambient air was enriched with bottled oxygen at a flow rate of 3.4-8.2 LPM.

Sodium bicarbonate was added to RWs to target 160 mg $CaCO_3$ L^{-1} . A small commercial foam fractionator (FF) (VL65, Aquatic Eco System, Apopka, FL, USA) and a homemade settling tank (ST) were operated intermittently in each RW, targeting culture water TSS concentrations between 200 and 400 mgL^{-1} and SS between 10 and 12 mL L^{-1} based on criteria used by Samocha *et al.* (2007). Settling tank flow rates varied between 8.5 and $12Lmin^{-1}$. Molasses was used as carbon source to raise the C:N ratio to 15 in order to stimulate growth of the bacterial biomass (Avnimelech, 2012). Raceways were maintained with no water exchange throughout the study. Municipal freshwater was added weekly to compensate for water losses due to evaporation and operation of the FF and ST.

Zootechnical performance

Sampling of the shrimp was carried-out twice a week. At each sampling, 100 animals were randomly selected and weighed. At the end of the experiment, the zootechnical performance of *P. vannamei* was evaluated by final weight (g), growth (g week-1), FCR, survival (%), yield (kgm^{-3}) and total biomass (kg).

Economic analysis

The indicators of profitability used in the present study were cost of production, net return, net present value, payback period, and internal rate of return. Ten-year cash flows and enterprise budgets were developed to provide those indicators for each dietary treatment using the shrimp production results of this study and extrapolating them into the context of a commercial facility. This hypothetical analysis assumed using one greenhouse system with ten 500 m^3 RWs: eight for the grow-out and two for the nursery phase to raise the PL10 to the 2.66 g juvenile shrimp. Analyses included a fixed cost component covering construction and equipment/machinery costs (initial investment approximately US$992,000). Other critical prices and costs used in the analysis include the heads-on selling price of shrimp (US$7.20 kg-1), the cost of the two feeds (HI-35: US$1.75 kg-1 and SI-35: US$0.99 kg-1), juvenile production costs (US$20 per 1000 to raise the PL10 to juvenile size), and an interest rate of 8% for operating, equipment and construction loans.

Statistical analyses

Daily and weekly water quality data from the two treatments was analyzed by Linear Mixed Models, using Factor Analytic (First Order, Heterogeneous). Shrimp mean final weight, weekly growth rate, survival (arcsine transformed), FCR, yield, and total biomass were analyzed using one-way ANOVA, which was preceded by evaluation of the statistical assumptions. SPSS statistical software (V. 20 for Windows, SPSS Inc., Chicago, Illinois) was used for all analyses. A significance level of $P< 0.05$ was used for all statistical tests.

Proximate composition of the feeds

Both feeds had similar protein, lipids, carbohydrate, ash levels and gross energy (Table 1). The SI-35 feed contained twice as much fiber as the HI-35 (2.69 and 1.61%, respectively).

Water Quality

Tables 2 and 3 summarize daily and weekly water quality parameters, respectively. There was no significant difference in all daily water quality parameters between the two

Table 3: Mean (±standard deviation; SD), minimum (Min) and maximum (Max) values of weekly water quality parameters for the treatments composed of the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi-intensive systems (SI-35) in a 67-day grow-out trial with Litopenaeus vannamei in hyper-intensive, biofloc-dominated, zero-exchange raceways.

	HI-35		SI-35	
	Mean (±SD)	**Min-Max**	**Mean (±SD)**	**Mm-Max**
Total ammonia nitrogen (mg L^{-1})	0.22 ±0.11	0.08-0.49	0.26 ±0.10	0.10-0.51
Nitrite-nitrogen (mgL^{-1})	0.40 ± 0.40	0.06-2.24	0.47 ±0.32	0.10-1.22
Nitrate-nitrogen (mg L^{-1})	140.64 ±79.01	39.53-358.72	136 ±65.45	45.54-285.71
Phosphate (mgL^{-1})	9.21 ±3.97	0.52-16.37	10.75 ±5.55	0.28-21.06
Five-day carbonaceous biochemical oxygen demand (mg L^{-1})	37.1 ±15.8	10.4-69.5	37.8 ±13.8	14.5-62.8
Settleable solids (mL L^{-1})	8.4 ±4.1	2.0-21.0	11.3 ±4.3	2.5-27.0
Total suspended solids (mgL^{-1})	223.8 ±69.8b	115.0-551.7	278.2 ±66.1a	155.0-460.0
Volatile suspended solids (mgL^{-1})	161.8 ±66.0b	92.0-435.0	205.3 ±44.2a	116.7-287.5
Turbidity (NTU)	90.8±22.6b	45.7-132.0	125.4±37.1a	67.9-246.0
Alkalinity (mgL^{-1})	208.1 ±43.3a	123.0-274.0	171.4±25.6b	102.0-230.0

Different superscript letters within each row indicate statistically significant differences ($P<0.05$).

treatments (Table 2). Among the weekly water quality parameters, TAN levels remained below 0.5 mg L^{-1} throughout the study, while NO_2–N levels remained below 2.24 mgL^{-1} with no significant differences between treatments. Likewise, the NO_3–N, PO_4, cBOD5 and SS levels did not show significant differences between treatments. However, TSS, VSS, and turbidity were significantly higher in the SI-35 treatment, whereas alkalinity was significantly lower in this treatment (Table 3).

Table 4: Summary of molasses, foam fractionator, settling tank, bicarbonate, oxygen and water usage in a 67-day grow-out trial with *Litopenaeus vannamei* using the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi-intensive systems (SI-35) in hyper-intensive, biofloc-dominated,zero-exchange raceways.

	HI-35	SI-35
Molasses (L)	10	10
Foam fractionator (h)	812	1253
Settling tank (h)	87	391
Total bicarbonate supplementation (kg)	41.6	53.6
Oxygen supplementation (m^3 kg-1 shrimp)	0.65	0.73
Water use (m^3 kg-1 shrimp)	0.13	0.14

Table 5: Zootechnical performance of *Litopenaeus vannamei* fed the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi-intensive systems (SI-35) in a 67-day grow-out trial in hyper-intensive, biofloc-dominated, zero-exchange raceways.

	HI-35	SI-35
Final Weight (g)	22.12 ±11.35	19.74 ±8.28
Growth per week (g week-1)	2.03 ± 0.01a	1.76 $\pm 0.10^b$
Feed Conversion Ratio	1.25 ±0.01b	1.43 $\pm 0.04^a$
Survival (%)	87.39 ±0.52	88.34 ±4.18
Yield (kg m^{-3})	9.74 ± 0.04a	8.71 $\pm 0.22^b$
Total Biomass (kg)	389.81 ±1.77a	348.49 $\pm 9.21^b$

Molasses supplementation was the same for both treatments and was used when TSS and SS were lower than the target levels from Day 2 to Day 5, and an algal growth observed via changing of water colour from Day 9 to Day 26. From Day 27, molasses supplementation was not necessary. The total bicarbonate supplementation was higher in the SI-35 than in the HI-35 treatment. Operation of the FFs and the STs was initiated on Day 7 and Day 44 for both treatments, respectively. However, the hours of operation of the FFs and STs were higher in the SI-35. In both treatments, oxygen supplementation was initiated on Day 17 and continued until the end of the trial. From Day 17 until Day 38 the supplementation was intermittent, whereas from Day 39 until the end of the study

supplemental oxygen was used continuously. The amount of oxygen used to produce 1 kg of shrimp was approximately 12% higher in the SI-35 treatment compared to the HI-35 treatment. The volume of water used to produce 1 kg of shrimp was slightly higher for the SI-35 treatment than the HI-35 (Table 4).

Zootechnical performance

Final weight and survival did not show significant difference between treatments. However, mean weekly growth, yield, total biomass were significantly lower, whereas FCR was significantly higher in treatment SI-35 than HI-35 (Table 5). The regression models for the relation between mean shrimp weights and days of culture were significantly different between the treatments (P< 0.002) (Fig. 1).

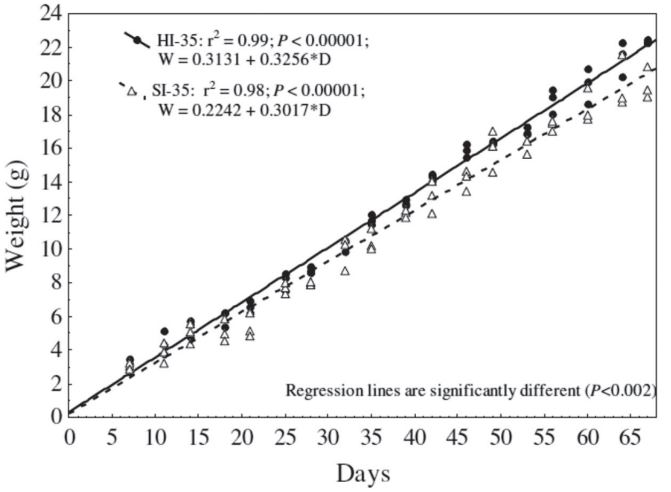

Fig. 1. Relation between mean weights of Litopenaeus vannamei (W) and culture time in days (D) for the treatments composed of the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi- intensive systems (SI-35) in a 67-day trial in super-intensive, biofloc-dominated, zero exchange raceways.

Table 6: Summary of assumed production and sales for the hyper-intensive recirculating shrimp production systems comparing the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi-intensive systems (SI-35).

	HI-35	SI-35
Production (kgcrop-1)	38,960	34,840
Crops year-1	5.5	5.5
Production (kgyear-1)	214,280	191,620
Selling price (US$ kg-1)	7.20	7.20
Total annual sales (US$)	1,542,816	1,379,664

Table 7: Summary enterprise budgets for the hyper-intensive recirculating shrimp production systems comparing the more expensive feed formulated for hyper-intensive systems (HI-35) and the cheaper feed designed for semi-intensive systems (SI-35), in USS kg^{-1}.

	HI-35	SI-35
Gross Receipts (USS kg-1)	7.20	7.20
Variable production costs (USS kg-1)	4.06	4.54
Income above variable Cost (USS kg-1)	3.14	2.66
Fixed cost (USS kg"1)	0.47	0.53
Total of all specified expenses (USS kg-1)	4.53	5.07
Net returns above all costs (USS kg-1)	2.67	2.13
Payback period (years)	1.4	1.9
Net present value (USS million)	2.9	2.0
Internal rate of return (%)	66.6	50.1

Economic analysis

The production per crop, crops per year, total production per year, and total sales per year for both treatments are presented in Table 6. The production parameters and total sales were approximately 10% lower for the SI-35 treatment over the HI-35. Table 7 provides a summary enterprise budget for both treatments. SI-35 treatment had variable production costs of US$0.48 kg-1 higher than the HI-35. The return was US$0.54 kg-1 lower in the SI-35treatment than HI-35. The payback period was 0.5 years more for the SI-35. The net present value was US$900,000 lesser for the SI-35 treatment than HI-35. Likewise, the internal rate of return was lower for SI-35 (Table 7).

Daily water quality variables did not show significant differences between treatments and were maintained within the recommended range throughout the trial. In biofloc systems, the oxygen consumption of the bacterial communities, the shrimp, and certain daily practices (e.g., molasses supplementation, feeding), usually result in a decreasing trend in DO concentration from culture initiation to the end (Avnimelech, 2012; Burford *et al.,* 2003). In the present study, the oxygen supplementation was used to avoid potential harmful effects of this decreasing trend on shrimp growth and to allow further intensification. From Day 17 until Day 38, supplementation was in response to molasses supplementation and feeding for both treatments. Beginning on Day 39, when shrimp biomass was estimated to be 6kgm-3, supplemental oxygen was used 24 h day-1; again for both treatments. Thus, feed treatment did not affect the total amount of oxygen used to keep DO level above 4.5 mg L-1. However, when it was considered the oxygen used to produce 1 kg of shrimp, it was possible to note that oxygen supplementation in the SI-35 was higher than in the HI-35 due to the lower total biomass produced in the first treatment.

The levels of TSS, turbidity, and VSS in the SI-35 treatment were significantly higher than the HI-35 treatment. In addition, it was necessary a higher number of hours of operation for the FFs and STs in the SI-35 treatment required to maintain the desired TSS and SS concentration compared to the HI-35 treatment. It is possible that these differences stemmed from the higher levels of non-digestible components in the SI-35 than the HI-35 feed (see Table 1). Relation between non-digestible components of feed and solids increase in biofloc system was previously suggested by Lopez-Ellas *et al.* (2015), who discussed that feed with higher levels of fiber could create higher amount of flocculated material that can be used as substrate by heterotrophic bacteria and other organisms.

In both treatments, alkalinity levels were maintained higher than 100 mg $CaCO_3$ L^{-1} via sodium bicarbonate supplementation, which is in agreement with the recommendations reported by other researchers for growing penaeid shrimp (Furtado *et al.,* 2011; Van Wyk and Scarpa, 1999). Despite this supplementation, it was recorded lower alkalinity level and higher amount of sodium bicarbonate used to maintain alkalinity in the SI-35 than in the HI-35 treatment. These differences between feed treatments actually may have been an effect of higher solids levels observed in SI-35 throughout the experimental period. Some researchers also have observed lower alkalinity levels under higher solids conditions in bioflocs systems (Ray and Lotz, 2012; Schveitzer *et al.,* 2013). Ebeling *et al.* (2006) have explained clearly the nitrification process that occurs in zero-exchange systems from the solids accumulation, resulting in the use of inorganic carbon from the alkalinity by the bacteria, which may explain the lower alkalinity and higher amount of sodium bicarbonate used to correct its level in SI-35 compared to HI-35.

The shrimp performance indicators for both feeds are generally better than those reported before for super-intensive, biofloc-dominated and no exchange operations (Ray and Lotz, 2012; Schock *et al.,* 2013; Wasielesky *et al.,* 2013). In addition, the results obtained in the current study showed continued improved shrimp performance compared to earlier studies at the Texas AgriLife Research Mariculture Laboratory (Samocha *et al.,* 2010, 2012). These comparisons with the results reported in previous studies evidence that the use of both HI-35 and SI-35 can provide a good *P. vannamei* production under hyper-intensive conditions.

Although shrimp production was improve in both HI-35 and SI-35 treatments in comparison to past studies, a clear response to the type of feed was found in the current trial. The effect of type of feed is presented by regression models, which are significantly different between the treatments, resulting in the lower weekly growth and yields and, consequently, higher FCR in the SI-35. Since all of the water quality parameters were maintained within the range recommended for shrimp culture throughout the study, the observed differences in shrimp performance are mostly associated to the feed.

The economic analysis of profitability indicated that both feeds would be commercially viable in a hyper-intensive, biofloc-dominated, zero-exchange system. However, the later growth recorded in the SI-35 directly affected the results. The production parameters and total sales were approximately 10%lower for the SI-35 treatment over the HI-35 (Table 6). The total production cost per kg of harvested shrimp was US$ 0.54 more for the SI-35 treatment than the HI-35, which is probably associated to higher amount of used feed and variable costs (e.g., use of sodium bicarbonate and oxygen supplementation, see Table 4) in the former. In addition, the net returns above all costs was US$ 0.54 kg-1 lower in the SI-35, resulting in a longer payback period, and lower net present value and internal rate of return for this feed (Table 7). Based on these results, we can conclude that the lower-price feed (SI-35) financially under performed the more-expensive feed (HI-35).

CONCLUSIONS

The results from this study suggest that both the HI-35 and the SI-35 feeds can be used for growing shrimp under high density, biofloc-dominated no water exchange conditions. However, feeding the shrimp a feed formulated for semi-intensive systems under hyper-intensive conditions, not only required more resources to maintain adequate water quality but also lessen shrimp growth, yield, and profitability compared to the feed specifically designed for the conditions experienced in the present study.

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CHAPTER

10

C/N RATIO AND ITS EFFECT ON BIOFLOC DEVELOPMENT, WATER QUALITY, AND PERFORMANCE OF PENAEUS VANNAMEI JUVENILES HIGH-DENSITY- ZERO-EXCHANGE - OUTDOOR TANK SYSTEM

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Increasing concerns over negative environmental impacts from shrimp farm effluent along with wide spread outbreaks of disease have led to the development of culture systems with minimal or zero water exchange (Browdy *et al.,* 2001; Burford *et al.,* 2003; Hopkins *et al.,*1993; Moss *et al.,* 2012; Samocha *et al.,* 2007). With little or no water exchange, properly managed Recirculating Aquaculture Systems (RAS) can reduce or eliminate the amount of nutrients and pathogens released into receiving streams. Biofloc systems are a unique type of RAS that maintain a community of suspended microalgae, autotrophic and heterotrophic bacteria which develop in limited-exchange systems (De Schryver *et al.,* 2008; Hargreaves, 2006; Ju *et al.,* 2008; Ray *et al.,* 2010). Operating high-density shrimp culture systems with limited or no water exchange results in production of large volume of suspended flocculated organic particles recently referred to as biofloc (Avnimelech, 2012; Burford *et al.,* 2003; De Schryver *et al.,* 2008). The promoted biofloc in these systems have been reported to confer many beneficial effects on shrimp culture to include: (1) improved water quality through removal of toxic nitrogen species such as ammonia and nitrite (da Silva *et al.,* 2013; Ebeling; 2006; Ray *et al.,* 2011; Xu *et al.,* 2012a); (2) improved feed utilization and shrimp performance from natural productivity (Ballester *et al.,* 2010; Wasielesky Jr. *et al.,* 2006; Xu and Pan, 2012; Xu *et al.,* 2012b); and (3) enhanced shrimp health through possible probiotic effects (Crab *et al.,* 2010; Haslun *et al.,* 2012;Xu and Pan, 2013; Zhao *et al.*, 2012).

Biofloc-based systems have three pathways for nitrogen conversion: photoautotrophic uptake by algae, chemoautotrophic bacterial conversion of ammonia-nitrogen to nitrate-nitrogen, and heterotrophic bacterial assimilation of ammonia-nitrogen directly to bacterial biomass (Ebeling, 2006). In any biofloc dominated system, all these three processes may be present to some degree depending on culture conditions and the applied management (Ebeling, 2006; Hargreaves, 2006:Ju *et al.,* 2008). The C/N ratio of the culture water is thought to be one of the critical factors affecting growth rate of different microbial communities, thereby generating different substrate utilization pathways and microbial biomass yields (Ebeling, 2006; Hargreaves, 2006). Previous studies showed that increasing the C/N ratio from feed and/or direct organic carbon supplementation can induce a shift of the biofloc community from photoautotrophic or chemoautotrophic to heterotrophic-dominated systems (Avnimelech, 1999; Browdy *et al.,* 2001;Ebeling, 2006). This transformation can have very significant impacts on water quality and biofloc biomass production (Ebeling, 2006), both of which can eventually affect feed utilization and shrimp performance. The Pacific White Shrimp *Penaeus vannamei* is a commercially important shrimp species currently being cultured in many parts of the world. Over the past decade, production of this species in high-density, biofloc-based no water exchange systems has achieved success (Avnimelech, 2012; Haslun *et al.,* 2012; Taw, 2010). However, biofloc development and its relationship to water quality dynamics and shrimp performance due to C/Nratio manipulation remains poorly understood. The current study was therefore conducted to investigate the effects of different C/N ratios on biofloc development and characteristics, inorganic nitrogen and phosphorous dynamics, feed utilization, and growth performance of *P. vannamei* juveniles raised in outdoor tanks at high-density and zero-exchange conditions.

EXPERIMENTAL DESIGN

Experimental shrimp and tank system

The study was conducted at the Texas A&M AgriLife Research Mariculture Laboratory at Flour Bluff, Corpus Christi, Texas. Eight-day-old postlarvae (PLg) *P.vannamei*, produced from a hybrid between Taura-Resistant and Fast-Growth breeding lines, were obtained from Shrimp Improvement System (Islamorada, FL, USA). Postlarvae were reared in a 40 m^3 biofloc raceway system operated with no water exchange. Molasses was added to the culture water any time measured TAN concentrations were above 1 mg L^{-1}. During the Nursery Phase Supplementation was based on the actual level of TAN in the culture water; 6 g of carbon were added for each 1 g of TAN found in the water (Ebeling *et al.,* 2006).

After the 35 days nursery period, juvenile shrimp were graded for similar weight (2.21 ±0.11 g) and stocked into the experimental tank system at 150 shrimp $tank^{-1}$ (equivalent to 300s hrimp m^{-3}). The experimental system consisted of twenty high-density black polyethylene circular tanks positioned within an open-air structure and under a semi-translucent roof. Each tank had a working volume of 500 L with 0.85 m^2 bottom surface area. Netting was used to cover the tanks to prevent the shrimp from escaping. Tanks were filled with brown-green biofloc- rich water from the previously described nursery raceway. Dissolved oxygen levels in each tank were maintained using two airstones and an air blower.

Feed feeding and carbohydrate addition

Shrimp were fed a commercial feed (HI-35, Zeigler Bros., Gardners, PA, USA) which was specifically designed and formulated for biofloc-based super-intensive zero-exchange systems. Feed sample analysis showed a crude protein content of 36.1 %, crude lipid content of 7.3%, fiber content of 1.6%, and ash content of 9.5%. Shrimp were fed twice daily (08:30 and 18:30 h) by hand in two equal portions. Initial rations were calculated based on an assumed growth rate of 1.2 g $week^{-1}$, feed conversion ratio (FCR) of 1.4, and 100% survival. Rations were later adjusted based on daily feed tray observations (1 tray per tank per treatment). The same amount of the feed was applied to all tanks, and feed input was recorded daily.

The molasses (24% w/w carbon and specific gravity of 1.3) was used as an organic carbon source to achieve the required C/N ratios. Input C/N ratios were calculated based on the carbon nitrogen contents of the feed and the carbon content of the molasses. The applied feed (Control treatment CN-9) had a calculated C/N ratio of 9:1, assuming the feed had 50% of carbon and 5.78% of nitrogen (36.1% crude protein in feed x 16% nitrogen in protein). The control treatment received no supplementation of organic carbon. To increase the C/N ratios to 12:1,15:1, and 18:1; 0.62 mL, 1.17 mL, and 1.73 mL of molasses were added for every 1 g of the feed offered, respectively. The resulting C/N ratios of 12:1, 15:1, and 18:1 were designated as treatment: CN-12, CN-15, and CN-18, respectively. One additional group in which molasses supplementation was based on the actual level of total ammonia nitrogen (TAN) in the culture water (6 g of carbon was added for each 1 g of TAN as measured weekly), was also included and referred to as treatment CN-adjust. The calculated C/N ratio of this treatment varied between 9:1and 12:1. Undiluted molasses was uniformly distributed over the tank's surface at 12:00 h daily. Each treatment had four randomly assigned replicate tanks, and the shrimp were cultured for 6 Weeks.

Tank management and biofloc development

All tanks were operated with no water exchange throughout the trial; and municipal freshwater was added to compensate for evaporative losses as needed. Sodium bicarbonate ($NaHCO_3$) was added to maintain pH above 7.2 (Furtado *et al.,* 2012). The color of bioflocs in all tanks were assessed visually. At the beginning, middle, and end of the trial, bioflocs were collected from each treatment tank for microscopic observation. For quantitative evaluation of the biofloc, settleable solids (SS), total suspended solids (TSS), and volatile suspended solids (VSS) were determined weekly following EPA methods (see Table 1). The biofloc volumetric index (BFVI) was defined as the volume of SS in mL occupied by 1.0 g of TSS after 30 min of settling (Xu *et al.,* 2012a). The VSS to TSS ratio (VSS/TSS) was calculated as the weight of VSS divided by the weight of TSS. Turbidity was measured using a portable turbidimeter (2100Q, Hach Company, CO, USA).

Table 1: Selected water quality indicators and monitoring procedures used in 6-week trial under different C/N inputs in biofloc-based, Zero-exchange tank system stocked with *Penaeus vannamei* juveniles at a density of 300 shrimp m^{-3}

Indicators	Methods
Dissolved oxygen (mg L^{-1}), Temperature (^oC), Salinity (ppt), pH	Multi parameter probe (Model 650,YSI Inc., Yellow Springs, OH, USA)
Total ammonia nitrogen (mg L^{-1})	FIAlab Autoanalyzer (EPA Method 350.1)
Nitrite-N (mg L^{-1})	FIAlab Autoanalyzer (EPA Method 353.2)
Nitrate (mg L^{-1})	FIAlab Autoanalyzer (EPA Method 353.2)
Phosphate (mg L^{-1})	FIAlab Autoanalyzer (EPA Method 353.1)
Alkalinity (mg L^{-1} $CaCO_3$)	Method #2320 B (APHA, 1995)
Total suspended solids (mg L^{-1})	Method # 2540 D (APHA, 1995)
Volatile suspended solids (mg L^{-1})	Method # 2540 E (APHA, 1995)
Settleable solids (mL L^{-1})	Imhoff Cone, Method # 2540 F (APHA, 1995)
Turbidity (NTU)	Turbidimeter (2100Q, Hach Company, Loveland, CO, USA)

Determination of water quality parameters

Water temperature, salinity, dissolved oxygen (DO), and pH were measured twice daily (~08:00 and 16:00 h) using a hand-held meter (YSI-650, Yellow Springs Instruments Inc., OH,USA). Water samples were collected weekly at 8:00 h from each tank; and were analyzed for alkalinity, TAN, nitrite-nitrogen ($N0_2$-N), nitrate- nitrogen ($N0_3$-N), and reactive phosphorus ($P0_4^{3-}$). A summary of the water quality indicators and the methods used is presented in Table 1. Total inorganic nitrogen (TIN) concentration was calculated as

follows: TIN = TAN + NO_2~-N + NO_3~-N. The results of selected water quality indicators are presented in Table 2

Table 2: The Overall means (minimum, maximum) of selected water quality parameters in 6-week trial under different C/N inputs in a biofloc-based, zero-exchange tank system stocked with *Penaeus vannamei* juveniles at a density of 300 shrimp m^{-3}

Treatment		Temperature (°C)	Salinity (ppt)	DO [a] (mg L^{-1})	pH	Alkalinity (mg L^{-1} as $CaCO_3$)
CN-9	Means	25.6	26.9	6.9	7.6	158.4
	Min, Max	15.6, 28.2	24.4, 29.8	5.5, 10.0	7.2, 8.0	128.5, 196.0
CN-adjust	Means	25.5	26.9	6.9	7.6	159.0
	Min, Max	16.1, 28.2	24.4, 29.6	4.9, 10.3	7.2, 8.0	116.0, 224.5
CN-12	Means	25.4	26.7	6.9	7.6	178.6
	Min, Max	15.6, 28.3	251, 28.3	5.5, 10.3	7.1, 8.0	114.5, 243.5
CN-15	Means	25.5	26.6	6.8	7.6	222.7
	Min, Max	15.1, 28.6	24.7, 28.6	5.2, 9.8	701, 8.0	133.0, 306.0
CN-18	Means	25.5	26.7	6.8	7.5	261.6
	Min, Max	15.5, 28.3	24.8, 28.6	5.8, 9.8	7.1, 8.0	133.0, 337.5

Shrimp harvest and calculations

After 6 weeks, tanks were drained and shrimp were harvested. Shrimp from each tank were counted and total wet weight was determined. Survival rate, growth rate (expressed as weekly weight gain), yield (expressed as yield per unit of water volume), and FCR were calculated using the following equations: Survival (%) = 100 x (final live shrimp count/initial stocking shrimp count), growth rate (g wk^{-1}) = (mean final weight of shrimp - mean initial weight of shrimp)/culture weeks, yield (kg m^{-3}) = total weight of shrimp harvested / water volume, FCR = total weight of feed offered/total shrimp weight gained.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 20.0 software for windows (IBM Corporation, NY, USA). Biofloc related parameters (SS, TSS, VSS, BFVI, VSS/TSS, turbidity) and weekly water quality indicators (TAN, NC_2-N, NCV-N, TIN, and PO_4^{3-}) were analyzed using two-way ANOVA with time and treatments as fixed factors. Data of shrimp mean final weight, growth rate, survival (arcsine transformed), yield, and FCR were analyzed using one-way ANOVA after conducting a homogeneity of variance test. Significant differences were considered at $P < 0.05$. When significant differences were found, Tukey's test was used to identify differences between treatments.

RESULTS OBTAINED

Biofloc development and characterization

One week after the trial initiation, the color of suspended bioflocs began to differentiate,appearing in green, brown-green, green-brown, brown, and brown in CN-9, CN-adjust, CN-12,CN-15, and CN-18, respectively. The green bioflocs were comprised of a loose flocculation interwoven with many filamentous microalgae, while the brown bioflocs appeared in aggregated particles colonized by dense microbial population. The biofloc development in terms of SS, TSS,and VSS over 6 weeks is presented in Fig. 1. Significant increases ($P < 0.05$) in SS, TSS, andVSS levels were found as the trial progressed, reaching their highest values at the end of the trial. SS, TSS, and VSS levels showed significant differences ($P < 0.05$) between the five treatments atmost of sampling times. SS levels in CN-9 and CN-adjust increased faster than those in CN-12, CN-15, and CN-18; and SS level was higher for CN-12, CN-15 and CN-18. TSS levels showed no significant differences between CN-12, CN-15, and CN-18; and they were higher than that in CN-9 but lower than that in CN-adjust after 2 weeks. VSS level was higher in the treatments with higher C/N ratios from 9:1 to 18:1 after 2 weeks.

BFVI values increased significantly ($P < 0.05$) over time and the differences in BFVI between the five treatments were consistent with the differences in SS after 3 weeks.VSS/TSS ratios in CN-9 and CN-adjust decreased significantly ($P < 0.05$) in the first 2 weeks,and then remained at relatively low values of no more than 60%. For CN-12, CN-15, and CN-18,no significant differences were found in VSS/TSS ratio as the trial progressed; and it showed higher value in treatments with higher C/N ratio at most of sampling times. Turbidity increased significantly ($P < 0.05$) over time; and the differences in turbidity between the five treatments were consistent with the differences in VSS during the trial period.

Inorganic nitrogen and phosphorus dynamics

The weekly changes of TAN, $N0_2$-N, $N0_3$-N, and TIN are shown in Fig. 4. TAN concentration fluctuated significantly ($P < 0.05$) between most sampling times while $N0_2$-N concentration increased significantly ($P < 0.05$) as trial time progressed; however, both of them were maintained at low levels in all treatments throughout the 6-week trial, not exceeding 0.67 mg LH and 1.12 mg L^{-1}, respectively. $N0_3$-N and TIN concentrations increased significantly ($P < 0.05$) in the first week, and then fluctuated significantly ($P < 0.05$) between most sampling times. Both $N0_3$-N and TIN concentrations showed decrease levels with the increase C/N ratio from 9:1 to 18:1 at most of sampling times; however, at the end of the trial only CN-18 showed lower $N0_3$-N and TIN concentrations compared to the trial initiation. Significant increases ($P < 0.05$) in the concentration of PO_4 were

detected as the trial progressed, reaching the highest value in the fourth week; and then it started decreasing to varying degrees in the different treatments during the last 2 weeks of the trial. The concentration of $P0_4^{3-}$ showed an increase in concentration with the increase in C/N ratio from 9:1 to 18:1 after 2 weeks.

Shrimp growth performance and feed utilization

The results of shrimp growth performance and feed conversion ratio are presented in Table 3. All five treatments showed high survival (above 95.5%) with no significant differences ($P > 0.05$) between treatments. Better shrimp final weights and growth rates, and significantly higher yields were found in CN-9, CN-adjust, and CN-12 than those in CN-15 and CN-18. Accordingly, lower FCR was found in CN-9, CN-adjust, and CN-12 than those in CN-15 and CN-18 ($P < 0.05$).

Table 3: Final weight growth, survival, yield and feed conversion ratio (FCR) of *Penaeus Vannamei* in a 6-week trial under different C/N inputs in biofloc-based, Zero-exchange tank system stocked with juveniles at a density of 300 shrimp m^{-3}

Treatment	Final weight (g)	Growth rate (g wk^{-1})	Survival (%)	Yield (g wk^{-3})	FCR
CN-9	9.84 ± 0.28 ^{bc}	1.27 ± 0.04 ^{c}	97.33 ± 0.36 ^{a}	2.81 ± 0.08 ^{b}	1.29 ± 0.08 ^{b}
CN-adjust	9.75 ± .36 ^{bc}	1.26 ± 0.02 ^{bc}	97.50 ± 0.76 ^{a}	2.79 ± 0.10 ^{b}	1.30 ± 0.10 ^{b}
CN-12	9.99 ± 0.22 ^{c}	1.30 ± 0.02 ^{c}	95.50 ± 2.12 ^{a}	2.83 ± 0.02 ^{b}	1.27 ± 0.02 ^{b}
CN-15	9.20 ± 0.16 ^{ab}	1.17 ± 0.06 ^{ab}	97.83 ± 0.30 ^{a}	2.64 ± 0.04 ^{a}	1.40 ± 0.04 ^{a}
CN-18	9.03 ± 0.36 ^{a}	1.14 ± 0.08 ^{a}	95.67 ± 1.64 ^{a}	2.53 ± 0.12 ^{a}	1.47 ± 0.12 ^{a}

Each value represents mean ± S.D. (N = 4). Values in the same row with different superscripts are significantly different ($P<0.05$).

Interpretation

Inoculation of culture water with biofloc-enriched water and the addition of carbohydrates are practical and effective means to accelerate the development of bioflocs in zero-exchange high-density shrimp culture systems (Krummenauer *et al.,* 2014; Xu *et al.,*. 2012a; Xu and Pan,2012). In this study the biofloc-rich water from the nursery raceway was used as the water source in all of the experimental tanks. The addition of molasses in all but the CN-9 treatment was aimed at increasing the C/N ratio and promoting development of heterotrophic bacteria in the culture tank water. One week after trial initiation, the color of the bioflocs in the tanks began to differentiate between treatments. It is safe to assume that with the increase in C/N ratio from 9:1 to 18:1 there was a shift in the microbial community from the original photoautotrophic microalgae to chemoautotrophic bacteria and further to heterotrophic bacteria. This study supports the

findings of other researchers which suggested that addition of molasses in no exchange systems favors the growth and production of heterotrophic bacteria. Heterotrophic bacteria have a maximum growth rate significantly higher than nitrifying bacteria, 5 day^{-1} compared to 1 day^{-1}, and are thus able to out compete autotrophs (Ebeling *et al.,*2006). Furthermore, the microbial biomass yield per unit substrate of heterotrophic bacteria is 40 times greater than that of nitrifying bacteria (Ebeling *et al.,* 2006; Hargreaves, 2006). This increase trend in heterotrophy with the increase in carbon supplementation was also noticed in the current study. In addition, more $NaHC0_3$ was added in the higher C/N ratio treatments to adjust the decrease in pH in these treatments resulting from the increased CO_2 production by the higher biomass of heterotrophic bacteria. The dominant heterotrophic bacteria in treatments CN-15 and CN-18 consumed a moderate amount of HCO_3 but produced a large amount of $C0_2$, while in CN-9 and CN-12 treatments, more HCO_3 was consumed and production of $C0_2$ was lower indicating higher dominance of the nitrifying bacteria (Ebeling *et al.,* 2006). The higher level of HCO_3^- supplementation in CN-15 and CN-18 treatments explains the higher alkalinity in these tanks.

SS, TSS, and VSS are often used as indicators for quantitative determination of biofloc (DeSchryver *et al.,* 2008), as their changes over time can reflect the development of the biofloc in the water. The BFVI value can generally reflect the structure and settling properties of bioflocs in culture systems (Lotito *et al.,* 2012), and the ratio of VSS to TSS can be considered as an indicator of the proportion of microbial biomass in the biofloc. In the present study, the biofloc level in terms of SS, TSS, and VSS increased gradually over the culture period in all experimental tanks. This increase is attributed to continued feed input and organic carbon supplementation in the form of molasses. The rapid increase in VSS level, from the second week on, was proportional to the level of carbon supplementation in the five treatments which was also accompanied by higher production of heterotrophic microbial biomass. According to the stoichiometric analysis of Ebeling *et al.* (2006), metabolism of 1.0 g of NH/-N results in the expected production of 15.85 g of VSS in algae biomass, 0.20 g of VSS in the form of nitrifying bacteria, and 8.07 g of VSS in the form of heterotrophic bacteria. Together with the analysis of inorganic nitrogen dynamics (see below), we can further confirm that the predominant microbes in bioflocs shifted from photoautotrophic microalgae to chemoautotrophic bacteria (nitrifying bacteria) and further to heterotrophic bacteria with the increase of C/N ratio from 9:1 to 18:1. However, the levels of SS and TSS during the experiment showed no direct correlation with regard to the carbon supplementation. This discrepancy may be related to different microbial communities and particulate composition of the bioflocs. In treatments with C/N ratio less than12:1, the biofloc community were dominated by filamentous microalgae and their spatial structure were loose, thereby resulting in very high settling volume of bioflocs in terms of SS; while in treatments with C/N ratio higher than 12:1, the biofloc community were dominated by dense bacteria and closely aggregated into flocculated

particles resulting in relatively low values of SS. The TSS is made of organic and inorganic particulate matter forming an irregular change among the treatments. The change patterns of BFVI and VSS/TSS values over the trial duration under the increased carbon supplementation further support these observations. The change pattern of BFVI value was similar with that of SS, and the change pattern of VSS/TSS value was basically correlates with that of VSS except for CN-adjust. It is interesting to note that the CN-adjust treatment had the highest TSS level and almost the lowest VSS level among the five treatments. This finding suggests that the biofloc in the CN-adjust treatment was dominated by nitrifying bacteria which yielded a smaller amount of microbial biomass with some inorganic particulate matter. The above information suggests that the VSS value is a superior quantifying parameter for quantitative determination of biofloc because of the more accurate representation of the microbial biomass and the development of biofloc. In addition, the pattern of turbidity change over the study under different carbon inputs was in agreement with that of VSS (e.g.,higher turbidity with the higher VSS level), indicating that the VSS in bioflocs was responsible for the turbidity of the culture water.

The community structure of biofloc and its development affect the microbial processes of metabolite assimilation and nutrient-recycling, creating different water quality dynamics in the culture system. In the present study, low TAN and NCV-N concentrations were observed in all five treatments and their dynamics didn't seem to be affected by the carbon supplementation. These results are in agreement with previous findings that there were no significant differences in the concentrations and dynamics of TAN and $N0_2$-N between treatments with C/N ratio of 10:1 and 14:1 (Xu *et al.,* 2012a) or 15:1 and 20:1 (Xu and Pan, 2012). Samocha *et al.* (2007) also reported no significant effect on TAN dynamic in the grow-out phase when the shrimp were fed a low protein feed (30%) under limited water discharge when culture water was supplemented with various levels of molasses. These results suggest that once mature mixtrophic bacteria were established in the culture water, they can effectively control TAN and NO_2-N concentrations by either heterotrophic assimilation (e.g., TAN assimilation into microbial biomass) or autotrophic nitrification (e.g., TAN assimilation to nitrite and then to nitrate) and keep them at acceptable ranges for shrimp culture. However, the change patterns of $N0_3$-N, TIN, and PO_4^{3-} concentrations over time showed significant differences between the various levels of carbon input. There was a decreasing trend in $N0_3$-N and TIN concentrations with the increase in carbon supplementation. Based on the characteristics of the three nitrogen conversion pathways in biofloc-based systems (Ebeling *et al.,* 2006; Hargreaves, 2006), we deduced that nitrification was the main TAN removal pathway in treatments with C/N ratios of 9 and 12, while TAN assimilation by heterotrophic bacteria with to bacterial biomass took place in treatments with C/N ratios of 15:1 and 18:1. It should be noted that only the concentrations of $N0_3$-N and TIN in the treatment with a C/N ratio of 18:1 were lower than the other treatments, indicating that only by increasing carbon

input to reach a C/N ratio of nearly 18:1, a net heterotrophic biofloc system could be achieved. Since the same amount of nitrogen (the same amount of feed) was provided throughout the trial to all of the tanks, the reduced accumulation of TIN with the increase in C/N ratio indicates that more waste nitrogen species were converted into biofloc biomass (especially heterotrophic microbial biomass), as demonstrated by the higher levels of VSS in treatments with higher C/N ratio. In addition, the increase in C/N ratio had significant impact on the concentration of PO_4^{3-} during this 6-week trial. After 2 weeks, the PO_4^{3-} concentration in the culture water significantly increased, showing a faster increase with the higher input of carbon. On one hand, more PO_4^{3-} was produced from uneaten feed over time, suggesting saturation of the microalgae PO_4^{3-} assimilation resulting in accumulation in the water. This was more obvious in treatments with higher input C/N ratio, in which the bioflocs were dominated by more heterotrophic bacteria and less microalgae. On the other hand, algal 'die-offs' that took place on cloudy days during Weeks 3 and 4 seemed to have resulted in a partial release of phosphorus into the water resulting in an abnormal increase in PO_4^{3-} concentration in all five treatments.

Many studies have demonstrated beneficial effects of biofloc on shrimp culture (Ballester et.al., 2010; Haslun *et al.,* 2012; Ray *et al.,* 2011; Wasielesky Jr. *et al.,* 2006; Xu and Pan, 2012; Zhao *et al.,* 2012). Several researchers suggested that apart from maintaining good and stable water quality, the established biofloc in the culture system can improve growth performance and feed utilization of different shrimp species to include: *Penaeus monodon* (Arnold *et al.,* 2009), *P.semisulcatus* (Megahed, 2010), *Farfantepenaeus paulensis* (Ballester *et al.,* 2010), *P.vannamei* (Xu and Pan, 2012), and *Marsupenaeus japonicas* (Zhao *et al.,* 2012). The degree to which these effects are realized may depend greatly on the biofloc type (e.g., the dominating microbial communities), which can be manipulated by adjusting input C/N ratio. In the present study although the water temperature showed large fluctuations (between 15 °C and 28 °C) and relatively low average value (25 °C), the cultured shrimp performed well in all treatments with growth rates between 1.14 and 1.30 g $week^{-1}$, yield between 2.53 and 2.83 kg m^3, FCR between and 1.47, and survival between 95.5% and 97.83%. Moreover, better growth rate, yield, and FCR were found in treatments with C/N ratio from 9:1 to 12:1 than those in treatments with C/N ratio of 15:1 and 18:1. As mentioned earlier, the bioflocs in the CN-9 and CN-12 treatments were dominated by microalgae and autotrophic bacteria while those in the CN-15 and CN-18 treatments were dominated by heterotrophic bacteria. The results suggest that the mixed type of biofloc dominated by both microalgae and bacteria are more beneficial for shrimp performance in high-density, zero-exchange culture systems. Moreover, this mixed algae-bacteria system produced moderate biofloc biomass. According to the study of Schveitzer *et al.* (2013), when *P.vannamei* were cultured in zero-exchange super-intensive tank systems with TSS concentration of higher than 800 mg L^{-1}, the final shrimp yield were lower than those cultured in concentration

of 200 mg L^{-1} to 600 mg L^{-1}. Furthermore, analysis of the shrimps' gills in the higher TSS concentration showed a greater degree of occlusion. The results from this study suggest that more studies are needed to optimize bacteria-algae mixed systems controlling biofloc densities to maximize beneficial effects. This will enable more efficient and healthy culture protocols can be developed for *P.vannamei* in biofloc-based high-density zero-exchange culture systems.

CONCLUSIONS

The application of biofloc technology (BFT) allows minimal or zero water exchange practice during the culture period, and thereby can improve sustainability, biosecurity and production in shrimp aquaculture (Moss *et al.,* 2012; Stokstad, 2010). The driving force of BFT systems is the development of biofloc, which is responsible for water quality control, waste assimilation, and nutrient recycling, which contribute to improved performance of cultured shrimp (Avnimelech, 2012). The results of the present study showed that manipulating the C/N ratio of the culture water had a significant influence on the development and characteristics of biofloc within a system by affecting the inorganic nitrogen and phosphorus dynamics, and eventually affecting shrimp performance and feed utilization. This study suggests that differences in organic carbon supplementations can lead to substantial disparity in system function and shrimp performance under high-density and zero-exchange conditions.

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CHAPTER

11

INTENSIVE NURSERY PRODUCTION OF THE PACIFIC WHITE SHRIMP *PENAEUS VANNAMEI* USING FEEDS OF HIGH AND LOW PROTEIN CONTENT IN A BIOFLOC-DOMINATED SYSTEM

Tzachi M. Samocha

Environmental damage associated with effluent discharge and massive crop losses due to disease outbreaks have created a need for more sustainable and biosecure shrimp production practices (Cowey and Cho, 1991; Samocha, 2009). Implementation of limited or no water exchange shrimp production systems has the potential to minimize these negative environmental impacts and disease outbreaks, while conserving water resources and not compromising profit. Several studies have shown that the Pacific white shrimp, *Penaeus vannamei*, can be cultured with reduced water exchange with no adverse affect on growth, survival and yield in the nursery and grow-out phases (Samocha *et al.,* 1998; Moss *et al.,*1998; Cohen *et al.,* 2005; Mishra *et al.,* 2008; Samocha, 2009).The output of intensive limited discharge systems can be improved when a nursery phase is included in the production scheme (Samocha, 2009, 2010). The shrimp nursery phase is defined as an intermediate step between the young postlarval (PL) stage and the grow-out phase. As nursery systems are stocked at high densities, this practice can improve facility utilization and provide better control over water quality and feed management that can lead to improved shrimp performance and profit in the grow-out phase (Sturmer *et al.,* 1992; Samocha and Lawrence, 1992; Yta *et al.,* 2004). Using greenhouse-enclosed limited-exchange systems can also be beneficial for shrimp nursery production in temperate climate areas to accommodate PL early season stocking ("headstart"), when the ambient water temperature in grow-out ponds is too low for the shrimp to survive and/orgrow. This

practice can extend the grow-out season to produce larger shrimp or to grow multiple crops per year (Samocha *et al.,*2000a,b; Samocha and Benner, 2001; McAbee *et al.,* 2003).

There is a need to develop diets for shrimp cultured in limited exchange nursery systems that will provide sufficient protein for shrimp production while minimizing the amount of nitrogen being introduced into the culture medium (Mcintosh *et al.,* 2001). Shrimp typically have a higher dietary protein requirement during the nursery phase than at later stages (Chen *et al.,* 1985; Velasco *et al.,*2000) However, there is a wide range in reported dietary protein requirements for *P. vannamei*, typically from 300 to 480 g kg-1 (30-48%), with an optimum for PL of 340gkg-1 (34%) (Hu *et al.,* 2008). In intensive nursery systems *P. vannamei* have been fed diets with protein levels as high as 40-55% (Samocha *et al.,* 1993; Velasco*et al.,* 2000).

The effluent water from intensive shrimp production systems often has high loads of nitrogen (N), phosphorus (P), particulate organic and inorganic matter, and oxygen demand (Cohen *et al.,* 2005). Much of the nitrogen input in culture systems enters the water column as total ammonia-nitrogen generated by feed which is not converted into shrimp tissue. Thakur and Lin (2003) showed that under no exchange *Penaeus monodon* assimilated only 23-31% of the nitrogen added to the system. The presence of microbial and algal communities in limited discharge systems helps with the recycling of the system's metabolites (Avnimelech, 1999; Burford *et al.,* 2003; Wang, 2003). Besides the nutrient recycling aspect, the dense bacterial community that develops in such systems plays a significant role in the production of single cell microbial protein ("biofloc") that can provide supplemental natural feed for the shrimp (Avnimelech *et al.,* 1994; Avnimelech, 1999; Browdy *et al.,*2001). Wasielesky *et al.* (2006) suggested that this enhanced natural production in zero exchange production systems allows the use of low protein feeds with no adverse effect on shrimp performance compared to high protein feeds.

As feed is the major driving force of intensive production systems, it is important to optimize its use to improve profitability, maximize growth, and minimize potential water quality deterioration. With this in mind, a 62-d trial was conducted with Pacific white shrimp, *P.vannamei*, PL under limited water exchange to improve feed management and water quality and optimize protein efficiency during the nursery phase. Specifically, the study had two major objectives: (1) to determine the effect of substituting high-protein (40%) with low-protein (30%) feed on shrimp growth, survival, protein efficiency ratio and selected water quality indicators in a biofloc-dominated system, and (2) to determine if molasses can be used to prevent ammonia and nitrite accumulation in a super-intensive shrimp nursery system .

EXPERIMENTAL DESIGN

Site and experimental system

The study was carried out in four 40 m^3 ethylene propylene diene monomer (EPDM, Firestone Specialty Products Company, Indianapolis, Indiana) lined greenhouse-enclosed raceways (RWs) at the Texas A&M AgriLife Research Mariculture Laboratory at Flour Bluff, Corpus Christi, Texas. Each RW (68.5 m^2 bottom area; 25.4 m x 2.7 m) had a center longitudinal fiber glass partition positioned over a 5.1 cm PVC pipe with spray nozzles every 0.5 m. Every RW had six banks, each with three 5.1 cm airlift pumps, and six 0.92 m long air diffusers (1.9 cm OD, Aero-TubeTM, Tekni-plex Aeration, Austin, Texas). Airlifts and air diffusers were positioned at equidistant on both sides of the partition and were operated continuously using a 3 HP regenerative air blower (Rotron, DR404, Area Inc., Homestead, FL) and a 7.5 HP positive displacement air blower (Model 4007-21L2, Tuthill Vacuum & Blower System, Houston, TX). In addition, each RW had a 2 HP centrifugal pump (Hydrostorm, Waterco Inc., Augusta, GA) and a Venturi injector (Model MIC-1583A, Mazzei Injector Co., Bakersfield, CA). To increase dissolved oxygen levels in the culture medium, the Venturi injector was set to operate using atmospheric air, pure oxygen or a mixture of the two. Water circulation through the nozzles on the pipe under the center partition was initiated on Day 17 with 20 min of operation in the morning and 40 min in the afternoon. This water circulation was increased gradually so that by Day 35 it was operated continuously. Each RW was equipped with an inline dissolved oxygen monitoring and alarm system (YSI 5200 multi-parameter system, Yellow Springs Instruments, Yellow Springs, OH). To control the levels of particulate matter, every RW had a small commercial foam fractionator (Model VL65, Aquatic Eco-systems, Inc., Apopka, FL) which was run periodically.

Prior to filling, RWs were sprayed with 500 ppm chlorinated freshwater. The trial was done with natural seawater from Upper Laguna Madre adjusted to 30 ppt salinity using municipal freshwater. Culture water was chlorinated to reach 10 ppm of active chlorine concentration 30 min after chlorination. Chlorine was removed by aeration only. Two days before stocking, water was fertilized using urea, phosphoric acid and sodium silicate to provide concentrations of 2.62 mgL^{-1}, 0.25 mgL^{-1} and 1.66 mgL^{-1} for N, P, and Si, respectively. On the day of stocking RWs water was inoculated with Chaetoceros muelleri to provide an initial algal concentration of 70,000 cells mL^{-1}.

Stocking and culture management

All four RWs were stocked with 10-day-old postlarvae (PL10, ~1 mg) at a density of 5000 m^{-3}. Postlarvae were produced from a specific-pathogen-free breeding population by Harlingen Shrimp Farms, Ltd., Los Frenos, TX. Each RW received 500 mL of molasses

(24% carbon, specific gravity of 1.3, according to Samocha *et al.,* 2007) every other day from Day 10 through Day 18 to promote development of heterotrophic bacteria. From Day 19 on, molasses was added to give 6 g of organic carbon for every 1 g of total ammo-nia nitrogen (TAN) found in the culture medium (Samocha *et al.,* 2007). No molasses was added from Day 30 until the end of the trial as TAN concentrations were consistently below 0.5 mgL^{-1}. Salinity was maintained at about 30 ppt by adding freshwater to offset losses due to evaporation. Some new water was also added to offset water loss associated with the use of the foam fractionator. Shrimp were sampled twice a week to monitor health, growth, and to adjust daily rations.

Feeds and feeding

For the first four days after stocking PL in all RWs were fed newly hatched live Artemia nauplii (~40PL^{-1} day^{-1}), and a combination of dry diets consisting of PL Redi-Reserve (400-600 µm, Zeigler Bros., Inc., Gardners, PA), SureStart #3 (300-500 µm), SureStart #4 (500-800 µm) (55% CP, Salt Creek, Inc., Salt Lake City, UT), 40% CP Fry #0 (420-590 µm), and Fry #1 (600-1000 µm) (Rangen Inc., Buhl, ID). This mixture of dry feeds was offered until Day 26. Beginning on Day 27, once biofloc was established, shrimp were fed two commercial diets made by Rangen Inc., with one containing 30% CP (LP30) fed to two RWs and one containing 40% CP (HP40)fed to the other two RWs (Table 1). These two diets were fed to shrimp as Fry #2 (1.0-1.41 mm) from Day 27 through 46, followed by Fry #3 (1.41-1.68 mm) from Day 47 through 59, and Fry #4 (1.68-2.83 mm) from Day 60 through 61. The transitions from one particle size to the next were done gradually, based on shrimp ability to handle the different particle sizes. Feeding rates ranged from 50% of the total estimated biomass (0.5 mg feed $shrimp^{-1}$ day^{-1}) during the first days after stocking and down to 4% of the estimated shrimp biomass during the final week of the trial (~30mg feed $shrimp^{-1}$ day^{-1}). Occasionally, rations were reduced when left over feed was observed prior to feeding. Feed was distributed by hand four times daily. During the last 18 days of the study an additional night feeding (30% of total daily ration) was provided using three 12-h belt feeders (Zeigler Bros., Inc., Gardners, PA) per 40 m^3 raceway.

Water quality analysis

Temperature, dissolved oxygen (DO), pH, and salinity were monitored at least twice daily. Turbidity, alkalinity, and settleable solids (SS) were monitored every other day. The target alkalinity level was higher than 160 mg L^{-1} $CaCO_3$, and after each analysis sodium bicarbonate was added to maintain this parameter above the target. Total ammonia-nitrogen, nitrite-nitrogen (NO_2-N), nitrate-nitrogen (NO_3-N), phosphate (PO_4), five-day carbonaceous biochemical oxygen demand ($cBOD_5$), total suspended solids (TSS), and

volatile suspended solids (VSS) were monitored once a week. In addition to the weekly monitoring, nitrite was measured daily when high concentrations were detected. From Day 19 (when TAN level >1 mgL^{-1} was first detected) and until Day 39 (when concentrations were <0.09mgL^{-1}), TAN was monitored daily. A summary of the procedures used for monitoring the different water quality indicators is presented in Table 2.

Table 1: Proximal analysis (g kg^{-1}) of the two commercial feeds used as experimental diets with low (LP30) and high protein (HP40) for a nursery trial of the Pacific white shrimp *Litopenaeus vannamei.*

Components	Shrimp production feed	
	30/0 (LP30)[a]	40/5 (HP40)[b]
Crude protein	300	400
Crude fat	70	80
Crude fiber	40	40
Phosphorus	10	10
Ash	150	150

[a] 30/0 (LP30) - Ingredient statement: animal protein products, plant protein products, processed grain-by-products, fish oil, soy lecithin, poultry fat, monosodium phosphate, lignin sulfonate (binder), L-ascorbic acid phosphate (source of vitamin C), choline chloride, vitamin E supplement, niacin supplement, d-calcium pantothenate, riboflavin supplement, thiamine mononitrate, biotin, pyridoxine, hydrochloride, folic acid, vitamin A supplement, vitamin D3 supplement, vitamin B12 supplement, manganese sulfate, zinc sulfate, ferrous sulfate, copper sulfate, sodium selenite, potassium iodate, propionic acid (preservative), ethoxyquin (preservative).

[b] 40/5 (HP40) - Ingredient statement: animal protein products, plant protein products, processed grain-by-products, grain products, squid meal, fish oil, soy lecithin, poultry fat, monosodium phosphate, ligninsulfonate (binder), L-ascorbyl-2-polyphosphate (source ofvitamin C), choline chloride, vitamin E supplement, niacin supplement, d-calcium pantothenate, riboflavin supplement, thiamine mononitrate, biotin, pyridoxine, hydrochloride, folic acid, vitamin A supplement, vitamin D3 supplement, vitamin B12 supplement, manganese sulfate, zinc sulfate, ferrous sulfate, copper sulfate, sodium selenite, potassium iodate, propionic acid (preservative), ethoxyquin (preservative).

Statistical analysis

Data was analyzed using SPSS statistical software (V. 15 for Windows, SPSS Inc., Chicago, IL). Repeated measures ANOVA was used to determine significant differences between treatments in water quality indicators.The Student 't-test' was used to determine differences between treatments in survival (arcsine transformed), mean final weight, specific growth rate (SGR), and protein efficiency ratio (PER), according to Zar (1996). All differences were analyzed at significance level of a = 0.05.

Table 2: Selected water quality indicators and monitoring procedures used in a 62-d nursery trial evaluating the performance of Penaeus vannamei fed low (LP30) and high protein (HP40) levels.

Indicators	Methods
Dissolved oxygen (mg L^{-1}),temperature (°C), salinity (ppt),pH	Multi parameter probe (Model650,YSI Inc., Yellow Springs, OH)
Total ammonia nitrogen (mg L^{-1})	FIAlab autoanalyzer (EPA Method350.1)
Nitrite-N(mg L^{-1})	FIAlab Autoanalyzer (EPA Method353.2)
Nitrate (mgL^{-1})	FIAlab Autoanalyzer (EPA Method353.2)
Phosphate (mg L^{-1})	FIAlab Autoanalyzer (EPA Method365.1)
Alkalinity (mgL^{-1} $CaCO_3$)	Method #2320 B (APHA, 1995)
$cBOD_5$ (mgL^{-1})	Method #5210 B (*aPHA,* 1995)
COD (mg L^{-1})	Method # 5220 D (APHA, 1995)
Total suspended solids (mgL^{-1})	Method # 2540 D (APHA, 1995)
Turbidity (NTU)	Method # 2130 B (APHA, 1995)
Volatile suspended solids (mgL^{-1})	Method # 2540 E (APHA, 1995)
Settleable solids (mLL^{-1})	Imhoff Cone, Method # 2540 F(APHA, 1995)

RESULTS

Growth, survival, FCR, and yield

The shrimp grew continuously over the culture period with growth rates of 114mg $week^{-1}$ and 104mg $week^{-1}$ for HP40 and LP30, respectively. Fig. 1 shows the shrimp growth curve in this trial. Shrimp fed the high protein diet (HP40) had significantly higher ($P<0.05$) mean final weight (1.03 vs. 0.94g, $P = 0.0393$) and specific growth rate (11.19 vs. 11.03% day^{-1}, $P = 0.0368$) than those fed the low protein diet (LP30). However no significant differences ($P>0.05$) were found in survival (84.13 vs. 82.29%, $P =0.8985$) or protein efficiency ratio (3.28 vs. 3.89, $P = 0.1389$) between HP40 and LP30 feeds, respectively (Table 3). In spite of the differences in growth rate, the higher protein efficiency ratio (PER) of shrimp in the LP30 treatment may suggest that in the presence of biofloc it is possible to reduce the protein content in the feed in this culture system. The biofloc provides an alternative protein source and supplemental feed to the shrimp (Avnimelech, 1999; Browdy *et al.,* 2001). However, the difference in PER was not significant due to under-replication in the present study.

P. vannamei has a reported dietary protein requirement between 30% and 48% (Guillaume, 1997; Rosas *et al.,* 2001; Hu *et al.,* 2008). McIntosh *et al.* (2001) fed juveniles of this species diets with 21% and 31% CP, using a small tank system under limited water exchange and medium stocking density (40 m^{-2}), and showed that shrimp

fed the high protein feed had better growth (14.04 vs.12.17 g), survival (96.2% vs. 90.6%), and FCR (1.75 vs. 2.15), as in the present study. On the other hand, Browdy *et al.* (2001), while working with the same species at a density of 104 PL m^{-2} in outdoor ponds in the presence of natural productivity, reported no differences in shrimp performance when shrimp were fed 30% and 45% protein diets.

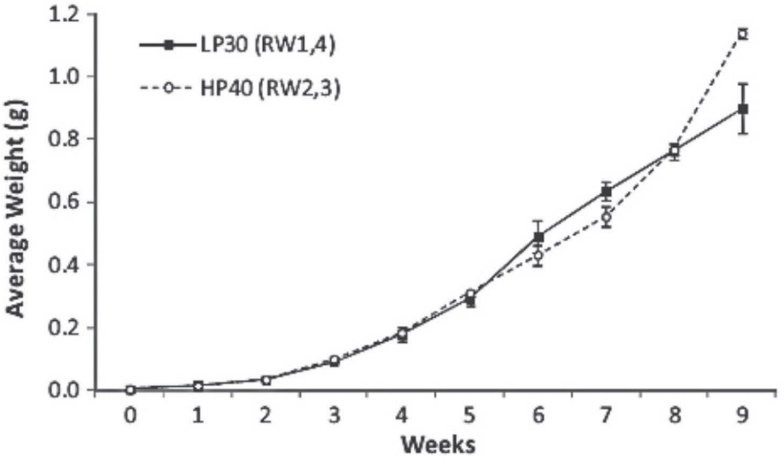

Fig. 1. Weekly growth of shrimp (~1 mg) fed low (LP30) and high protein (HP40) diets during the 62-d nursery study.

Table 3: Performance of Litopenaeus vannamei postlarvae in a 62-d high-density nursery trial (5000 PLm^{-3}) and high protein (HP40) diets in a biofloc-dominated system (mean ± SE).

Treatments	Final weight (g)	SGR[a] (% day^{-1})	Survival (%)	PER[b]
Low-protein (LP30)	0.94 ± 0.08a	11.03 ± 0.01a	82.29 ± 11.26a	3.89 ± 0.17a
High-protein (HP40)	1.03 ± 0.02b	11.19 ± 0.05b	84.13 ± 6.07a	3.28 ± 0.19a

[a] SGR (specific growth rate) = 100 (In final weight – In initial weight) / days of culture.

[b] PER (protein efficiency ratio) = gain of biomass / protein intake.

Values in the same column with different letters are significantly different (P<0.05).

Environmental factors

Table 4 summarizes the means and ranges of the daily and weekly water quality indicators monitored in this study. As settleable solids (SS) concentrations in the culture medium were low during most of the trial, foam fractionators were operated only during the final two weeks when SS levels were >15mLL-1 and/or TSS were >400mgL-1. The only significant differences (P>0.05) found in daily and weekly water quality indicators between the two treatments were in nitrite-N, nitrate, and phosphate which were significantly higher in HP40 than in LP30 treatments (Fig. 2B-D).

Table 4: Summary of daily (AM & PM) and weekly water quality indicators for raceways operated in a biofloc system with *Litopenaeus vannamei* fed low (LP30) and high protein (HP40) diets over a 62-d nursery trial.

Variables		LP30 (RW1,4)		HP40 (RW2,3)	
		AM	PM	AM	PM
Temperature (°C)	Mean ± SE	27.6 ± 0.2	28.7 ± 0.2	27.7 ± 0.2	28.7 ± 0.2
	Range	23.0-32.09	24.0-31.9	23.6-30.4	24.6-31.5
Dissolved oxygen	Mean ± SE	5.7 ± 0.1	5.7 ± 0.1	5.7 ± 0.1	5.7 ± 0.1
(mg L^{-1})	Range	4.2-7.4	4.4-11.4	3.7-7.3	4.3-11.0
pH	Mean ± SE	7.5 ± 0.0	7.4 ± 0.0	7.4 ± 0.0	7.3 ± 0.0
	Range	6.8-8.7	6.7-8.9	6.7-8.5	6.6-8.7
Salinity (ppt)	Mean ± SE	31.2 ± 0.1	31.2 ± 0.1	31.5 ± 0.1	31.5 ± 0.1
	Range	29.4-32.9	29.4-32.8	30.3-32.6	30.1-32.6
Total ammonia	Mean ± SE	0.84 ± 0.18		0.76 ± 0.10	
Nitrogen (mg L^{-1})	Range	0.0-3.46		0.0-2.93	
Nitrite-N (mg L^{-1})	Mean ± SE	4.13 ± 2.22^{a}		5.78 ± 0.67^{b}	
	Range	0.0-20.25		0.0-24.38	
Nitrate-N (mg L^{-1})	Mean ± SE	65.77 ± 1.19^{a}		96.10 ± 0.30^{b}	
	Range	0.04-288.94		0.10-420.76	
Phosphate (mg L^{-1})	Mean ± SE	3.06 ±0.29^{a}		3.91 ± 0.06^{b}	
	Range	0.0-10.91		0.0-13.11	
Alkalinity (mg L^{-1}	Mean ± SE	151.14 ± 5.0		149.56 ± 4.7	
$CaCo_3$)	Range	146.1-156.2		144.8-154.3	
Turbidity (NTU)	Mean ± SE	163.8 ± 15.1		144.9 ± 14.8	
	Range	44.7-237.5		49.0-256.2	
Settleable solids	Mean ± SE	5.9 ± 1.0		6.8 ± 1.1	
(mLL^{-1})	Range	0.2-13.5		0.4-15.0	
Algae (cell mL^{-1} x 10^4)	Mean ± SE	242.7 ± 90.4		193.8 ± 66.0	
	Range	2.6-1481.0		1.4-1170.0	
$cBOD_5$ (mgL^{-1})	Mean ± SE	18.0 ± 7.91		19.2 ± 11.10	
	Range	3.7-50.3		3.9-28.7	

Values within each row with different superscript letters are significantly different (P>0.05).

Total ammonia nitrogen (TAN) peaked at the third week, averaging 3.46 mg TAN L^{-1} and 2.93 mg TAN L^{-1} for LP30 and HP40 treatments, respectively (Fig. 2A). Although TAN concentrations in the RWs increased until Day 26 (reaching a maximum level of 4.16 mgL^{-1} in RW1), molasses supplementation helped keep them under control (Samocha *et al.,* 2007). From Day 30 until the end of the study, TAN concentrations remained below

0.5 mgL^{-1} requiring no additional organic carbon supplementation besides the contribution from the feed. The data suggest that by the fifth week the autotrophic nitrifying bacterial community, mainly ammonia oxidizing bacteria (AOB), became established and converted the ammonia to nitrite in all four RWs. The difference in protein content of the feed (30/40%) had no significant impact on the TAN concentration in the culture medium (Table 4 and Fig. 2A). Higher protein content in feed has resulted in higher ammonia concentrations in closed systems in other studies (Samocha *et al.,* 1998; Mcintosh *et al.,* 2001). The difference is likely due to the addition of molasses in the present study. The highest TAN concentration recorded in our study was 4.16 mg TAN L^{-1} (0.082mgL^{-1} NH_3 at pH 7.4, temp. 28.2 °C, and salinity 31 ppt). This was much lower than the concentration at which Mishra *et al.* (2008) reported high survival (96.2%) of shrimp PL when exposed to TAN concentration of 27mgL^{-1} (0.189mgL^{-1} NH_3 at pH of 6.8, temp. of 28°C, and salinity of 25 ppt) and half the safe level of unionized ammonia for *P. vannamei* juveniles (0.16 mgL^{-1} NH_3) reported by Lin and Chen (2001). Furthermore, as we did not observe any dead PL during periods of high ammonia concentration it is probable that TAN did not affect shrimp survival in our trial.

On the other hand, as nitrite oxidizing bacteria (NOB) are slow to develop, the levels of nitrite in the culture medium increased continuously until Day 46, but the conversion to nitrate began after the fifth week (Fig. 2C). This indicates that, in spite of the addition of molasses, the rates of N uptake and nitrification processes by the microbial biomass were lower than the rate of N addition with shrimp feed for the majority of the trial. It is interesting to note that the patterns of nitrite concentration increase and decrease were different for the HP40 and LP30 protein treatments. Although the increase in nitrite concentrations started after the third week for all four RWs (Fig. 2B), the maximum nitrite-N levels in the HP40 were higher than the LP30 protein treatments (34 vs. 29mg NO_2-NL^{-1}, Fig. 3). This higher concentration was expected since the high-protein feed would have generated more TAN than the low-protein feed and AOB developed faster than NOB. Because organic carbon supplementation was discontinued from Day 30, all of the TAN had to be converted into nitrite. The plot of daily changes in nitrite in each separate raceway (Fig. 3) showed that it took 29 and 31 days (from Day 23 until Day 52 and Day 54) for the nitrite-N concentrations in the HP40-protein RWs to drop to below the 0.5 mgL^{-1} level, while for the low-protein treatment, it took 24 and 36 days (from Day 23 to Days 47 and 59). Nitrate concentrations averaged 96.1 mgL^{1} and 65.8 mgL^{-1} for the 40% and 30% CP feed, respectively, with final concentrations increasing to 420.8 mg L^{-1} and 288.9 mgL^{-1} by the end of trial (Fig. 2C and Table 4). Since no additional organic carbon was added to any of the RWs from Day 30 on, and because the high-protein feed NO_3 L^{-1}) over six weeks did not affect growth and survival but did have a negative impact on shrimp biomass and antennae length, with greater impact at lower salinities (2-18 ppt). In our study exposure to a maximum NO_3 concentration of 421 mg L^{-1} or mean

of 95 mg NO_3-N L^{-1} had no adverse effect on shrimp. This level was far lower than the value (220mgNO_3-NL-1) cited by Kuhn *et al.*,(2010).

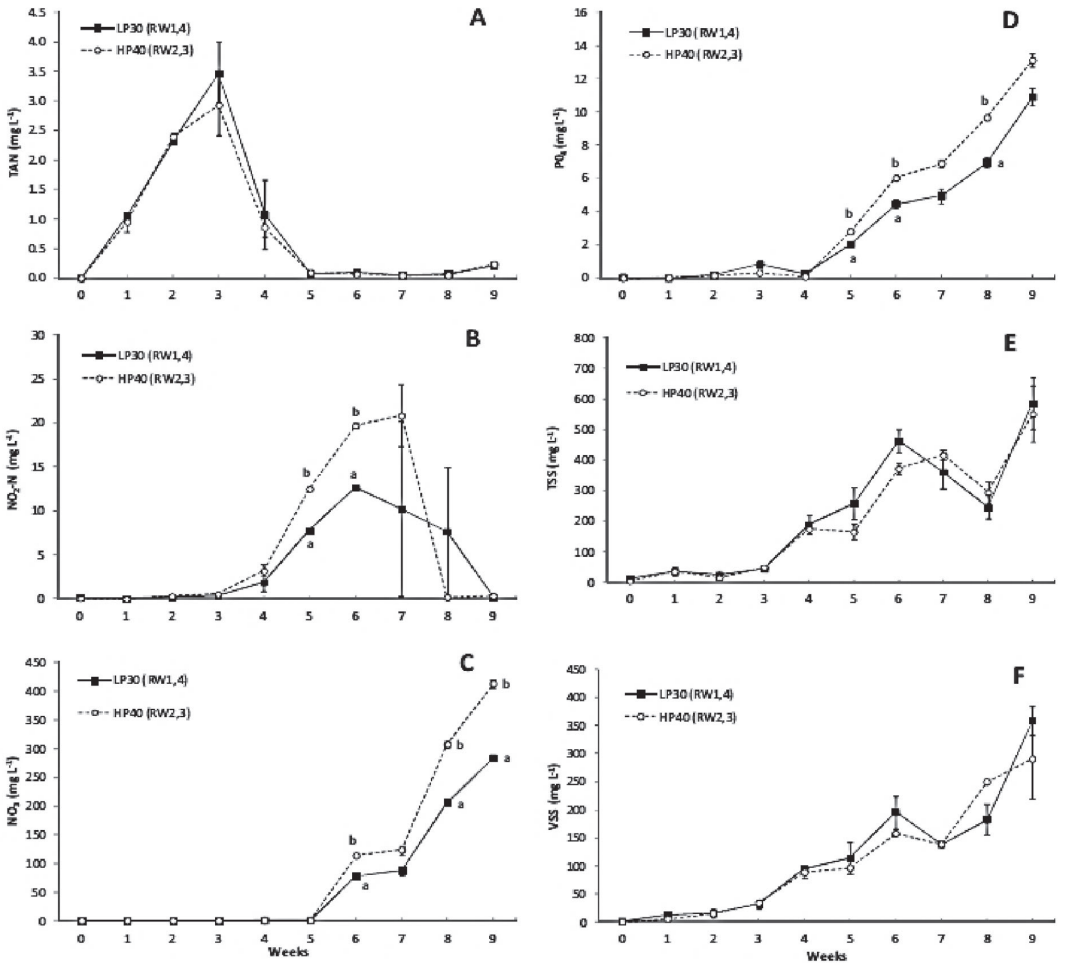

Fig. 2. Weekly variations in water quality of the raceways during 62 culture days using low (LP30, RW1,4) and high protein (HP40, RW2,3) feeds (±SD).

Phosphate, TSS, and VSS changes followed the same increasing trend as nitrate, reaching levels higher than 13 mg L^{-1}, 500 mg L^{-1}, and 300mgL^{-1}, respectively (Fig. 2D, E and F) by the end of the study. However, only PO_4 levels of the high-protein treatment were significantly higher than the low-protein treatment (Table 4). Handy *et al.* (2004) also reported that PO_4 increased over the duration of a study in a nursery trial in the same system from 1.30 to 25.57mgL^{-1}. In a minimal water exchange study on 1.31 g *P. vannamei*, Ray *et al.* (2010) found that the use of settling tanks resulted in significantly lower phosphate concentration (40.4 vs. 104.6 mgL^{-1}) than tanks operated without the removal of solids. In our study the foam fractionators were also very effective reducing

the phosphate levels with reduction from maximum levels of 10.91 mgL^{-1} PO_4 and 13.11 mgL^{-1} PO_4, to 3.06mgL^{-1} PO_4 and 3.91 mgL^{-1} PO_4, for LP30 and HP40, respectively.

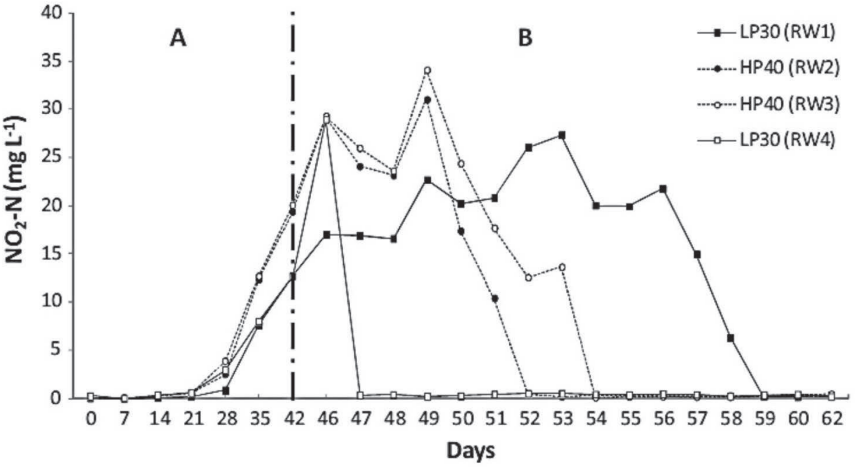

Fig. 3. Weekly (A) and daily (B) changes in water nitrite concentration in raceways using low (LP30, RW1,4) and high protein (HP 40, RW2,3) feeds during the 62-day nursery study.

The five-day carbonaceous biochemical oxygen demand in our RWs ranged from minima of 3.7 and 3.9 to maxima of 28.65 mg L^{-1} and 50.32mgL^{-1} for the 30% and 40% protein diet treatments, respectively (Table 4). The cBOD5 mean remained below 30 mg L^{-1} for the majority of the trial in all RWs. However, cBOD5 increased at the seventh week (averaging 45mgL^{-1}) in the two RWs fed the high-protein diet (40% CP) with a subsequent decrease to 30mgL^{-1}. Handy *et al.* (2004) and Cohen *et al.* (2005) reported similar increases in cBOD5 (from 8.0mgL^{-1} to 33.6mgL^{-1} and 5.04mgL^{-1} to 28.60mgL^{-1}, respectively) during shrimp nursery studies in the same system, operated with a foam fractionator or pressurized sand filter. On the other hand, Mishra *et al.* (2008) documented a reduction in cBOD5 level from about 96 to 60 mgL^{-1} after using a foam fractionator for 71 days in a limited exchange nursery study in the same system. Our study demonstrated that foam fractionators can help reduce the levels of PO4, TSS, VSS, settleable solids, turbidity and cBOD5, as documented by other studies (Handy *et al.,* 2004; Mishra *et al.,* 2008).

Since each RW was equipped with online dissolved oxygen monitoring and alarm system the average dissolved oxygen levels in the RWs were above 5.6 mgL^{-1}. The AM and PM maxima and minima DO values reported in Table 4 suggest that DO never dropped below 3.6 mgL^{-1}. This DO monitoring system provided us with a tool to regulate feeding in order to avoid drops in DO after feeding.

CONCLUSIONS

The increase in biofloc in the system was provided by the development of heterotrophic bacteria which feed on ammonia and organic carbon from molasses and the autotrophic bacteria that consume nitrogen compounds (e.g., ammonia and nitrite), reducing their concentration in the water while consuming inorganic carbon (e.g., alkalinity). This biofloc, formed by the bacterial biomass and other microorganisms present in the medium, has substantial nutritional value that contributed to the diet of the cultured shrimp in the present study, providing a feed supplement. The results of this work suggest that substituting high (40%) with low-protein (30%) feed in the shrimp nursery phase in a biofloc dominated system may provide an alternative to improve biofloc technology. Shrimp fed the high protein diet had significantly higher SGR and final weight than those fed the low protein diet, but there were no significant differences in shrimp survival or PER between the two diets. Several advantages to using the low protein feed can be shown. Firstly better water quality, as nitrite, nitrate and phosphate were lower in the LP30 than in the HP40 treatment; second, feed with lower protein content is cheaper; and third the use of lower protein feed in this system can reduce the environmental impacts from shrimp culture, through lower protein use and water exchange requirement.

CHAPTER

12

INTENSIVE NURSERY SYSTEM FOR THE PACIFIC WHITE SHRIMP, *PENAEUS VANNAMEI,* UNDER LIMITED DISCHARGE CONDITION

Jeet K. Mishraa, Tzachi M. Samochaa , Susmita Patnaika, Mike Speed, Ryan L. Gandya and Abdul-Mehdi Alid

INTRODUCTION

Global shrimp farming traditionally relied on expansion of extensive practices as strategies to increase production. Nevertheless, recent industry expansions were associated with increased in stocking densities, aeration and use of specially formulated commercial dry feeds. Operating an intensive culture system with consistent output has been a major challenge for shrimp producers in recent years. Among others, the year-round production depends on predetermined shrimp stocking density and careful feed management to maximize growth and survival. Over or under stocking practices can result in suboptimal production (Garza *et al.,* 2004). Also, incorporation of nursery phase should improve the system's production predictability. The nursery phase is defined as the intermediate step between the early postlarval stage and the grow-out phase. The integration of an intermediate nursery phase has also been found to improve efficiency of intensive limited discharge shrimp production systems (Samocha *et al.,* 2000; Cohen *et al.,* 2005). Previous studies have reported several benefits from incorporation of nursery phase in the shrimp production cycle to include; increased survival, improved feeding efficiencies, and enhanced growth performance (Apud *et al.,* 1983; Sandifer *et al.,* 1991; Samocha *et al.,* 2000, 2002). This phase is usually characterized by high water renewal rates, high stocking densities, and the use of high quality artificial diets (Speck *et al.,* 1993).

In most aquaculture systems, the incoming water is the common pathway of pathogen introduction into the culture medium (Lotz and Lightner, 1999). Operational management of intensive biosecure shrimp production systems requires a strict enforcement of disease control and prevention (Moss *et al.,* 1998). About two decades ago, intensive shrimp production practices used high water exchange to maintain suitable water quality (Wang, 1990; Hopkins *et al.,* 1993; Moss *et al.,* 1999). Flushing is not only wasteful of water resources but also a potential source of environmental pollution. To minimize the release of nutrients into the adjoining coastal ecosystems, governmental agencies have imposed strict effluent discharge guidelines (Ibrekk and Elvestad, 1990; Mathiesen, 1990; Hopkins, 1992;Samocha *et al.,* 2002).

The effluent waters from intensive shrimp production systems are typically characterized by high loads of nitrogen (N), phosphorus (P), particulate matter, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) (Hopkins *et al.,* 1993; Mcintosh *et al.,* 2001; Jackson *et al.,* 2003; Cohen *et al.,* 2005). Most of the N input in shrimp culture systems enters the water column as ammonium (NH_4^+) generated by feed and is not converted into shrimp tissue. For example, Thakur and Lin (2003) showed in a study with *Penaeus monodon*, where high protein feed (42% crude protein) was used, that shrimp could only assimilate 23-31% and 10-13% of N and P, respectively. In many cases the culture waters from these systems have been released into low-recharge streams without prior removal of excess nutrients and other particulate matter (Wang, 1990, 2003; Avnimelech *et al.,* 1994; Cohen *et al.,* 2005). However, these nutrients can be easily taken up by microorganism and serve as a fuel for operating ‘’floc system’’ in limited discharge culture practice (Burford *et al.,* 2004). Operating sustainable biosecure shrimp production facilities dictate the use of nutrient recycling methods for the metabolites generated by these systems. With the combined aim of increasing the production and reducing nutrient releases, several studies described different filtration methods used in recirculating aquaculture systems (RAS) including; biological filtration by oyster, macroalgae and aquatic plants (Corpron and Armstrong, 1983; Shpigel and Neori, 1996; Jones *et al.,* 2002; Wang, 2003), and mechanical filtration (Maruyama and Suzuki, 1998; Suzuki *et al.,* 1999, 2003; Timmons *et al.,* 2001). Foam fractionation was also listed as another effective tool to remove fine suspended solids (5-10 |x) from the culture water (Timmons, 1984; Hussenot and Lejeune, 2000). This method is particularly suitable for intensive production systems operated in a closed recirculating mode, or for extensive and semi-intensive systems, where water renewal is low (Hussenot, 2003). In limited discharge production systems, the phytoplankton and the microbial communities can play a major role in recycling the autochthonous nutrients accumulating within the system (Avnimelech *et al.,* 1994; Funge-Smith and Briggs, 1998; Avnimelech, 1999; Wang,2003; Jackson *et al.,* 2003; Burford *et al.,* 2003). These microbial and phytoplankton communities are the major driving forces behind the increase in the carrying capacity of these systems.

In conventional flow-through production systems, shrimp derive the majority of nutrition from supplemental feed, hence high quality commercial feed has been used to improve the yield (Kureshy and Davis,2002; Thakur and Lin, 2003). In limited discharge systems, because of their detritivorous and continuous grazing behavior, shrimp depend on the supplemental feed, benthic fauna, and other detritus as their nutrient sources (Hunter *et al.,* 1987; Moriarty, 1997; Leber and Pruder, 1988; Moss *et al.,* 1999; Burford *et al.,* 2004). Furthermore, in the presence of carbonaceous substrate, microbial community can produce single cell protein that can serve as supplemental feed for the culture species (Avnimelech *et al.,* 1994; Avnimelech, 1999;Browdy *et al.,* 2001). Previous studies suggested that the Pacific white shrimp can be reared with reduced water exchange without adverse effect on the growth, survival and yield (Hopkins *et al.,* 1993; Moss *et al.,* 1998;Cohen *et al.,* 2005).

Only limited information is available regarding the management of intensive nursery production systems of the Pacific white shrimp under limited discharge mode(Davis and Arnold, 1998; Wang and Leiman, 2000;Samocha *et al.,* 2000, 2001, 2002, 2006, 2007; Handy *et al.,* 2004; Cohen *et al.,* 2005). Furthermore, although the concept of biosecurity in shrimp production was developed many years ago, it still requires some improvements (Davis and Arnold, 1998; Pruder, 2004). The current study expands the knowledge-base of operating limited discharge intensive nursery system while evaluating the effect of different methods for particulate matter control on selected water quality indicators and performance of *P. vannamei* PL in a greenhouse-enclosed nursery raceway system.

The primary objectives of this study were: (1) to evaluate the effect of limited water discharge on selected water quality indicators and shrimp performance; (2) to evaluate the potential use of foam fractionation as a tool to reduce particulate matter load; and (3) to evaluate the feasibility of implementing biosecurity measurements in limited discharged nursery raceway system.

EXPERIMENTAL DESIGN

Experimental system and design

A 71-day nursery study was conducted in four 40 m^3 HDPE lined greenhouse-enclosed raceways at the Texas Agricultural Experiment Station (TAES), Shrimp Mariculture Research Facility (SMRF), Corpus Christi, Texas. All four raceways were operated with high pressure rapid sand filters (TR-60, 473 L min-1 capacity, Purex Triton Pac Feb Inc., Sanford, North Carolina). Every raceway had a central longitudinal fiberglass partition placed over a 5.1 cm (2 in.) schedule 40 PVC pipe designed to provide oxygen-rich water across the bottom of the raceway. Water oxygenation in each raceway was generated by a 2 hp pump connected into a 5.1 cm Venturi injector (Mazzei Injector Co., Bakersfield,

California). This setup increased dissolved oxygen levels of the water going back into the raceway by mixing it with air or pure oxygen based on the oxygen demand of the system. In addition, each raceway was provided with eighteen 5.1 cm airlift pumps arranged in three equidistance banks on each side of the partition and six 1 m long air diffusers (Bio-WeaveTM, Aquatic Eco-Systems Inc., Apopka, Florida). Two raceways were each equipped with an external home-made foam fractionator (3.05 m long and 30.48 cm in diameter) operated by a 3.81 cm Mazzei Venturi injector and powered by 1 hp pump with a flow rate of 37.9 L min^{-1}. These raceways were operated with 3.35% average daily water exchange while the other two were operated with a daily water exchange of 9.37%. The daily water exchange rates were calculated based on the total water used in each raceways over the duration of the trial. The control of the particulate matter load in the culture medium during the first 45 days of the study was done by the pressurized sand filters. With the increase in shrimp biomass, these filters became ineffective in controlling particulate matter load. At that point the control of the particulate matter load in the raceways operated with no foam fractionators was done by water exchange while the control in the other two raceways was done primarily by foam fractionation.

Water source and treatment

Natural seawater (35-40 ppt) was pumped from the Upper Laguna Madre into a storage pond where salinity was adjusted to about 30 ppt using chlorinated municipal freshwater. Incoming water was filtered through 350 mm filter bag and then chlorinated using 12% sodium hypochlorite solution to achieve a concentration of 10 ppm active chlorine 30 min post-application with a targeted residual chlorine level of 1.0 ppm after 24 h. Raceways were filled from the storage pond after chlorine concentration was below 0.05 ppm. Water was fertilized before stocking using urea, phosphoric acid and sodium silicate to provide calculated concentrations of 2.25, 0.138, and 1.55 ppm for N:P:Si, respectively. Pure culture of Chaetoceros muelleri (initial inoculation concentration of 3.8 104 cells ml^{-1}) was added following the fertilization. Previously, chlorinated seawater from the storage pond or municipal freshwater served to offset water losses from sand filter backwashes, evaporation and operation of the foam fractionators.

Stocking

Nauplii (N_{4-5}) from specific-pathogen-free (SPF) broodstock of *P. vannamei* were donated by a commercial shrimp hatchery (Harlingen Shrimp Farms, Ltd., Los Fresnos, Texas). Four to five-day-old postlarvae (PL_{4-5}) were produced at the TAES facility and were stocked in four raceways (4050 PL m^{-3}).

Water quality analysis

With the exception of dissolved oxygen (DO) that was measured on as needed basis (e.g. more than twice a day during the last 4 weeks of the study), pH, water temperature and salinity were monitored twice daily (morning and afternoon). Turbidity, settleable solids (SS) and algal cell densities were monitored daily. Ammonium-nitrogen, Nitrite-nitrogen, nitrate-nitrogen, reactive phosphorus (RP), 5-day carbonaceous biochemical oxygen demand ($cBOD_5$), chemical oxygen demand, total suspended solids (TSS), and volatile suspended solids (VSS) were monitored weekly. A summary of the procedures used for monitoring the different water quality indicators is provided in Table 1.

Feed management

Postlarvae were fed newly hatched Artemia nauplii (50 nauplii PL day-1) for the first 3 days. The nauplii were replaced by two types of crumble feeds: a 50% crude protein diet (Redi Reserve™, Zeigler Bros., Gardners, Pennsylvania) that was fed for the first 7 days, and a 45% CP feed (45/10 Swim-up, Fry #1, #2, #3 & #4, Rangen Inc., Buhl, Idaho) that was fed from the 8 day until the harvest. Feed was provided manually four times a day 7 days a week. Daily rations for the first week were based on a fixed percentage of the estimated total shrimp biomass in each raceway. Rations from the second week on were adjusted based on feed consumption, gut fullness, shrimp mean weights, and the estimated survival and FCR. Shrimp group samples were collected randomly and weighed every 2-3 days to determine the mean body weight in each raceway. An analytical balance (Monobolc - PB 303-S Delta Range,0.001-310 g, Mettler Toledo Inc., Columbus, Ohio) was used to monitor shrimp growth. Feed consumption was monitored prior to each feeding by scooping the raceways' bottom with a fine-mesh dip net.

Biosecurity substantiation

To minimize the introduction of pathogens with the incoming water, the natural seawater was chlorinated to provide targeted residual chlorine of 1 ppm 24 h post chlorination. Raceways were enclosed in a greenhouse structure equipped with an electric wire shocker to keep predators out (e.g., raccoons, birds etc.). Nets and other equipments that were used in each raceway were washed with freshwater and left to dry after each use to minimize introduction of diseases. No attempts were made to disinfect the equipment after each use.

About 100 animals were collected daily from each raceway in a glass beaker to observe shrimp behavior and gut fullness. In addition, at least 10 shrimp from each raceway were checked daily for signs of fouling or lesions using a dissecting scope. Viral-pathogen-free status, namely absence of Taura syndrome virus (TSV), white spot syndrome virus (WSSV), yellow head virus (YHV), and infectious hypodermal and hematopoetic

necrosis virus (IHHNV), was substantiated by two-step nested PCR procedure conducted by Texas Veterinary Medical Diagnostic Laboratory (TVMDL, Texas A&M University System, College Station, Texas) on samples (>30 shrimp) taken from all four raceways and preserved in 95% ethanol. Shrimp samples (25 shrimp from each raceway) were also preserved in Davidson's fixative at the end of the study for histology evaluation that was carried out by the same diagnostic lab.

Table 1: Selected water quality indicators and the monitoring procedures used in a 71-day nursery trial with the Pacific white shrimp, *P. vannamei*, under limited discharge condition

Indicators	Methods
Algal cell counts (cell mL^{-1})	Hemocytometer
Ammonium (NH_4-N) (mg L^{-1})	Artiola (1989)
$cBOD_5$ (mg L^{-1})	Method # 5210 B APHA (1995)
COD (mg L^{-1})	Method # 5220 D APHA (1995)
Nitrate (mg L^{-1})	Hach method
Nitrite (mg L^{-1})	Method # II.15.II.2-NO_2. Strickland and Parson (1992).
Reactive phosphorous (mg L^{-1})	Artiola (1989)
Settleable solids (mg L^{-1})	Imhoff Cone, Method # 2540 F APHA (1995)
Dissolved oxygen (mg L^{-1}), temperature (^oC), salinity (ppt), pH	Multi parameter probe (YSI Model 650, YSI Inc., Yellow Springs, Ohio)
Total suspended solids (mg L^{-1})	Method # 2540 D APHA (1995)
Turbidity (NTU)	Aquafluor turbidity meter, Turners Design, Model # 8000-001, Sunnyvale, California
Volatile suspended solids (mg L^{-1})	Method # 2540 E APHA (1995)

Table 2: Mean weights, yield, survival and PCR values (±standard error) or Pacific white shrimp, *P. vannamei*, in a 71-day nursery trial

Treatments	Wt_i[a] (mg)	Wt_f[b] (g)	Yield (kg m^{-3})	Survival (%)	FCR
Foam Fractionator-2[c]	0.6	1.91±0.34[a,d]	7.23±0.56[a]	96.2±9.54[a]	1.03±0.15[a]
Foam Fractionator-3[c]	0.6	2.00±0.34[a]	7.23±0.56[a]	96.2±9.54[a]	1.03±0.15[a]
Water Exchang-1[e]	0.6	1.73±0.34[c]	4.33±0.56[b]	68.9±9.54[a]	1.50±0.15[a]
Water Exchang-4[e]	0.6	1.43±0.34[d]	4.33±0.56[b]	68.9±9.54[a]	1.50±0.15[a]

[a]Mean body weight at stocking.
[b]Mean final body weight at harvest.
[c]Raceway operated with foam fractionator and 3.35% average daily water exchange.
[d]Columns with same superscript letters suggest no statistically significant differences (α = 0.05).
[e]Raceway operated without foam fractionator and 9.37% average daily water exchange

Statistical analysis

The data was analyzed using the SPSS statistical software (Version 12 for Windows, SPSS Inc., Chicago, Illinois). Repeated Measures ANOVA test was used to compare differences in daily and weekly water quality indicators between treatments followed by the Student-Newman-Keuls (SNK) multiple range test for mean separation. One-way ANOVA test was used to determine significant differences between treatments in survival (arcsine transformed), mean final weights, FCR and yields. All differences were analyzed at significance level of a = 0.05.

RESULTS OBTAINED

Growth, survival, and yield

At harvest (71 days from stocking), the mean body weights of the shrimp in the raceways operated with FF were significantly higher than in the other raceways (1.91 i 0.47 and 2.0 i 0.59 g vs. 1.73 i 0.67 and 1.43 i 0.57 g, respectively Table 2). Furthermore, the yields in the raceways operated with the FF were also significantly higher than the other two raceways (7.64 and 6.89 kg m^{-3} vs. 3.92 and 4.74 kg m^{-3}). Although not statistically significant, survival (100 and 92.4% vs. 55.9 and 81.8%), and FCR (0.97 and 1.08 vs. 1.64 and 1.36) of the shrimp reared in the raceways operated with FF were better than those raised in the other two raceways.

Physicochemical factors

Tables 3 and 4 and Figs. 1-10 summarize the changes in daily and weekly water quality indicators over the 71-day period in the four raceways. These graphs were provided to show the changes over time in the above listed water quality indicators. Except for Nitrite-nitrogen, reactive phosphorus, algal cell density, and turbidity, no other significant differences were found in daily and weekly water quality indicators between treatments. Nitrite-nitrogen levels in the raceways operated with limited water exchange and with FF were significantly lower than the other raceways. On the other hand, the RP, turbidity, and algal cell density in these raceways were significantly higher ($P < 0.05$).

No signs of lethargic behavior, empty gut or external fouling were observed during daily monitoring of shrimp collected from all four raceways. Furthermore, all shrimp samples submitted for disease diagnosis showed no signs of infections by any of the viruses of concern. However, histological observations of the samples collected at harvest suggest that shrimp raised with increased water exchange regime showed greater levels of external fouling and intestinal bacterial infections compared to shrimp raised in the raceways operated with FF with a reduced water exchange.

Table 3: Summary of daily water quality indicators for raceways operated with two water exchange regimes over a 71-day nursery trial with Pacific white shrimp, *P. vannamei*

RW	Temprature (°C)		DO (mg L^{-1})		pH		Salinity	Turbidity	SS	Algae
ID	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	(ppt)	(NTU)	(mg L^{-1})	(cell $mL^{-1} \times 10^4$)
FF[a]	26.3[a,b]	27.4[a]	6.0[a]	5.9[a]	7.2[a]	7.2[a]	27[a]	39[a,b]	1.5[a]	403[s]
Std.	1.61	1.23	1.61	1.57	0.70	0.91	1.82	17.38	1.90	283.53
WE[c]	26.2[a]	27.4[a]	6.2[a]	6.3[a]	7.3[a]	7.3[a]	25[a]	20[b]	3.4[a]	220[b]
Std.	1.53	1.60	1.11	1.61	0.63	0.82	2.33	7.73	2.13	174.22

[a]Raceways operated with foam fractionator and daily water exchange of 3.35%.

[b]Columns with same superscript letters suggest no statistically significant differences (α = 0.05).

[c]Raceways operated without foam fractionator and daily water exchange of 9.37%

Table 4: Summary of weekly water quality indicators for raceways operated with two water exchange regimes over a 71-day nursery trial with Pacific white shrimp, *P. vannamei*

RW	mg L^{-1}							
	$cBOD_5$	COD	NH_4–N	NO_2–N	NO_3–N	RP	TSS	VSS
Foam fractionators[a]	48[a,b]	478[a]	2.56[a]	4.00[a]	6.54	4.7[a]	126[a]	65[a]
Standard deviation	31	328	6.46	4.50	3.39	4.4	98	74
Water exchanges[c]	40[a]	593[a]	0.26[a]	6.40[b]	7.24	3.4[b]	110[a]	60[a]
Standard deviation	37	321	0.57	6.43	7.59	2.8	85	56

[a]Raceways operated with foam fractionators and 3.35% daily water exchange.

[b]Columns with same superscript letters suggest no statistically significant differences (α = 0.05).

[c]Raceways operated without foam fractionators and 9.37% daily water exchange.

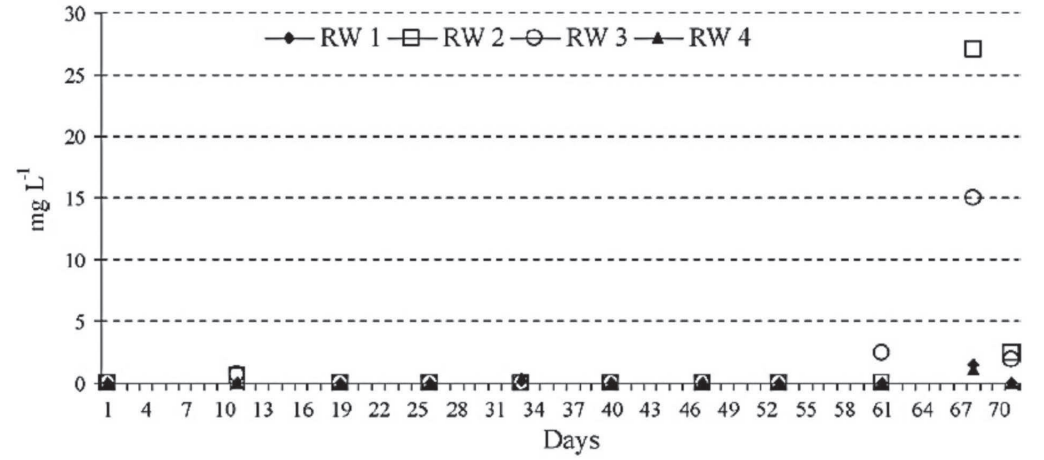

Fig. 1. Weekly changes in water ammonium–nitrogen levels in raceways operated with (RW 2, 3)/without (RW 1, 4) foam fractionators in a 71-day nursery study

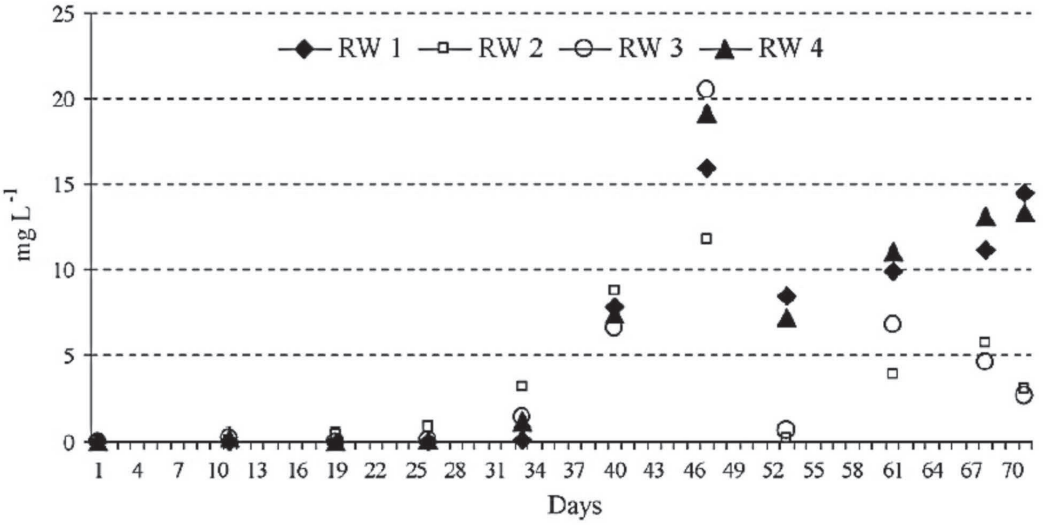

Fig.2. Weekly changes in water nitrite–nitrogen levels in raceways operated with (RW2,3)/ without (RW1,4) foam fractionator in a 71-day nursery study

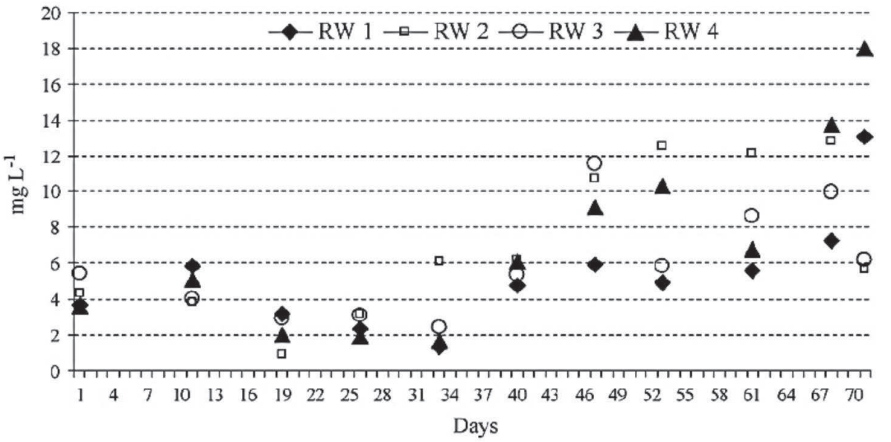

Fig. 3. Weekly changes in water nitrate–nitrogen levels in raceways operated with (RW 2, 3)/without (RW 1, 4) foam fractionators in a 71-day nursery study

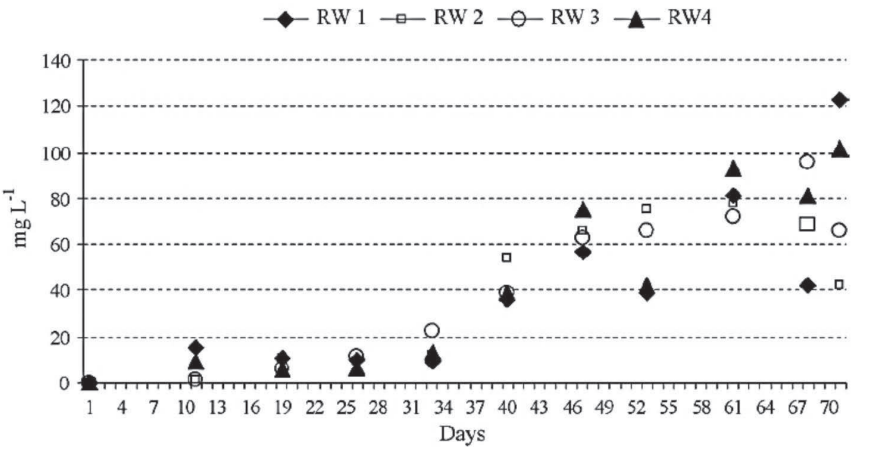

Fig. 4. Weekly changes in water 5-day carbonaceous biochemical oxygen demand levels in raceways operated with (RW 2, 3)/without (RW 1, 4)foam fractionators in a 71-day nursery study

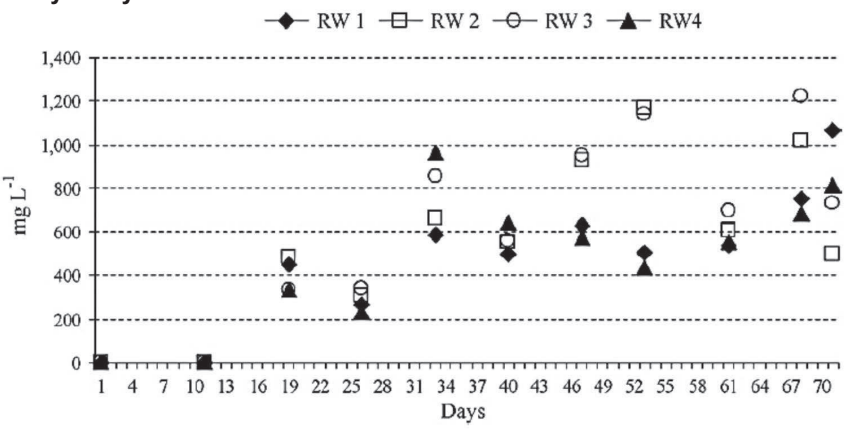

Fig. 5. Weekly changes in water chemical oxygen demand levels in raceways operated with (RW 2, 3)/without(RW 1, 4) foam fractionators in a 71-day nursery study.

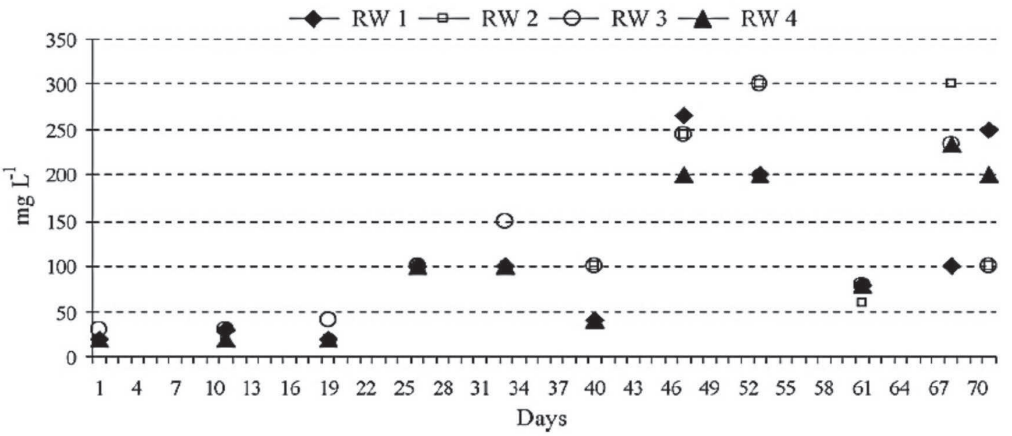

Fig. 6. Weekly changes in water total suspended solids levels in raceways operated with (RW 2, 3)/without (RW 1, 4) foam fractionators in a 71-day nursery study.

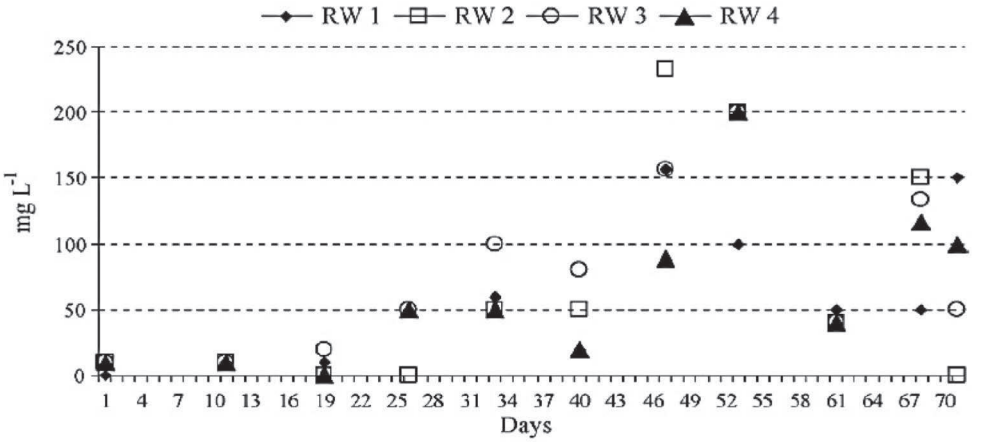

Fig. 7. Weekly changes in water volatile suspended solids levels in raceways operated with (RW 2, 3)/without (RW 1, 4) foam fractionators in a 71-day nursery.

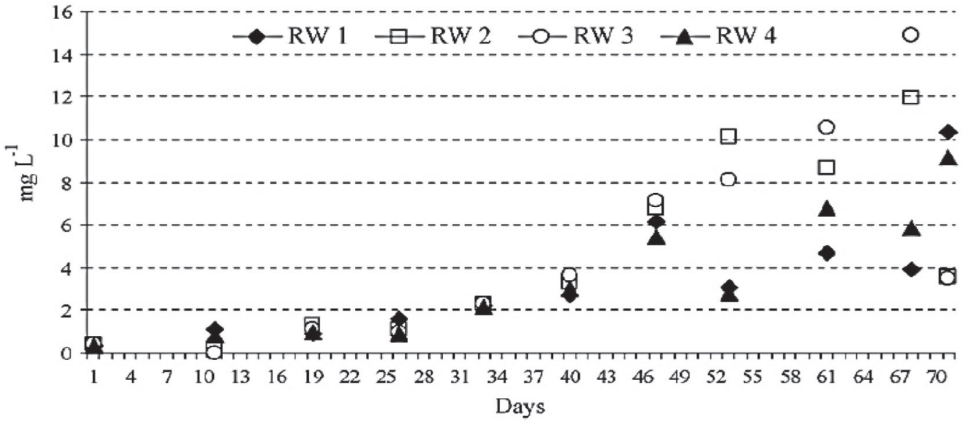

Fig. 8. Weekly changes in water reactive phosphorus levels in raceways operated with (RW 2, 3)/without (RW 1, 4) foam fractionators in a 71-day nursery study.

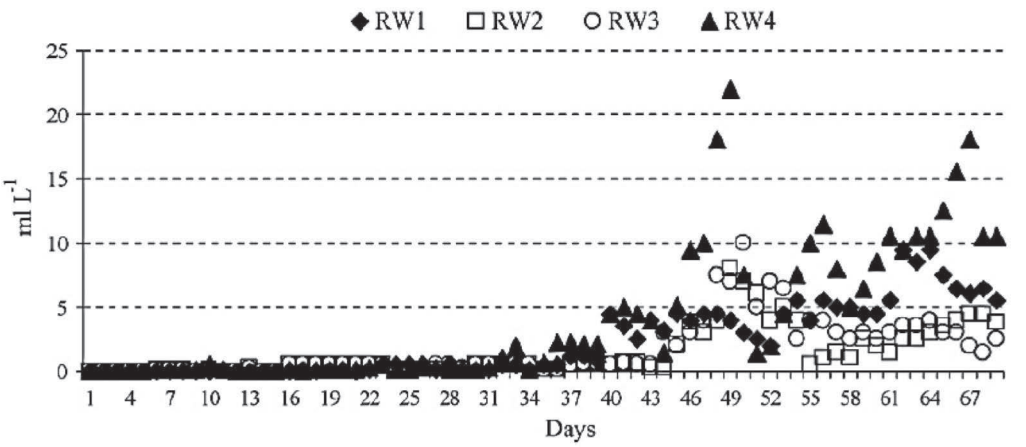

Fig. 9. Weekly changes in water settleable solid levels in raceways operated with(RW2,3)/ without (RW1,4) foam fractionators in a 71-day nursery study.

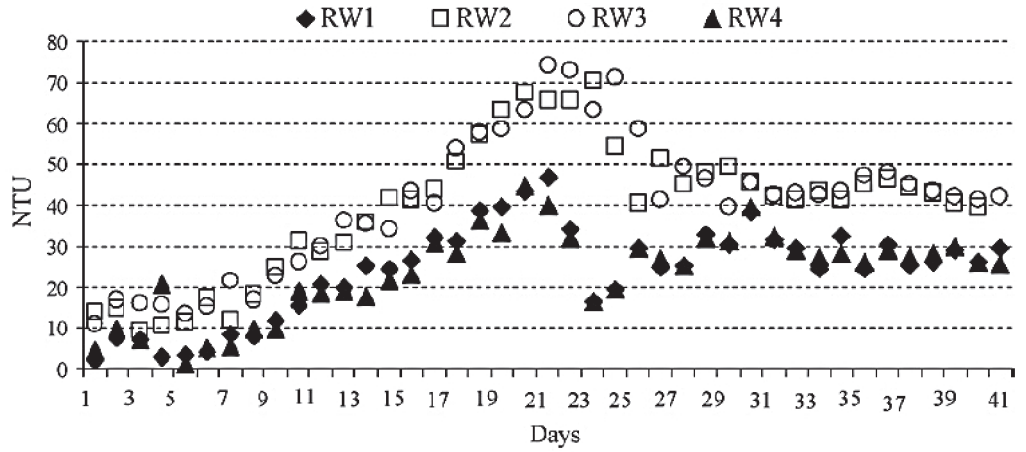

Fig.10. Daily changes in water turbidity levels in raceways operated with (RW2,3)/without (RW1,4) foam fractionators in a 71-day nursery study

INTERPRETATION

Growth, yield, and survival

The maximum yields (6.89 and 7.64 kg m^{-3}) observed in the raceways operated with FF were much higher than the 4.3 kg m^{-3} reported by Cohen *et al.* (2005) in a 50-day trial with *P. vannamei* in the same raceways which were operated with no FF under lower water exchange (1.1% day-1) and stocking density (3300 PL m^{-3}). On the other hand, Handy *et al.* (2004) in another 74-day nursery trial in this system reported a yield of only 3.18 kg m^{-3} while operating the raceways with FF at higher stocking density (5010 PL m^{-3}) and lower water exchange (2.06% day-1). The results from the current study showed that higher yields are feasible when raceways are operated with FF and a slightly

higher water exchange (3.35% day-1) than those used in the previous studies. Furthermore, the low FCR values (0.97 and 1.08) obtained in the two raceways operated with FF suggest that under the conditions of this study, natural productivity supported large portion of the shrimp nutritional requirements as documented by several other researchers working with limited discharge systems (Avnimelech, 1999; Burford *et al.,*2003, 2004).

Water quality indicators

After 48 days, the dissolved oxygen concentrations in all four raceways dropped below the 5 mg L-1 level. To keep up with the oxygen demands, culture water was circulated through the Venturi injectors using atmospheric air, oxygen-rich air and finally pure oxygen from compressed oxygen cylinders. Decreasing trend in pH levels also noticed overtime reaching levels below 6.0 about 7 weeks from the study initiation. Previous studies by Chen *et al.* (2006), Ebeling *et al.* (2006), and Rijn *et al.* (2006) documented a decrease in pH during the chemolithotrophic nitrification process as a result of alkalinity ($CaCO_3$) consumption and the release of CO_2 and H^+ into the culture medium. The changes in ammonium-N and nitrite-N levels in our culture medium (see Section 4.2.1) suggest that the same processes might have taken place in the current study. In our study, pH control (between 6.5 and 7.00) was done either by water exchange or by operating the FF (e.g., no chemical was added to the culture medium).

Algal cell density and reactive phosphorus

Throughout the culture period, algal cell densities,and reactive phosphorus in raceways operated with the foam fractionators were significantly higher than raceways operated without. A gradual increase in RP concentration was noticed in all raceways reaching a maximum level of 13.4 mg L^{-1} at the end of week 7 in raceways operated with FF. It is interesting to note that the RP levels dropped to 3.52 mg L^{-1} in these raceways towards the end of the study while levels in the other two raceways remained high (9.74 mg L^{-1}). Hopkins *et al.* (1993), while working with outdoor lined ponds with soil on the bottom and operated with limited water exchange, documented significantly higher reactive phosphorus levels in these ponds than those operated with increased water exchange. These authors also suggested water exchange as the only effective way to prevent accumulation of this nutrient. Burford *et al.* (2003) also documented high accumulation of RP in lined ponds (without soil) operated with limited discharge compared to earthen ponds in which RP could bind to the sediments. The constant increase in RP in the raceways operated without FF suggests that the water exchange rate was not high enough to significantly reduce its levels in these raceways. Hargreaves (2006) showed that algal assimilation could temporarily reduce phosphate; although levels increased again following crashes of the algal blooms. Burford *et al.* (2003) while working with limited discharge shrimp ponds

also observed higher algal density in lined ponds compared to earthen ponds. Since high algal density was noticed in the raceways operated with foam fractionators, it is safe to assume that this increase in algal biomass was responsible for the decrease in RP in these raceways towards the harvest when algal concentrations were at their peaks.

Ammonium, nitrite and nitrate

Throughout the study, soluble nitrogen species followed typical nitrification dynamics (see Figs. 1-3). Nitrogen plays an important role in the limited discharge aquaculture system due to its dual role, as a nutrient and toxicant (Burford and Lorenzen, 2004). Nitrogen in the form of ammonia (NH_3) and nitrite is highly toxic to shrimp, however, the toxicity depends on various factors including species tolerance, water characteristics (e.g., pH, temperature, salinity, DO) and exposure duration (Hargreaves, 1998; Barajas *et al.,* 2006). Wajsbrot *et al.* (1990) reported a 96 h LC50 of 1.43 mg L^{-1} for ammonia (23.7 mg NH_4^+ L^{-1} at pH 8.1, temperature 27 °C, and salinity 40.5 ppt) for juvenile (0.55-2.45 g) of Penaeus semisulcatus Similarly, Frias-Espericueta *et al.* (2000) reported a 12 h LC50 value of 0.70 mg L^{-1} ammonia (22.5 mg NH_4^+ L^{-1} at 28 C, pH 7.92, salinity 34 ppt) for PL12 and early juveniles of *P. vannamei*. Barajas *et al.* (2006) reported low mortality (>1%) in a short-term exposure (4 h) to 0.549 mg L^{-1} ammonia (18.0 mg NH_4^+ L^{-1} at pH 8.0, temperature 26 °C, and salinity 38 ppt) for PL30 of the same species. In the current study, shrimp in the FF operated raceways were exposed for 2 days to ammonia concentrations of 0.189 and 0.105 mg L^{-1} (27.04 and 15.01 mg L^{-1} of NH_4^+ at pH 6.8, temperature 28.0 °C, salinity 25 ppt). These levels of ammonia were far below the 96 h LC50 levels reported by Wajsbrot *et al.* (1990) for *P. semisulcatus*, and the 12 h LC50 levels reported by Frias-Espericueta *et al.* (2000) for *P. vannamei*. Furthermore, high survival rates and yields in the current study in the raceways which experienced the high ammonia levels suggest that these levels had no adverse effect on the shrimp under the conditions of this study.

Nitrite is another nitrogen species that can be toxic to shrimp. Sowers *et al.* (2004) reported a 96 h LC50 of 8mgL^{-1} NO_2-N (uncorrected for salinity, temperature and dissolved oxygen) for *P. vannamei* juveniles (0.27 g). On the other hand, when working with the same nursery system under similar conditions, Handy *et al.* (2004) showed that a short exposure (about a week) of juvenile *P. vannamei* to concentration of 29.0 mg L^{-1} of NO_2-N still resulted in high shrimp yield and survival. Similarly, Cohen *et al.* (2005), while working with this species using the same nursery system, reported good yields (4.25 and 4.33 kg m^{-3})of juveniles (>1 g) with high survival (97.5 and 106%) after 1 week exposure to NO_2-N level of 26.4 mg L^{-1}. The high survival (100%) and yield (7.64 kg m^{-3}) of the shrimp in the raceway that experience exposure to NO_2-N level >20 mg L^{-1} for 2 weeks

suggests that this high level didn't affect the shrimp performance under the conditions of our trial.

Nitrate, unlike ammonia and NO_2-N is less toxic to shrimp however, high concentration (100 mg L^{-1}) was reported to be lethal to shrimp (Muir *et al.,* 1991; Rijn *et al.,* 2006). The highest NO_3-N encountered in the study remained between 13 and 18 mg L^{-1} in all four raceways. It is safe to assume that this concentration did not affect shrimp performance as Wickins (1976) also reported no adverse effect on growth of *P. monodon*.

Chemical and biochemical oxygen demand

Under the limited water exchange regime, the demand for dissolved oxygen increased over time. A similar increase was noticed in the levels of COD and cBOD5. After 2 weeks into the study, the COD concentrations in the culture medium reached the 400 mg L^{-1} range. As expected, these COD values were far higher than the 40 mg L^{-1} level reported for the Corpus Christi Bay in Texas (Warnken *et al.,* 2001). Further increase in concentration to the 1100 mg L^{-1} range was noticed 8 weeks into the study. Starting on week 7, the concentrations of COD in the raceways operated with FF were higher than the other two raceways. A significant drop (to the 400 mg L^{-1} range) in the COD level was noticed on the week of the harvest in the two raceways with the FF. The levels found in our study were much lower than those reported by Cohen *et al.* (2005) for the same nursery system (2430 mg L^{-1}) in raceways operated with rapid sand filters and with no FF. More observations are needed to find out to which extent the FF were the driving force in reducing the COD levels in our study.

The cBOD5 levels in our study remained below 96 mg L^{-1} for great part of the trial in all four raceways. However, a significant decrease to the 60 mg L^{-1} range was found towards the harvest in the raceways operated with FF. This decrease suggests that the FF may have helped reduce the cBOD5 levels. However, more studies are needed to confirm this finding as other observations made by Samocha (unpublished data) in a previous study (Handy *et al.,* 2004) showed an increase in cBOD5 (from 8.0 to 33.6 mg L^{-1}) towards the harvest in a raceway operated with FF. It should be noted that the control of cBOD5 in the raceways operated without FF in our study was achieved by water exchange. Although the sharp increase (123 mg L^{-1}) in concentration found in one of these raceways can be explained by shrimp mortality triggered by mechanical failure, the level in the other raceway was still higher than in the raceways operated with FF.

Solid management and foam fractionators

Throughout the study, the particulate matter was kept in suspension by the bottom spray bars and the airlift pumps. Whenever settling of particulate matter was found, a dip net

was used to disperse it in the water column. During the early phase of the study, control of particulate matter was done mostly by the sand filters and occasional limited water exchange. From week 6 on, the sand filters could not effectively remove particulate matter from the culture medium as filter cycles were extremely short. From that time on, the levels of TSS, VSS, turbidity, and suspended solids (SS) showed significant increase in all four raceways (Fig. 10). Operating the FF helped reduce the concentrations of all of the above constituents to far below the levels found in the raceways operated without FF and with higher water exchanges. Previous studies also reported large removal of fine suspended solids when FF were used (Lomax, 1976; Lawson, 1978; Weeks *et al.,* 1992; Suzuki *et al.,* 2003; Handy *et al.,* 2004). Weeks *et al.* (1992), while working with fish recirculating system equipped with biological filter and foam fractionators, found the FF to be a very effective tool in reducing TSS, VSS, total dissolved solids (TDS), and cBOD5 in the culture water. These findings are for the most part in agreement with our finding for the current study.

Biosecurity and shrimp health

Daily visual examination of shrimp samples from each raceway showed large part of the population with full guts through the study. Lack of abnormal or lethargic behavior may imply an overall healthy environment for the shrimp in this study. The limited water exchange and the careful monitoring of feed consumption helped excluding bacterial diseases. Use of larvae from specific-pathogen-free brood stock enable stocking of the system with high quality seedstock. All samples sent to the disease diagnostic lab showed no sign of infection by viral pathogen of concern. Furthermore, histopathology results of shrimp collected during the harvest showed higher levels of external fouling and intestinal bacterial load in shrimp from the two raceways operated with higher water exchange and with no foam fractionators than those from the other two raceways.

CONCLUSION

This study showed that PL of the Pacific white shrimp, *P vannamei*, can be raised in limited water exchange regime with excellent health condition, low feed conversion rates, high survival rates, good growth, and high biomass loads. Culture tanks operated with foam fractionators showed better results than those operated without. The low FCR values obtained in the raceways operated with foam fractionators and reduced water exchange suggest significant contribution to shrimp nutrition from natural productivity. Nevertheless, more studies are needed to better define this contribution and the beneficial effects of the management practices used in this study on system performance. Furthermore, as the PL in this study were fed a high protein diet (45% CP), it will be interesting to study the effect of lower protein feed on shrimp performance under similar conditions.

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CHAPTER

13

INTENSIVE AND SUPER-INTENSIVE NURSERY SYSTEMS FOR *PENAEUS VANNAMEI*

Tzachi M. Samocha

According to the Food and Agriculture Organization (FAO, 2009), the world's seafood supply from aquaculture increased from 6% in the 1970s to almost 50% in 2006. With the world population estimated to increase to about eight billion people by 2030. and assuming seafood consumption at current rate of 17 kg/capita/y, an additional 29 million tones of seafood will be needed to meet the world demand. Recent FAO estimates suggest that about half of all wild fish stocks are considered "fully exploited'" while 30% are deemed "overexploited. depleted or recovering." Nevertheless, even if we assume that supply from fisheries can stay at the current level, most of the future seafood needs will have to be supplied by aquaculture (Nature, 2009). According to the FAO (2003), shrimp is the most important and profitable commodity amongst all seafood trade. In the U.S., shrimp was the most popular seafood in 2004 with imports reaching 500,000 tons and a value of $3.7 billion (FAO, 2006). Furthermore, according to Cascorbi (2004) and the FAO (2004), approximately 50% of the shrimp traded internationally in recent years have been produced by aquaculture. These sources also stated a significant increase in world shrimp production from 900,000 tons in 1994 to 1,600,000 in 2003. Current projections by the FAO (2004). suggest a 15% annual growth in the world's shrimp farming industry over the next decade. Although there are several shrimp being cultured in different parts of the world, the production of the Pacific White Shrimp (*Penaeus vannamei*) is the most important and extensive industry in the Eastern and the Western Hemispheres, accounting for approximately 47% of the world farmed shrimp production (FAO, 2004; Harvey, 2004).

REVIEW OF SHRIMP PRODUCTION PRACTICES

Although shrimp grow-out systems have existed for centuries (Bardach *et al.,* 1972) the last twenty years have significant quantities of shrimp been produced in ponds. This increase is mainly attributable to the rapid development of more dependable production technologies. Shrimp production methods can be classified under five categories based on yield and management: extensive, semi-extensive, semi-intensive, intensive, and super-intensive. Production in outdoor ponds can vary from about 100 to 70.000 kg/ha/crop for the extensive and the super-intensive systems, respectively. Although there is no clear definition for each category, the extensive level is characterized by low stocking densities and no active supplementation of food or aeration devices. Marketable shrimp production can take place in a single-, two-, or multi-phase system. The single-phase system calls for direct stocking of the postlarvae (PL) into the grow-out ponds in which they stay until the harvest. When production involves only one transfer of the juvenile shrimp from small tanks, where they stay for a short period, into the grow-out ponds this system is defined as a two-phase system (Lawrence *et al.*, 1985; Lawrence and Iluner. 1987; New and Rabanal. 1985). On the other hand, a three-phase system is characterized by two transfers of the juvenile shrimp before they are stocked in the grow-out ponds (Parker el ai. 1974). To maximize carrying capacity, the three-phase system requires greater organizational capacity. Furthermore, since shrimp are stocked at higher densities, one can anticipate greater expertise, more equipment, and handling stress when transporting larger shrimp into the grow-out ponds (Duenas *et al.,* 1983). For these reasons, the two-phase system is used more commonly among producers. The growing of the young shrimp in these small vessels is defined as the primary and secondary nursery phases. In most cases, the extensive and semi-extensive management practices typically do not use the nursery facilities. Although the use of the nursery phase is more common in semi- intensive, intensive and super-intensive production systems, this is not always the case. For example, high-density, family operated ponds in the Far Fast are often times directly stocked with PL skipping the nursery phase altogether. It is also interesting to note that Wang and Leiman (2000), in their analysis of different shrimp production systems, concluded that the two-stage system, with a prolonged first stage, is more efficient than either the single-stage or multi-stage systems.

Advantages and disadvantages of using shrimp nursery systems

The advantages of two-stage production systems have been summarized by several authors (Cao and Jiang. 1990; Fast, 1991a; Sandifer *et al.,* 1991; Sturmer *et al.,* 1992; Samocha and Lawrence. 1992; Stem and Letellier. 1992). In general, two-stage shrimp production systems can improve PL inventory-control and reduce losses to production. Furthermore, since nursery tanks can he stocked at high densities, it can provide for more efficient

utilization of the grow-out system with greater control over water quality and feed consumption. These improved conditions generally result in high IM. survival (85-95%) that can lead to a greater profit (llirono, 1983; Aquacop, 1985a; Fast. 1991b; Sturmer M al. 1992; Samocha and Lawrence. 1992). Nursery systems can help produce larger and hardier shrimp for stocking the grow-out ponds. Furthermore, stocking these larger juveniles can also result in shorter production cycles and a higher number of crops per year (Clifford. 1985; Briggs and Brown. 1991; Fast, 1991b. Peterson and Griffith. 1999; Samocha *et al.,*. 2001). Because of the shorter production cycle, grow-out ponds should have a better bottom quality that can support higher natural productivity, which can reduce feed cost. Duenas *et al.* (1983) and llirono (1989) also mentioned the potential use of these nursery facilities for PL holding when grow-out ponds were mostly stocked with wild PL. Use of nursery facilities can also improve the farm performance as shrimp can be kept in the nursery tanks to help with the grow-out ponds dry-out process. Furthermore, in areas known to be severely affected by viral diseases, nursery facilities can be used as primary quarantines to prevent the introduction of the diseases into the grow-out ponds. During this waiting period, PL can be inspected and if found to be infected, stock can be destroyed with limited effect on the grow-outponds. Several authors (Samocha and Lawrence, 1992; Sturmer *et al.*, 1992; Samocha *et al.,* 1993a; Peterson and Griffith, 1999; Samocha *et al.,* 2000a; McAbee *et al.,* 2003) suggested the potential use of greenhouse-enclosed nursery systems in temperate climate areas to stock PLduring the carl) spring season ("headstart"), when ambient water temperature in outdoor ponds is too low for the PL to survive and/or grow. This early stocking can provide for extension of the grow-out season to facilitate harvesting larger shrimp or for the production of two crops a year (Sturmer *et al.,* 1992; Samocha and Lawrence, 1992; Samocha *et al.,* 2000a,b; Samocha and Benner, 2001; McMahon *et al.,* 2001. 2002; McAbee *et al.*, 2003).

It is also important to mention some of the disadvantages associated with the use of intensive nursery facilities. These systems are more labor-intensive and require a greater amount of handling of the shrimp. With very little room for errors, especially when working with high densities well trained biologists are needed to monitor the water quality, shrimp growth) feed consumption, shrimp health and condition of the culture tanks to ensure smooth operation (Samocha *et al*., 1993 a, b). in addition, construction costs per square meter of these systems are much higher than for the traditional grow-out ponds. Finally, in viral infected areas, the use of a two-stage system can result in increased shrimp stress and mortality as observed by Stem and Letellier (1992).

A short review of small scale early nursery studies

Mahler *et al.* (1974) provided a description of a three-stage system used by the Environmental Research Laboratory (ERL), at the University of Arizona in Puerto Penasco.

Sonora, Mexico, for the production of marketable shrimp. Culture tanks, which were housed in inflated plastic structures, had an elongated raceway shape (23 m x 3 m) with concrete walls lined with a polyvinyl membrane. To control algal growth and reduce heat losses, the inflated covers were painted white. Optimal water temperatures were maintained with well water and a heat exchanger. Pure oxygen was used to meet the system's oxygen demand (Mahler *et al.,* 1974). A daily water exchange rate as high as 300% was needed to maintain water quality. Salser et al (1978) provided a brief description of the methodology used at the facility. An operation manual published by the University of Sonora (CICTUS, 1983) provides a detailed description of the day to day activities at the facility. The shrimp nursery was done in two stages. In the first stage, five to seven-day-old PL (PL5.7) were cultured for 22 days in 3,000 L flat bottom tanks at densities up to 20 PL/L with survival up to 80%. In the second stage, PL harvested from the small tanks (0.01 to 0.02 g) were transferred into a 75 m* raceway with a water depth of 60 cm at a density of 2.000 to 2,500/m^3 (1,200 to 1,500/m2). Shrimp were kept in this raceway for about seven weeks before they were transferred into the grow-out raceway. The average weight of the shrimp at the end of the second nursery period varied between 1.5 and 2 g with 80% survival and a biomass load as high as 2 kg/m^2. The research initiated in Sonora was continued in Hawaii by Marine Culture Enterprises (MCE). A detailed description of the system and the management used at the Hawaii facility was provided by Moore and Brand (1993). It is interesting to note that although yield as high as 5.5 kg/m^2 of marketable size shrimp have been reported from this system, the facility ceased to operate due to chronic viral disease outbreaks.

Cheong *et al.* (1988) described another six-raceway system (each: 20 m x 2 m x 1 m) used for the production of marketable size *Penaeus merguiensis*. Each raceway was equipped with a submergible pump, a settling tank, and a biodrum filter for particulate matter control. Nursery raceways were stocked with PL45 (0.3 g) at moderate stocking densities of 500-625 PL/m . The low survival (16.3-22.5%) after 92 days with a biomass load of about 1.4 kg/m^2 suggest unfavorable growing conditions. Briggs and Brown (1991) used twelve 25 m^3 concrete tanks for growing PL15.50 of *P. monodon* in another intensive nursery system. The authors found that better results were obtained when tank bottoms had a thin layer of river sand. Their findings also suggested that reducing water exchanges had adverse effect on growth and survival when shrimp stocking density was increased from 1,000 to 2,000 PL/m^2. Mock et al (1973) described yet another greenhouse-enclosed nursery system. Their preliminary work was done in two 16 m^2 raceways with water levels of 0.75 m. Each raceway was provided with a center longitudinal partition and airlift pumps to create a circular water flow and to facilitate waste removal. Growth from 0.1 to 9.0 mg and survival of 90% were reported for L. setifems in a 22-day nursery study. Other studies by these authors were conducted in raceways similar to the prototype

developed for the Puerto Penasco project. Unlike the ERL prototype, this raceway bottom had a V shape (0.5% slope) and a longitudinal partition 5 cm off the central line. Aeration and water circulation were provided by airlift pumps. Particulate matter control was done by a variety of filters. Water temperature was maintained by space heaters and a heat-exchanger. Questionable survival of 100% was reported for a nursery period of 181-day for *Farfaniepenaeus aztecus,* the Atlantic Brown Shrimp, stocked at a density of 12,500 PL/m^2 (Mock *et al.,* 1977). According to these authors, particulate matter accumulation and constant need for manual waste removal were the major obstacles to system commercialization.

The two-phase production practice was also used by the Oceanic Institute in Hawaii. Nursery trials were conducted in circular 30 m^3 concrete flat-bottom tanks with stocking densities of up to 1,200 PL/m^2. Suitable dissolved oxygen was maintained solely by air blower driven airstones. Tanks were equipped with a 10 cm central drainpipe that was connected into a harvest basin. Other OI nursery studies were conducted in a 340 m^2 round center drain pond with paddlewheel aerators to provide aeration and water circulation (McSweeny *et al.*, 1988, Kanna *et al.,* 1991). Culture water was fertilized and inoculated with diatoms prior to stocking using PL5.10 for a nursery trial period of 30 to 50 days. Water exchange (0 - 60%/d) and periodic siphoning of the waste from the tank's center helped maintain water quality. Shrimp survival rates at harvest were reported to be high (85-95%). Aquacop (1985a,b) described another nursery system for *P.vannamei* using 10 to 100 m^3 culture tanks. This system was operated with heavy aeration and no filtration or water exchange. The authors mentioned that the primary nutrition source for the PL in this system were bacterial floc which developed within the water column. A mean weight of 0.1 g was reported in 30-day nursery trials when the stocking density varied between 1 and 10 PL/L.

Sturmer *et al.* (1992) provided a short summary of an intensive nursery system used in Israel for early PL of P. semisulcatus. Nursery trials were conducted under shaded structures using circular high density polyethylene (HDPE) and concrete tanks with working water volumes between 2 and 15 m^3 (bottom area of 3-20 m^2). Each tank was equipped with airstones for aeration and airlift pumps to create circular flow and to facilitate particulate matter removal through a center filter pipe. Nursery trials were conducted with stocking densities between 5 and 70 PL/L. The reported survival rates for these studies varied between 41% and 100% with mean final weights between 2 and 374 mg, depending on PL stocking age, nursery duration, and water temperature. It is interesting to note that in a controlled study, where nursery was performed with three algal species (*Tetraselmis snecica, Chaetoceros gracilis,* and *Isochyrisis galbana*) and without algae, better shrimp survival and growth where found compared in the presence of algae.

Small and large scale use of traditional nursery ponds

Published information regarding management of shrimp nurseries in outdoor ponds is very limited. Sturmer *et al.* (1992) provided a brief summary of nursery trials with several shrimp species. Citing several authors (Parker *et al.,* 1974; Apud *et al.,* 1979; Duenas *et al.,* 1983; Hirono, 1983; Pretto, 1983; Aquacop, 1984; Issar *et al.,* 1987; Seidman and Issar, 1988; Villalon,1991), the above authors showed a wide range of pond sizes (0.02-4 ha), stocking densities (28-200 PL/m^2), and nursery durations (25-69 days) being used with variable survival (55-85%) and shrimp size at harvest (0.25-5.3 g). A more extensive review of nursery practices in outdoorponds was provided by Samocha and Lawrence (1992). The paper describes different practices associated with operation of nursery ponds including: preparation, fertilization, PL evaluation,transportation, acclimation and stocking, feed and feed management, water quality monitoring and control, water exchange, shrimp sampling, harvest procedures and transfer of the juveniles into the grow-out ponds.

One nursery method that has been used in Texas called for stocking PL during early spring in greenhouse enclosures built inside the grow-out ponds (Cook et ah, 1988). These enclosures had a tunnel shape with a lined bottom and sides. The sizes of these structures varied based on the grow-out pond size with 350 m" and 520 m" enclosures being used for a 0.8 ha and 1.2 ha grow-out ponds, respectively. Nursery culture water was fertilized to enhance algal blooms using 1:1 ratio of N:P (0.5 ppm N to 0.07 ppm P) and silicate at a concentration of 1.6 ppm. Water exchange (0% in the 1st week after stocking up to 100% after the 4^{th} week) was performed by pumping water from the grow-out pond and filter pipe, which was placed inside the nursery structure. Two air-diffusers and 1 hp paddlewheels were used to maintain adequate dissolved oxygen levels. Postlarvae were fed three to four times a day with a 45% protein diet at a level starting at 25% of the total estimated biomass and reduced to about 10% of the total body weight toward the release of the juveniles into the grow-out pond. Stocking densities varied between 2,000 and 2,300 PL/m^2. Within eight weeks, the average juvenile size obtained was 1g. Although no determination of the number of PL released into the grow-out ponds was done, the authors mentioned at least 80% survival in well-managed enclosures.

Mr. Chamorro (Camaco, Panama) provided current procedures for managing nursery corrals in grow-out ponds in Panama. The size of the corral, which was built with 1.5 mm mesh, is defined based on size and expected stocking density in the grow-out ponds and in most cases can vary between 200 m and 1,250 m^2. The corrals are round and located in areas of good water exchange rates and minimal effect from siltation. The water in the corral receives organic and inorganic fertilizers, just like the rest of the grow-out ponds. The bottom of the corral is covered with $Ca(OH)_2$ three days before filling and a minimum of 9 pieces of the vertical netting AquaMat* is place in each enclosure. Every corral has at least two fine-mesh feeding trays to evaluate consumption and to adjust

rations before each feeding. Rations are based on a feeding table constructed based on the corral size and stocking densities. Shrimp are fed a 40% crude protein diet seven days a week every four hours by broadcasting the feed by hand. Feed particle size varies with the size of the shrimp. Selected water quality indicators are monitored in the morning and in the afternoon. Shrimp are generally kept in this enclosure for at least 21 days.

Several other producers (Mr. Drazba, Camanica, Nicaragua; Mr. Aguirre, Aqualab, Ecuador; Mr.Cordova, Naturisa, Ecuador, personal communication) provided a short summary of nursery ponds used in Central and South America and stressed that these ponds have been an effective production tool in these countries. In early dates, these ponds were used for nursery of wild PL (100-300 PL/m^2) and for storage to provide shrimp for stocking the grow-out ponds when wild PL are in short supply. Maintaining adequate water quality was done with water exchange. Nevertheless, after the TSV outbreak which resulted in heavy losses in the nursery ponds, producers switched to direct stocking to minimize losses. With the development of TSV resistant PL, nursery ponds began once more to gain acceptance as a production tool. A renewed interest in using greenhouse-enclosed nursery ponds emerged after the producers realized that keeping the PL at water temperatures above 30° C prevented the massive losses associated with the WSSV outbreaks. The animals are raised to about 0.5 g before they are transferred into the grow-out ponds. Traditional nursery ponds in Ecuador have been used for the last 40 years to grow juveniles to be stocked in the grow-out ponds, this practice did not go through many changes over the years. As mentioned earlier, nursery ponds were very useful for storing PL for out of season stocking. In some cases farmers kept juveniles in these nurseries for several months. Of significance is the fact that when juveniles were transferred into the grow-out ponds, they showed a compensatory growth rate. The main change in nursery practices, over the last decade, was in seed supply coming from commercial hatcheries rather than from natural nursery grounds. Other noticeable recent changes that improved shrimp survival and health were the use of more balanced and stable diet formulations, along with greater use of probiotic, "bokashi (a decompose organic matter under anaerobic condition)," molasses, and molasses-fermented bacteria (mostly *Lactobacillus sp.*). Postlarvae are stocked into nursery ponds (average 1 ha in size) at a density of 10 to 200 PL/m^2 in which they stay up to 20 days when they reach a size of 0.15 g. Nursery ponds are filled with screened water which is fertilized to promote phytoplankton bloom. Water level is gradually increased over two to three days to an average depth of about 1 m right before stocking. For the most part, PL are fed manually from the levees two times daily using a 35% crude protein pelleted diet (starting with 1 kg/ha/d up to about 10 kg/ha/d). Very little water exchange is performed throughout the cycle (about 5%). Transfer of juveniles is performed by lowering the water level in the nursery ponds to a point where juveniles can be harvested with a fine mesh bag placed at the outlet gate. Shrimp are slowly pulled out (1-2 kg at a time) from the bag,

weighed and transferred into a meshed basket inside a well-oxygenated hauling tank. A sample (about 0.22 kg) is taken for every 22 kg of shrimp harvested to get a better estimate of shrimp survival. For the most part, shrimp are released from a wooden walkway structure inside the grow-out pond to avoid stirring the pond bottom.

Nursery ponds are usually positioned all over the farm close to the grow-out ponds to avoid long transfer trips. In many cases, nursery ponds are built adjacent to the grow-out ponds so that the shrimp are transferred without the need for hauling tanks. Thus nursery ponds continue to help assess seed quality, provide better facility utilization with more control over the environment.

Nevertheless, these facilities are more expensive to build and require better trained operators. As production companies become more vertically integrated, the fit is natural.

Use of intensive nursery systems

The increased stocking densities in the nursery phase have spawned several studies to evaluate the potential use of vertical substrates as a tool to improve shrimp performance in these systems.

Sandifer *et al.* (1987), in their nursery tank study with *P.vannamei* supplemented with vertical netting, reported better survival rates (82 vs. 58%) but not better growth rates. On the other hand, in a six-week small-tank nursery trial with this species, Moss and Moss (2004), while working at three densities (778, 1,167, and 1,556 shrimp/m^2), found a significant improvement in shrimp growth at all tested densities in the presence of vertical netting with no statistically significant improvement in shrimp survival. Arnold et al (2006) found better growth and survival of *P.monodon* PL in a small scale nursery study when tanks were supplemented with artificial substrates compared to tanks without artificial substrates. These authors also found lower FCR for shrimp raised in the presence of artificial substrates. Although reduced growth and survival rates were noticed when density was increased from 1,000 to 2,000 PL/m1, shrimp performance was significantly better than in tanks without vertical substrates. Peterson and Griffith (1999) also reported improvements in both survival and growth rates of shrimp reared in the presence of vertical netting. More recent nursery studies with *P. monodon* by Arnold et al (2009) also showed better shrimp performance with improved FCR and lower levels of total ammonia nitrogen (TAN) in the presence of vertical substrates than without. On the other hand. Kumlu *et al.,* (2001) in their work with PL of *Metapenaeus monoceros* found no beneficial effect on survival or growth when tanks were supplemented with vertical or horizontal netting. Similarly. Samocha et al (1993a). while working with *P. vannamei* in a 40 m nursery raceway system at the AgriLife Research Mariculture Lab did not find beneficial effects on growth. FCR or survival rates when PL were cultured in the

presence of vertical netting. It is interesting to note that similar high survival and low FCR values were reported for other trials in the same nursery system even in the absence of vertical netting (Handy *et al.,* 2004; Cohen *et al.,* 2005; Samocha *et al.,* 2007; Mishra *et al.,* 2008). Several researchers documented an inverse relationship between stocking density and shrimp growth in the nursery phase (Palomino *et al.,* 2001; Coman *et al.,* 2004; Moss and Moss. 2004; Arnold *et al.,* 2006; Mays *et al.,* 2006). Other researchers (Rodriguez *et al.,* 1993; Ray and Chien, 1992; Nga *et al*., 2005) were able to document not only negative impact on growth but also lower survival with increase densities during the nursery phase. Furthermore, the study by Nga and co-workers showed that physical interference stress and cannibalism could be excluded as causal factors, suggesting that the negative impact of crowding (at 50 and 100 PL/L) on shrimp growth and survival was due to some chemical compounds or other water quality variables. According to their findings, pH, temperature, salinity, dissolved oxygen, chlorine, nitrite and nitrate all had a minor influence, whereas ammonium toxicity could not be excluded as the driving factor for the lower growth and survival rates observed in their study.

Use of intensive nursery facilities was also found to be a useful tool to improve production in shrimp farms in Ecuador. According to one source (Mr. Aguirre, Aqualab, Ecuador, personal communication), raceways are currently being used in both shrimp farms and hatcheries. An increase in use was noticed during the WSSV epidemic because farmers thought it could provide better nourished, healthier, stronger, and more uniform postlarvae. Furthermore, since hatcheries and raceways in this country require temperature control for most of the year, farmers found out that the PL would remain healthy even during the worst period of the WSSV outbreak as long as the temperature was kept above 30° C. Most nursery raceways are of very simple construction. They basically consist of a rectangular box, with a sloping -sometimes V shaped bottom, enclosed by boards or concrete panels, semi buried in sand or soil, and then lined with HDPE. In some cases, raceways are large larval rearing tanks. Typical dimensions are 2 m wide by 1.4 m deep by 10, 20, or more meters long, with a final working volume of 20 m^3, 40 m^3, or more. Raceways are covered in some manner, usually with a transparent greenhouse clear polyethylene membrane. During the colder months, a gas or diesel fired boiler with a heat exchanger and coils of black HDPE tubing are used for supplemental heating. Aeration is provided by regenerative blowers, air tubing, and suspended airstones; some have perforated tubing and others use a combination of both airstones and perforated tubing. Some of the larger hatcheries use raceways to increase production capacity. They move young postlarvae (PL5.7) to holding tanks until the customers buy them. Meanwhile, they have freed-up their available larval rearing capacity. Hatcheries rarely keep PL in raceways for more than 15 days because of the cost (power, labor and feed) and losses associated with this practice.

Better harvest size was also reported when grow-out ponds were stocked with juveniles (2 to 3 g larger size) compared with directly stocked ponds. Mr. Cordova (Naturisa, Ecuador, personal communication) also stated that better harvest sizes were obtained in juvenile stocked ponds for the same period of grow-out time compared to direct stocked ponds. Furthermore, by having juveniles ready to be stocked right after the grow-out ponds harvest, restocking of these ponds can be accelerated; thus minimizing the pond down-lime, minimizing production cost, and maximizing the number of crops per year. As stated earlier, since weaker animals are eliminated during the nursery phase, these facilities help stock healthier shrimp into to the grow-out ponds.

According to Mr. Aguirre (Aqualab, Ecuador personal communication), some farmers have raceways at their farms where they keep the PL for up to 15 days. It was estimated that about 20% of the PL in Ecuador go through a raceway nursery phase before being transferred into the grow-out ponds. By using these nursery raceways, the farmers were able to stock their ponds with hardier and larger shrimp that resulted in shorter production cycles in the grow-out ponds.

Raceways are stocked at a density of about 60 PL/L or less and are fed up to 24 times per day, with 12 or less feedings in most cases. Shrimp are fed basic shrimp grow-out feed together with Artemia flakes and starter feed (35 to 40% protein). When available, algae are added, especially upon stocking. Water is kept constantly aerated and most operators perform a daily water exchange. In only a few cases farmers use limited biofiltration to reduce water exchanges. Most farms use some type of bacteria, probiotic, enzyme, or different combinations of these additives.

Operators change the type of treatment based on the system requirements. Many biologists try to implement biofloc practices for water management; however, because of the short nursery duration the use of this practice is limited. Farmers like the advantages that come with the use of nursery facilities as it gives them a better tool to operate and manage their farms. One farmer in Ecuador was able to obtain 5 to 6 crops per year (10 g shrimp) using nurseries. For farmers harvesting 13-16 g shrimp, the use of nurseries facilities provide an increase of at least a half crop cycle per year. Nevertheless, it is important to remember that nursery performance depends on how well the system is designed as well as the design of the rest of the farm. Although only small portion of the farms in Ecuador use nursery facilities, 75% of the farm managers in well- organized operations prefer to operate their farms with nursery facilities. Their reasons in order of importance are: 1) Better survival, 2) Shorter use of the grow-out ponds, 3) Greater accuracy when stocking the grow-out ponds, and 4) Use of stronger and healthier juveniles for stocking the grow-out ponds. It is interesting to note that when farm managers were

asked for their choice if they had to build a farm from scratch, most preferred to have some type of nursery capabilities. More technically inclined biologists preferred raceways and biofloc techniques.

A similar beneficial effect on shrimp performance in the grow-out ponds was described by Samocha et al (2001) for another shrimp farm in Ecuador (Pesquera Bravito). This farm used several 50 m^3 greenhouse-enclosed raceways; most of them had no center partition or Venturi injectors to enhance oxygen delivery into the culture medium. Early nursery productions were conducted with relatively high water exchange (50 - 60%/d). Stocking of the grow-out ponds with these juvenile shrimp significantly improved the farm's performance. The improvement in the farm's performance resulted in further expansion of the nursery system. A significant reduction in water exchange was achieved (25 to 30%/d) when the system was equipped with improved water polishing capabilities, which included particulate removal, biofiltralion, and foam fractionation. It is interesting to note that the dechlorination of the water (5 mg/L) used for the nursery was accomplished by aeration and adding vitamin C to the water (5 ppm). Before stocking, culture water was treated with EDTA (5 ppm) and inoculated with different algal species (including Chaeioceros, Tetraselmis, Amphora, Navicula, Chlorella) and probiotic. This nursery system and management consistently produced healthy PL with a good survival rate (>80%) and no need for antibiotic.

Samocha *et al.* (2001) described another Ecuadorian farm (Rmpacadora National - ENACA) which used a nursery facility. This farm had four 70 m3 HDPE-lined greenhouse-enclosed raceways. Each tank was equipped with a perforated bottom PVC pipe grid for mixing and water oxygenation. As was the case for the previous two facilities, culture water was chlorinated (10 mg/L) before use to minimize the risk of viral disease outbreak. Tanks were stocked with PL10-15 at a density between 15 and 18 PL/L for nursery duration of up to four weeks with no water exchange. Molasses was added to prevent increase in ammonia and nitrite concentrations. In most cases, FCR were below 1 with yield between 0.28 and 0.55 kg/m^3 and shrimp survival between 70.4 and 97.8%.

A short survey of several shrimp farms in Nayarit, Sinaloa and Sonora in Mexico documented the use of intensive nursery raceway systems (Mr. Matsumoto. Seapro, Mexico personal communication). Most of the farms use round or raceway type tanks inside greenhouse structures to provide passive temperature control. The number of the culture tanks in these farms varies from 4 to 40 with a working volume between 80 and 150 m^3. Reported stocking densities vary between 7 and 250 PL/m^3 depending on the nursery duration (14 - 30 days). In most cases, culture water is filtered (1-5 micron) and treated by chlorine or ozone before use. A large number of the producers are using

several probiotics and molasses together with water exchange (10 - 30%/d) to maintain suitable water quality. Only a few farmers are working with zero or limited water exchange. Since harvest biomasses rarely exceed 1 kg/m^3, dissolved oxygen levels are maintained using air blower and air diffusers. Shrimp survival rates vary between 65 and 95% with harvest sizes between 14 and 25 mg in size. Producers that work with low stocking densities may keep the shrimp until they reach the 0.2 g in size.

Research initiated by the AgriLife Research Mariculture Laboratory, formerly known as the Texas Agricultural Experiment Station Shrimp Mariculture Research Facility (TAES-SMRF), in 1986 focused primarily on the development of intensive nursery systems for *P. vannamei*. Studies were conducted in six 68.3 m greenhouse-enclosed raceways. Sturmer et al (1992) reported an average water exchange between 10 and 280%/d during the early nursery studies.

Although Sturmer and Lawrence (1987a) reported yields higher than 2.2 kg/m2 for this system, waste accumulation and removal were major operational problems. Samocha et al (1993a)showed significant improvements in waste management of this system by installing a center partition and airlift pumps in each raceway.

The integration of a nursery phase has also been found to improve the efficiency of intensive limited discharge shrimp production systems (Samocha *et al.,* 2000a,b; Cohen *et al.,* 2005; Mishra *et al.,* 2008). In most aquaculture operations, the incoming water is the common pathway of pathogen introduction into the culture system (Lotz and Lightner, 1999). Management of intensive biosecure shrimp production systems requires a strict enforcement of disease control and prevention (Moss *et al.,* 1998a). About two decades ago, intensive shrimp production practices used high water exchange to provide suitable water quality (Wang, 1990; Hopkins *et al.*, 1993; Moss *et al.,* 1999). This practice is not only wasteful of water resources but also a potential source of environmental pollution. To minimize the release of nutrient-rich water into the adjoining coastal ecosystems, governmental agencies have imposed strict effluent discharge guidelines (Ibrekk and Elvestad, 1990; Mathiesen, 1990; Hopkins, 1992; Samocha *et al.,* 2002).

The effluent waters from intensive shrimp production systems are typically characterized by high loads of nitrogen (N), phosphorus (P), particulate matter, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) (Hopkins *et al.,* 1993; Mcintosh, 2001; Jackson *et al.,* 2003; Cohen *et al.,* 2005). Most of the nitrogen contribution in shrimp culture systems enters the water column as ammonium (NH_4) generated by feed that is not converted into shrimp tissue. For example, Thakur and Lin (2003), while working with Penaeus monodon that was fed high-protein dry feed (42% crude protein), showed that shrimp could only assimilate 23-31% and 10-13% of N and P, respectively. In many cases, the culture waters from these systems have been released into low-

recharge receiving streams without prior removal of excess nutrients and other particulate matter (Wang, 1990; Avnimelech *et al.,* 1994; Wang, 2003; Cohen *et al.,* 2005).

Burford *et al.* (2003) suggested that these nutrients can be easily taken up by microorganisms and serve as a fuel for operating a "floc system" in a limited discharge culture practice. Operating sustainable biosecure shrimp production facilities under conditions of limited or no discharge dictates the incorporation of nutrient recycling methods for the metabolites generated by these systems. With the combined aim of increasing production and reducing nutrient releases, several studies described different filtration methods used in recirculating aquaculture systems (RAS). These include biological filtration by oysters, macroalgae and aquatic plants (Corpron and Armstrong, 1983; Shpigel and Neori, 1996; Jones *et al.,* 2002; Wang, 2003), and mechanical filtration (Maruyama and Suzuki, 1998; Suzuki *et al.,* 1999; Timmons *et al.,* 2001; Suzuki *et al.,* 2003). Foam fractionation was also listed as another effective tool to remove dissolved organic matter and fine suspended solids (5-10) from the culture water (Timmons, 1984; Hussenot and Lejcune, 2000). This method is particularly suitable for either intensive production systems operated in a closed recirculating mode, or for extensive and semi-intensive systems where water renewal is low (Hussenot, 2003). In limited discharge production systems, the phytoplankton and the microbial communities can play a major role in recycling the autochthonous nutrients accumulating within the system (Avnimelech *et al.,* 1994; Funge-Smith and Briggs, 1998; Avnimelech, 1999; Wang, 2003; Avnimelech, 2006; Bratvold and Browdy, 1998; Hargreaves, 2006). These microbial and phytoplankton communities are the major driving forces behind the increase in the carrying capacity of these systems.

In conventional flow-through intensive production systems, shrimp derive the majority of their nutrition from supplemental feed, hence high quality commercial feed has been used to improve the yield (Kureshy and Davis, 2002; Thakur and Lin, 2003). In limited discharge systems, because of their detritivorous and continuous grazing behavior, shrimp depend on the supplemental feed, benthic fauna, and other detritus as their nutrient sources (Hunter *et al.,* 1987; Moriarty, 1997; Leber and Pruder, 1988; Moss *et al.,* 1999; Burford *et al.,* 2004; Moss *et al.,* 2006; Wasielsky *et al.,* 2006; Ray *et al.,* 2009). However, in the presence of a carbonaceous substrate, the microbial community can produce single cell proteins that can serve as supplemental feed for the culture species (Avnimelech *et al.,* 1994; Avnimelech, 1999; Browdy *et al.,* 2001a; Ray et al 2009). Previous studies suggested that the Pacific White Shrimp, *Penaeus vannamei*, could be cultured with reduced water exchange without an adverse effect on the growth, survival and yield (Hopkins *et al.,* 1993; Moss *et al.,* 1998a; Cohen *et al.,* 2005).

Davis and Arnold (1998) describe a closed recirculating intensive nursery system that was used for several penaeid shrimp including *P. vannamei*. The system was equipped with airlift pumps for water circulation and vertical screen filter plates for a biological

filter. The authors provided a summary of the results from six years of research working with this system. Both bait shrimp (*L. setiferus*) and marketable size shrimp (*P. vannamei*) have been produced with final biomass loads as high as 10 kg/m3 in 100 to 120-day and 160 to 175-day production cycles, for the bait and the food shrimp, respectively. Shrimp nursery was conducted in a 10 m circular closed recirculating tank with a biofilter. Good survival rates (84.8 and 96.7%) and mean final weights (0.16-0.45 g) were reported for nursery duration between 21 and 32 days when stocking densities varied between 2.1 and 8.3 PL/L.

Use of biosecure limited water exchange nursery systems by the AgriLife Research Mariculture Laboratory

Much of the published information on the use of intensive nursery systems for *P. vannamei* under limited discharge management has been published by researchers from the AgriLife Research Mariculture Laboratory in Corpus Christi, Texas (Samocha *et al.*, 2000a,b; 2002; Handy *et al*., 2004; Samocha *et al.*, 2004; Cohen *et al*., 2005; Samocha *et al.*, 2006; 2007; Mishra *et al.*, 2008). Although the concept of biosecurity in shrimp production was developed many years ago, it still requires some refinements (Davis and Arnold, 1998; Pruder, 2004). The successful use of limited or no water exchange management in shrimp culture should further facilitate the implementation of improved biosecurity practices by this industry.

As most of the nursery systems used in the U.S. are built inside greenhouse structures, biosecurity practices are easier to implement so that catastrophic losses to infectious disease outbreaks can be minimized (Moss *et al.*, 1998b; Ogle and Lotz, 1998; Bratvold and Browdy, 1999; Leung and Moss, 1999; Moss *et al.*, 1999; Lotz and Lightner, 2000; Mishra *et al.*. 2008). In addition to biosecurity issues, when these systems arc operated with minimal or no water exchange, the environmental impact from nutrient-rich effluent waters on receiving streams is reduced or eliminated (Browdy *et al.*, 2001b). Enclosed minimal-exchange systems use considerably less land than that required for open pond culture, can serve as an excellent model for sustainability in aquaculture, and provide an opportunity for inland culture operations(Browdy and Moss, 2005).

Sturmer *et al.* (1992) and Samocha *et al.* (1993a) described the first nursery prototype system developed at the Texas AgriLife Research Mariculture Laboratory at Flour Bluff, formerly known as the Texas Agricultural Experiment Station, Shrimp Mariculture Research Facility (TAES-SMRF), Corpus Christi, Texas. This intensive nursery prototype system has six 40 raceways enclosed in about 1,000 m~ Quonset-style greenhouse structures with translucent fiber glass roofs. Each raceway is 30.48 m long (bottom length only 28.04 m), 3.38 m wide (bottom width 2.44 m), and has a mean depth of 0.55 m and mean water

depth of 0.4 m. The raceways have sloped concrete reinforced walls and sand bottoms, all covered by an impermeable membrane (e.g., HDPE & EPDM). Every raceway is equipped with a 2 hp centrifugal pump, a pressurized rapid sand filter (PRSF), and a center partition positioned over a 5.1 cm schedule 40 PVC pipe. The bottom pipe has spray nozzles to enhance water circulation near the bottom. Eighteen 5.1 cm airlift pumps, grouped into six banks, are positioned on bothsides of the partition to enhance raceway water circulation. Additional aeration is provided by six1 m long air diffusers and a Venturi injector operated by the above mentioned pump.

While striving to reduce water exchange and improve biosecurity in the nursery phase, Samocha *et al.* (2002) described system modifications and new management practices that led to a significant reduction in water usage without compromising shrimp health and performance. As mentioned before, early studies with this nursery system used a pressurized rapid sand filter (PRSF) for filtration of the incoming water and for particulate matter removal from the culture medium. One of the main characteristics of this system was the high daily water exchange used during the nursery phase.

Cohen *et al.* (2005), while working with two raceways that were stocked at a density of 3,300 PL/m^3, reported weekly changes in selected water quality indicators over a seven-week nursery period. It is interesting to note that although high levels of nitrite-nitrogen (> 26 mg/L) were found in the culture water during the last two weeks of the trial, no adverse effects on shrimp survival were found in this study. Furthermore, the authors reported (Table 1) good shrimp growth (1.1 g juveniles), high survival rate (>98%), low FCR (<1), and high yields (>4 kg/m3), while using only low water exchange (1.1%/day). Keeping these shrimp in the nursery system for 50 days had no adverse effects on growth and survival in the grow-out phase. Good growth (1.32 g/wk with 21.2 g av. wt. after 106 d) and survival (80%) were reported when the shrimp were stocked (50/m^2) in HDPE-lined outdoor ponds operated with limited water exchange (Cohen *et al.,* 2005).

Table 1: Harvest results from a 50-day nursery trail at the AgriLife Research Mariculture laboratory in two nursery raceways stocked with *Litopenaues vannamei*.

RW	FCR	Mean Weight (g)	Yield (kg/m^3)	Survival (%)	Water Added (% Total Volume/d)
#1	0.86	1.12	4.25	97.5	1.1
#2	0.98	1.01	4.33	106.0	1.1

The transition into low water exchange practices in operating the nursery system required implementation of more efficient methods than sand filtration to regulate particulate matter concentrations in the culture medium. An early study (Handy *et al.,* 2004; Samocha *et al.*, 2004) evaluated three methods for particulate matter removal from the culture

medium (bead filtration, foam fractionation, and pressurized rapid sand filtration) in a 74-d nursery trial. Although high concentrations of total ammonia-nitrogen and nitrite-nitrogen were found in the culture water of the raceway operated with the PRSF during the trial, no adverse effects were noticed on shrimp health, growth, and survival. Furthermore, better shrimp results (e.g., higher mean final weight, greater yield, lower water usage, and lower FCR) were reported using the PRSF for particulate removal compared with the other two methods (Table 2). Although bead filters have been used successfully in the past for particulate matter control in recirculating aquaculture systems, the bead filter used in this study had numerous operational problems that prevented adequate particulate matter control. Thus it is safe to assume that the inferior results observed in the raceway operated with the bead filter can be attributed to the poor performance of the bead filter. Unlike the bead filter, the foam fractionator was very effective in removing particulate matter from the culture medium. Because of its high particulate removal rate the operation of the foam fractionator had to be halted about a week before the study termination as concentrations of the particulate matter in the water column became extremely low. It is interesting to note that this high removal rate of the particulate matter resulted in heavy unicellular algal bloom reaching concentrations as high as 5 x 106 cells/ml.

Table 2: Summary of 74-d intensive nursery study with Litopenaeus vannamei using three particulate matter (PM) removal methods under limited-discharge.

PM Control	PL/m^3	Initial Wt. (mg)	Days	Final Wt. (g)	Yield (Kg/m^3)	Survival (%)	New Water	FCR
Bead[1]	3,780	0.6	74	0.65	2.42	96.3	1.35	1.70
PRSF[2]	6,540	0.6	74	0.85	5.26	100.1	0.47	1.09
FF[3]	5,010	0.6	74	0.69	3.18	97.8	2.06	1.50

[1]R W operated w/ Bead Filter

[2]R W operated w/ Pressurized Rapid Sand Filter

[3]R W operated w/ Foam Fractionator

These high algal concentrations were associated with elevated nitrite-nitrogen levels which required more frequent water exchanges than the other two raceways to alleviate shrimp stress. As mentioned earlier, the particulate matter and the bacterial floc in particular can serve as supplemental food for the shrimp and help with the nitrification process (Avnimelech, 2006; Bratvold and Browdy, 1998: Hargreaves, 2006). When this system is operated with limited water exchange, the concentrations of particulate matter in these systems can reach very high levels (e.g., > 100 ml/L). Furthermore, since a significant amount of additional oxygen is required to support these bacterial floc, and because oxygen supplementation is expensive, special attention should be placed to maintain the correct balance between the bacterial floc and the amount of oxygen needed.

Samocha *et al.* (2006; 2007) reported successful use of molasses to prevent an increase in total ammonia-nitrogen (TAN) in the culture medium during a nursery study under limited discharge conditions. In this study, the amount of molasses added into the nursery tanks was calculated based on the assumption that 6 g of carbon are needed to convert 1 g of TAN into bacterial biomass (Avnimelech, 1999; Ebeling *et al.*, 2006). The authors cautioned that shortly after adding molasses, dissolved oxygen level greatly decreased, especially under limited water exchange practices. Careful monitoring and capacity to increase oxygen supply are strongly recommended to prevent oxygen depletion. Although adding a carbon source into the culture medium can be used to prevent the increase of TAN, a full cost-benefit analysis is strongly recommended for each system to determine the potential use of this management practice.

In an effort to improve nursery system management under limited discharge conditions, Mishra *et al.* (2008) performed another study on the effect of suspended-solids removal on system performance. In their 71-d study, four raceways were filled each with 40 m^3 and stocked with young (four to five-day-old) PL of *P. vannamei* at a density of about 4,000 PL/m^2 Two of the raceways were equipped with a home-made foam fractionator (FF) and were operated with a daily average water exchange of 3.35%, while the other two were operated without a FF and with a daily water exchange of 9.37%. Unlike the earlier studies, in order to avoid excessive removal of the particulate matter and bacterial floes, the foam fractionators were operated. It is interesting to note that under the conditions of this study, the mean nitrite-N concentration in the raceways operated with the FF and the reduced water exchange was significantly lower than the raceways operated with a higher water exchange (4.0 vs. 6.4 mg/L). Table 3 summarizes the shrimp performance in the two treatments. Shrimp in the raceways operated with a FF and the lower water exchange had a higher mean final weight (1.91 and 2.0 g vs. 1.73 and 1.43 g), greater survival (100 and 92.4% vs. 55.9 and 81.8%) and yield (7.64 and 6.89 kg/m^3 vs. 3.92 and 4.74 kg/m^3), and with lower FCR values (0.97 and 1.06 vs. 1.36 and 1.64) than those operated without FF and with higher water exchange rate. Furthermore, disease diagnostic results of samples collected before the harvest showed no signs of viral pathogen infection. However, histological preparations showed greater external fouling and higher intestinal bacterial loads in shrimp which were cultured in the higher water exchange raceways than those maintained at lower water exchanges.

Table 3: Mean weights, yield, survival and FCR values (± Standard Error) of *Litopennaeus vannamei* in a 71-d nursery trial.

Treatments	Initial Wt. (mg)	Final Wt. (g)	Yield (Kg m^3)	Survival (%)	FCR
Foam Factors[1]	0.6	1.96±0.34[a 5]	7.23±0.56[a]	96.2±9.54[a]	1.03±0.15[a]
Water Exchange[2]	0.6	1.58±0.34[d]	4.33±0.56[b]	68.9±9.54[a]	1.50±0.15[a]

[1]Raceways operated with foam fractionators and 3.35% average daily water exchange.
[2]Raceways operated without foam fractionator and 9.37% average daily water exchange.
[3]Columns with same superscript letters suggest no statistically significant differences (α=0.05)

The following summary describes several other intensive nursery systems that were built according to the basic AgriLife Research conceptual design and were used by shrimp producers in the U.S. and in other countries. Harlingen Shrimp Farms, Los Fresnos, Texas, built one of the first intensive nursery-system prototypes in 1990. The system was constructed based on encouraging results from the stocking of grow-out ponds at this farm with juveniles of *P.vannamei* from the intensive nursery facility of AgriLife Research Mariculture Laboratory. Of significance was the fact that good growth and survival were observed at harvest of the grow-out ponds stocked with these juvenile shrimp although they experienced six hour transfer from the lab to the farm site. The farm's nursery system had a total of four 210 m2 (260 m3) raceways which were built inside a greenhouse covered HDPE-lined pond. Every raceway was equipped with a center partition. Water circulation and aeration was provided by one 1 hp paddlewheel aerator, a bank of four 5.1 cm airlift pumps and airstones. Good survival and FCR results were obtained when PLwere stocked at low (4,000-4,400 PL/m^2) and high densities (11,200 PL/m^2). As documented earlier, the higher stocking density resulted in smaller shrimp than those raised at lower density (Table 4). Although the use of the "headstart" program enabled the farm to produce two shrimp crops per year, because of the low prices of the smaller size shrimp, the farm is not using this system anymore.

Table 4: Performance of *Litopenaeus vannamei* PL in a green house-enclosed nursery raceway system, Harlingen Shrimp Farms

Indicator	High Density	Low Density
Stocking Density (PL/m^2)	11,200	4,000-4,400
PL Age at Stocking (days)	10	30
Nursery Duration (Days)	27	36-44
Harvest Density (PL/m^2)	10,000	3,400-4,100
Mean Final Weight (mg)	94.0	666-920
Biomass Load (Kg/m^2)	0.94	2.29-3.35
Survival (%)	89.4	85.6-94.0
FCR	0.87	0.77-0.84

Samocha and Benner (2001) described two greenhouse-enclosed nursery raceways (each, 65 m, 71m), which were built by R&G Shrimp Farm, Port Lavaca, Texas. Although a greenhouse-enclosed raceway system can be expensive to construct, the farmer was able to build the greenhouse, the two raceways and the needed equipment (two pressurized

sand filters, two pumps, an air blower etc.) for less than $10,000. The raceway structure was made from 10 cm x 10 cm wooden posts, plywood sheets, and an HDPE liner. A double-layer polyethylene inflated cover, using a small air blower, provided an additional inexpensive thermal insulation for the system during the nursery period.

Table 5 summarizes the production results from a 34-d trial conducted with two stocking densities where low FCR with high survival and yields were obtained under a daily water exchange of about 15%/d. This system setup enabled the producer to purchase PL early in the season, when prices were still low, and also benefit from stocking his grow-out ponds with nursed PL which translated into shorter grow-out cycle and higher sell price.

Table 5: Summary of a 34-day intensive nursery production in greenhouse- enclosed raceways, R&G Shrimp, Port Lavaca, Texas.

Stocking		Harvest			FCR
PL/m^2	Wt. (mg)	Wt. (g)	kg/m^2	Survival (%)	
42,000	1	0.1	5.62	101	<1
39,000	1	0.1	5.59	98	<1

Samocha *et al.* (2003) reported another successful use of nursery raceways for a low salinity farm (2.5-3.0 ppt). at Loma Alta Aquaculture, near San Perlita, Texas. A heavy infestation of dragonfly larvae that preyed on the young PL was the primary reason for the poor survival of the shrimp in the grow-out ponds. To overcome the problem the farm built small greenhouse-enclosed lined raceways at the corner of each pond to keep the shrimp for 30 to 35 days prior to the release into the grow-out pond.

The farm constructed and operated several nursery raceways of two sizes (90 m^2 and 180 m^2 with 114 m and 228 m, respectively). These raceways were filled with diluted seawater to facilitate the transition from 30 ppt water to the farm low salinity water. Air diffusers and airlift pumps, which were positioned on both sides of the center partition, generated aeration and water circulation. As was the case for the AgriLife Research nursery system, each raceway had a Venturi injector that was operated solely with atmospheric air. Since each raceway had only a limited number of airlift pumps, raceways bottoms had to be swept two to three times per week to re-suspend settled particulate matter. Except for makeup water that was used to offset seepage and evaporation, no water was exchanged for the first two weeks after stocking. About a week before moving the juveniles into the pond, new water was pumped from the farm's distribution canal to acclimate the shrimp to the farm's low salinity water. Raceways were stocked at densities between 10,000 and 15,000 PL/m^3 (11,700 to 19,400 PL/m). Throughout the production cycle, dissolved oxygen was maintained at 5 mg/L or above. Injection of CO_2

via air diffusers was used to control pH in the raceways during heavy algal blooms. When needed, kerosene heaters were used for short periods to prevent drop in temperature below 20° C. Ammonia and nitrite concentrations in the raceways were generally low. However, on several occasions, ammonia levels as high as 13.5 mg/L were recorded. For better estimation of survival, before releasing the juveniles into the grow-out pond, shrimp biomass in each raceway was determined along with the mean weight of the shrimp. The average survival in all nursery production trials varied between 80% and 97% with an average shrimp weight between 0.1 and 0.25 g, and FCR between 0.7 and 1.0. Of significance was the fact that shrimp survival in grow-out ponds stocked with juveniles from the nursery raceways was 71% compared with the 50% for the direct stocked ponds. In addition to the improved survival, the farm could take advantage of low PL prices extended during periods of low demand. The use of this nursery system enabled the farm to produce two crops a year of small size shrimp; however, one crop yielded larger size shrimp. As was the case for the R&G Shrimp Farm, the early stocking enabled the farm to harvest its ponds before farms without greenhouse-enclosed nursery systems and thus benefitted from the higher selling prices of its product.

An improved design of the AgriLife Research prototype was described by Samocha *et al.,*(1993b). This prototype was constructed out of concrete and tested in an inland commercial shrimp farm in Arizona. Samocha *et al.* (2004) provided a detailed description of the four 97.5 m (147.6 m) greenhouse-enclosed raceways that were used for nursery and grow-out of *P. vannamei* using low salinity ground water (1.8-2.6 ppt).

Table 6: Stocking and harvest data for postlarvae of *Penaeus vannamei* reared in greenhouse-enclosed raceways using low-salinity well water (Woods Brothers Shrimp Farm, Gila Bend, Arizona, USA).

PL Age (d)	RW	Stocking	Time (d)	Harvest	Survival (%)	FCR
		PL/m^2		Av. Wt. (g) kg/m^2		
15	1	19,200	34	0.10±0.08 2.34	100	0.70
15	2	20,400	35	0.09±0.07 2.10	100	0.7

The use of the nursery raceways provided the farm with off-season low PL prices and extended the acclimation period of the PL to the local water. It also added at least a month and a half to the production cycle in an area where temperature can be a limiting factor for shrimp production. Table 6 summarizes some of the data obtained in an early nursery trial. The data suggested that nursery of *P. vannamei* can be done in an inland facility using low salinity ground water with good survival, low FCR, and high yields. A follow up grow-out study showed that good survival (86%) and yield (4.39 kg/m) were obtained when juveniles harvested from the nursery trial were stocked in the same system.

The intensive nursery prototype of the AgriLife Research was also adopted by several producers outside the US including Panama (Camaco Shrimp Farm), Ecuador (Empagran, Semacua Hatchery & Ficacua), and Mexico (Industrias Pecis, Yucatan). Samocha *et al.* (2000a,b) described one of the large hatcheries in Ecuador, which was retrofitted with twelve 120 m^2 HDPE-lined ponds through intensive greenhouse-enclosed nursery raceway systems. The new modifications and the management protocols were based on the design and practices used by the AgriLife Research Mariculture Laboratory. Every raceway had a center partition that was positioned over a bottom pipe with 80 spray nozzles. This bottom pipe was connected to a Venturi injector and a 3 hp centrifugal pump for delivery of oxygen rich water across the length of the raceway. A rapid 454 LPM sand filter served for water filtration. Water circulation around the center partition was generated by six banks of 7.6 cm PVC airlift pumps. In addition to the aeration provided by the airlift pumps, every raceway had six 0.9 m long air diffusers. To increase biosecurity and to minimize the risk of viral-pathogen introduction, nursery water was chlorinated (10-15 ppm) before use. Nursery water was fertilized and inoculated with the diatom Chaetoceros gracilis, with initial density of 40.000 cells/ml few days before stocking. Operating the nursery system under this new management enabled the producer to cut back on daily water exchange from 300% to about 5%/d. The reduction in daily water exchange along with the water and feed management resulted in the harvest of very healthy shrimp with good survival and elimination of the old practice, which called for a routine antibiotic treatment of the shrimp. Table 7 summarizes some of the physical and chemical characteristics of culture water found while working with the system.

Table 7: Physical and chemical characteristics of culture water in an intensive nursery system used by a commercial hatchery in Ecuador.

Indicator	DO (mg/L)	Temp. (°C)	pH	TAN (mg/L)	NO_2 (mg/L)	NO_3 (mg/L)
Normal Range	3.0-8.5	25-33	7.0-8.5	0-15	0-20	25-35
Range (Max-Min)	1.5-15.00	23-34	7.0-9.0	0-15	0-25	25-45

Table 8 summarizes some of the PL performance information from these trials. Although most of these trials were conducted with stocking densities below 66 PL/L, higher stocking densities (83 PL/L) were tested in a few trials.

Table 8: Stocking and harvest data from intensive nursery production trials with the Pacific White Shrimp in a commercial hatchery in Ecuador.

Indicator	Stocking Size (mg)	Stocking (PL/L) PL Age (d)	Harvest Size (mg)	Survival (%)	FCR	Nursery Duration (d)
Average	2.4	38.4 9.1	18	70.1	1.23	14
Minimum	0.9	12.1 6	11	33.6	0.72	8
Maximum	4.7	64.4 11	24	98.9	3.86	19

The effect of stocking densities on the average weights of the shrimp harvested from the nursery raceway system. As documented by other researchers, the increase in stocking densities resulted in reduction in the final mean weight of the shrimp at harvest. It is important to note that since better survival and yields were found in the grow-out ponds that were stocked with PL from the nursery raceway system, compared with direct stocking, and because of the limited nursery space, the hatchery had to keep the PL for a short duration (14 to 17 days only). The beneficial effect of stocking the grow-out ponds with PL from the nursery system compared to direct stocking. A 16% improvement in survival was reported for ponds stocked with PL from the nursery system. Because of the short nursery cycles, biomass load at harvest for the most part was below 2.5 kg/m^3. However, the system was designed to handle a much higher biomass load as demonstrated in several longer nursery trials (50 days) with lower stocking densities (6.3 PL/L), where high yields (4.1 kg/m^3) with good survival (85.7%) and low FCR values (1.1) were reported.

One of the first farms to use a greenhouse-enclosed intensive nursery raceway system in Mexico was Industrias Pecis, Yucatan, Mexico. This facility had 24 raceways in six separated greenhouse structures. Raceways were lined with 1 mm HDPE membrane with a bottom area of about 130 m^2 and working volume of about 80 m^3. A full description of the culture system, the system process, stocking procedure and the day-to-day management is provided by Samocha *et al.* (2001). The farm used this nursery system mostly to eliminate the weak PL before stocking the grow-out ponds. In most cases, raceways were stocked at a density of 35,000 PL/m^3 (25,000 PL/m^2) with a typical nursery duration of three to four weeks. Raceways bottoms were siphoned daily with a water exchange of up to 50%/d. A heat exchanger was used during the cold months of the year to maintain adequate water temperature in the raceways. Table 9 summarizes the typical concentrations of selected water quality indicators during the nursery phase. Table 10 shows the beneficial effect of using this nursery system on the farm performance.

Table 9: Concentrations of selected water quality indicators during nursery production trials in Industrials Pecis Shrimp Farm, Mexico.

DO (mg/L)	Temp (°C)	Salinity (ppt)	Algae (cell/ml×10^3)	NO_2 (mg/L)	NO_3	NH_3-N
>5	26-30	8-9	450-750	<1	<5	<0.4

Table 10: Effect of postlarvae source (nurse facility vs. hatchery) on farm performance (Industrials Pecis, Mexico, courtesy of Mr. Figueras)

Postlarvae Origin	Stocking size (mg)	Av. Survival (%)	Av. FCR	Cycles per Year
Nursery raceways	150-200	75	1.4	3.2
Hatchery	1-5	55	1.9	2.3

CONCLUSIONS

According to FAO (2006) there are different factors which are driving the aquaculture industry to intensify. One of the main forces is the unavailability of sites. Water availability, site environmental carrying capacity, and regulatory issues all have to be addressed. The industry continuously looks for more efficient ways to use available resources. This intensification requires more education and training, greater institutional support, specialized services, and skilled people to manage these systems. In addition, there is a trend for development and implementation of safety and quality standards for the industry. A good example is the implementation of the Hazard Analysis and Critical Control Point (HACCP) by the US which is designed to improve aquatic animal health management and food safety records. Obviously,there will be further development of codes of practice and better management practices (BMPs) in collaboration with producers. It should be mentioned that in many countries, instead of high yield per unit area, aquaculture is now aiming more on economic sustainability and overall competitiveness.

This paper briefly describes nursery systems used by commercial farmers and researchers indifferent parts of the world. Special emphasis was placed on the use of intensive nursery systems and how these systems can increase biosecurity, sustainability and optimize outputs. The major driving forces of these intensive nursery systems arc water circulation, high oxygenation, and suspension of the particulate matter along with careful monitoring of feed consumption. The data presented suggests that good survival, high yields, and low FCR of the Pacific White Shrimp can be expected when using viral-pathogen-free PL under limited water exchange conditions. Certainly, the use of intensive nursery systems can help improve shrimp production practices,especially when combined with better health management, use of high quality postlarvae, and higher quality feed.

CHAPTER

14

SMPs FOR PROFITABLE AND SUSTAINABLE VANNAMEI FARMING IN INDIA

S. Felix and M. Menaga

INTRODUCTION

Shrimp farming sector in India is highly remunerative and a fast growing sector with 50240.77 ha area under the culture which yields an export production of 353413.1 MT with an enormous scope for increasing the foreign exchange. Although higher production levels are needed, increased aquaculture production is limited globally by the availability of suitable land and water. Most of the Southeast Asian countries like Thailand, Vietnam, Indonesia were also culturing *Penaeus monodon* and since 2001-02 onwards most of them have shifted to culture of exotic White leg shrimp, *Penaeus vannamei* because of the availability of Specific Pathogen Free (SPF) and Specific Pathogen Resistant (SPR) broodstock. In India, Pilot-scale introduction of *P. vannamei* was initiated in 2003 and after a risk analysis study large-scale introduction has been permitted in 2009. The commercial farming of Pacific white shrimp production started from the year 2009-10 is the largest cultured shrimp in terms of production and productivity. Andhra Pradesh tops in the area under culture and production followed by Tamil Nadu and Gujarat

Though the scientific measures are useful to boost production, the first and foremost consideration, to promote shrimp farming in the country is to adopt standard management practices. Despite the huge water area that can be brought under shrimp farming and the technological break-through in the production of post larvae and their culture to a marketable size, there are a number of problems to be solved before any large scale commercial shrimp farming can be undertaken in this country. It is heartening that the vast potential of shrimp production has awakened not only the shrimp farmers but also the development agencies of Government and private sectors.

Standard Management Practices

Standard Management Practices (SMP) in the aquaculture context outline norms for responsible farming of aquatic animals, the implementation of which is voluntary. SMPs are not a certification standard. SMP implementation improves the quantity, safety and quality of products taking into consideration animal health and welfare, food safety, environmental and socio-economical sustainability. Implementation of SMPs can help to achieve compliance with quantifiable standards and indicators set by international agencies and third party certification bodies.

SMP defines the code of conduct of responsible aquaculture with possible critical control points to prevent the outbreak of diseases. The management of the shrimp farms by adopting the standard management practices will enhance the shrimp production and mimicking of the natural ecosystem will be more easier. These SMPs provide a voluntary set of standards and procedures for improving production while helping to preserve the environment. They are a key in the factor that has come to be known as "sustainability".

PRINCIPLE OF SMP

- Emerging through experience to make sure that aqua farm do not negatively impact the environment, and become a sustained economic activity with significant positive impact on social wellbeing.
- Farming activity are to be technologically sound, environment friendly economically viable and socially acceptable.
- SMPs are the guidelines to meet these requirements of sustainability.

Categories of SMP are in Shrimp Farming

SMPs were designed to be simple, practical, affordable and cost-effective. Special attention was given to addressing the needs of small scale producers. In developing SMP, several categories of possible impacts and suggested for each category in shrimp farming. Producers can select as per the sites and operations

- SMP for site selection
- SMP for Farm Design and Construction
- SMP for pond preparation
- SMP for Water quality and its management
- SMP for Seed Selection and stocking
- SMP for Nursery rearing and Management
- SMP for Feed and its management
- SMP for Health management of shrimps

- SMP for use of chemicals and drugs
- SMP for Wastewater management

SMP for site selection

- Large-scale shrimp aquaculture may bring in excessive demand on land resources, resulting in multi-user conflicts. Construction of shrimp farms may make inroads into agricultural land. The conversion of agriculture land for aquaculture should be avoided. Construction of shrimp ponds on marginal land not fit for cultivation alone can be adopted for shrimp farming.
- Generally clayey loam soils are preferred. High capital and operational cost will be involved in maintaining a farm in sandy area, which is also to be avoided owing to the high water percolation through the sandy soils, and possible environmental damage which could arise from it.
- Further, the topography of the soil and its contour should be ascertained in relation to the water intake and drainage points as well as construction costs. A better site is the one, which involves lesser capital investment for constructing fully drainable ponds.
- The quality of soil should be ascertained for soil pH, permeability, bearing capacity and heavy metal content. Soil with low pH of below 5 (example acid sulphate soils) should be avoided. Similarly, soils with high concentrations of heavy metals also should be avoided. Use a soil pH meter to test the soil pH.
- Soil should be wet while using this equipment. Do not turn the soil (tilling) in acidic soil. If done, the soil will be more acidic. Wash the soil by water intake and drain 2-3 times to reduce the soil acidity. Use the dolomite or agricultural lime to neutralize soil acidity

The suitable soil characteristics ideal for construction of a shrimp farm are as follows:

pH	Organic Carbon	Calcium carbonate	Available nitrogen	Available Phosphorus	Electrical conductivity
7-8	1.5 – 2.5%	> 5%	50–75 mg/100 g soil	4–6 mg/100 g soil	> 4 µmhos

SMP for Farm Design and Construction

A site-specific approach to design and construction of shrimp farms is necessary, as site characteristics vary greatly from place to place. The following standard management practices should be considered while designing and constructing shrimp farms:

- Embankments should be designed to prevent flooding and erosion, after taking into consideration the tidal amplitude, water current, wind direction, wave action and the past histories of flooding in the area during cyclones/ storms.

- In soils, which are seepage prone, design should include an inner clay core in the dykes with greater compaction and trench around the farm to reduce saline water intrusion into the neighbouring lands.
- The elevation of the pond bottom, drainage canal and the outlet should be designed in such a way that the water in the farm can be drained fully and easily through gravity.
- Ponds should have separate intake and outlet structures to permit control of filling and draining.
- A minimum water depth of 80-100 cm should be maintained in the ponds.
- Inlet and discharge canals should be separate so that water supply and wastewater are not mixed. In areas where such a provision cannot be made, it is advisable that waste treatment pond should be included in the design.
- The farm design should not alter natural water flows, or impound floodwater.
- The sluice gates should be watertight and provided with net filters.
- Where possible, vegetative buffer zones, riparian vegetation and habitat corridors should be maintained and vegetative cover provided on exposed earthwork.
- Pump intakes should be screened, vegetative buffers provided around pump stations, and containments installed to prevent fuel spills.

SMP FOR POND PREPARATION

- Dry the pond completely for the next crop. It helps in removing the disease carrying crustaceans from previous crops in the pond. Sun drying kills fish/ crustacean and their eggs in the soil .Helps in oxidizing the organic matter thus reducing the sludge
- Remove the organic waste from the pond bottom and it is easy to remove when the soil is semi dried (slightly wet). If the organic waste can't be removed at one time (e.g. because of high costs of removal) remove a proportion of it.
- The removed organic bottom soil should be disposed at outside the pond. Ponds with acid-sulphate soils are repeatedly dried and flushed, to remove the acids formed by pyrite oxidation.
- The tilling and ploughing of the pond bottom should be done either manually or by machines
- Application of lime is useful in correcting the pH of the soil and water, as a disinfectant and for increasing the mineralisation process. If the soil pH is not below 7.5, a basal dose of 300-500 kg/ ha can be applied.

SMP FOR WATER QUALITY AND ITS MANAGEMENT

The following standard management practices are to ensure that the harmful effects of these practices are reduced.

- Good water quality should be maintained by using water stable feed with minimal wastage.
- Water quality parameters should be monitored regularly and periodical water exchange is necessary to maintain optimal water quality conditions.

Optimal levels of water quality parameters for shrimp farms

Sl. No.	Water Quality Parameters	Optimal Level
1.0	Temperature (^{o}C)	28 – 33
2.0	Transparency (cm)	25 – 45
3.0	pH	7.5 - 8.5
4.0	Dissolved oxygen (ppm)	5 – 7 (above 50% air saturation)
5.0	Salinity (ppt)	15 – 25
6.0	Total alkalinity (ppm)	200
7.0	Dissolved inorganic phosphate (ppm)	0.1 - 0.2
8.0	Nitrate - N (ppm)	< 0.03
9.0	Nitrite - N (ppm)	< 0.01
10.0	Ammonia - N (ppm)	< 0.01
11.0	Cadmium (ppm)	< 0.01
12.0	Chromium (ppm)	< 0.1
13.0	Copper (ppm)	< 0.025
14.0	Lead (ppm)	< 0.1
15.0	Mercury (ppm)	< 0.0001
16.0	Zinc (ppm)	< 0.1

- Fill the water using water filter nets. Always use double layer of fine mesh filter net (300 micron mesh size) to filter the water at water inlet point (water gate).
- After filling the pond, hold water for 10 to15 days before stocking the seeds.
- If filling pond from reservoir, hold water in reservoir 7 days before shifting to grow-out pond
- Maintain a water depth of more than 80 cm in shallowest part of pond.
- Fertilisers and lime should be used in a responsible manner only when it is actually required. Do not apply lime and fertilizers together

SMP in Seed Selection and Stocking

- Prefer PL-12 stage or older (total body length should be more than 12 mm). Smaller sizes may not be ready for stocking and may quickly die in the pond.
- Shrimp seed should be uniform in size and dark or light brown colour. Seed with red, blue or green colour must be rejected.
- Shrimp seed should be strong and active (swimming against the water current). Shrimp seed should pass a salinity stress test
- Shrimp seed should have full gut and well developed hepatopancreas. Collect 10-20 seed. Observe under a magnifying glass or microscope. The gut should not be empty. The hepatopancreas should not be small and light in colour.
- Stock the seed in deeper part of the pond, not in shallow water.
- Acclimatize the seed in pond water before releasing
  - Keep the seed bags floating in water for 20-30 min.
  - Slowly mix the pond water in seed bag during next 30 min, and then release the seeds to pond.

SMP in Nursery rearing and its management

Nursery ponds are usually positioned in farms close to the grow-out ponds to avoid long transfer trips. In many cases, nursery ponds are built adjacent to the grow-out ponds so that the shrimp are transferred without the need for hauling tanks. Thus nursery ponds continue to help assess seed quality and provide better space utilization with more control over the environment.

- Do not use the juveniles from poorly managed commercial nurseries. It may lead to high chances of importing disease.
- Avoid juveniles from the poorly managed commercial nurseries.
- Maintain on-farm nursery. i.e., small nurseries within the farms.
- Nurse the hatchery seed in a small earthen enclosure within the farm (on-farm nursing) for 10-15 days or with the Nursery raceways for the super intensive shrimp farming

SMP in Feed and feed management

- Feed ingredients should not contain contaminants, anti-nutritional factors, microbial toxins, banned antibiotics or other adulterating substances.
- Farm-made wet diets should not be used. However, when wet feeds are used crustaceans should be avoided as an ingredient.

- Only dry, nutritionally balanced pelleted feed with optimal water stability should be used.
- Freshly obtained feed should be used to the extent possible. In any case feed stored for more than two months should not be used. Feed should be stored in cool, dry areas to prevent mould and other contamination.
- Feeding rates should be determined from standard feed curves/charts (Table 3 above) and adjusted for shrimp biomass on a weekly basis.
- Feed check trays should be used to regulate feeding rates. Feed trays should be widely distributed in the pond.
- Since the shrimps require about 4 hours for digestion of feed, feeding frequency should be 4 – 6 times in a day. Since shrimps are nocturnal, more than 60 percent of the feed should be fed during night.

SMP in Health management of Shrimps

- Any disease should be diagnosed immediately with the help of trained pathologists/ microbiologists.
- On a daily basis record the number of diseased and dead shrimp.
- Remove the diseased and dead shrimp and burry them far away from the pond site.
- Do not throw away the dead and diseased shrimp in water canal or on open places.
- Do not apply any chemicals/medicines without advice from shrimp health specialists.
- Chemical treatments that can stress the animals should not be employed.
- For non-infectious diseases related to pond conditions, treatment of animals should be carried out or pond conditions corrected.
- For serious infectious diseases that may spread widely, the pond should be isolated, remaining shrimp should be net harvested and the pond should be disinfected without discharging any water.
- Dead and diseased shrimp should be disposed off in a sanitary manner that will discourage the spread of disease.

SMP FOR USE OF CHEMICALS AND DRUGS

Use of chemicals: Chemicals should be avoided in shrimp ponds for prevention or treatment of disease, as feed additives, disinfectants, for removal of other fish or for treatment of soil or water. However, chemicals may be required in hatcheries. The hatchery operators should carefully monitor entry of such chemicals into the natural waters from the hatcheries and they should take steps to remove such materials from the wastewaters.

Use of fertilizers: Both organic and inorganic fertilizers are used widely in shrimp culture for promoting the growth of fish food organisms, particularly for the early post-larval stages. This may contribute to the nutrient load in waters receiving the effluents. Therefore, as far as possible only organic manure/ fertilizers and other plant products should be used for such purposes.

Use of antibiotics/ drugs: The use of antibiotics in shrimp culture is strictly prohibited as their use may result in development of pathogens resistant to such drugs and the transfer of these pathogens into human beings might result in development of resistance in human pathogen

SMP in waste water management

- Proper designing of the farm with independent intake and outfall will reduce the nutrient loading.
- Proper compaction of bunds with vegetative cover should be provided which will reduce erosion.
- Proper pond preparation methods will reduce nutrient loads.
- Proper water and soil quality management in the culture ponds will reduce the nutrient loading of wastewater
- Responsible feed management will reduce feed wastage.
- During harvest, water should be drained carefully avoiding re-suspension of sediment.
- Shrimp pond wastewater should not be discharged into freshwater areas or onto agricultural land.
- Removing of sediments from the pond bottom should be avoided. It should be corrected in situ.

Effluent Treatment System (ETS) is mandatory for farms above 5 ha. At least 10 per cent of the total pond area should be earmarked for the ETS which may be used for secondary aquaculture projects, particularly for culture of mussels, oysters, seaweed, other fin fishes, etc. Such integrated projects would help improving the wastewater quality, reducing the organic and nutrient loads and producing an additional cash crop.

Use about 10-hp of aeration for each 10 kg/ha of daily feed input. The key is to avoid dissolved oxygen concentration below 3 mg/L – preferably below 4 mg/L. In the case of EMS, the disease occurs early when aeration usually is not so critical. Position the aerators to avoid erosion. Aeration can be less at the beginning of culture period and increased over time. Less aeration usually may be used during the day.

CONCLUSION

Developments in aquaculture are gaining considerable momentum in India through innovative and advanced technologies. However, we need to be cautious and conscious of the "Eco friendly approaches" while we direct our energies on improved and enhanced yield through aquaculture. Sustainable development has come to stay and it will be accepted as a viable concept to eradicate poverty and to improve the quality of human life while living within the carrying capacity of supporting ecosystem. Needless to emphasise that it is paramount importance in aquaculture that it has to be practised within the boundaries of its sustainable limits by adopting standard management practices.

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