Development of Zero Water Exchange Shrimp Culture System Integrated with Bioremediation of Detritus and Ammonia- Nitrogen

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by

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Ph.D. Thesis under the Environmental Studies

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Certificate

This is to certify that the research work presented in the thesis entitled "**Development of zero water exchange shrimp culture system integrated with bioremediation of detritus and ammonia- nitrogen**" is based on the original work done by Mr. Haseeb M under my guidance, at the National Centre for Aquatic Animal Health, School of Environmental Studies, Cochin University of Science and Technology, Cochin – 682016, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** and that no part of the work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the work presented in the thesis entitled "**Development of zero water exchange shrimp culture system integrated with bioremediation of detritus and ammonia-nitrogen**" is based on the original work done by me under the guidance of Dr. I.S. Bright Singh, Professor, School of Environmental Studies and Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin - 682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Dedicated to

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Haseeb.M

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Chapter 1

General Introduction

Globally, stagnating yields from capture fisheries and an increasing demand for fish and fishery products have raised expectations for increased contribution from aquaculture (Manoj and Vasudevan, 2009; Cullis-Suzuki and Pauly, 2010). The Food and Agriculture Organization of the United Nations (FAO) defined aquaculture as "the farming of aquatic organisms in inland and coastal areas, involving intervention in the rearing process to

enhance production" (FAO, 2011). More than 220 species of Shell fish and Fin fishes are farmed.

Since 1970 the global aquaculture production has increased 40 times and is expected to quintuple in the coming 50 years (Avnimelech *et al*., 2008; Bosma and Verdegem, 2011). Higher productivity has been made possible through technological changes and economizing production costs. Aquaculture has an important role in the development of national economies and plays a key role in rural development. It is expected that aquaculture will contribute and strengthen food security and alleviate poverty in many developing countries. The reported global production of food fish from aquaculture, including fin fishes, crustaceans, molluscs and other aquatic animals for human consumption, reached 52.5 million tons in 2008. The contribution of aquaculture to the total production of capture fisheries and aquaculture continued to grow, rising from 34.5 per cent in 2006 to 36.9 percent in 2008. In the period 1970–2008, the production of food fish from aquaculture increased at an average annual rate of 8.3 per cent, while the world population grew at an average of 1.6 per cent per year. World aquaculture output has increased substantially from less than 1 million tons of annual production in 1950 to the 52.5 million tons reported for 2008, increasing at three times the rate of world meat production (2.7% from poultry and livestock together) in the same period (FAO, 2010).

Globally penaeid shrimp culture ranks 6th in terms of quantity and second in terms of value amongst all taxonomic groups of aquatic animal cultivated. Over the past three decades, shrimp farming in Asia has been expanding rapidly to a vibrant export industry currently valued to more than US $ 8 billion (FAO, 2006).

1.1 Shrimp farming systems

1.1.1 Traditional systems

This is an age old practice with a variety of polyculture systems with a large component of miscellaneous fish and a small component of shrimps. These systems are fully tide – fed with salinity variations depending on the monsoon regime. Neither supplementary feeding and nor fertilization is done. Traditional culture is also known as trap and culture method. The entry of unwanted predators and other undesirable species, which compete for food with the commercial species, is one of the main disadvantages. Average production is low and ranges from 200 to 500 Kg/ha/year. The most well known of these systems are the bheries of West Bengal and the paddy-cum aquaculture systems of Kerala, Goa and Karnataka (Hein, 2002).

1.1.2 Improved traditional systems

In improved traditional system, the entry of unwanted organisms is controlled, supplementary stocking is done with the desired species of shrimp seed with the adoption of improved environment friendly technology. The production and productivity of the system can be increased with the yield levels varying between 1000 and 1500 kg/ha/season (Hein, 2002).

1.1.3 Extensive systems

Commonly found in Bangladesh, India, Indonesia, Myanmar, Philippines and VietNam. Extensive grow-out systems are established in tidal areas where water pumping becomes unnecessary. Ponds with an irregular shape according to land boundaries are generally larger than five hectares and easily constructed by manual labor for cost reduction. Wild seed, which either enters the pond through the sluice gate by the tide or are purchased from

collectors, are usually stocked at a density 2-3 / m². Shrimp feeds on natural foods that enter the pond regularly along with the tide and are subsequently enhanced by organic or chemical fertilizers. If available, fresh fish or molluscs may be used as supplementary feed. Due to the low stocking densities, larger sized shrimp (>50 g) are commonly harvested within six months or more. The yield is lowest in these extensive systems, at 50-500 kg/ha/yr.

Modified extensive systems lay out is as extensive systems, but involving pond preparation with tilling, liming, and fertilization and application of higher stocking densities, of the order of 5 to 10/m^2. Farmers often use a combination of local feeds or locally produced or imported pellet feeds. One or two crops of 600 to 1100 kg/ha can be harvested per year. Water quality is maintained at optimum levels through daily water exchange through concrete sluices fixed at appropriate places or through pumps.

1.1.4 Semi intensive systems

This system is dependent upon reliable shrimp seed supply, preferably from hatcheries, well formulated shrimp feed in addition to the natural food. Ponds of 2-3 ha with 1 – 1.5 m depth are used and commonly stocked with hatchery-produced seeds at the rate of 5 to 20 PL/m². Water exchange is regularly carried out by tide and supplemented by pumping. Natural food organism in the ponds are enhanced by applying organic manures such as cow dung, poultry droppings and inorganic fertilizers like super phosphate, urea etc. Extraneous materials such as water conditioners, probiotics etc, are used in this system to enhance the survival and growth. The duration of culture period is 4 - 5 months. Production yields range from 500 to 4000 kg/ha/yr. Semi intensive system is no longer recommended due to nutrient loading resulting in

eutrophication of recipient water bodies, environment degradation and emergence of diseases.

1.1.5 Intensive systems

It is the most sophisticated system requiring high financial and technical inputs. This culture system is found in all shrimp producing countries and is commonly practiced in Thailand, Philippines, Malaysia and Australia. Ponds are generally small (0.1 to 1.0 ha) with a square or rectangular shape. Stocking density ranges from 25 to 100 PL/m². Daily water exchange is carried out by pumping. Heavy aeration, either powered by diesel engines or electric motors, is necessary for internal water circulation and oxygen supply for both animals and phytoplankton. Feeding with artificial diets is carried out 4-5 times per day followed by feed tray checking. Production yields of 4000 to 15000 kg/ha/yr are common in place. High stocking densities and heavy feeding lead to over crowding and stress on the culture stock accompanied by nutrient loading. These factors not only affect the final production but also lead to serious environmental and health problems manifested through a host of diseases. The trend towards intensive shrimp aquaculture has been developed due to the anticipated high profit from farmed shrimp but high capital cost and operating costs make intensive shrimp farming a risky proposition.

1.2 Brief account of shrimp farming in India

India is in the 5th position in terms of aquaculture production of Penaeid shrimp. The potential area available in the coastal region of the country for shrimp farming is estimated between 1.2 million to 1.4 million hectares. Presently an area of about 157,000 hectares is under farming, with a production of about 100,000 metric tons of shrimp per year. The productivity

has been estimated at 660 kg per hectare per year. India's coastline is 8118 km with 2.02 million km^2 of exclusive economic zone, leaving large areas still unexploited efficiently for aquaculture.

Aquaculture practice in India is invariably extensive farming. Relatively smaller groups of farmers practice semi-intensive and intensive farming. Traditional methods of shrimp farming such as prawn filtration in Kerala, Bhasa Bhada Fisheries in W. Bengal have been in practice in India for a longer period of time before the advent of modern shrimp farming. Much of shrimp farming is still practiced mainly as an enterprise of small and marginal farmers who depend on backwaters and estuaries on the coastal zone. About 91% of the shrimp farmers in the country have a holding of less than 2 ha, 6% between 2 to 5 ha, and the remaining 3% have an area of 5 ha and above. The major methods of shrimp farming practiced are traditional, improved traditional in farms within the coastal regulation zone and extensive and modified extensive farms outside the coastal regulation zone. A predominant percentage of culture production is that of Tiger shrimp (*Penaeus monodon*), followed sparsely by white shrimp (*Penaeus indicus*), banana shrimp (*Penaeus merguiensis*) and *Litopenaeus vannamei*. Development of shrimp farming has also led to a substantial rise in the number of shrimp hatcheries and development of accessory industries such as feed mill and production of farm equipments. Shrimp farming provides direct employment to about 0.3 million people, and ancillary units provide employment to 0.6 – 0.7 million people. There is a definite close-knit relationship between the community and shrimp farming (Manoj and Vasudevan, 2009). Since much of shrimp farming in India is practiced by farmers, dependence on the governing agencies and independent help groups for resources, technology, and technical knowledge is greater.

Over the last decade Indian shrimp farming industry has transformed from a traditional shrimp trapping system to a capital oriented semi intensive system due to ever increasing consumer demand, high foreign exchange and stagnation in the wild catch (Abraham and Debasis, 2009).

1.3 Environmental problems of shrimp culture

Shrimp aquaculture is fraught with environmental problems that arise from: (i) the consumption of resources, such as land, water, seed and feed; (ii) their transformation into products valued by society; and (iii) the subsequent release of waste into the environment (Kautsky *et al*., 2000). The direct impacts include release of eutrophicating substances and toxic chemicals, the transfer of diseases and parasites to wild stock, and the introduction of exotic and genetic material into the environment. The environmental impact can also be indirect through the loss of habitat and niche space, and changes in food webs. Nutrient input to shrimp ponds by adding fertilizer and feed can result in the eutrophication of waters receiving the shrimp pond effluents (Funge-smith and Briggs, 1998; Chopin *et al*., 2001; Marinho-Soriano *et al*., 2011).

The global growth of aquaculture industries has resulted in competitions for natural resources such as water and land (Piedrahita, 2003). Apart from strong annual growth, the culture of the fish over the past few decades has also been strongly intensified. This intensification leads to the negative environmental impacts through the discharge of substantial amounts of polluting nutrients into adjacent waterways; a high proportion of these nutrients originate from the commercial feed and fertilizers (Heining 2000; Xu *et al*., 2008; Chavez-crooker and Obreque-Contreras, 2010).

Semi intensive and intensive production systems are heavily dependent on formulated feeds based on fish meal and fish oils. Formulated diets fed to shrimp consist of high amounts of protein (35–50%), nitrogen (6%) and phosphorus (2%). However, only 20-22% of feed assimilated in to shrimp tissues while the remaining is discharged as dissolved and particulate waste (Hanh *et al*., 2005). Based on a food conversion ratio (FCR) of 2.0 with a stocking density of 30-50 shrimp m^{-2}, 2 tons of shrimp feed would generate 1 ton of shrimp and waste of 900 kg of organic matter, 87 kg of nitrogen and 28 kg of phosphorus (Hanh *et al*., 2005) . Shrimps are poor converter of feed. Of the total feed given (dry weight), 15% is left unconsumed, 20% is lost as feces and 48% as metabolites with only 17% being incorporated into the flesh of shrimp (Primavera, 1994). Hence, more than 50% of the feed input into the pond goes to waste. This further leads to deterioration of the environment by reducing dissolved oxygen and increasing the ammonia and hydrogen sulphide levels (Wickins, 1976; Mevel and Chamroux, 1981).

Fertilization through organic and inorganic sources has become a management protocol in aquaculture. It compensates for the specific nutrient deficiencies and augments biological productivity mediated through autotrophic and heterotrophic pathways (Das and Jana, 1996).

The discharge of waste nitrogen from shrimp farms directly into adjacent environments has raised concerns globally about adverse environmental impacts stemming from such practices (Naylor *et al*., 1998, 2000; Cripps and Bergheim, 2000; Tacon and Forster, 2003; Crab *et al*., 2007; Amirkolaie, 2008). Effluents from aquaculture ponds contain living and dead particulate organic matter, dissolved organic matter, ammonia, nitrite, nitrate, phosphate, suspended soil particles, and other substances that can be considered potential

pollutants (Boyd, 1990; Hopkins *et al*., 1992; Avnimelech *et al*., 1994; Hargreaves, 1998; Samocha *et al*., 2004). Therefore, agencies responsible for water pollution abatement consider the aquaculture industry as a potential polluter (Samocha *et al*., 2004).

In addition to the environmental issues, the discharge of untreated pond effluent represents an economic loss of costly nutrients, thereby reducing farm profitability (Smith *et al*., 2002). One of the major challenges facing the shrimp farming industry is to overcome environmental concerns and to improve economic efficiency by developing and implementing an integrated approach to reducing nutrient waste. However, most of the nitrogen and phosphorous in shrimp culture ponds are organic and/or in particulate form. (Shimoda *et al*., 2005, 2007).

1.4 Risk associated with the use of chemicals.

Chemicals used in shrimp culture may be classified as therapeutants, disinfectants, water and soil treatment compounds, algicides and pesticides, plankton growth inducers (fertilizers and minerals) and feed additives. Excessive and unwanted use of such chemicals results in problems related to toxicity to non-target species (cultured species, human consumers and wild biota), development of antibiotic resistance and accumulation of residues (Primavera, 1998). Constraints to the safe and effective use of chemicals including misapplication of some chemicals, insufficient understanding of mode of action and efficacy under tropical aquaculture conditions, as well as uncertainties with regards to legal and institutional frameworks to govern chemical use in aquaculture (Barg and Lavilla-Pitogo, 1996). Pesticides and other products used in shrimp farming for their toxic properties can pose a risk

to wild flora and fauna, but also to the health of the shrimp culture (Graslund and Bengtsson, 2001).

The extensive use of antibiotics in shrimp farming contributes to the development of antibiotic resistant pathogens and these microbes infect both humans and domesticated animals (Holmstrom *et al*., 2003). Several studies have shown that number of antibiotics are moderate to highly toxic to aquatic organisms (Holten *et al*., 1999; Wollenberger *et al*., 2000). The abuse of antimicrobial drugs, pesticides, and disinfectants in aquaculture has caused the evolution of resistant strains of antimicrobial agents of pathogenic bacteria such as *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *E. ictaluri*, *Vibrio anguillarum*, *V. salmonicida*, *Pasteurella piscida* and *Yersinia ruckeri.* (Cabello, 2006).The antimicrobial agents chloramphenicol and nitro furan groups are no longer permitted in aquaculture industry because of their bioaccumulation and carcinogenic effects (Boontahi *et al*., 2011).

1.5 Diseases

Rapid development of shrimp culture has been accompanied by the occurrence of diseases induced both by natural and fabricated environmental changes. The important pre-disposing factors leading to disease outbreaks in shrimp culture are adverse environment, high stocking density, nutritional deficiency, inadequate aeration, insufficient water exchange, heavy algal blooms, physical injury and presence of high numbers of virulent pathogens (Alavandi *et al*., 1995; Guan *et al*., 2003; Sanchez *et al*., 2008). The majority of current shrimp diseases can be categorized into two major groups: viral and bacterial. Viral diseases are the most devastating because they are often difficult to be detected and impossible to be treated in ponds (Brock *et al*., 1997).

Of the more than 20 known penaeid viruses, five viruses cause the greatest economic loss. They are yellow head virus (YHV), white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and taura syndrome virus (TSV). In most cases, the only viable solution to viral diseases is to keep away the diseases from entering the culture system, and if infected clean up and disinfect.

Among all viral pathogens the most lethal one is the white spot syndrome virus (WSSV) (Flegel, 2009) which has no treatment, and prevention is the best way to avoid its outbreak (Menasveta, 2002). Studies done on WSSV risk factors were with respect to the carrier organisms (Lo *et al*., 1996; Kanchanaphum *et al*., 1998; Suppamataya *et al*., 1998; Corsin *et al*., 2001; Hossain *et al*., 2001; Yan *et al*., 2004; Liu *et al*., 2006), mode of transmission (Suppamataya *et al*., 1998; Peng *et al*., 1998; Corsin *et al*. 2001), effect of physico-chemical parameters of water and sediment (Vidal *et al*., 2001; Guan *et al*., 2003; Rahman *et al*., 2006; Reyes *et al*., 2007) and general conditions of the ponds (Tendencia *et al*., 2011).

The causative agents of bacterial diseases in aquaculture are mostly *Vibrio* spp. (Ruangpan and Kitao, 1991; Vandenberghe *et al*., 2003; Austin and Austin, 2007; Cano-Gomez *et al*., 2009). Among them luminous *Vibrio harveyi* is a major pathogen to the black tiger shrimp *Penaeus monodon* (Austin and Zhang, 2006). Even though, the primary etiological agent of luminous vibriosis is *V.harveyi* (Jayasree *et al*., 2006), *V.campbelli*, a non-luminous bacterium, also has been implicated in vibriosis (de la Pena *et al*., 2001). The acute infection usually occurs when shrimps are one month old and therefore farmers call it one month mortality syndrome. However, chronic *Vibrio* infections can occur during later stages of the culture as well, that too

till harvest, especially due to poor water and pond bottom quality conditions. When the problem occurs later in the crop cycle, it is often associated with "loose shell syndrome". In higher salinity, the severity is usually greater and is often caused by luminous species. "Loose shell syndrome" is probably a result of chronic bacterial infection. The affected shrimps usually are bigger in size and have a paper like carapace with a gap in between muscle tissue and carapace. The predisposing factor of this chronic disease is likely toxic pond bottom conditions. Shrimp diseases can easily enter a shrimp pond by one or more means, including seed, water, pond intruders, birds and mammals, feeds, people, equipment, frozen and fresh shrimp.

Viral and bacterial disease together with poor soil and water quality turns out to be the main cause of shrimp mortality (Liao, 1987; Chamberlian, 1997), although deficient environment management of shrimp farms is yet another important determinant (Flegel, 1996). The risk of disease seems to increase with intensity of farming, precisely the density of shrimp in the pond. It has been reported that disease occurrence in shrimp ponds in various places was closely associated with excessive stocking and poor water quality (Spaargaren, 1998), and there appears to be a clear linkage between environmental conditions and diseases (Krishnani *et al*., 1997; Kautsky *et al*., 2000; Gupta *et al*., 2002; Joseph *et al*., 2003).

1.6 Accumulation of nutrients and organic matter in pond soil

Pond bottom conditions change with time gets affected to a large extent by the accumulation of organic matter residues, such as dead algae, feces and feed residues, leading to high oxygen consumption and the development of reducing conditions (Boyd, 1995; Avnimelech and Ritvo, 2003). Pond

sustainability is determined, to a large extent, by the capacity of the pond bottom to metabolize the organic load before reaching a point of deterioration, when excessive accumulation of reduced components affects fish or shrimp growth. Excessive accumulation of organic residues leads to the development of anaerobic conditions, formation of reduced species, high sediment oxygen demand, deterioration of the pond and fish (or shrimp) growth inhibition (Avnimelech and Ritvo, 2003).

Organic matter settles to the bottom and is decomposed by microorganisms. Easily decomposable organic matters, such as simple carbohydrates, protein, and other cellular constituents, are degraded quickly. More resistant materials, such as complex carbohydrates and other cell wall components, accumulate because they degrade slowly. There is a continuous input of organic matter to the bottom, so microorganisms are continuously decomposing both fresh, easily degradable (labile) organic matter and older, resistant (refractory) organic matter as well. Because there is a more or less continuous resuspension and redeposition of particles and stirring of the surface sediment by fish and other organisms, the organic matter becomes rather uniformly mixed in the upper layer of sediment. Nevertheless, there usually is a layer of fresh organic matter at the sediment surface that has not been completely mixed into the sediment. Organic matter concentration usually is greatest near to the sediment surface (Munsiri *et al*., 1995). The ratio of labile organic matter to refractory organic matter also is greatest near to the sediment surface (Sonnenholzner and Boyd, 2000).

The nitrogen concentration of the organic matter is the net result of deposition of organic nitrogen, decomposition of the organic matter and flux between the water and sediment (Montealegre *et al*., 2005). The main sources

of organic nitrogen are uneaten feed, faeces and dead phyto and zooplankton. The amount of uneaten feed is difficult to be assessed in ponds. For fish cage culture, Philips *et al*. (1985) estimated feed spills of 15-30%. Boyd (1995) stated that uneaten feed was usually less than 5-10%, but more conclusive measurements were scarce; faeces generally accounts for 5-15% of the nitrogen ingested by fish of which 30-40% being incorporated in to fish biomass and 25–80% of which gets excreted as ammonia or dissolved organic nitrogen (Kaushik, 1980; Lovell and Konopka, 1985; Lovell, 1988).

Organic enrichment also can lead to an increased presence of pathogenic bacteria. Studies on surface sediments of a well-established fish farm showed that benthic bacteria levels were closely related to organic enrichment and their concentration was three times higher in stations beneath the cages. Counts (colony forming units (CFU)) of heterotrophic bacteria indicated a shift toward Gram negative bacteria, with a predominance of *Cytophaga/ Flexibacter*-like bacteria, and the occurrence of pathogenic bacteria (such as *Vibrio*) in sediments beneath the cages. In contrast, Gram-positive bacteria were more prevalent in the control site, where they represented up to 90% of total isolates (Vezzuli *et al*., 2002).

A small amount of organic matter in pond soil is beneficial, as it contributes to the cation exchange capacity of bottom soil, chelates trace metals, provides food for benthic organism, and releases inorganic nutrients upon decomposition. However, too much organic matter in pond soils can be detrimental because microbial decomposition can lead to the development of anaerobic conditions at the soil- water interface.

1.7 Improved shrimp grow out system

Number of methods have been used or suggested to minimize impacts of shrimp pond effluents and sustainable aquaculture (Porrello *et al*., 2003; Martinez-Cordova and Enriquez-Ocana, 2007; Ehler *et al*., 2007; Carvalho *et al*., 2009) in the environment. Wetland ecosystems have the ability to remove aquatic pollutants through a variety of physical, chemical and biological processes (Hussenott, 2003; Sindilariu *et al*., 2007). Constructed wetlands have been shown to have broad applicability as wastewater treatment systems. Schwartz and Boyd (1995) evaluated constructed wetlands for treatment of channel catfish pond effluents. Suggested advantages of such wetlands include low cost of construction and operation, elimination of chemical wastewater treatment, stabilization of local hydrologic processes and contribution of excellent wildlife habitat. Mangrove forests have also been used as nutrient sinks (Rivera-Monroy *et al*., 1999; Primavera *et al*., 2007). The main disadvantage in using constructed wetlands for treating aquaculture pond wastes is the necessity of large amount of space.

Integrated multi trophic aquaculture strategies have been described as a key development factor for aquaculture sustainability (Barrington *et al*., 2010). It integrates a number of complementary organisms at a farm site in order to optimize nutrient utilization and reduce solid waste that goes to sediments. Bioremediation of aquaculture effluents (Paniagua-Michel and Garcia, 2003; Troell *et al*., 2003), mainly by the use of filter feeding bivalves, microalgae and sea weeds (Jones *et al*., 2007; Muangkeow *et al*., 2007) have been experimented. Moreover, low or zero water exchange (Balasubramanian *et al*., 2005) and recirculation systems (Lezama- Cervantes *et al*., 2010) have also been tried in different places.

1.7.1 Recirculating aquaculture systems (RAS)

During the last 20 years, recirculation systems have been applied commercially to marine shrimp grow out on much larger scales than previously envisioned. Reasons for these applications include: control of diseases from source water and organisms in source water, scarcity of high-quality source water, control of water quality problems with source water, improved growth performance due to greater control over water quality parameters, including temperature, in some cases, and concerns about environmental degradation caused by shrimp pond effluents.

RAS has numerous advantages over the flow through and pond culture systems, including tighter control of water quality, temperature, and biosecurity, which improves survival and growth; enhanced effluent handling and discharge; water conservation; and reduction in the risks of introducing disease and pollutants (Skjølstrup *et al*., 2000; Menasveta, 2002). RAS with microbial floc would enhance the production characteristics of shrimp and fish and the culture becomes sustainable (Moss *et al*., 2000, 2001).

However, the RAS also has disadvantages. The most important is the deterioration of the water quality if the water treatment process within the system is not controlled properly. This can cause negative effects on fish growth, increase the risk of infectious diseases, increased fish stress, and other problems associated with water quality that result in the deterioration of fish health and consequently loss of production (Timmons *et al*., 2002). The water quality in RAS depends on different factors most importantly the source, the level of recirculation, the species being cultured and the waste water treatment process within the system (Losordo *et al*., 1999). Most water quality problems

experienced in RAS were associated with low dissolved oxygen and high fish waste metabolite concentrations in the culture water (Sanni and Forsberg, 1996). Waste metabolites production of concern includes total ammonia nitrogen (TAN), unionized ammonia (NH_3-N), nitrite (NO_2-N), nitrate (NO_3-N) (to a lesser extent), dissolved carbon dioxide (CO_2), suspended solids (SS), and non-biodegradable organic matter. Of these waste metabolites, fish produces roughly 1.0-1.4 mg/ L TAN, 13-14 mg / L CO_2, and 10-20 mg/ L TSS for every 10 mg/ L of DO that they consume (Hagopian and Riley, 1998).

1.7.2 Zero water exchange shrimp culture system

Zero water exchange aquaculture systems have become common in shrimp aquaculture because such methods can control disease outbreaks and environmental pollution more easily (Samocha *et al*., 2001; Mckinnon *et al*., 2002; Burford *et al*., 2003; Wasielesky *et al*., 2006; Ballester *et al*., 2010). Minimal exchange aquaculture system offers an environmentally attractive means of shrimp and fish production (Ray *et al*., 2010; Vinatea *et al*., 2010). Several successful examples of the implementation of zero water exchange shrimp culture systems can be cited including Belize Aquaculture Ltd in the western hemisphere (McIntosh *et al*., 2000). BAL developed a zero water exchange and recycle strategy to reduce the effluents and sediments that would be released in to the environment by a typical intensive shrimp farm. Lawrence *et al*. (2001) reported on the successful intensive culture of *Litopenaeus vannamei* on the White Spot Syndrome Virus infected farm in Panama. Balasubramanian *et al*. (2005) studied the zero water exchange shrimp culture system in the periphery of Chilka lagoon, Orissa, India. This approach addresses environmental questions raised by both society and the scientific community regarding sustainable development concepts which

demand a convergence of ecological prudence, economic efficiency and social equity in all human activities (Bailey, 1988; Mcintosh and Phillips, 1992; de Kinkelin and Michel, 1992; Currie and David, 1994; Primavera, 1994; Kestemont, 1995). However, production costs of closed systems are much higher than those of open systems. Moreover, environmental problems still remain when waste water is discharged from such farming systems.

Earlier common knowledge was that high rates of water exchange were necessary with shrimp culture to remove nitrogenous and other potentially toxic metabolic waste products and to prevent accumulation of potentially toxic organic sediments. With intensive culture, these wastes were thought to be one of the main limiting factors for shrimp production. Waste concentrations are related to feed input rates (Brune and Drapcho, 1991), or more specifically to feed quantity and protein content (Westerman *et al.*, 1993).

However, the water volume needed for even small to medium aquaculture systems can reach up to several hundreds of cubic meters per day. For instance, penaeid shrimp requires about $20m^3$ fresh water per kg shrimp produced (Wang, 2003). For an average farm with a production of 1000 kg shrimp ha^{-1} yr^{-1} and total pond surface of 5 ha, this corresponds with a water use of ca. 270 m^3 day^{-1}.

High water exchange rates through shrimp ponds, however, are not always environment friendly and do not always benefit shrimp culture. Water intake can entrain and/or impinge on biota, which are then lost. Pond influent/effluent waters may also carry shrimp diseases into ponds and discharge diseases from ponds into the environment. Nutrients and suspended

solids in effluents may cause eutrophication and sedimentation in receiving waters. High sediment loads in source waters may lead to pond depth shoaling, increased operating costs, and lost culture time to remove sediments. Exotic shrimp species may also escape to the environment with wastewater, and potentially become established and/or cause disease transmission.

When genetic improvements are made shrimp culture stocks, and these stocks are more widely used, "improved" shrimp stocks could jeopardize population genetics of wild stocks through escapement and interbreeding. Drain harvest typically contributes to the highest concentrations of solid materials in to receiving waters, especially in the last portion of the drain when sediments are resuspended and carried out with drain waters. The last 10 to 20% of pond drain waters have been reported to contain >60% of total settlable solids and >40% of suspended solids discharged during harvest (Teichert-Coddington *et al.*, 1999). Other discharges result from heavy rainfall, which overflows the outlet weir. In some cases, such as in arid areas or during dry seasons, water must be exchanged to maintain pond salinity within acceptable ranges for shrimp (Hopkins *et al.*, 1995).

In conventional flow through production system, 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; Burford *et al.*, 2004; Mishra *et al.*, 2008).

In limited discharge production system, the phytoplankton and the microbial communities can play a major role in recycling the auctochthonous nutrients accumulating within the system (Avnimelech *et al*., 1994; Wang, 2003; Burford *et al*., 2003). These microbial and phytoplankton communities are the major driving force behind the carrying capacity of these systems.

The most promising features of the zero water exchange culture systems are what they offer to both increased biosecurity and reduced feed costs and water use (Chamberlian and Hopkins, 1994), and by doing so increase the possibility of moving the shrimp culture industry along a path of greater sustainability and environmental compatibility (Tacon *et al*., 2002).

The major problem associated with decrease of water exchange is rapid eutrophication of the system resulting in increasing concentration of nutrients and organic matter over the culture period. Phytoplankton bloom crash will be common and re blooming will be comparatively fast, and there is still a period of stress to the shrimp due to the nutrients and organic matter enrichment over the culture period and oxygen drops could be ultimately making the environment toxic to shrimp (Lemonnier and Faniniz, 2006). The super eutrophic pond water can decrease the carrying capacity of the pond (Lin, 1995). After 120 days, stressful condition develops and growth rates are slower compared to open system. Obviously the balance between waste production and assimilative capacity in pond environment is of paramount importance for the success of closed system.

1.8 Water quality management

Shrimp culture production is frequently determined by the environmental condition of the pond especially water quality. It is the core of the

environmental problems experienced on farms since shrimps are strongly affected by the conditions in the water and on the pond bottom (Boyd and Fast, 1992; Chen, 1992) and that will influences optimal shrimp health, growth and yield (Jones *et al*., 2001; Case *et al*., 2008). Water quality in the ponds is determined by the initial water quality used for the culture, as well as the organic loadings in to the pond in the form of feeds and fertilizers during the culture cycle (Yusoff *et al*., 2011). It can be described as "physical, chemical and biological factors that influence the beneficial use of water" (Hernandez *et al*., 2011). The physical, chemical and biological phenomena present in the water dictate the dynamics of the culture (Hopkins *et al*., 1992; Sandifer and Hopkins, 1996; Browdy *et al*., 2001; Li *et al*., 2006).

Physico–chemical changes such as temperature, pH, dissolved oxygen, salinity and environmental pollutants have been reported to increase vulnerability to invading pathogens in decapod crustaceans; and the results of which have been reviewed by Cheng *et al*. (2003) and Cheng and Chen, (2002). pH is an important chemical parameter to be considerd because it affects the metabolism and other physiological processes of culture organisms. A certain range of pH (pH 6.8 – 8.7) should be maintained for acceptable growth and production. A diurnal fluctuation pattern that is associated with the intensity of photosynthesis occurs for pH. This is because carbon dioxide is required for photosynthesis and accumulates through nighttime respiration. It peaks before dawn and is at its minimum when photosynthesis is intense. All organisms respire and produce carbon dioxide (CO_2) continuously, so that the rate of CO_2 production depends on the density of organisms. The rate of CO_2 consumption depends on phytoplankton density. Carbon dioxide is acidic and it decreases the pH of water. Also, at lower pH, CO_2 becomes the dominant

form of carbon and the quantity of bicarbonate and carbonate would decrease. The consumption of CO_2 during photosynthesis causes pH to peak in the afternoon and the accumulation of CO_2 during dark causes pH to be at its minimum before dawn. It is interdependent with other water quality parameters, such as carbon dioxide, alkalinity, and hardness. It can be toxic in itself at a certain level, and also known to influence the toxicity of hydrogen sulfide, cyanides, heavy metals, and ammonia (Klontz, 1993).

Methods used to control high pH have included algicides (Osunde *et al*., 2003), chemical intervention with acids or buffers (Boyd *et al*., 1978; Pote *et al*., 1990), water exchange (McGee and Boyd, 1983), and mechanical stirring (Paerl and Tucker, 1995; Tucker and Steeby, 1995). However, each of these methods has serious risks or practical limitations. Algicides may be toxic (Osunde *et al*., 2003), flushing is commercially impractical, and buffers or acids temporarily address the symptoms but not the actual problem.

Oxygen is one environmental parameter that exerts a tremendous effect on growth and production through its direct effect on feed consumption, maturation and metabolism and its indirect effect on environmental conditions. Fluctuation of dissolved oxygen concentration and the possible oxygen depletion of aqua farm waters are clearly dependent on the size and intensity of the aquaculture operation (i.e. the oxygen demand of both the cultured stock and oxygen released) and on the topography - hydography of the water body. DO is always listed among the most important variables to be mandatory and measured. This relies on two basic principles. 1) a higher total weight of fish per unit volume of water can lead to increased activity and thus increased respiration as a result of overcrowding (Svobodova *et al*., 1993) and (2) oxygen depletion implies alterations in the ecosystem structure such as during

dystrophic crisis in stagnant shallow conditions like shrimp ponds. Oxygen affects the solubility and availability of many nutrients. Low levels of dissolved oxygen can cause changes in oxidation state of substances from the oxidized to the reduced form. Lack of dissolved oxygen can be directly harmful to culture organisms or cause a substantial increase in the level of toxic metabolites (Li *et al*., 2006). Low level of oxygen tension hampers metabolic performance in shrimp and can reduce growth, moulting and cause mortality (Soundarapandian *et al*., 2009). Le Moullac *et al*. (1998) reported that 24 h after the exposure to low oxygen concentration (1 mg O_2/L), there was a significant decrease in hemocyte count (reduced by 7.6%) compared to *L. vannamei* held in water with optional oxygen concentration. At the same time, hypoxic *L. vannamei* suffered higher mortality after infection with *Vibrio alginolyticus* compared to shrimp held in water with adequate oxygen levels. It is therefore important to continuously maintain dissolved oxygen at optimum levels of above 3.5 ppm.

In aquaculture, alkalinity is the measure of the capacity of water to neutralize or buffer acids using carbonate, bicarbonate ions, and in rare cases, by hydroxide, thus protecting the organisms from major fluctuations in pH. Without a buffering system, free carbon dioxide will form large amounts of a weak acid (carbonic acid) that may potentially decrease the night-time pH level to 4.5 (Boyd, 1990; Zweig *et al*., 1999; Shinde *et al*., 2011). During peak periods of photosynthesis, most of the free carbon dioxide will be consumed by the phytoplankton and, as a result, drive the pH levels above 10.0. Numerous inorganic (mineral) substances are dissolved in water. Among them, calcium and magnesium, along with their counter ion carbonate (CO_3^{-2}) comprise the basis for the measurement of 'hardness'. Hard waters have the

capability of buffering the effects of heavy metals such as copper or zinc which are in general toxic to fish. The hardness is a vital factor in maintaining good pond equilibrium.

The hardness is an important water quality parameter because a direct relation between water metal content and pH variations exists. When the concentration of Ca and Mg trends to be higher, the buffering capacity of the water becomes higher too, and is more capable to smooth pH variations. In other words, hard water is more stable than soft water. Calcium has an important role in the biological process of fish. It is necessary for bone formation, blood clotting, and other metabolic reactions (Wurst and Durborow, 1992). Fish can absorb calcium for these needs directly from water. The presence of free calcium at relatively high concentration in culture water helps reduce the loss of other salts (eg. Sodium and potassium) from fish body fluids. In the case of magnesium, it is used by photosynthetic organism because it is embedded in the center of the chlorophyll molecule, and it is also required as prosthetic group in proteins (Alatorre – Jacome *et al*., 2011)

Aquaculture organisms are cold-blooded animals. They can modify their body temperature to the environment in normal condition, unlike the warm-blooded animals, which can react to maintain the optimum body temperature. Temperature is probably the most important physical variable on aquatic ecology. It affects directly the growth, metabolism, oxygen consumption, moulting and survival (Boyd, 1995; Wyban *et al*., 1995; Henning and Andreatta, 1998; Ramanathan et al., 2005). Das and Saksena (2001) noted an inverse significant correlation of temperature with the growth and the low temperature negatively impacted shrimp metabolism.

As a consequence, temperature set the growth, development and reproduction rates in biological species. This fact is very useful in aquaculture: because fish do not expend energy on corporal temperature regulation, they can assimilate almost the food nutrients into muscular tissue (Soto-Zarazúa *et al*., 2011). As results of an adequate temperature condition, the biomass production and final yield of the fish farm can increase. The optimum range of temperature for the Black tiger shrimp is between 28^0C-30^0C.

It is important to remark the influence of temperature in fish respiration rate. A rise of temperature causes more oxygen consumption in bacteria, algae and fish. Because respiration implies carbon dioxide release and energy consumption, the gas balance can be dangerous for fish. If there are enough inorganic nutrients in the water, the algae biomass can increase to considerable levels. Even some algae species can double their biomass with in 3.5 hours (Brennan and Owende, 2010). Then, the elevated rates of nutrient assimilation will produce significant impacts on the water quality. On the other hand, a higher temperature results higher metabolic rates. The increment in fish metabolism enhances the protein breakdown. As a consequence, the release of NH_3 by fish will be high, too. The resulting combination of high temperatures with high concentration of NH_3 is very a toxic environment (Eshchar *et al*., 2006). Finally, if the exposure to high temperatures is very long, the structure of the proteins begins to break, causing fish death. Cheng *et al*. (2003) have shown that the appetite, survival rate, growth rate, and the digestive enzyme activities increase with the temperature from 18^0 to 30^0 C.

Salinity is another, important parameter to control growth and survival of shrimps. Even though *P. monodon* is an euryhaline animal it is comfortable when exposed to optimum salinity. At high salinity the shrimps will grow slow

but they are healthy and resistance to diseases. If the salinity is low the shell will be weak and prone to diseases. Muthu (1980), Karthikeyan (1994) and Soundarapandian and Gunalan (2008) recommended a salinity range of 10 - 35 g/L ideal for *P. monodon* culture. According to Chen (1980) salinity ranges of 15 -20 ppt are optimal for culture of *P.monodon*. There are a few reports (Ramakrishnareddy, 2000; Collins and Russel, 2003), which stated that *P. monodon* adapted quite well in fresh water conditions also because of its wide range of salinity tolerance. Salinity plays an important role in the growth of culture organisms through osmoregulation of body minerals from the surrounding water. The early life stages of both shrimp and prawn require standard seawater salinities but while growing they can with stand to brackish water or even to freshwater conditions. However, for better survival and growth optimum range of salinity should be maintained in the aquaculture ponds. Decreasing salinity may affect shrimp physiology and water quality parameters such as concentration of ammonia and nitrite. In *L.vannamei* ammonia – N excretion rate is reported to be lower at salinities of 25 g/L than at salinities of 10 g/L or 40 g/L and ammonia-N excretion of *P.chinensis* juveniles decreases with increased salinity, pH and ambient ammonia level (Decamp *et al*., 2003).

Phosphorus (P) is found in the form of inorganic and organic forms (PO_4) in natural waters. Inorganic phosphates include orthophosphate and polyphosphate while organic forms are those organically-bound phosphates (Van wazer, 1973). Phosphorous is a limiting nutrient needed for the growth of all plants - aquatic plants and algae alike (Hu *et al*., 1989; Barak *et al*., 2003; Neill, 2005; Khoi *et al*., 2008). However, excess concentrations especially in rivers and lakes can result to algal blooms. A lake with a concentration of

below 0.010 mg/L is considered as oligotrophic, while concentrations between 0.010 and 0.020 mg/L are indicative of mesotrophy, and concentrations exceeding 0.020 mg/L are considered eutrophic (Muller and Helsel, 1999).

Phytoplankton and zooplankton make excellent indicator of environmental conditions and aquatic health with in ponds because they are sensitive to changes in water quality. They respond to low dissolved oxygen levels, high nutrient levels, toxic contaminants, poor food quality or abundance and predation. A good picture of the current conditions in the ponds can be derived by looking at plankton indicators, such as their biomass and abundance and species diversity. Phytoplankton affects oxygen levels, nutrient concentrations, light levels, and zooplankton biomass (Chien, 1992). These blooms shade stock, prevent growth of benthic algae (by shading the benthos), oxygenate water, reduce toxic ammonia levels and provide a food source for zooplankton which in turn can provide a food source for higher trophic levels that may be eaten by the stock (Burford, 1997). Chlorophyll *a* is present in all photosynthetic organisms including algae. Its measurement as an index of water quality (Papista *et al*., 2002) and phytoplankton biomass (Desortova, 1981) is widely accepted. Generally, higher chlorophyll *a* concentrations translate into higher individual cell counts and biomass of phytoplankton, though not always, as not all algal cells produce equal amounts of chlorophyll *a* (Felip and Catalan, 2000). For this reason it is also important to identify and count phytoplankton cells in water samples.

Phytoplankton communities undergo a continual succession of dominant species due to dynamic changes in growth factors such as light, temperature and nutrient concentrations (Case *et al*., 2008). In areas where temperature is high and light is abundant, nutrient concentrations and ratios become

important environmental factors influencing the dominance of various taxonomic groups (Hecky and Kilham, 1988; Yusoff *et al*., 2002; Yusoff and McNabb, 1997). Sanders *et al*. (1987) reported that nutrient – loading ratios can exert a strong selective effect on natural communities of phytoplankton. Various forms of algae, most notably diatoms, are nutritious and can benefit shrimp production by contributing qualities such as essential amino acids and highly unsaturated fatty acids (Moss *et al*., 2001). However, potential harmful algae can also be found in aquaculture system. One group that has been problematic for shrimp culture is cyanobacteria, also known as blue green algae. Some cyanobacteria are capable of producing toxins that may diminish shrimp growth or directly cause mortality (Alonso- Rodriques and Paez-osuna, 2003; Zimba *et al*., 2006).

A lack of phytoplankton in the water column can lead to excess light reaching the bottom of ponds, resulting in the proliferation of benthic algae (Burford, 1997). This process has been associated with reduced growth in cultured Penaeid prawns (Allan and Maguire, 1994). Eutrophication of waters by increased nutrient inputs is a serious problem in marine and freshwater, and natural and aquaculture habitats. Increased nutrients can stimulate phytoplankton blooms to detrimental levels which can be directly harmful through the toxins they may produce (Anderson *et al*., 2002) or cause anoxic conditions when blooms crash and decompose, which has the potential to kill animals living in these anoxic conditions (Burford, 1997).

Managing stable phytoplankton populations is a major challenge in earthern pond aquaculture; especially if the cultured species does not graze phytoplankton directly (Danaher *et al*., 2007). Dense phytoplankton bloom with high photosynthetic rates can result in elevated pH level in the after noon

causing physical and physiological stress (Boyd and Tucker, 1998) and even prawn mortality (Straus *et al.*, 1991).

Zooplankton, as an essential component of all aquatic ecosystems, represents a key link in aquatic food web. In aquaculture, zooplankton are not only a good indicator of trophic status of rearing water (Conde – Porcuna *et al.*, 2002; Hietala *et al.*, 2004) but also an important regulator for water quality through interactions among zooplankton, phytoplankton, bacterioplankton, benthos, and fish metabolism within food webs. Additionally, zooplankton is a good food source for reared fish, especially for fry, fingerlings, and juveniles. Therefore, zooplankton may closely relate to both quality and quantity of fish production in aquaculture industry.

1.9 Ammonia Toxicity (Total ammonia nitrogen, TAN)

TAN is composed of NH_3-N (unionized ammonia) and NH_4^+ (ionized ammonia) (Losordo *et al.*, 1992; Masser *et al.*, 1992). It is the unionized form that is most toxic to aquatic organisms as it can readily diffuse through cell membranes and is highly soluble in lipids (Chin and Chen, 1987). There is an equilibrium between NH_4^+ and NH_3-N in the water. This equilibrium is controlled by pH, temperature, and salinity. The higher the pH and temperature, the higher the proportion of NH_3-N, while an increase in salinity will lead to lower proportions of the unionized form (Losordo *et al.*, 1992; Masser *et al.*, 1992; Sampaio *et al.*, 2002).

Ammonia is the initial product of the decomposition of nitrogenous organic wastes and respiration. Nitrogenous organic wastes come from uneaten feeds and excretion of fishes. Thus, the concentration of ammonia-N is positively correlated to the amount of food wastage and the stocking

density. The physiology of aquatic stocks is also partly responsible for ammonium and nitrite accumulation because the animals are able to metabolize, on average, only 25 – 30% of proteins available in feeds while the rest is released in the form of ammonia (Avnimelech and Ritvo, 2003). High concentrations of ammonia causes an increase in pH and ammonia concentration in the blood of the fish which can damage the gills, the red blood cells, affect osmoregulation, reduce the oxygen-carrying capacity of blood and increase the oxygen demand of tissues (Lawson, 1995; Crab *et al*., 2007). Increased ammonia nitrogen has been reported to cause growth and moulting and Na, K^+, ATP ase activities in penaeids. Generally, NH_4^+ is harmless, however, NH_3 can be extremely toxic.

TAN is produced by microbial remineralization processes within the sediment, and by shrimp excretion (Burford and Williams, 2001; Burford and Longmore, 2001). TAN is rapidly utilized by the phytoplankton community, and N turnover in shrimp ponds is about 1 to 2 days (Burford and Glibert, 1999). As a result of high rates for both production and consumption, variations in primary productivity, e.g. due to bloom crashes, can have major effect on TAN concentration. Bacteria transform the nitrogen in uneaten feed to ammonia, and fish excretes ammonia through their gills. Ammonia is toxic at low concentrations and should therefore be removed from the water. The short-term (48 – 96 h) LC_{50} values for unionized ammonia (NH_3) range from about 0.2 to 3 mg /L, suggesting that its safe levels in aquaculture ponds should be lower than 0.02 – 0.3 mg / L (Boyd and Tucker, 1998; Crab *et al*., 2007). In intensive recirculating aquaculture ponds, ammonia is oxidized by bacteria in biological filters to nitrite, which is also toxic, and to nitrate, which is not toxic, in a process called nitrification (Losordo and Westers, 1994). The

biological conversion of ammonia to nitrite is carried out by ammonia oxidizing bacteria (AOB) and the subsequent oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). Both bacterial groups are obligate autotrophs, grow slowly (Hooper, 1989) and have different sensitivities to environmental constraints such as salinity, light intensity and pH. This may lead to imbalanced nitrification and an accumulation of toxic ammonia or nitrite.

For this reason, farmers are forced to exchange water from external sources at high rates more frequently in order to dilute toxic nitrogenous concentrations and this practice tremendously magnifies the risk of disease outbreaks (Sesuk *et al.*, 2009).

1.10 Nitrite-Nitrogen (NO_2 - N)

Nitrite is the intermediate product of the nitrogen cycle and is often caused by ammonia biological oxidation.The stochiometric reaction is as follows.

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

The presence of nitrite in the environment is a potential problem due to its well documented toxicity to animals (Kroupova *et al.*, 2005). It disrupts multiple physiological functions including regulatory, respiratory, cardiovascular, endocrine and excretory processes. However, if high levels do occur, it can cause hypoxia, due to deactivation of hemoglobin in fish blood, a condition known as the “brown blood disease” (Lawson, 1995).

The toxicity of nitrite is dependent on chemical factors such as the reduction of calcium, chloride, bromide and bicarbonate ions, levels of pH, dissolved oxygen and ammonia. High nitrite concentrations plus low chloride levels can result to reduced feeding activities, poor feed conversions, and

lower resistance to diseases and susceptibility to mortality (Lawson, 1995). An inverse relationship between salinity and nitrite toxicity is accepted in literature, yet it has been shown that nitrite is also toxic to marine shrimps at fairly low concentrations (Boyd and Tucker, 1998).

1.11 Nitrate - Nitrogen (NO_3 - N).

Nitrate is formed through nitrification process, i.e. oxidation of NO_2 into NO_3 by the action of aerobic bacteria. The following stochiometric reaction shows the over all process.

$$NO_2^- + 1.5O_2 \rightarrow NO_3^-$$

In total, when a nitrite molecule is oxidized, a free energy yield (ΔG) of -18 kcal mole^{-1} is released. In aquaculture ponds, the most representative bacterial genus that can perform the nitrite conversion to nitrate is *Nitrobacter*, but other genera of bacteria are commonly present during nitrification (Camargo *et al.*, 2005).

A natural pathway to remove the nitrate in an aquaculture system is done by denitrification. The reaction is carried out by bacteria in the absence of oxygen and in the presence of methanol as a carbon source. The general reaction has two steps (Rijin *et al.,* 2006).

$$NO_3 + 5/6\ CH_3OH \rightarrow \tfrac{1}{2}\ N_2 + 5/6\ CO_2 + 2/3\ H_2O + OH^-$$

Generally, it is stable over a wide range of environmental conditions and is highly soluble in water. Compared with other inorganic nitrogen compounds, it is also the least toxic. However, high levels can affect

osmoregulation, oxygen transport, eutrophication and algal bloom (Lawson, 1995).

1.12 Feed management

Feed management is a key factor affecting water quality and production economics in aquaculture (Jolly and Clonts, 1993). Survival and Growth have the greatest impact on the economic performance of shrimp production, and correct feeding is essential for both (Wyban *et al*., 1989). To estimate feed input. Feed utilization should be planned and daily feed input Feed management strategies should there for be aimed at optimizing feed input, reducing feed conversion ratio and reducing the potential impact on the culture and effluent water (Jorry *et al*., 2001). There are varieties of methods used should be limited. Daily input must be reasonable and should consider the growth of the shrimp as well as the nutrient recycling capacity of the pond ecosystem. Growth is easily estimated based on weekly sampling of the shrimp. However, feed inputs are often overestimated due to unrealistic expectations of future shrimp growth as well as survival. Placing upper limits on daily feed inputs based on historical growth rates helps limit expectations (Davis *et al*., 2006). Another feed option method is multiple daily feeding. It is desirable because shrimp eat slowly and almost continuously, (Lovett and Felder, 1990). Increased feeding frequency reduces nutrient leaching and improves feed utilization efficiency (Wyban and Sweeney, 1989; Nunes and Parsons, 2000). Environmental aspects should also be considered when determined daily feed input. Water temperature and dissolved oxygen levels primarily influence feeding activity, metabolism and thus influences both the types and quantities of feed used (Goddard 1996). Natural food also plays an important role in shrimp culture as it provides an important yet available food

source (Chanratchakool *et al*., 1994). Feeding trays also improves feed management in shrimp culture systems (Nunes and Suresh 2001).

1.13 Soil quality management

Pond bottom conditions are more critical for shrimp than for other aquaculture species because shrimps spend most of their time at the bottom, burrow into the soil and ingest pond bottom soil (Boyd, 1989; Chien, 1989). The distribution of the Penaeid shrimp in the natural environment can be influenced by sediment characteristics (William, 1958). Penaeid shrimp commonly burrows into the substratum to hide from predators. Soil conditions of pond bottoms influences water quality and production. Banerjea (1967) revealed that the potential for fish production in ponds was influenced by pH and concentrations of organic matter, nitrogen and phosphorus in soils. Concentrations of nutrients and phytoplankton productivity in pond waters are related to pH and nutrient concentrations in soils (Boyd, 1995; Boyd and Munsiri, 1996).

Nutrients and organic residues tend to accumulate at the bottom and are thus to some extent removed from the water phase. However, an excessive accumulation beyond what could be defined as the carrying capacity of the sediments may result in the deterioration of the pond system. Such a development seems to be of special importance for shrimp culture, since shrimps live in the soil-water transition zone. Reactions and fluxes within and across the water-soil interface are very significant in natural aquatic system and even more in intensive aquaculture systems. Organic matter settles and accumulates on the pond bottom in extensive, semi intensive and intensive ponds. Anaerobic conditions develop in the sediments of intensively stocked and fed shrimp ponds, the process being more pronounced with the increase in

pond intensification. The development of anaerobic conditions constrains production and is a barrier to further intensification (Munsiri *et al*., 1995; Steeby *et al*., 2004; Thunjai *et al*., 2004).

1.14 Sediment oxygen demand in shrimp pond

Dissolved oxygen concentration is one of the critical factors affecting processes and conditions at the sediment water interface. Sediment oxygen demand (SOD) is an indicator of the intensity of the mineralization process and benthic community metabolism. Oxygen supply to the sediments is by diffusion from the water column and by mechanical infusion of water into sediments where it will be consumed through respiration of living organisms or through chemical oxidation in sediments. Bioturbation increases gas exchange between water and sediments and supply of oxygen to the sediments as well (Brooks *et al*., 2003). Accumulation of organic matter in sediment increases both biological and chemical oxygen demands (BOD & COD) (Brooks *et al*., 2003). Increase of BOD is predominantly due to aerobic, heterotrophic bacterial activity. Oxygen uptake by sediments under different Danish fish farms was 5 to 15 times higher than at control sites (Shakouri, 2003). While oxygen demand is equal to influx of oxygen, the sediments have the capacity to assimilate organic matter (Brooks *et al*., 2003) and its productivity will increase (Pearson and Black, 2001). If demand for oxygen exceeds the oxygen diffusion rate, sediments become anoxic and anaerobic processes will predominate (Redox Discontinuity Level, RDL).

1.15 Microbial activity in pond bottom soil

Sediments are enriched with nutrients and organic matter by sedimentation of organic materials to the pond bottom. The concentration of

the nutrients (including organic carbon compounds) in the pond bottom soil are typically several orders of magnitude higher than these found in the water. Bacteria consume large amount of oxygen and sediments become anoxic below the surface. Ram *et al*, (1981, 1982) found that the density of aerobic and anaerobic bacteria to the pond bottom soil is two to four orders of magnitude higher than the density of these groups in the water column. Allan *et al*. (1995) reported that density of the bacteria in the pond sediment is two fold greater than the water column. Burford *et al*. (1998) reported a bacterial count of 15.5 x 10^9 cells/g in the centre of the ponds where the sludge accumulated and 8.1 x 10^9 cells/gm at the periphery of the ponds. From this it was found that the bacterial count increased with the nutrient concentrations and with the smaller sediment grain size.

1.16 Redox reactions in the pond bottom soil

Redox Potential is an index indicating the status of oxidation or reduction. It is correlated with chemical substances, such as O_2, CO_2 and minerals composed of aerobic layer, whereas H_2S, CO_2, NH_3, H_2SO_4 and others comprised of anaerobic layer. Microorganisms are correlated with the status of oxidation or reduction. With the degree of E*h*, it is indicative of one of the parameters that show the supporting ability of water and soil to the prawn biomass (Boyd, 1995)

During oxygen depletion, other terminal electron acceptors can be used to mediate the decomposition of organic matter. Many anaerobic processes taking place in the pond bottom lead to the production of reduced and potentially toxic compounds. Anaerobic condition may affect aquaculture production both due to the unfavorable conditions at the pond bottom, or affect it through the diffusion of

reduced compounds from the sediment upward to the water column. The redox system in the pond bottom is made of both organic components as well as reduced inorganic species (such as sulfides, Fe and Mn ions), yet, the driving force to the development of the high oxygen demand and anoxic conditions is the organic matter accumulated in the sediment (Bratvold and Browdy, 2001). The sequence of redox reactions as listed in Table 1. When oxygen is depleted denitrification occurs, with nitrate as an electron acceptor. Subsequently iron, manganese, sulphate and CO_2 serve as electron acceptors. Burford and Longmore, (2001) reported that 50 – 80% of the carbon degradation in shrimp pond soil was anaerobic, mostly coupled with sulfate reduction.

Reduced inorganic species may affect biological activity; reduced divalent manganese is toxic to fish (Nix and Ingols, 1981). The un-dissociated species H_2S is highly toxic to fish and shrimp. Hydrogen sulphide may inhibit aerobic respiration by binding to heme of cytochrome c oxidase in place of molecular oxygen (Smith *et al*., 1977). Hydrogen sulphide toxicity is inversely related to dissolved oxygen concentration; consequently, sulphide increases the sensitivity to hypoxia (Adelman and Smith, 1970).

A very important sequence of reactions is the fermentation of organic substrate. The fermentation of organic substrate leads to the production of large variety of reduced organic materials such as organic acids, ketones, aldehydes, amines and mercaptans. A significant fraction of those compounds are easily detected by their offensive odour and few of these are toxic. Ram *et al*. (1981, 1982) found that the number of acid forming bacteria increases when a bottom become anaerobic. Organic enrichment and microbiological process in the sediment could be summarized as follows (Pearson and Black, 2001; Brooks and Mahnken, 2003):

- Aerobic respiration, ammonium oxidation (to nitrite) and nitrite oxidation to nitrate. Sediments are in oxic condition.
- Denitrification (production of N_2 from nitrate by aerobic bacteria).
- Nitrogen reduction (producing ammonium from nitrate) and manganese reduction.
- Iron reduction.
- Sulfate reduction and production of H_2S. The sediment is anaerobic/ aerobic.
- Methanogenesis, producing of methane by fermentative bacteria. The sediment is extremely anoxic.

Table 1.1. Redox reactions in pond bottom (adapted by Reddy et al., 1986; Avnimelech and Ritvo, 2003

Electron acceptor (oxidizing system)	Process	Approximate redox potential (mV)
$O_2 \rightarrow CO_2$	Aerobic respiration	500 – 600
$NO_3 \rightarrow N_2$	Denitrification	300 - 400
Organic components		
$Fe^{3+} \rightarrow Fe^{2+}$	Fermentation	< 400
$Mn^{4+} \rightarrow Mn^{2+}$	Reduction	200
$SO_4 \rightarrow S^{2}$-	Sulfate reduction	-100
$CO_2 \rightarrow CH_4$	Methanogenesis	-200

The commonly used treatment of organic sludge is drying and the addition of lime. Drying of pond soil between crops is commonly used and accelerates degradation of pond organic sludge (Boyd and Teichert-Coddington, 1995). Liming neutralize acidity and facilitate microbial activity (Boyd, 1995). Bioremediation or bacterial augmentation is the recent approach

because it constitutes an environment friendly approach to minimize environmental degradation (Nimrat *et al.*, 2008).

1.17 Bioremediation

Bioremediation is the use of individual or combined organisms (animal, bacteria, etc.) to minimize the polluting discharge of effluents from aquaculture or any other activity, taking advantage of the natural or modified abilities of those organisms to reduce and/or transform waste products (Chavez- Crooker and Oberque-Contreras, 2010). The process can be conducted in different forms: in situ, ex situ, bioaugmentation and biostimulation. One of the main concerns of bioremediation is that the degradation products whatever it may be should be non toxic to the stocked animals. Major advantages of bioremediation are:

1) It can be done on site.
2) The process does not lead to any site disruption.
3) There is every possibility for permanent waste elimination.
4) Being a biological process it will be comparatively too inexpensive.
5) Can be effectively coupled with other treatment technologies.

Bioremediation can be used to reduce organic and inorganic compounds and nutrients from aquaculture effluents, mainly by the use of filter-feeding bivalves, microalgae and seaweed (Jones *et al.*, 2002; Muangkeow *et al.*, 2007). There are many mollusk species that have the potential to bioremediate aquatic environments; of which some of them are commercially important (Martinez- cordova *et al.*, 2011; Peharda *et al.*, 2007) and others have been observed to reduce luminous bacteria in shrimp ponds (Tendencia, 2007). Biofilters (polymer spheres with immobilized microorganisms), biofilims, bioflocs (De Schryver *et al.*, 2008; Kuhn *et al.*, 2009) or combined systems

including two or more of these practices have been published. Although it has been demonstrated that some bivalves and micro- or macro algae are capable of bioremediating effluents, many of these studies have been focused on the bioremediation of fish effluents (Hussenot and Martin, 1995; Zhou *et al*., 2006; Liu *et al*., 2010). The use of green water technology was a good biological control for luminous vibriosis in *P.monodon* culture system.

Microorganisms are known to play an important role in nutrient cycling and decomposition (Anderson *et al.,* 1987; Coleman and Edwards, 1987; Rheinheimer, 1992). Hence, water quality in aquaculture systems is to a great extent controlled by microbial degradation of organic matter (Avnimelech *et al*., 1995). Organic matter is degraded by a wide array of microorganisms. For instance, heterotrophic microorganisms oxidize organic matter consuming oxygen and releasing carbon dioxide in the process, whilst autotrophic nitrifying and sulphur bacteria consume oxygen and carbon dioxide during the process of oxidizing ammonia, nitrite and sulphide. In shrimp culture systems, ammonia and sulphide are the forms of nitrogen and sulphur that are toxic to shrimp. Hence, nitrogen and sulphur cycle bacteria are most important in recycling lethal forms of nitrogen and sulphur.

1.18 Bioremediation of detritus

The dissolved and suspended organic matter contains mainly carbon chains and is highly available to microbes and algae. A good bioremediator must contain microbes that are capable of effectively clearing carbonaceous wastes from water. Additionally, it helps if these microbes multiply rapidly and have good enzymatic capability. Members of the genus *Bacillus,* such as *Bacillus subtilis, Bacillus licheniformis*, *Bacillus cereus*, *Bacillus coagulans,*

and of the genus *Phenibacillus*, like *Phenibacillus polymyxa,* are good examples of bacteria suitable for bioremediation of organic detritus. When certain *Bacillus* strains are added to the water in sufficient quantities, they can make an impact. They compete with the bacterial flora naturally present for the available organic matter, like leached or excess feed and shrimp faeces (Sharma, 1999). As part of bio-augmentation, the *Bacillus* can be produced, mixed with sand or clay and broad casted to be deposited in the pond bottom (Singh *et al*., 2001). *Lactobacillus* is also used along with *Bacillus* to break down the organic detritus. These bacteria produce a variety of enzymes that break down proteins and starch to small molecules, which are then taken up as energy sources by other organisms. The removal of large organic compounds reduces water turbidity (Haung, 2003). Bioaugmentation has been reported as effective in eliminating disease-causing pathogens by dominating the pond bottom and it is capable of waste digestion and sludge clean-up as well (Walker and Clymo, 1996). Bioaugmentation agents improve water quality by enhancing the mineralization process and reducing the accumulation of organic wastes (Thomas *et al*., 1992; Shariff *et al*., 2001).

Bioremediation of organic rich sediments aims at the mobilization and removal of organic macro elements from the accumulation area (Vezzuli *et al*., 2002). Many examples in which both singular bacterial strains and microbial systems have been successfully utilized to reduce and or transform selected pollutants to nontoxic molecule in laboratory conditions (Eschenhagen *et al*., 2003; Gallizia *et al*., 2003). Gallizia *et al*, (2005) have been used to evaluate the different bioremediation protocols to enhance the decomposition of organic polymers.

1.19 Bioremediation of nitrogenous compounds

Nitrogen applications in excess of pond assimilatory capacity can lead to deterioration of water quality through the accumulation of nitrogenous compounds (e.g., ammonia and nitrite) with toxicity to fish and shrimp. The principal sources of ammonia are fish excretion and sediment flux derived from the mineralization of organic matter and molecular diffusion from reduced sediment, although cyanobacterial nitrogen fixation and atmospheric deposition are occasionally important (Ayyappan and Mishra, 2003). Nitrification proceeds as follows:

$$NH_4^+ + 1\ 1/2\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

$$NO_2^- + 11/2\ O_2 \rightarrow NO_3^-$$

Bacteriological nitrification is the most practical method for the removal of ammonia from closed aquaculture systems and it is commonly achieved by setting of sand and gravel bio-filter through which water is allowed to circulate. The ammonia oxidisers are placed under five genera, *Nitrosomonas, Nitrosovibrio, Nitrosococcus,* and *Nitrospira,* and nitrite oxidizers under three genera, *Nitrobacter, Nitrococcus* and *Nitrospira.* There are also some heterotrophic nitrifiers that produce only low levels of nitrite and nitrate and often use organic sources of nitrogen rather than ammonia or nitrite. Nitrifiers in contaminated cultures have been demonstrated to nitrify more efficiently. Nitrification not only produces nitrate but also alters the pH slightly towards the acidic range, facilitating the availability of soluble materials (Ayyappan and Mishra, 2003). The vast majority of aquaculture ponds accumulate nitrate, as they do not contain a denitrifying filter. Denitrifying filters helps to convert nitrate to nitrogen. It creates an anaerobic region where anaerobic bacteria can

grow and reduce nitrate to nitrogen gas (Rao, 2002). Nitrate may follow several biochemical pathways following production by nitrification.

$$NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Unlike the limited species diversity of bacteria mediating nitrification, at least 14 genera of bacteria can reduce nitrate. Among them, *Pseudomonas, Bacillus* and *Alkaligenes* are the most prominent numerically (Focht and Verstraete, 1977).

1.20 Bioremediation of ammonia nitrogen

Sequestering ammonia from intensive culture systems has been achieved by chemical (Gräslund and Bengtsson, 2001) and biological filters (Malone and Pfeiffer, 2006) and through *in situ* application of microbial amendments (Rombaut *et al*., 2003). In small scale shrimp grow out systems, commonly found in developing tropical countries, zero or limited water exchange based on chemical and biological filtrations and recirculating aquaculture systems are practically and economically not viable (De schryver *et al.,* 2008). Main constraints are high capital cost, and technical problems related to their establishment and operation (Shan and Obbard, 2001). A recent alternative is the bio-flocs technology (BFT) (Avnimelech, 2006 ; Crab *et al*., 2007) where heterotrophic bacteria and algae are grown into flocs under controlled conditions within the culture ponds. The intensive growth of heterotrophic bacteria immobilizes inorganic nitrogen depending on the C/N ratio. However, factors responsible for their dynamics and its effects on growth and survival of cultured species warrant further investigations to exploit the merits of BFT (Crab *et al.,* 2007). Under such situations use of nitrifying bacteria as bioaugmentor has been found to be a better option and biological nitrification

can be sustained by maintaining optimal conditions for their proliferation. Both the groups of nitrifiers (ammonia and nitrite oxidizers) involved in nitrification are obligate autotrophs, slow growers and have different levels of sensitivities to environmental factors such as salinity, light intensity and pH. However, immobilization techniques help overcome these limitations to a large extent (Seo *et al*., 2001), and while doing so, maintenance of a high cell density of viable culture of nitrifying bacteria in the active growth phase turns out to be a key factor for providing an effective *in situ* treatment for aquaculture (Shan and Obbard, 2001).

1.21 Probiotics

The concept of biological disease control, particularly using microbiological modulator for disease prevention, has received widespread attention. A bacterial supplement of a single or mixed culture of selected non-pathogenic bacterial strains is termed probiotics. Probiotics' the term was firstly coined by Parker (1974) and originated from two Greek words 'pro' and 'bios' which mean 'for life'. According to his original definition, probiotics are "organisms and substances which contribute to intestinal microbial balance". Probiotics include a broad spectrum of live microorganisms consisting of yeasts, photosynthetic bacteria, lactic acid bacteria, other Gram-positive and Gram-negative bacteria (Vine *et al*., 2006), particularly *Bacillu*s sp. (Vaseeharan and Ramasamy, 2003; Ziaei-Nejad *et al*., 2006; Liu *et al*., 2010). Probiotics were developed and used in aquaculture for consumer health reasons as well as for environmental concerns (Wang *et al*., 2005). When administered in adequate amounts to ponds or tanks, they may confer health benefits to the organisms being cultured and replace or reduce prophylactic antimicrobial agents in aquaculture (Balcazar *et al*., 2006). Probiotics are thought to improve water quality, particularly by reducing ambient

ammonia (Shariff *et al*., 2001) and increasing dissolved oxygen (Wang *et al*., 2005). They are known to activate immune responses, reduce pathogenic problems and increase survival and growth of cultured hosts including fish and shrimp (Verschuere *et al*., 2000; Irianto & Austin., 2002; Balcazar *et al*., 2006). Recently, in an effort to sustain and ultimately enhance shrimp production, the use of probiotics has increased (Gatesoupe, 1999).

Among the large number of probiotic products in use today are the bacterial spore formers, mostly of the genus *Bacillus* (Hong *et al*., 2005). The introduction of *Bacillus* sp. in close proximity to pond aerators reduces chemical oxygen demand and increases shrimp harvest (Porubcan, 1991). Among 80 bacterial strains isolated from healthy wild shrimp *L. vannamei*, *Vibrio* P62, *V.* P63, and *Bacillus* P64 showed inhibitory effects against *V. harveyi* (S2) at 54%, 19%, and 34%, respectively. Moreover, *Bacillus* P64 showed both probiotic and immunostimulatory features, while *Vibrio* P62 only showed good probiotic properties (Gullian *et al*., 2004). *Bacillus* spores have been used as biocontrol agents to reduce vibrios in shrimp culture facilities (Skjermo and Vadstein, 1999; Rengpipat *et al*., 2000). *Bacillus* spp. is often antagonistic against other microorganisms, including fish and shellfish pathogenic bacteria (Rengpipat *et al*., 2000). *Bacillus fusiformis* improved the survival and accelerated the metamorphosis of *P. monodon* and *L. vannamei* (Guo *et al*., 2009).

The use of probiotics influenced digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity and improved feed utilization (Bomba *et al*., 2002). Rengpipat *et al*. (1998) reported that appropriate probiotic applications were shown to improve intestinal microbial balance, thus leading to improved food absorption. An improved survival and immune system was also reported by Kumar *et al*.

(2006) in Indian major carp after the fishes were fed with probiotics (*Bacillus subtilis*). This could best explain the low levels of luminous vibrios and high shrimp survival in ponds with four times per week application.

According to recent publications, in aquaculture, the mechanism of action of probiotic bacteria have several dimensions (Verschuere *et al*., 2000; Ziaei-Nejad *et al*., 2006; Wang *et al*., 2005).

Probiotic bacteria competitively exclude the pathogenic bacteria or produce substances that inhibit their growth (eg. Bacitrocin and polymyxin produced by *Bacillus* sp.).

- Provide essential nutrients to enhance the availability of sufficient nutrients to the cultured animals.
- Provide digestive enzymes to enhance digestion
- Probiotic bacteria directly uptake or decompose the organic matter or toxic materials.
- Improving the quality of water.

The potential benefits of probiotics in aquaculture ponds are, they

- Regulate the micro flora
- Control pathogenic microorganisms.
- Enhance decomposition of the undesirable organic substances and improve the environmental quality minimizing toxic gases like ammonia, hydrogen sulfide, methane.
- Increase the population of food organisms.
- Improve the nutrition level and immunity.
- Prevent the frequent outbreaks of diseases

Bioaugmentation has been reported as effective in eliminating disease-causing pathogens by dominating the pond bottom and it is capable of waste digestion and sludge clean-up as well (Walker and Clymo, 1996). Bioaugmentors improve water quality by enhancing mineralization and reduction in the accumulation of organic wastes (Thomas *et al.,* 1992; Shariff *et al*., 2001). By splitting the weekly dosage of the bioaugmentor to twice or four times per week, significant enhancement in the efficiency of waste removal, reduction in the level of luminous *Vibrio*, and stabilization in phytoplankton density could be achieved. Janeo *et al*. (2009) demonstrated enhanced survival and production of tiger shrimp (*P. monodon*) on application of probiotics. According to Ehrlich *et a*l, (1988), the addition of bacterial products in aquaculture ponds accelerated nitrification and rapid decomposition of organic solids.

Based on the above understanding on shrimp culture systems and the culture environments and their management the following objectives have been identified in the present work.

1.22 Need for Research

Considering all above, for according protection to the reared crop from horizontal transmission of diseases, to maintain health of the culture environment quality through the application of bioaugmentors and to protect the external environment quality from the discharge of effluents from culture systems, the best option available was to adopt zero water exchange technology with moderate stocking density. Even though zero water exchange systems in aquaculture may not be a novel concept much standardization are required for every species being cultured in each region. In the present context *P. monodon* was selected as the species of choice and the probiotics and bioaugmentors were indigenous,

developed by this Centre (National Centre for Aquatic Animal Health). Obviously, studies were required to evaluate the efficacy of both probiotics and bioaugmentors *in vivo* in attaining a healthy crop free from diseases, with the required environmental quality. Accordingly, it was required to develop a protocol for zero water exchange technology in a shrimp culture system with *P.monodon* as the species suitable to Indian conditions, validate it in different seasons and find out an appropriate stocking density for a system with out artificial aeration. Among the bioaugmentor used nitrifying bacteria were not incorporated, as an indigenous product was not available. However, a technique of producing immobilized nitrifying bacterial consortia has been developed already in this lab (Manju et al., 2009). Considering the requirement of an indigenous commercial product a mass production technology of the nitrifiers was also required. Considering all above requirements the following objectives were identified to work on, and the thesis deals with the same.

Objectives.

1) Standardization of bioremediation technology for zero water exchange shrimp culture system
2) Validation of the zero water exchange technology in different seasons and its comparison with open shrimp grow out systems
3) Effects of stocking density on water and sediment quality, and on growth of Penaeus monodon cultured in brackish water earthen ponds under zero water exchange.
4) Mass production of nitrifying bacteria immobilized on wood powder and validation.

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Chapter

2

Standardization of bioremediation technology for zero water exchange shrimp culture system

2.1 Introduction

Aquaculture has been experiencing faster growth rate world over during the last two decades. Among the cultured species Crustaceans as a group demonstrated the highest growth rate (almost 17% per year from 2000 to 2006) and among them penaeid shrimp was by far the most dominant in terms of volume and value (FAO, 2009). However, the rapid expansion of aquaculture has brought in serious problems with respect to competition for water and land (Paez ozuna *et al.,* 2003), environmental degradation such as destruction of mangrove forests, eutrophication of receiving waters, modifications of habitats of terrestrial and aquatic animals and alterations in landscape and hydrological pattern (Martinez Cordova *et al.,* 2010). Nutrient discharge from grow out ponds has been one of the most serious problems in shrimp aquaculture. It has to be pointed out that approximately just 30% of nitrogen (Shimoda *et al*., 2007) and 15% of phosphorous (Shimoda *et al.,* 2005) in the feed input alone gets incorporated into shrimp biomass and the rest gets wasted in to the surrounding environment.

Another crisis faced by shrimp industry is the onset of diseases caused by both potential and opportunistic pathogens. Among them the most serious one was the outbreak of white spot viral disease caused by white spot virus (WSV) predisposed by poor water quality and sharing of same source water (Burford *et al.,* 2003). Therefore, it has become imperative to develop measures to protect the crop from the onslaught of diseases as well as to prevent the discharge of waste water in to the culture environment as a long term strategy. It has been pointed out that at present there are no therapeutic measures available for shrimp viral diseases. However, several farm management strategies have been developed to prevent viral entry into the culture system avoiding subsequent disease outbreak. The strategy includes augmented biosecurity and attainment of the required effluent quality. In several countries this could be accomplished by implementing biosecured, closed shrimp production systems, including zero water exchange and recirculating culture packages (McIntosh, 1999; Balasubramanian *et al*., 2005; Lezama - Cervantes *et al.,* 2010). Introduction of effluent quality management measures, reduction in the availability of space required for culturing and reduction in the introduction of infectious diseases were the highlights of these developments (McAbee *et al*., 2003; Sowers *et al.,* 2005; Azim and Little, 2008).

The major problem associated with zero water exchange shrimp culture system is the rapid eutrophication resulting in a phytoplankton bloom followed by bloom crash and re- blooming after a time lag. This imposes tremendous stress to the cultured stock, and reduces the carrying capacity of the pond (Lin, 1995). Likewise, according to Tacon *et al.,* (2002) closed zero-water exchange culture systems can support biologically only a limited level of nutrient input

and shrimp biomass without getting compromised with shrimp growth and survival or the system getting crashed altogether. Generally after 120 days, stressful conditions get developed and growth rates slow down compared to the open system. Obviously, the balance between waste production and assimilative capacity in the pond environment is of paramount importance for the success of closed systems.

Success of shrimp production in a culture system is frequently determined by the environmental conditions prevailing in the pond (Case *et al.*, 2008). Water quality in the ponds is determined by the initial quality of intake water, as well as the organic loading in the form of feeds and fertilizers during the culture cycle (Yusoff *et al.*, 2011).

Pond bottom conditions are more critical to shrimp than to other cultured species, as shrimp spends most of their life time on the bottom soil (Boyd, 1989). Distribution of penaeid shrimp in the natural environment is influenced largely by sediment characteristics. From chemical stand point, particularly in closed systems with minimal or no water exchange, sediment can be a source or sink for macronutrients such as Cu, Fe, Mn and Zn (Horowitz *et al.*, 1999). Aquaculture pond bottom soil is the recipient of large amount of nitrogen, phosphorus and organic matter (Boyd, 1990) and these substances tend to accumulate in the bottom soil. High load of organic matter in the bottom soil results in anaerobic conditions in the surface layers of soil and soil water interface. When oxygen is depleted, other electron acceptors mediate the decomposition of organic matter and the anaerobic processes taking place in the pond bottom leading to the production of reduced and potentially toxic compounds like sulphide and methane (Boyd, 1990).

Bioremediation has two facets, such as bioaugmentation and biostimulation. In bioaugmentation, novel organisms are added in to the system and modify the microbial environment. However, in biostimulation the existing organisms are stimulated to act upon the pollutant (Chavez–Crooker and Oberque-Contreras, 2010). Various environment friendly techniques to maintain good water quality and to control disease outbreaks have been developed. The application of bioaugmentors and probiotics, for instance, is a better alternative to administrating antibiotics in culture systems (Havenaar and Hius intveld, 1992; Nogami and Maeda, 1992; Douillet and Langdon, 1994; Garriquez and Arevalo, 1995; Vine *et al*., 2006; Castex, 2008) and the process is rapidly being accepted as a management strategy to prevent diseases and to protect the environment (Gatesoupe, 1999; Corre *et al*., 2000). This is accomplished through a series of enzymatic processes being carried out by the bioaugmentor. The mechanism of action is through competitive exclusion in which the bioaugmentor proliferate in the environment and compete for existing nutrients resulting in the displacement of pathogenic bacteria (Stravric and D Aoust, 1993). Bioaugmentation has been reported as effective in eliminating disease causing pathogens by dominating the pond bottom and it is simultaneously capable of waste digestion and sludge clean up as well (Walker and Clymo, 1996).

Bioaugmentors improve water quality by enhancing mineralization and reducing the accumulation of the organic waste (Thomas *et al*., 1992; Shariff *et al*., 2001). Moriarty (1998) reported displacement of luminous *Vibrio* species present in pond water when an antagonistic *Bacillus* species was introduced in to the culture system. Spores of genus *Bacillus* have advantages over vegetative cells, as they are stable for long periods, can be formulated into useful commercial products, widely used as biological agents, possess

antagonistic effects on pathogens and are naturally ingested by animals (Hong *et al.*, 2005). As part of bioaugmentation, King (1986) observed an increased removal of ammonia from pond effluents when a biofilter containing chemo litho autotrophic bacteria were used; while Ehrlich *et al*. (1988) reported that the use of bacterial consortia in aquaculture ponds accelerated both nitrification and rapid decomposition of organic solids. Application of bioaugmentors and probiotics in aquaculture settings have been increasing over the years with increasing demand for more environment friendly aquaculture practices (Gatesoupe, 1999). Our previous studies have shown that Detrodigest^{TM} an indigenous probiotic preparation containing *Bacillus* MCCB 101 (Gene bank accession no. EF 062509) and Enterotrophotic^{TM} a gut probiotics preparation (Jayaprakash *et al.*, 2005) could maintain water quality and aquatic animal health and control *Vibrio* population in penaeid and non penaeid culture systems. The present study aims at developing a zero water exchange shrimp culture technology coupled with the application of the bioaugmentor Detrodigest^{TM} for detritus degradation and Enterotrophotic^{TM} to modify the gut microbial flora.

2.2 Materials and Methods

2.2.1 Location and description of the pond

Six *Penaeus monodon* grow-out ponds with an area of 1 ha each, located at Pancham Aquaculture Farms Ltd, Mumbai, India were used for the experiment. Three ponds were set apart for the implementation of the bioremediation protocol and designated as ‘test’ ponds and the rest three as ‘controls’.

2.2.2 Pond preparation

Five sampling stations were fixed in each pond, and 100 g soil samples each were collected from each station and divided into two parts. One part was

separated for E*h* and pH and the other component from all the sampling stations of each pond were mixed together, dried, packed separately as composite samples for each pond, for total soil analysis. E*h* and pH were measured immediately and the other analysis subsequently. The ponds were eradicated of weed fishes by applying lime (1275 kg/ha) and ammonium sulphate (255 kg/ ha) at a ratio of 5:1 and tea seed powder at the rate 100 kg/ha after maintaining the water level to 10 cm. On completing the eradication, water was pumped into the ponds to have maximum 5-10 cm at the topmost portion of the pond through 100 micron bag net fitted to the sluice gate. The sluice gate was then closed by mud packing. After recording salinity and pH of the input water, 'Detrodigest' (a bioaugmentor preparation developed by National Centre for Aquatic Animal Health for bioremediation of detritus) was brewed and applied to the ponds. Brewing was done at the pond site by inoculating an aliquot of 300 ml Detrodigest/ hector for 24- 48 h in 100 L medium composed of filtered (through muslin cloth of 100 mesh size), chlorinated (7% chlorine as sodium hypochlorite) and dechlorinated (after 6 hours by adding 20 g sodium thiosulpahte/ 100L) pond water from the respective pond and supplemented with cooked 100 g rice bran and 500g shrimp feed. Generation of fermented smell and froth were the signs of brewing. For the best results the preparation was aerated from an air pump, passed through a bacterial filter of 45 µm or is manually agitated intermittently to get final cell count of 1 x 10^9 CFU/ ml. The preparation was subsequently diluted with pond water and sprayed over the ponds during morning hours. This will provide an approximate cell count of $1x10^4$/ml in 1 hectare pond with 70cm water column. This is the dosage which can be obtained considering the bacterial count in the preparation.

Periodically (once in 24 hours) pH and E*h* of the soil were measured in all the five stations to examine the impact of the treatment in improving E*h*. The application of Detrodigest was continued once in a week till the total organic carbon dropped to 2% and E*h* of the sediment rose to around -100 mV. For filling up the pond, water was pumped from the creek through 100µ mesh to get minimum 70 cm water column. All these processes were done only in test ponds and the control ponds were just filled with water after the eradication and were analyzed for the parameters as described above.

2.2.3 Fertilization

Water samples collected from the test ponds were subjected for analysis of chemical parameters such as ammonia, nitrite, nitrate, phosphate and total phosphorus. Based on the nutrient status, inorganic fertilizers were supplemented as per requirement to initiate bloom. A combination of nutrients such as Nutrimix, 650 g (NCAAH, CUSAT), triple super phosphate, 15 kg/ha (FACT, Kerala) and 100 L cow dung extract prepared from 50 kg cow dung, were applied to the ponds during morning hours after regulating pH to 7 – 7.5 by applying dolomite. Nutrimix per kg contained sodium nitrate (588 g), sodium phosphate (39 g), sodium silicate (270 g), copper sulphate (25 g), and ferric chloride (78 g). In addition, another nutrient preparation 'Micromix' containing ferric chloride and magnesium chloride at a ratio of 1:1 was also applied at a rate of 300 mL/ ha. Under this situation, wherever there was delay in the production of phytoplankton within three days of application, an additional dosage of 40 L extract from 40 kg cow dung mixed with 200 g Nutrimix, 620 g single super phosphate, and 200 mL Micromix was applied per hectare. At this stage one more comprehensive analysis of water was made to asses the suitability of the ponds for stocking.

2.2.4 Stocking and management of the ponds

When the water and sediment pH remained between 7- 8.5, sediment E*h* between -100 to -125 mV, alkalinity between 70 to 120 mg/L in the test ponds both test and control ponds were stocked with *Penaeus monodon* seed, tested nested PCR negative for WSSV and qualified for stocking with regard to all other health parameters such as color, swimming behavior, muscle to gut ratio, pigmentation and intactness of appendages, recorded with the help of microscope. The post larvae were subjected for salinity and temperature stress tests as well. To make an assessment of the survival of post larvae subsequent to stocking 100 of them were maintained in a nylon hapa and counted after 48 hours presuming it to reflect the over all survival in the pond. In cases where alkalinity happened to be less than 50 mg/L in the ponds, it used to be elevated by applying dolomite or $CaCO_3$ in small doses. Henceforth, regular monitoring and management of the system was carried out. The bioaugmentor, Detrodigest, was applied into the ponds regularly at seven days interval. All physical and chemical parameters of water and soil were measured weekly. Whenever the phytoplankton bloom did not develop readily, inorganic fertilizers were applied. After one month of the culture 100 animals were randomly sampled from different parts of the pond using cast net and the body weights assessed and the health parameters examined. Feeding was regulated according the body weight. On completion of 30 days Enterotrophotic (a gut probiotics preparation of National Centre for Aquatic Animal Health) (having cell count of $1x10^8$ cfu/ml) application commenced by coating on to the diet at a ratio of 50 mL for 10,000 animals spread over one day's meal for 70 days after which 100 ml/10,000 animals coated on to the feed to provide per animal minimum of $5x10^5$ cells per day during 70 days of culture

and $1x10^6$ cells per animal afterwards. This is the dosage which could be obtained considering the bacterial count in the preparation.

2.2.5 Probiotics

2.2.5.1 Detrodigest™

The organism used for the preparation of Detrodigest is *Bacillus cereus* sensu lato MCCB 101 (Genbank Acc. No. EF 062509) isolated from aquaculture fields of Kerala by this Centre and subjected to rigorous screening procedures. Extensive field level demonstrations and validations over a period of five years have been made in Kerala and Tamil Nadu to ascertain its suitability in shrimp culture systems and it has proven to be an appropriate preparation for prolonged and safe detritus management in any aquaculture system. An extensive study on the salinity preference of the organism in the Detrodigest revealed its euryhaline nature by growing and adequately producing hydrolytic enzymes at all salinities tested ranging from fresh water to high saline seawater (0 - 45 g/L). The organism in the Detrodigest is highly versatile with the potential to produce a variety of enzymes such as protease, lipase, chitinase etc. Another outstanding property of the organism is its capability to bring down ammonia, which gets liberated as a result of deamination and excretion by shrimp. This was very much reported during the field level demonstration programme as the ammonia content in all ponds, which received Detrodigest, was below toxic level (<0.01mg/L NH_3). Rapid degradation of detritus as soon as formed by Detrodigest makes more dissolved oxygen available at the pond bottom. As a result of the digestion of detritus, mineralization proceeds at faster rate resulting in phytoplankton bloom, generally three days after the addition of Detrodigest. The bloom may remain for 10 days and for perpetual blooming repeated addition once in 10 days has been found to be fruitful.

2.2.5.2 Enterotrophotic™

Enterotrophotic is a scientific blend of *Bacillus cereus* sensu lato (MCCB 101) (Genbank accession. no. EF 062509) and *Arthrobacter nicotianae* (Genbank accession no. EU402968). (MCCB 104) (Jayaprakash *et al.*, 2005) isolated by this Centre from marine environment and characterized for application as gut probiotic. They control *Vibrio* in shrimp/prawn intestine by way of antagonistic activity mediated by an extracellular anti-*Vibrio* molecule and enhance feed acceptance and digestion by way of producing hydrolytic enzymes.

The dose of application was 50 mL/ 10000 animals up to 70 days of culture and after that 100 ml/10000 animals coated with the feed.

2.2.6 Water quality – Physico -chemical analysis

2.2.6.1 pH, Temperature and Salinity

pH was measured using digital pH meter, temperature using thermometer and salinity using refractometer.

2.2.6.2 Estimation of alkalinity

Alkalinity of the water samples were measured by titrating with standard hydrochloric acid (APHA, 1995). Titration to pH 8.3 or decolorisation of phenolphthalein indicator indicate complete neutralization of OH^- and half of the CO_3^-, while pH 4.5 or sharp change from yellow to pink of methyl orange indicator, indicate total alkalinity (complete neutralization of OH^-, CO_3^- and HCO_3^-). Alkalinity is commonly expressed in mg $CaCO_3$/ L.

An aliquot of 100 mL sample was taken in a conical flask and added 3 drops of phenolphthalein indicator. If there has been no colour produced, the

phenolphthalein alkalinity turns out to be zero. If a pink colour gets developed it is titrated against 0.1 N hydrochloric acid (8.3 mL of concentrated hydrochloric acid in 1 L distilled water) till the colour disappears or pH 8.3 is attained. Subsequently, an aliquot of 3 drops of methyl orange was added to the same flask, and continued titration till pH dropped to 4.5 or the yellow colouration changed to orange. The volume of hydrochloric acid used was noted. In case the pink colourations did not appear after addition of phenolphthalein, the estimation of methyl orange alkalinity was continued.

Calculation

Phenolphthalein alkalinity (mg/L $CaCO_3$) = (A x 1000) / mL sample

A = Pink to colorless end point

Total alkalinity of sample in mg $CaCO_3$ /L

= (Normality of HCl x volume of HCl consumed/volume of sample taken) x 50 x 1000

Phenolphthalein and total alkalinities are determined, then three types of alkalinity can be calculated from Table.1.

Table.2.1. Relationship between hydroxide (OH^-), carbonate (CO_3^-) and bicarbonate (HCO_3^-) alkalinities

Result of titration	OH alkalinity	CO_3 alkalinity	HCO_3 alkalinity
P = 0	0	0	T
P < ½ T	0	2P	T – 2P
P = ½ T	0	2P	0
P > ½ T	2P -T	2 (T-P)	0
P = T	T	0	0

P = Phenolphthalein alkalinity, T = Total alkalinity

2.2.6.3 Estimation of hardness

EDTA forms a chelated soluble complex when added to a solution of certain metal cations (APHA, 1995). Metal cations in the presence of Eriochrome Black T at a pH of around 10.0 become wine red. When EDTA is added as a titrant, the calcium and magnesium will be complexed, and when complexation is complete, the solution turns from wine red to blue, marking the endpoint of titration.

An aliquot of 50 mL sample was taken in a conical flask, added 2 mL buffer solution (1.179 g disodium salt of EDTA, 780 mg magnesium sulphate in 50 mL and 16.9 g ammonium chloride dissolved in 100 mL distilled water and added with 143 mL concentrated ammonium hydroxide, diluted to 250 mL) and a pinch of Eriochrome Black T indicator (Ground 100 mg of the Eriochrome Black T indicator with 100 g of sodium sulphate). Titrated against 0.01 M EDTA (18.1612 g EDTA dissolved in 1L distilled water) till wine red colour changed to blue, and calculated the hardness as follows:

Total Hardness (mg $CaCO_3$/L) = Equivalent hardness x 100

Equivalent hardness of water sample (mg/L)

= Volume of titrant x molarity of titrant x 1000 / volume of sample taken.

2.2.6.4 Estimation of Calcium

Calcium in water samples was measured by EDTA titrimetric method (APHA, 1995). Calcium can be determined directly, with EDTA, when the pH was made sufficiently high that the magnesium is largely precipitated as the hydroxide and an indicator is used that combines with calcium only. Several indicators give a color change when all of the calcium has been complexed by

EDTA at a pH of 12 to 13. Magnesium is estimated as the difference between hardness and calcium.

An aliquot of 50 mL water sample was taken in a conical flask, 1 mL 1N sodium hydroxide (40 g NaOH dissolved in 1 L distilled water) and pinch of murexide indicator were added. Titrated immediately against 0.05 M EDTA solution (18.612 g dissolved in 1 L distilled water) till pink colour changed to purple and calcium and magnesium were calculated as follows:

Calcium (mg Ca/L)	=	A x normality of EDTA x 40 x 1000/ volume of sample
Where A	=	titer value for calcium
Magnesium	=	Total hardness - calcium

2.2.6.5 Estimation of dissolved oxygen (Idometric method) (APHA, 1995)

Oxygen present in the sample oxidizes the divalent manganese to its higher valency, which gets precipitated as brown hydrated oxidizes after addition of sodium hydroxide and potassium iodide. Upon acidification, manganese reverts to divalent state and liberates iodine from potassium iodide equivalent to dissolved oxygen content in the sample. The liberated iodine is titrated against sodium thiosulphate using starch as an indicator. If no oxygen is present, a pure white precipitate of Mn(OH) forms when manganous sulphate and alkali reagents are added to the sample. If oxygen is present the divalent Mn(II) is oxidized to higher valency Mn(IV) and precipitate as a brown hydrated oxide.

Water samples were collected in BOD bottles taking care to avoid air bubbles getting trapped. An aliquot of 1 mL Winkler A (480 g manganous sulphate dissolved in 1L distilled water) followed by 1 mL Winkler B (500 g

sodium hydroxide and 10 g sodium azide dissolved in 1L distilled water) were added immediately after collection and placed the stopper carefully excluding air bubbles, and mixed the solution by inverting the bottles repeatedly. The stopper of the bottle was carefully removed and added 1 mL concentrated sulphuric acid and mixed by gentle inversion until the precipitate was completely dissolved. An aliquot of 50 mL of the preparation was titrated against 0.025 N sodium thiosulphate (6.205 g dissolved in 500 mL distilled water and added 0.4 g solid sodium hydroxide), using starch as the indicator untill the blue colour turned to colourless.

Calculation

Oxygen content of the sample (mg/L)

= (8 x volume of titrant x normality of titrant x 1000)/volume of sample

2.2.6.6 Estimation of Ammonia (Solorzano, 1969)

Ammonia reacts in moderately alkaline solution with hypochlorite to monochloramine, which, in the presence of phenol, catalytic amounts of nitroprusside ions and excess hypochlorite, gives indophenol blue. The formation of monochloramine requires a pH between 8 and 11.5. At a pH higher than 9.6, precipitation of Mg and Ca ions as hydroxides and carbonates occurs in seawater. However, these ions can be held in solution by complexing them with citrate.

An aliquot of 10 mL sample was taken in a test tube, added 0.4 mL phenol solution (20 g of crystalline phenol dissolved in 95% V/V ethyl alcohol), 0.4 ml sodium nitroprusside (1 g dissolved in 200 mL distilled water) and 1.0 mL oxidizing solution [alkaline reagent (100 g sodium citrate and 5 g sodium hydroxide dissolved in 500 mL distilled water) and sodium

hypochlorite 4: 1 ratio]. Absorbance was taken at 640 nm after 1 hr incubation at room temperature. A series of standards (4.714 mg ammonium chloride dissolved in 100 mL double distilled water gave 10µg/mL ammonia - nitrogen) were prepared and the factor value was calculated.

Ammonia nitrogen in mg/L = Factor Value x Absorbance of the sample

Factor Value = Concentration of standards/ absorbance.

2.2.6.7 Estimation of Nitrite and Nitrate (Bendschneider and Robinson, 1952)

Under acidic condition (pH 2.0 to 2.5) nitrite ion (NO_2^-) as nitrous acid (HNO_3) reacts with sulphanilamide to form a diazonium salt, which combine with N- (1-naphthyl)-ethylene diamine dihydrochloride (NED dihydrochloride) to form a bright coloured pinkish red azo dye. The colour produced is directly proportional to the amount of nitrite present in the sample.

Nitrate is determined by converting nitrate to nitrite using a mixture of phenol – sodium hydroxide and copper sulphate - hydrazine sulphate. Reagents are added and incubated at dark for 18 hours, added acetone and complexed with sulphanilamide and NED.

Nitrite analysis

An aliquot of 10 mL sample was taken in a test tube, added 0.2 mL sulphanilamide (5 g dissolved in a mixture of 50 mL concentrated hydrochloric acid and 450 mL distilled water) and 0.2 mL of NED (0.5 g dissolved in 500 mL distilled water). The absorbance was taken after 8 minutes at 543 nm. Series of standards (4.925 mg sodium nitrite dissolved in 100 mL gave 10µg/mL) were prepared and calculated the factor value.

Nitrate analysis

To the 10 mL sample added 0.4 ml phenol – sodium hydroxide solution [This solution was prepared by the mixing of Phenol solution (46 gm dissolved in 1 L distilled water) and sodium hydroxide (30 g dissolved in 2 L distilled water) at 1:1 ratio] and 0.2 mL hydrazine sulfate – copper sulphate solution [this solution prepared by the mixing of hydrazine sulphate (14.5 g hydrazine sulphate dissolved in 1 L distilled water) and copper sulphate (0.1 g copper sulfate dissolved in 1 L distilled water) at 1:1 ratio], incubated in dark for 18 - 24 hours. After incubation 0.4 mL acetone, 0.2 mL sulphanilamide and 0.2 mL NED were added. Absorbance was measured after 8 minutes at 543 nm. A series of standards (6.0707 mg sodium nitrate dissolved in 100 mL gave 10 µg/mL Nitrate - nitrogen) were prepared and calculated the factor value.

Calculation

Concentration of nitrate in sample in mg/L = [(x-y) x 100/ efficiency]

Where x = Absorbance of nitrate x Factor value of nitrate

y = corresponding concentration of nitrite

Efficiency = (A/B) x 100

Where A= Observed concentration of standard (absorbance x factor value of nitrite)

B= Original concentration of standard prepared.

Efficiency measures the percentage of nitrate converted into nitrite

2.2.6.8 Estimation of Inorganic Phosphate (Ascorbic acid method) (Strickland and Parson, 1972)

Ammonium molybdate and potassium antimonyl tartarate react in acid medium with orthophosphate to form a heteropoly acid - phosphomolybdic

acid- that is reduced to intensely coloured molybdenum blue by ascorbic acid. Intensity of the colour can be measured photometrically and this is related to the concentration of phosphate by Beer-Lambert's law.

An aliquot of 10 mL sample was taken in a test tube, 1.6 mL combined reagent [mixed 5 mL 5 N sulphuric acid (14 mL concentrated sulphuric acid diluted to 100 mL distilled water), 0.5 ml potassium antimonyl tartarate (0.686 g dissolved in 250 mL distilled water), 1.5 mL ammonium molybdate (4 g dissolved in 100 mL distilled water) and 3 mL ascorbic acid (1.76 g dissolved in 100 mL distilled water)] were added. Absorbance was measured at 880 nm after 10 minutes incubation. A series of standards (54.87 mg anhydrous potassium dihydrogen phosphate dissolved in 250 mL distilled water gave 50 μg PO_4^{3-}/ mL) were prepared and calculated the factor value.

Concentration of phosphate ions in sample in mg/L = factor value x absorbance.

2.2.6.9 Estimation of total phosphorus (Persulphate digestion method) (Strickland and Parsons, 1972)

Organically bound phosphorus is completely decomposed to phosphate by a strong oxidizing agent (alkaline persulphate).

An aliquot of 40 mL sample was taken in a 100 mL screw capped glass bottle, added 8 mL potassium per sulfate reagent (5 g potassium persulphate and 3g boric acid dissolved in 100 mL 0.375 M sodium hydroxide solution). The solutions were mixed well, autoclaved for 30 minutes under 15 lbs pressure, adjusted the pH and analysed inorganic phosphate.

Concentration of phosphate ions in sample in mg/L = factor value x absorbance

2.2.6.10 Estimation of total Kjeldhal nitrogen (APHA, 1995)

In the presence of concentrated sulphuric acid, potassium sulphate and cupric sulphate catalysts, organically bound nitrogen gets converted into ammonium bisulphate. Potassium sulphate was added to raise the boiling point of sulphuric acid from 345°C to 375° C. The digest was diluted, made alkaline with sodium hydroxide and distilled. The liberated ammonia was absorbed in boric acid. The absorbed ammonia was determined by titration.

Digestion of the sample.

An aliquot of 50 mL sample was taken in 800 mL Kjeldhal flask with few glass beads to which 50 mL digestion reagent (134 g potassium sulphate and 7.3 g copper sulphate dissolved in 800 mL distilled water and carefully added 134 mL concentrated sulphuric acid and made up to 1L with distilled water) was added and boiled the solution. As digestion continued, coloured – turbid sample became transparent and pale green. After the digestion, cooled the solution and diluted to 300 mL with distilled water. After that carefully added 50 mL sodium hydroxide – thiosulphate reagent (500 g sodium hydroxide and 25 g sodium thiosulphate dissolved in 1 L distilled water) to form an alkaline layer at the flask bottom. Swirled the Kjeldhal flask to ensure complete mixing.

Distillation

A quantity of 50 mL of boric acid (20 g boric acid dissolved in 1L distilled water) with mixed indicator solution (100 mg of methyl red and 100 mg of methyl blue dissolved in 100 mL 95% ethyl alcohol) (pink colour solution) was taken in a 500 mL flask and placed it below the condenser of the distillation unit. Collected 200 mL of distillate in flask (pink solution turns to green due to absorption of ammonia) and titrated the distillate against 0.2 N sulphuric acid till the original colour appeared (i.e. change of green to pink

colour indicated the end point of the titration). Distilled water blank was also run in the same procedure as above.

Calculation

Total –N (TKN) mg/L = (A-B) x 280/ mL of sample

Where

A = Volume of H_2SO_4 required of sample, mL

B = Volume of H_2SO_4 required for blank, mL

2.2.6.11 Estimation of Silicate (Strickland and Parson, 1972)

The sea water sample is allowed to react with molybdate under conditions that result in the formation of silicomolybdate, phosphomolybdate and arsenomolybdate complexes. A reducing solution, containing metol (4 Methylamino phenol sulphonate) and oxalic acid, is then added which reduces the silicomolybdate complex to give a blue reduction compound and simultaneously decomposes any phosphomolybdate or arsenomolybdate, so that interference from phosphate, arsenate is eliminated.

An aliquot of 25 mL sample was taken in a stoppered conical flask, 10 mL of the molybdate solution (4 g ammonium paramolybdate dissolved in 300 mL distilled water and 12 mL of concentrated sulphuric acid were added and made up to 500 mL) was added and mixed the solution and allowed to stand for 10 minutes. Then 15 mL reducing reagent was added immediately and made the volume to 50 mL. After three hours of incubation absorbance was measured at 810 nm. The reducing reagent was prepared by mixed 100 mL metol sulphite solution [6 g sodium sulphite dissolved in 500 mL distilled water and then added 10 g metol (p-methyl aminophenol sulphate)], 60 mL oxalic acid solution (50 g of oxalic acid dehydrate dissolved in 500 mL distilled water) and 60 mL 50% sulphuric acid and made up to 300 mL with

distilled water. A series of standards of silicate (313 mg sodium hexa fluorosilicate dissolved in 1000 mL distilled water gave 0.1 mg SiO_2/mL) were prepared and calculated the factor value.

Concentration of silicate ions in sample in mg/L = factor value x absorbance.

2.2.6.12 Estimation of Hydrogen Sulphide (Grasshoff, 1983)

An aliquot of 10 mL sample was taken in a test tube, added 0.2 mL N, N-dimethyl-p-phenylene diamine dihydrochloride (2 g dissolved in 500 mL 6 M hydrochloric acid) and 0.2 mL ferric chloride (8 g dissolved in 500 mL 6 N hydrochloric acid). Absorbance was measured at 670 nm after 1-hour incubation. A series of standards of sulphide (0.750 g sodium sulphide was dissolved in 1 L distilled water) were prepared. An aliquot of 25 mL of the above solution and 5 mL zinc acetate (10.44 g zinc acetate dissolved in 1 L distilled water) pippeted out to 500 mL volumetric flask and made up to 500 mL with distilled water (which gave 5 µg sulphide/mL) and calculated the factor value.

Concentration of sulphide in sample in mg/L = factor value x absorbance.

2.2.6.13 Estimation of BOD (Biochemical Oxygen Demand) (APHA, 1995)

Biochemical oxygen demand is defined as the amount of oxygen required by bacteria in decomposing organic material in a sample under aerobic conditions at 20^0C over a period of 5 days.

Dilution method

1. Preparation of dilution water
   a. Aeration – Aerated 1 L distilled water by bubbling compressed air for 1 day to attain dissolved oxygen saturation. Tried to maintain temperature near 20°C.

b. pH –neutral pH (7.2) .

c. Addition of nutrients –1 mL each of phosphate buffer (8.5 g potassium dihydrogen phosphate, 33.4 g disodium hydrogen phosphate and 1.7 g ammonium chloride dissolved in 1 L distilled water) magnesium sulfate (22.5 g dissolved in 1 L distilled water), calcium chloride (27.5 g dissolved in 1 L distilled water) and ferric chloride (0.25 g dissolved in 1 L distilled water) solution were added to 1 litre of dilution water and mixed well.

Dilution method (without seeding)

Samples were diluted in standard dilution water in a graduated cylinder of 1000 mL capacity and thoroughly mixed together. Transferred the diluted sample in to 4 labelled BOD bottles and closed immediately. One bottle was used for determination of the initial dissolved oxygen and other three bottles were incubated at 20^0C for 5 days. After incubation 1 mL winkler A and 1 mL winkler B reagents were added into the bottle, initial and final dissolved oxygen contents of the samples were determined.

Calculation

BOD mg/L = D1 - D2/P

Where

D1 = DO of the sample bottle on 0 day

D2 = DO of the sample bottle after 5th day

P = Volume of sample / volume of sample + dilution water.

2.2.6.14 Estimation of phytoplankton (APHA, 1995)

Qualitative and quantitative estimates of phytoplankton were undertaken at weekly intervals. Plankton samples were collected from the

ponds through fine-meshed plankton net (0.025 mm). The samples were preserved immediately with 5% buffered formalin in plastic bottles. Plankton density was estimated by using a sub-sampling technique. A Sedgwick–Rafter (S–R) cell was used under a calibrated binocular compound microscope for plankton counting. Plankton were identified to genera and were counted using the formula proposed by Clesceri *et al*. (2005) and was expressed as the number of cells per liter of water.

2.2.6.15 Primary productivity (light and dark bottle method) (Wetzel and Likens, 1991)

Productivity is defined as the rate at which inorganic carbon is converted to organic form. Primary productivity can be determined by measuring the changes in oxygen and carbon dioxide concentration. In this method clear (light) and black (dark) bottles are filled with water samples and suspended in the water column for an incubation period of six hours. Alternatively, the samples can be incubated under controlled conditions in environmental growth chambers also. The dissolved oxygen concentration is determined at the beginning and the end of the incubation period. Productivity is calculated on assuming that one atom of carbon is assimilated for each molecule of oxygen released.

Water sample was taken in three BOD bottles from the ponds. One of the clear bottles was immediately fixed to calculate the initial dissolved oxygen while the remaining two bottles, one light bottle and the other dark bottle (covered to protect from light) were hung at the depths from where the water samples were collected with the help of stack and rope. The samples were incubated in situ for 6 hours and then removed to measure the oxygen concentration.

Calculation

The increase in oxygen concentration in the light bottle during incubation period is a measure of net production, which is because of concurrent use of oxygen in respiration. It is somewhat less than the total production. The loss of oxygen in dark bottle is used as an estimate of total plankton respiration.

Net Photosynthesis = Light bottle DO – Initial DO

Respiration = Initial DO – dark bottle DO

Gross photosynthesis = Light bottle DO – dark bottle DO

Thus

NPP = DO in light bottle – initial DO x 0.375 / T x12

R = Initial DO – DO in dark bottle x 0.375 / T x 12

GPP = DO in light bottle – DO in dark bottle x 0.375 / T x 12

Where

GPP = Gross primary productivity in gC/m^3/day or mg C/L/day

NPP = Net primary productivity in gC/m^3/day or mg C/L/day

R = respiration on gC/m^3/day or mg C/L/day

T = Time period of incubation (h)

0.375 is a factor used to convert oxygen to carbon.

2.2.6.16 Estimation of Chlorophyll (APHA, 1995)

Known volumes of water samples were taken from the ponds and filtered through micro fibre glass filter paper (GF/C Whatman) using a vacuum pressure air pump. Placed the filter paper in the tissue grinder added 3 mL 90% acetone and ground until the filter fibers were separated. Saved the acetone and put the ground filter in to a centrifuge tube and made up the total

volume of 10 mL. The filter paper was kept in dark at 4°C for 12 hours. After incubation centrifuged the content at 3000 rpm for 15 minutes. Decanted the clear supernatant in to cuvettes. Absorbance was measured at 664 nm, 647 nm, 630 nm and 750 nm.

Calculation

Chlorophyll a (mg/L) = 11.85 (Abs_{664}) – 1.54 (Abs_{647}) – 0.08 (Abs_{630})

Chlorophyll b (mg/L) = 21.03 (Abs_{647}) – 5.43 (Abs_{664}) – 2.66 (Abs_{630})

Chlorophyll C (mg/L) = 24.52 (Abs_{630}) – 7.60 (Abs_{647}) – 1.67 (Abs_{664})

2.2.7 Soil quality

2.2.7.1 Estimation of soil pH

Weighed 20 g fresh sediment, from which stones, twigs and larger materials were removed and placed in a beaker. An aliquot of 40 mL distilled water was added and stirred the preparation vigorously on a magnetic stirrer. The suspension was incubated for 30 minutes. The clear supernatant was decanted and saved and the pH determined using a pH meter (Scientific tech, India).

2.2.7.2 Redox potential

Redox potential determines the geochemical mobility of pollutants and nutrients in various parts of the environment and consequently their influence on ecosystems. Microbial respiration in soil provides electrons that drive most redox reactions and these act to reduce available oxygen. However, if the rate of oxygen used up in respiration exceeds oxygen availability, reduction of other substances takes place and this can affect the speciation of nutrients either directly or indirectly.

The sediment sample were taken from four sides of the pond bottom and placed in an ice box and immediately transferred to the laboratory. Stones, twigs and larger materials were removed from the sample and dipped the ORP meter (Eutech instruments, Japan) electrode and noted the E*h* value.

2.2.7.3 Determination of total organic carbon

Soil organic matter is oxidized under standard conditions with excess of potassium dichromate in sulphuric acid solution, and the excess dichromate is determined by titration against standardized ferrous ammonium sulphate solution. From the value of dichromate consumed the organic carbon content can be calculated.

Weighed 0.2 g of finely divided ground soil in to a 500 mL conical flask. Pipetted out 10 mL of 1 N potassium dichromate solution (24.52 g dry potassium dichromate dissolved in 500 mL distilled water) in to the flask. The flask was swirled and mixed the dichromate with soil. Subsequently, 20 mL concentrated sulphuric acid was added and mixed by gentle rotation for 1 minute to ensure complete contact. The flasks were incubated 30 min, then added 200 mL distilled water and 3 mL ferroin indicator. Titrated the contents of the flask against 0.25 N ferrous ammonium sulphate solution (98 g ammonium ferrous sulphate dissolved in 400 mL distilled water and 10 mL concentrated sulphuric acid made up to 500 mL). The color of the solution changed from blue to green and finally to grayish red at the end point (as the dichromate is reduced, the solution becomes greener and just before the end point clear bluish green color develops. As soon as there is slight excess of ferrous ion, color changes to grayish red which is being the reduced ferrous orthophenathrloine complex masked by the green chromium ion). The distilled

water blank were also done in an identical manner using the same reagents alone

Calculation

Total Organic carbon in soil (%) = (A-B) x normality of FAS x 0.003 x 100 / weight of soil taken

Where

A = Volume of blank

B = Volume of titrant

FAS = Ferrous ammonium sulphate

2.2.7.4 Total Nitrogen

Nitrogen in soil is present mostly in organic form, together with small quantities of ammonium and nitrates. Kjeldhal method measures only organic nitrogen and ammonium excluding nitrates.

The soil is digested with concentrated sulphuric acid in the presence of a catalyst. As the proper digestion takes place at higher temperature (360 – 410ºC), sodium sulphate is added to raise the boiling point of sulphuric acid, finally after digestion, the nitrogen gets converted to ammonium sulphate and determined after distillation under alkaline conditions.

A quantity of 5 g sediment sample was taken in 300 mL Kjeldhal flask, added 25 mL distilled water to moisten the soil. A quantity of 20 g catalyst mixture (This was composed of ground 20 g copper sulphate, 3 g mercuric oxide and 1 g selenium powder. One part of this mixture was mixed with 20 parts of sodium sulphate or potassium sulphate) and an aliquot of 35 mL concentrated sulphuric acid were added and mixed gently. The solution was

heated initially at low heat for first 10 to 30 minutes, until frothing stopped and subsequently the temperature was elevated. The digestion was continued until the content became light yellow. The solution was heated further for about one and half hour to release all the residual nitrogen. The digest was cooled and added 100 mL distilled water mixed thoroughly and transferred the supernatant to 800 mL distillation flask. An aliquot of 100 mL 40% sodium hydroxide and few pieces of zinc were added to the distillation flask and swirled gently. A 500 mL conical flask containing 25 mL of boric acid (4 g boric acid dissolved in 100 mL distilled water) with mixed indicator (Mixed alcoholic solution of bromocresol green (0-5%) and methyl red (0-1%) in 2:1 ratio) was placed below the condenser and started the distillation. During distillation colour of the solution changed to blue due to the dissolution of ammonia. An aliquot of 150 mL of the condensate were collected and removed the flask before stopping the heating to prevent back sucking. The contents were titrated against 0.1 N hydrochloric acid until the colour changed to pink.

Calculation

a. When whole digest is distilled:

$$\text{Total nitrogen \%} = \frac{\text{A} - \text{B x Normality of HCL x 1.4}}{\text{S}}$$

Where

A = mL of hydrochloric acid used with sample

B = mL of hydrochloric acid used with blank.

S = weight of soil taken.

2.2.8 Shrimp Health parameters

2.2.8.1 Selection of shrimp post larvae

Examined the post larvae for their morphological features such as rostral spine numbers, length of carapace and sixth abdominal segment to differentiate between PL 20 and early stages of post larvae. The color, visual swimming behavior, shell, muscle, gut, muscle to gut ratio, pigmentation and appendages were observed under a microscope.

Table 2.2. Quality of Post larvae of *P.monodon*

Quality	Healthy seeds
Colour	Light grey/black/ brown/ transparent
Activity	Swimming actively, they do not clump together
Feeding	Readily accept and consume feed
Shell	Clean Shell
Muscle	Clear, smooth and thick muscle
Gut	Full gut
Pigmentation	Chromatophores well defined and located along the mid ventral line
Appendages	Intact, with out any deformity

2.2.8.2 Stress test

Subject the shrimp post larvae to following stress tests.

2.2.8.2.1 *Salinity test*

Exposed a sample of seeds reared at 30 g/L salinity to 15 g/L salinity for 2 hours and transferred to the initial state. Healthy fry survived the sudden change in salinity and resumed feeding with in 24 hours of the test.

2.2.8.2.2. Formalin test

About 100 seeds were exposed to 100 ppm formalin for 2 hours and shifted back to fresh seawater. Healthy fry were found surviving.

2.2.8.3 Health assessment

The animals were regularly observed for the parameters such as black spot on the shell and carapace, tail spreadness, blisters, antenna cut, gut fullness, black gill, stunted growth, milky shrimp/cotton shrimp, weak shrimp, intestine fullness, hind gut swelling and white gut, body cramp, muscle necrosis and melanosis, red discolouration, physical damage and, blisters on the body.

Growth performance and factors affecting growth were analysed. Weekly health condition factors such as mean body weight, average daily growth, survival rate, total biomass, feed requirement, % feed used, feed increment and FCR were estimated.

Daily feed requirement = Standing crop x percentage of feed

$$\text{Survival rate} = \frac{\text{Actual feed consumed per day}}{\text{No. of animal stocked x Averagebody weight x \% feeding rate}}$$

$$\text{Growth per day} = \frac{\text{Averagebody weight x feed \%}}{\text{FCR}}$$

$$\text{FCR} = \frac{\text{Quantityof feed consumed}}{\text{Totalweight gain.}}$$

$$\text{Feed efficiency} = \frac{\text{Total weight gain}}{\text{Quantityof feed consumed}}$$

2.3 Results

2.3.1 Stress test of the larvae and screening

On subjecting, the larvae for formalin and salinity stress tests all the larvae were found surviving and consuming feed suggesting that they were healthy enough to be stocked. Such batches of larvae were used for stocking.

2.3.2 Water quality analysis - Physico - chemical analysis

Water quality parameters during the culture period are summarized in Table.3. pH, alkalinity, transparency and dissolved oxygen did not show significant difference between the control and test ponds and they were at the optimum range for *Penaeus monodon* culture. The average morning pH of the test ponds was 8.2 ± 0.07 and in the control it was 8.04 ± 0.08, while in the evening 8.6 ± 0.18 and 8. 5 ± 0.14 respectively (Fig.1 & 2). Evening pH of the test ponds was 9.2 at times due to the presence of heavy phytoplankton bloom.

Total alkalinity here refers to carbonate and bicarbonate alkalinity, as hydroxide was not present during the entire period, which ranged between 54 ± 1.41 to 106 ± 0 mg/ L and 53 ± 2.94 to 87 ± 1.88 mg/ L in the test and control ponds respectively. Addition of dolomite helped maintain the level of alkalinity in the ponds (Fig.1& 2, Table 3).

Water transparency or Seechi disc visibility, an indicator of phytoplankton density, was highest in the beginning of the culture that gradually declined towards the end of the cycle, due to the increased phytoplankton production as evidenced by the increased chlorophyll content. (Fig. 3).

Salinity in both the category ponds ranged between 8 to 15 g/L. Calcium and magnesium concentration showed an increasing pattern corresponding to the increase of salinity. Average of water temperature was similar among the ponds during the experimental period and varied with in a narrow range. At the initial period of the culture water temperatures was 26 ± 0.02 and gradually increased and reached 30 ± 0.03° C at the end of the study. The gradual increase in water temperature during the growing period was associated with the gradual rise in air temperature during summer season which affected the thermal pattern in water through out the culture period (Table 3).

Dissolved oxygen levels in the test ponds were above 4 mg/L during the culture period (Fig. 4). Four paddle wheel aerators were used in both sets of ponds. In the test ponds aeration started after 120 days of culture, however, in the control ponds it was required to give aeration after 30 days of culture. In the control ponds dissolved oxygen below 3 mg / L could be observed at one or two occasions

In the case of total ammonia nitrogen there was a significantly lower concentration in the test ponds compared to the control ($P<0.001$). Total ammonia nitrogen concentrations in the test ponds ranged from 0 to 0.06 mg/L with the mean value of 0.02 ± 0.007 mg/L and in the control ponds from 0 to 0.21 mg/L with the mean value of 0.08 ± 0.02 mg/L respectively. The concentrations fluctuated largely throughout the culture period in the control ponds and there was a significant difference between the initial and final phase of the culture ($P = 0.002$) (Fig. 5). The highest ammonia value recorded in the control ponds was 0.21 ± 0.02 mg/L during the 12th day sampling. The lowest recorded value was 0 mg/L in the test ponds which was recorded during several occasions. The

concentrations of nitrite nitrogen in the test ponds were generally low throughout the culture period and ranged between 0.006 ± 0.0007 to 0.02 ± 0.0027 mg/L with the mean value of 0.02 ± 0.005 mg/L. However, in the control ponds it varied between 0.006 ± 0.003 to 0.09 ± 0.024 mg/L with the mean value of 0.04 ± 0.008 mg/L and significantly lower values were observed in the test ponds (P = 0.002) (Fig. 6). There was a significant difference between nitrate nitrogen in the two category ponds and it ranged between 0.02 to 0.13 mg/L with the mean value of 0.07 ± 0.01 mg/L in the test ponds and 0.03 to 0.24 mg/L with the mean value of 0.12 ± 0.02 mg/L in the control ponds respectively (P<0.01) (Fig. 7). The highest nitrate concentration was observed in the control ponds during the 6th sampling.

The mean value of total nitrogen in the test ponds was 0.17 ± 0.034 mg/L and varied between 0.08 ± 0.022 to 0.31 ± 0.046 mg/L. In the control ponds the concentration was observed an increasing pattern up to 6th week, since thereafter, a reduction up to 10th week, then gradually increased and reached 0.47 ± 0.065 mg/L. The average concentration during the entire cultural period was 0.3 ± 0.05 mg/L and the mean was significantly different (P<0.001) (Fig. 8).

There was no significant difference between the concentration of inorganic phosphorous and total phosphorus in both the category ponds. The maximum concentration occurred at the 6th week and the pattern of variation was the same in both the systems (Fig. 9). Maximum total phosphorus (0.39 mg/L) occurred in the control ponds during the sixth week which corresponded to the inorganic phosphorus (0.229 mg/L) during the same period (Fig. 10).

Silicate showed a declining trend in the test ponds. The maximum (1.1 ±0.115 mg/L) during the initial weeks and the minimum 0.6 ± 0.34 mg/L

towards the end of the culture, while the control ponds did not show a definite pattern. In the control ponds it ranged between 0.51 ± 0.05 to 0.91 ± 0.04 mg/L, significantly lower compared the test ($P < 0.001$)(Fig. 11).

There was not any significant difference of hydrogen sulphide and biochemical oxygen demand between both the category ponds. Hydrogen sulphide concentration was observed low throughout the culture period (Table 3.)

2.3.3 Biological analysis

Concentration of chlorophyll *a*, a quantitative measure of the amount of phytoplankton, was significantly higher in the test ponds compared to the control ponds ($P<0.05$)(Fig. 12) which in the test ponds ranged between 5.54 to 44.22 µg/L with the mean value of 24.17 ± 6.8 µg/L. However, in the control ponds they were between 5.3 to 33.95 µg/L with the mean value of 18.09 ± 5.03 µg/L. Average concentration of Chlorophyll *b* was found 4.485 ± 1.3 µg/L and 2.356 ± 0.9 µg/L in the test and control ponds respectively. Gross primary productivity with significantly higher values were observed in the test ponds ($P<0.05$) (Fig. 13, Table 3). Net primary productivity in the test ponds ranged between 0.05 ± 0.03 to 2.2 ± 0.078 g C/m^3/day and in the control ponds it ranged between 0.5 ± 0.04 to 1.7 ± 0.067 g C/m^3/day.

2.3.3.1 Phytoplankton.

Phytoplankton count in the test ponds ranged from 4.8 x 10^4 cells/mL to 71 x 10^4 cells/mL and in the control ponds from 4.2 x 10^4 cells/mL to 57.3 x 10^4 cells/mL. Phytoplankton population density showed a tendency to increase

when culture progressed. The major families present in the culture ponds were Cyanophyceae (6 genera) Bacillarophyceae (7 genera) Chlorophyceae and Pyrrophyceae (2 genera) (Table 4).

2.3.3.2 Zooplankton

The major groups of zooplankton recorded in the test and control ponds were almost similar and among them protozoans were the prominent group. In the test ponds 30% was occupied by protozoans followed by unidentified crustaceans (28%), copepods (20%), rotifers (10%), cladocerans (2%) and 10% could not be identified. In the control ponds protozoans and unidentified crustaceans were the most prominent groups (28%) followed by copepods (22%), Rotifers (12%), cladocerans (4%) and 6% could not be identified (Table 5).

2.3.4 Microbiological analysis

Total bacterial count ranged from 1.3 x 10^4 to 3.5 x 10^7 CFU/mL in the test ponds and in the control ponds it ranged from 2.3 x 10^4 to 2.1 x 10^7 CFU/mL. Total *Vibrio* count on TCBS plates was significantly lower ($P<0.05$) in the test ponds compared to that of the controls. *Vibrio* counts ranged between 60 to 262.5 CFU/mL with the mean of 157.59 ± 20.5 CFU/mL and 62.5 to 410 CFU/mL with the mean of 236.7± 28.7 CFU/mL in the test and control ponds respectively.

2.3.5 Sediment quality

Sediment quality parameters measured during the culture period are summarized in Table 6. Sediment pH ranged between 7.5 ± 0.556 to 8.34 ± 0.678 and there was no significant difference between the test and control ponds (Fig. 14). Soil E*h* in the test ponds ranged between -72 to -166 mV

with the mean value of -140 ± 10.8 mV and in the control ponds between -96 to -226 mV with the mean value of -174 ± 12.16 mV which was significantly different (P<0.0001)(Fig.15). The lower redox potential occurred in the control ponds at the final phase of the culture with significant difference between initial, mid and final phases of the culture period, while in the test ponds significant difference occurred between the initial and mid phase of the culture, however the mid and final phase did not show significant difference.

The concentrations of ammonia- nitrogen, nitrite- nitrogen and nitrate-nitrogen on the 120th, 150th, and 170th days of culture in the pond sediment treated with probiotics were lower than that of the control ponds (Fig. 16).

The concentration of total organic carbon and organic matter showed a significantly lower value in the test ponds compared to control (Fig. 17). The maximum TOC content in the sediment was 2.3 ± 0.09 % at 170th day in the control ponds. Total organic matter in the test ponds ranged from 0.55 ± 0.27 % to 2.24 ± 0.157% with the mean value of 1.197 ± 0.175, while in the control ponds it ranged from 0.55 ± 0.27% to 3.96 ±0.09% with the mean value of 2.04 ±0.28%.

The concentrations of total nitrogen and total phosphorus in the test ponds were lower than that of the control ponds. The amount of total phosphorus in the test ponds ranged from 2.15 ± 0.017 mg/g to 5.7 ± 0.014 mg/g, while that of the control ponds 2.37± 0.12 mg/g to 7.6 ± 0.134 mg/g (Fig. 18). Total nitrogen concentration in the test ponds ranged from 0.12 to 0.19% with the mean of 0.14 ± 0.035 and in the control ponds it ranged from 0.13 to 0.4% with the mean of 0.2 ± 0.08 respectively (Fig. 19). Total

heterotrophic bacteria in the sediment of the test ponds ranged from 1.33 x 10^4 CFU/g to 2.4 x 10^6 CFU/g and in the control ponds ranged from 1.22 x 10^4 CFU/g to 1.85 x 10^6 CFU/g.

2.3.6 Health assessment

Results of health assessment in the test and control ponds are shown in Table.7. During health assessment colour of the shrimps in both test and control ponds was normal. The exoskeleton in the animals of test ponds was quite normal but in the control ponds 4 to 15% of animals showed fouling with algae, 5 to 10% exhibited necrosis, 5 to 10% physical damage (breakages of exoskeleton). Antennae of the animals were normal in test ponds whereas in the control ponds 5 to 12% of animals exhibited antennae cut. Appendages were normal in colour in the animals of both the systems but partial fouling with algae was observed in those of control ponds. In the tail region, 5 to 30% of animals of control ponds exhibited blisters and 10 to 20% showed fouling with algae. Gills were normal in colour in the animals in both the ponds; however, 5% showed swelling in the control ponds. The fullness of intestine was comparatively higher in the animals in test ponds. Towards the terminal part of the culture around 20% of the animals in the control pond were with loose shell.

2.3.7 Growth parameters

Results of overall production of shrimp in both the category of ponds are summarized in Table 8. The average survival of post larvae in hapa maintained in both the two category ponds were 85% after 24 hours of stocking. On completion of the study period (160 days of culture), the shrimps of average size of 37.2 ± 0.88 g were harvested from the test ponds. They registered an

average daily growth of 0.23 g/day. In the control ponds the average daily growth was 0.18 g/day and the harvested shrimps had an average body weight of 33.4 ± 0.98 (182 days of culture). The survival was significantly higher in the treated ponds (74 ± 0.99%) compared to the control ponds (54 ± 0.89%). However, the feed conversion ratio of the test ponds was significantly lower (1.9) than the control groups (2.7).

2.4 Discussion

The study was focused on the performance of a zero water exchange shrimp culture system with the addition of bioaugmentor and probiotics. This application significantly enhanced the efficiency of waste removal and enhanced the survival and production of the shrimp. Wang *et al*. (2005) investigated the effect of commercial probiotics on water quality in shrimp, *P. vannamei*, and showed that probiotics could significantly reduce the concentrations of nitrogen and phosphorus in pond water compared with those of the control, and enhanced the survival and production.

pH and alkalinity did not show significant difference between the test and control ponds and they were at the optimum level for *Penaeus monodon* culture. Ramakrishna (2000) and Soundarapandian (2010) recommended pH of 7.5 to 8.5 for *P. monodon*. Fluctuation of pH around the neutral value during culture period has been reported earlier, and it does not affect the growth of the shrimp (Chen and Wang 1990). Evening pH was slightly higher than the morning due to the changes in the rate of photosynthesis by phytoplankton and other aquatic plants in response to the daily photoperiod. Transparency mainly depends on the presence of phytoplankton and some times it is influenced by the presence of particulate matter in the water column.

During the study period average transparency in the test ponds was 34.9 cm and in the control ponds 27.03 cm. However, in the control ponds transparency reached below 20 cm during the end of the culture period which may be due to the presence of particulate matter in the water column.

Dissolved oxygen level in the ponds could be maintained through the use of paddle wheel aerators in the control ponds throughout the culture period and in the test ponds after only 120 days of culture without significant difference. Aerators are commonly operated almost continuously to both supply oxygen and to maintain water under circulation in ponds (Martinez-Cordova *et al.*, 1998). Hopkins *et al.* (1996) successfully farmed shrimp in ponds with reduced or no water exchange, and with appropriate aeration level. In the present study the slightly higher level of dissolved oxygen recorded in the test ponds even in the absence of aeration could be attributed to the beneficial effect of probiotics which paved the way for more phytoplankton production and increased photosynthetic activity. The early morning dissolved oxygen levels in both the category ponds were negatively correlated with productivity (-0.741,-0.631) and chlorophyll (-0.919, -0.95).

Nitrogen plays an important role in the limited discharge of aquaculture system due to its dual role, as a nutrient and a toxicant (Burford and Lorenzen, 2004). Nitrogen in the form of ammonia (NH_4^+) and nitrite (NO_2^-) are toxic to shrimp, however the toxicity depends on various factors including species tolerance, water characteristics (pH, temperature, salinity and dissolved oxygen) and exposure duration (Hargreaves, 1998; Barajas *et al.*, 2006). Mean Total Ammonia Nitrogen (TAN) concentration was significantly lower in the test ponds and it never exceeded the safe range during the culture period. This may be due to the fact that deleterious nitrogenous wastes were effectively

removed by phytoplankton and microbial activity. Burford *et al.* (2003) reported in a zero water exchange system of Belize Aquaculture Ltd, USA, with plastic lining and high aeration rates, perpetual high nitrite, ammonia and phosphate concentrations. Moreover, it was reported earlier that in *P. monodon* grow out system, even with frequent water exchange ammonia might increase up to 6.5 mg/L. (Chen and Tu, 1991). Nitrite and nitrate were significantly lower in the test ponds compared to those of the control. Nitrite accumulation is a typical cycling of nitrogenous compounds that occurs as a result of the establishment of nitrifying bacteria that carryout the oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) and nitrate (NO_3^-) (Krishnani *et al.,* 2009) and has significant effects on the shrimp performance (Lin and Chen, 2003; Vinatea *et al.,* 2010). In the study of Vinatea *et al*. (2010) nitrite concentration had significant inverse correlation with shrimp growth rate. Nitrate, unlike ammonia and nitrite is less toxic to shrimp, however, high concentration (100 mg/L) was reported to be lethal (Muier *et al.,* 1991, Rijin *et al.,* 2006). The average nitrate levels in the test ponds recorded were also lower than what was recorded by Briggs and Fungesmith (1994) in the shrimp ponds of Thailand. In the present study total nitrogen was found positively correlated with chlorophyll (r = 0.689, 0.684), and productivity (r = 0.537, 0.792) in both the test and control ponds respectively

The concentrations of inorganic and total phosphorus in the test and control ponds did not show significant differences. This result is in contrast with the findings of Boyd (1990) who reported that dissolved orthophosphate concentrations were usually not greater than 5-20 μg/L and seldom exceeded 100 μg/L even in highly eutrophic waters. However, the studies of Burford *et al.,* (2003) in a closed system showed phosphate concentrations between 0.07 - 1.17

mg/L. In the present study the nutrient concentrations in the water column was found linearly correlated with the cumulative feed input. In a closed system nutrients keep on accumulating overtime which might be an advantage as the high concentrations of nitrogen and phosphorus with in the system could support the growth of natural food organism contributing ultimately to the shrimp growth. According to Thakur and Lin (2003), the total nitrogen and total phosphorus concentrations in water were found increasing with the progress of rearing.

The concentration of hydrogen sulphide did not show significant difference between both sets of ponds because a layer of photosynthetic green and purple sulphur bacteria might have developed at the pond bottom especially in the hypolymnion which utilized H_2S as the source of hydrogen ions for photosynthesis there by oxidizing it to hydrogen and sulphur. This phenomenon might have prevented H_2S from entering in to the water column.

The ranges of total heterotrophic bacterial (THB) counts recorded in this study were within the levels reported earlier in India (Sharmila *et al.,* 1996; Otta *et al.,* 1999) and South-East Asia (Peranginangin *et al.,* 1992; Sung *et al.,* 2001). There was a significant difference in *Vibrio* counts between test and control ponds and the mean value in the test pond was 157 CFU/mL and in the control ponds it was 236 CFU/ mL. There was significant positive correlation between *Vibrio* and salinity in the ponds. The high saline modified extensive pond had more *Vibrio* including luminous vibrios (Abraham and Debasis, 2009). These results are in conformity with Abraham *et al.* (2003) who reported salinity dependant distribution of luminous vibrios in shrimp farms.

According to Moriraty (1998) the luminescent vibrios present in the pond water were displaced when a strain of antagonistic *Bacillus* sp. was added.

Moreover, the use of probiotics influences digestive processes by enhancing the population of beneficial microorganisms in the gut, which enhances microbial activity and improves feed utilization (Bomba *et al.*, 2002). Rengpipat *et al.* (1998) reported that appropriate probiotics applications were shown to improve intestinal microbial balance, leading to improved food absorption.

In the case of primary productivity, there is significantly higher value in test ponds compared to control ponds. It is a classical pathway to remove mineral nutrients from water and helps pond digestion, which leads to reduction of mineral nitrogen as pollutant in the discharge (Hopkins *et al.*, 1995). In the present study, these values were lower than the studies of Hepher and Pruginin (1981) and Ali (1986).

Chlorophyll *a* concentration was generally low at the initial phase of the culture which gradually increased over time. In the test ponds it was significantly higher than that of the control ponds ($P>0.005$). In general these values were lower than those in the studies of Cremen *et al.* (2007) who reported chlorophyll a concentrations between 54 µg/L to 75 µg/L in ponds run under green water technology. There was a high positive correlation between productivity and chlorophyll in both the category ponds.

A steady phytoplankton count was observed in the test ponds throughout the culture period, and significantly higher compared to control groups. During the initial period of the culture, diatoms such as *Nitzschia*, *Pluerosigma* and *Navicula* etc., were dominant in the test ponds and when culture progressed they were replaced by Cyanophyceae. According to Case *et al.* (2008) diatom dominance gets replaced with Cyanobacteria as nutrient concentration increases and silica gets depleted within the culture period. Yusoff *et al.* (2002) also

reported that shrimp ponds which received unpolluted water were dominated by diatoms in the beginning of the culture period and Cyanobacteria appeared after the first month of the culture. The presence of diatoms in the beginning of the culture period is crucial as they formed the beneficial natural feed for post larvae stocked. In addition good water quality in the initial phase of the culture was also important to allow growth and proliferation of beneficial macrobenthos, which would become major natural food items for juvenile and post larvae of shrimp. Studies carried out on phytoplankton on shrimp farms in Bangladesh by Islam *et al*. (2004) identified that Bacillariophyceae was the largest group being represented by up to 13 genera, while Pyrrophyceae was the smallest group, represented by only two genera. In shrimp ponds from Mexico, Rodrıguez and Paez-Osuna (2003) listed 48 genera of phytoplankton that commonly occurred in coastal areas. They also reported Bacillariophyceae, Cyanophyceae, Chlorophyceae, and Euglenophyceae as the dominant groups in coastal waters and in the shrimp farming systems. High phosphate usually encourages the growth of Cyanophyceae, whereas high nitrate concentration encourages diatom growth (Vanni and Findley, 1999).

Pond bottom soil containing black and glutinous organic sludge is required to be treated before the commencement of the next crop as the accumulated toxic material and high density of shrimp pathogens (*Pseudomonas* and *Vibrio*) may turn out to be harmful to shrimp. Therefore, appropriate pond preparation and maintenance of optimum soil pH, E*h* and organic carbon are more vital to sustainable shrimp culture. Liming is considered as one of the most important procedures for maintaining soil at a satisfactory pH (Charman and Murphy 2000). Nimrat *et al.* (2008) showed that

addition of probiotics with drying and tilling of the sediments was effective in significantly maintaining the soil physical and chemical characteristics.

Pond sediment plays an important role in nutrient cycling by retaining or releasing nutrients. During the study period the sediment pH stood at optimum range for the culture operation. Boyd and Pipoppinyo (1994) reported that sediments were to be at a pH range of 7.5-8.0 because microbial activity was most rapid at that pH range. Application of probiotics to the pond soil can accelerate decomposition of undesirable organics and other waste products (Gatesoupe, 1999). Microbial decomposition of organic matter recycles nutrients and prevents accumulation of organic matter in pond bottom. The optimum range of organic carbon in pond sediment is 1-3% (Banerjea, 1967). However, during the study period organic matter concentrations in both the category ponds were lower than the optimum range. Lower concentrations are unfavorable for the growth of benthic organisms that are important food for many species and higher concentrations favor anaerobic conditions at the sediment - water interface. Soil organic matter contains about 50-58% carbon (Nelson and Sommers, 1982). Redox potential of the test ponds was significantly lower compared to control ponds. Previous studies reported that E*h* in fish farm sediments was as low as -200 mV (Pawar *et al.*, 2002).

Total phosphorus and nitrogen concentrations in the test ponds were lower than the control ponds. Wang and He (2009) reported decreased concentrations of total phosphorus, total inorganic phosphorus, total nitrogen and total organic carbon in sediments after the ponds were treated with commercial probiotics. During shrimp culture, it was common to accumulate organic material in the pond bottom originated from unused feed, feces and plankton die-off (Avnimelech *et al.*, 1995). As a consequence, nutrients (N, P

and C) levels in pond sediment are usually higher in the final phase compared with the starting phase. In summary, the probiotic application significantly increased the redox potential, and brought down TN, TP and TOC in the test pond sediment.

The high bacterial count in the sediment may be attributed to the presence of high organic matter and nutrients in the sediment than in the water column. The observed total heterotrophic bacterial level in the sediment during the culture period was similar to those found in other shrimp culture ponds (Devaraja *et al.,* 2002). Devaraja *et al*. (2002) reported that total heterotrophic bacterial levels were 3.75 x 10^5 CFU/g in the pond bottom soil collected from a shrimp culture pond. Tendencia *et al*. (2006) reported levels of THB in shrimp bottom soil, ranging from 5.4 x 10^5 CFU/g to 6.8 x 10^5 CFU/g.

It was clear from the study that the application of bioaugmentor 'Detrodigest' to water and sediment and gut probiotic 'Enterotrophotic' had beneficial effect on the survival and growth of *P.monodon*. Previous studies showed that the application of the probiotic, *Bacillus coagulan*s SC8168 to water had beneficial effects on the survival rate of shrimp (*P. vannamei*) larvae (Zhou *et al.,* 2009). Besides, supplementation of the commercial probiotic containing *Bacillus* sp as the ingredient significantly increased the survival rate of Indian white shrimp (*Fenneropenaeus indicus*) compared to untreated controls (Ziaei-Nejad *et al.,* 2006). A similar finding was obtained by Nogami and Maeda (1992), who isolated a bacterial strain PM-4 (*Thalassobacter utilis*) from seawater and inoculated into blue crab (*P. trituberculatus*) larval rearing tanks at concentrations of 10^6 cells /mL and observed a survival of 27.2% in the test tanks compared to 6.8% in the control tank. The application of probiotics improved the survival, growth rates, and

FCR of shrimps (Balcázar *et al.*, 2007; Hai *et al.*, 2009). The positive effects of probiotics in FCR have also already been pointed out in *P. indicus* larvae fed with *Lactobacillus plantarum* (Uma *et al.*, 1999).

The FCR values in the test ponds were significantly lower than that of the control ponds. Average food conversion ratios varied between 1.5 to 1.75 in Indian culture systems (Paul Raj, 1999). In the study by Castex (2008) probiotic treatment improved the final biomass. McIntosh (2002) reported that the dietary crude protein level could be decreased from 31% to 24% with FCR decreasing from 2.2 to 2.0 and body N retention efficiency increased from 23 % to 37% in shrimp reared under commercial zero water exchange culture conditions. Krantz and Norris (1975) stated that survival rates of 60 to 80% are to be expected for *P. monodon* under suitable rearing conditions. Average survival of 70-80% is quite possible if ideal conditions are maintained for *P. monodon* (Ramakrishan, 2000).

The total shrimp yield in the test ponds was 20% more than that of the control ponds and took less than 20 days compared to that of the control to attain the maximum growth. In the test ponds final shrimp weight was 37 g/animal but in the control ponds it was 33 g/animal and the cost benefit analysis showed that the technology applied to the ponds were economically viable and profitable. The shrimps in the test ponds were healthy throughout the culture period without any ailments such as, zoothamium infestation, vibriosis, loose shell syndrome, detritus accumulation, necrosis, melanosis and, antenna cut.

The high survival in the test pond could be due to the beneficial effects of bioaugmentor and probiotics. The bioaugmentor Detrodigest containing

Bacillus cereus sensu lato might have hastened the breakdown of organic wastes into available nutrients and augmented the production and maintenance of phytoplankton in the test ponds. This can be pointed out as the reason for the lower concentration of ammonia, nitrite and nitrate in the same system. The steady algal growth indicated by the higher chlorophyll content and primary productivity showed that the system was in equilibrium and productive.

Poor growth and low survival in the control ponds could be due to the deterioration of sediment quality which lead to high redox value and low oxygen level in the pond bottom. Unhealthy signs such as fouling of exoskeleton, necrosis, physical damage, blisters, antenna cut, swelling of gills and loose shell syndrome towards the terminal part of the culture were characteristic of the animals in the control ponds. Overall there was reduction in feed intake of the shrimp in the control ponds.

The lower FCR in the test ponds could be due to the beneficial effects of probiotic Enterotrophotic which helped digest the food materials and maintain the beneficial bacterial flora in the gut of the animals.

Zero water exchange Technology – A package of practice

Pond Preparation

- Eradicate weed finfishes and shell fishes by applying a mixture of Lime : Ammonium sulphate (5:1 ratio) at the rate of 1275 Kg and Tea seed Powder at the rate 100Kg/ha
- Measure pH, Eh and total organic carbon of the soil.
- Fill the pond from the water source to have maximum 5-10 cm
- Record salinity and pH of the water

- Apply 'Detrodigest' 300ml/ hector after brewing in 100 litre filtered pond water. (Brewing protocol has been given in Page 54, Section 2.2.2 Pond preparation)
- Measure Soil E*h*, pH and TOC.
- Continue the process till the TOC drops to 2% and E*h* rises to -100 mV.

Fertilization

- Fill the pond with water during high tide through the sluice gate fitted with 100 micron bag net till 70cm water column is obtained.
- Calculate the total volume of the water and fix the water level indicator.
- The ponds are subjected to the analysis of nutrients of water and decision is taken the extend to which fertilization is required.
- Apply Detrodigest, Cow dung juice, Nutrimix and Micromix. (Page: 55, section 2.2.3)
- The process is continued once in five days till bloom develops to a range of 10^4 to 10^6 phytoplankton cells/ml.
- At this stage one comprehensive analysis of water and sediment is made to assess the suitability of ponds for stocking.

Stocking

- When the water and sediment pH gets stabilized between 7-8.5, sediment E*h* between -100 to -125 mV, alkalinity between 70-120 ppm the ponds are stocked.
- Stocking: 6 PL20/m^2, PCR negative for WSSV after the following tests.
- Colour, swimming behaviour, muscle to gut ratio, pigmentation and intactness of appendages are recorded with the help of microscope.
- The post larvae are subjected to salinity and formalin stress tests. (For more details Pages 76-77)

To make an assessment of the survival of post larvae subsequent to stocking, 100 of them are maintained in a nylon hapa and counted after 48 hours presuming it to reflect the over all survival in the pond (For more details Page 56; 2.2.4 Stocking and management of the ponds)

Management of the ponds

- All physical, chemical and biological parameters are measured once in a week.
- If the phytoplankton blooms does not remains stable, which indicates deficient nutrient replenishment, either addition of Detrodigest and/or application of nutrients are resorted.
- If the bloom production is in excess feeding reduced.
- If the pH rises to above 9 in the evening hours, maintain alkalinity above 70 ppm by the addition of Dolomite at the rate 50 Kg per hectare
- After one month, body weight is assessed and feeding regulated along with health assessment.
- Detrodigest^{TM} application is continued at the rate once in a week (7 days).
- Application of 'Enterotrophotic', the gut probiotics, is initiated from 30th day onwards at the rate 50ml/10000 animals.

Validation

Having standardized the bioremediation technology for zero water exchange shrimp culture system based on the Pancham Aquaculture Farms Ltd, Mumbai the next attempt was to validate the same elsewhere in different seasons demarcated by monsoon such as pre monsoon and post monsoon. To accomplish the task 16 ponds were selected in Kerala and the protocol was implemented and data generated as presented in the next chapter (Chapter 3).

Table 3. Water quality parameters of test and control ponds during the culture period (Ranges, Mean ± SD)

	Test ponds	Control ponds
pH (Morning)	7.9 – 8.7 (8.2 ± 0.07)	7.8 – 8.4 (8.04 ± 0.08)
pH (Evening)	8.3 – 9.2 (8.6 ± 0.18)	8.02 – 8.9 (8.5 ± 0.14)
Transparency (cm)	24.04 – 46.7 (34.9 ± 2.17)	13.8 -53.3 (27.03 ± 5.52)
Salinity (g/L)	8 – 15 (10.9 ± 0.42)	8 – 15 (10.5 ± 0.47)
Temperature	26 - 30	26 - 30
Dissolved Oxygen (mg/L)	4 – 7.2 (5.3 ± 0.47)	2.6 – 5.8 (4.5 ± 0.5)
Alkalinity (mg/L)	54 – 106 (72.42 ± 1.4)	53 – 87 (67.6 ± 2.3)
TAN (mg/L)**	0 – 0.06 (0.02 ± 0.007)	0 – 0.21 (0.08 ± 0.02)
NO_2^-- N (mg/L)*	0.006 – 0.02 (0.02 ± 0.005)	0.006 – 0.09 (0.04 ± 0.008)
NO_3^- - N (mg/L)*	0.02 - 0.13 (0.07 ± 0.01)	0.03 – 0.24 (0.12 ± 0.02)
PO_4^- - P (mg/L)	0.07 – 0.22 (0.11 ± 0.02)	0.09 – 0.23 (0.13 ± 0.03)
Total Phosphorus (mg/L)	0.11 – 0.34 (0.2 ± 0.02)	0.11 – 0.39 (0.23 ± 0.04)
Silicate (mg/L)**	0.6 – 1.1 (0.9 ± 0.2)	0.51 – 0.91(0.7 ± 0.08)
Total Nitrogen (mg/L)**	0.08 – 0.31 (0.17 ± 0.03)	0.08 – 0.47 (0.3 ± 0.05)
H_2S (mg/L)	0.001 – 0.006	0.001 – 0.008
BOD (mg/L)	4.75 – 24.05 (15.43 ± 0.8)	5.46 – 26.23 (17.31 ± 1.13)
Chlorophyll a (µg/L)*	5.54 – 44.22 (24.17 ±6.8)	5.3 – 33.95 (18.09 ±5.03)
Chlorophyll b (µg/L)	0 – 10.49 (4.48 ± 1.3)	0 – 7.6 (2.36 ± 0.9)
GPP (g/C/m^3/day)*	0.08 – 3.8 (2.1 ± 0.48)	0.08 – 2.8 (1.7 ± 0.45)
NPP (g/C/m^3/day)	0.05 – 2.2	0.5 – 1.7
Phytoplankton	4.8 x 10^4 -71 x 10^4	4.2 x 10^4 – 57.3 x 10^4
Total bacteria (cfu/ mL)	1.3 x 10^4 – 3.5 x 10^7	2.3 x 10^3 – 2.1 x 10^7
Vibrio (cfu/mL)*	60 – 262.5 (157.3 ± 20.5)	62.5 – 410 (236 ± 28.7)

** $P < 0.001$
* $P < 0.05$
TAN – Total Ammonia Ntrogen
GPP – Gross Primary Productivity
NPP – Net Primary Productivity

Table 4. Major families and genera of phytoplankton recorded from the shrimp ponds during the culture period.

Cyanophyceae	Chlorophyceae	Bacillariophyceae	Pyrrophyceae
Anabaena	*Chlorococcus*	*Chaetoceros*	*Gymnodinium*
Oscillatoria	*Closterium*	*Melosira*	*Eridinium*
Nostoc		*Navicula*	
Polycystis		*Pleurosigma*	
Trichodesmium		*Nitzchia*	
Arthospira		*Skeletonema*	
		Coscinodiscas	

Table 5. Major groups of zooplankton recorded in the shrimp ponds during the culture period

Zooplankton groups	Test Ponds	Control Ponds
Protozoans	30%	28%
Copepds	20%	22%
Unidentified Crustaceans	28%	28%
Rotifers	10%	12%
Cladocerans	2%	4%
Others	10%	6%

Table 6. Sediment quality parameters of test and control ponds through out the culture period

(Ranges, Mean ± SD)

	Test ponds	Control ponds
pH	7.5 – 8.12 (7.9 ± 0.14)	7.34 – 8.34 (7.9 ± 0.15)
Eh**	- 72 - ¯166 (-140 ± 10.8)	-96 - ¯226 (-174 ± 12.16)
TOC (%)*	0.3 – 1.3 (0.7 ±0.09)	0.32 – 2.3 (1.17 ± 0.08)
TOM (%)	0.55 – 2.24 (1.19 ± 0.17)	0.55 – 3.96 (2.04 ± 0.19)
TAN	0.21 – 0.47 (0.4 ± 0.09)	0.37 – 0.69 (0.53 ± 0.89)
NO_2^- -N	0.07 – 0.19 (0.13 ± 0.06)	0.09 – 0.21 (0.16 ± 0.05)
NO_3^- -N	0.09 – 0.39 (0.27 ± 0.08)	0.13 – 0.4 (0.29 ± 0.07)
Total Nitrogen (%)	0.12 – 0.19 (0.14 ± 0.04)	0.15 – 0.4 (0.2 ± 0.08)
Total Phosphorus (mg/g)	2.15 – 5.7 (3.7 ± 0.9)	2.37 – 7.6 (4.7 ± 0.8)

** P < 0.001
** P < 0.05
Eh – Oxidation Reduction potential
TOC – Total Organic Carbon
TOM – Total Organic Matter

Table 7. Health assessment of test and control ponds

DOC	Pond No	Colour	Exoskeleton				Antenna	Appendages		Tail			Gills			Intestine		
			Fouling	Necrosis	Melanosis	Physical damage	cut	Fouling	Colour	Spreadness	Blisters	Fouling	Colour	Swelling	Degeneration	Full gut	Half Gut	Empyt Gut
30	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	100%	0	0
	Control	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	100%	0	0
40	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	100%	0	0
	Control	Normal	4%	Nil	Nil	5%	5%	5%	Normal	Normal	Nil	10%	Normal	Nil	Nil	90%	10%	0
50	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	92%	8%	0
	Control	Normal	Nil	Nil	Nil	Nil	5%	Nil	Normal	Normal	Nil	12%	Normal	Nil	Nil	80%	10%	10%
60	Test	Greenish	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	5%	Nil	Normal	Nill	Nill	100%	0	0
	Control	Normal	Nil	Nil	Nil	Nil	Nil	10%	Normal	Normal	5%	Nil	Normal	Nil	Nil	86%	12%	25
70	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	88%	10%	2%
	Control	Normal	10%	Nil	Nil	Nil	10%	12%	Normal	Normal	Nil	Nil	Normal	Nil	Nil	82%	12%	6%
80	Test	Greenish	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	78%	20%	2%
	Control	Normal	Nil	Nil	Nil	Nil	Nil	10%	Normal	Normal	Nil	12%	Normal	Nil	Nil	78%	10%	12%
90	Test	Greenish	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	100%	0	0
	Control	Normal	Nil	5%	Nil	Nil	4%	Nil	Normal		22%		Normal	Nil	Nil	67%	33%	0
100	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	5%	Nil	Normal	Nill	Nill	70%	20%	10%
	Control	Normal	10%	Nil	Nil	5%	Nil	15%	Normal		30%		Normal	5%	Nil	76%	20%	4%
110	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	20%	Normal	Nill	Nill	95%	5%	0
	Control	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal		12%		Normal	Nil	Nil	64%	32%	4%
120	Test	Greenish 30%	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	70%	20%	10%
	Control	Normal	12%	10%	Nil	Nil	4%	Nil	Normal	Normal	6%	Nil	Normal	Nil	Nil	70%	30%	0%
131	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	80%	12%	8%
	Control	Normal	Nil	Nil	Nil	Nil	Nil	10%	Normal		10%		Normal	Nil	Nil	76%	20%	4%
140	Test	Normal	10%	Nil	Nil	Nil	Nil	Nil	Normal	Normal	7%	Nil	Normal	Nill	Nill	92%	4%	2%
	Control	Normal	10%	Nil	Nil	Nil	12%	Nil	Normal		18%		Normal	Nil	Nil	68%	15%	17%
151	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	12%	Nil	Normal	6%	Nill	75%	15%	10%
	Control	Normal	10%	10%	Nil	10%	10%	12%	Normal		12%		Normal	Nil	Nil	72%	20%	85%
160	Test	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nill	Nill	76%	20%	4%
	Control	Normal	15%	Nil	Nil	10%	10%	10%	Normal		10%		Normal	Nil	Nil	65%	20%	15%

Table 8 . Details of the cultures in the test and control ponds

	Test ponds	**Control ponds**
Days of culture	160	182
Average Body Weight (g)	37.2 ± 0.88	33.4 ± 0.98
Growth rate (g/day)	0.23	0.18
Survival (%)	74 ± 5.6	54 ± 10.55
FCR	1.9	2.7

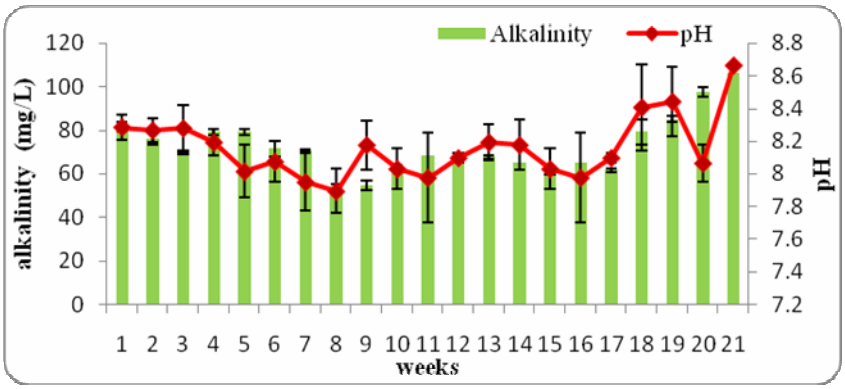

Fig. 1. Variation of pH and total alkalinity in the test ponds during the culture period

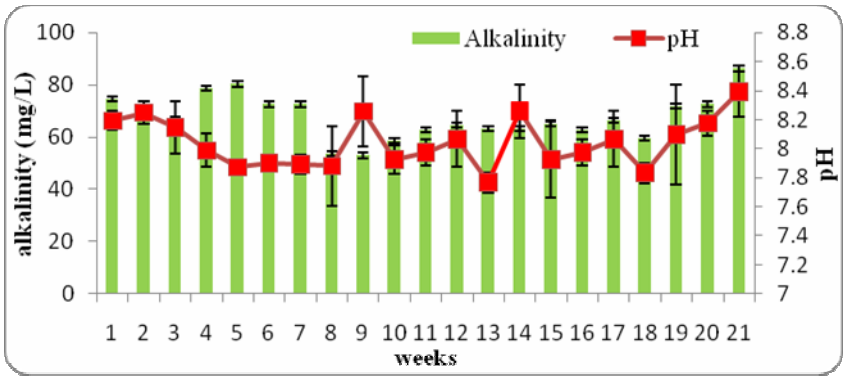

Fig. 2. Variation of pH and total alkalinity in the control ponds during the culture period.

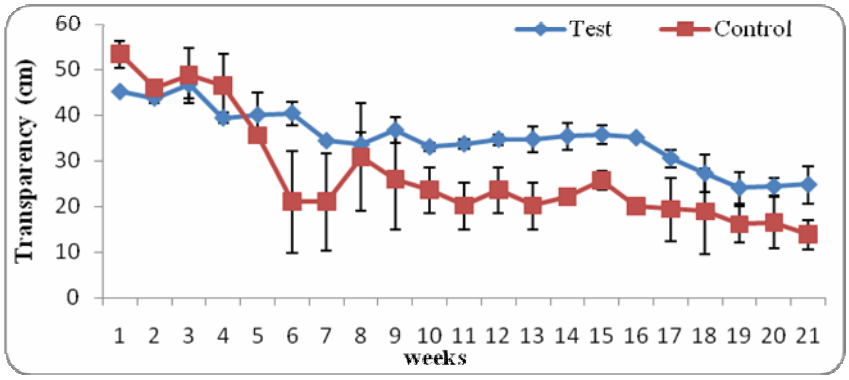

Fig. 3. Variation of water transparency in the test and control ponds during the culture period

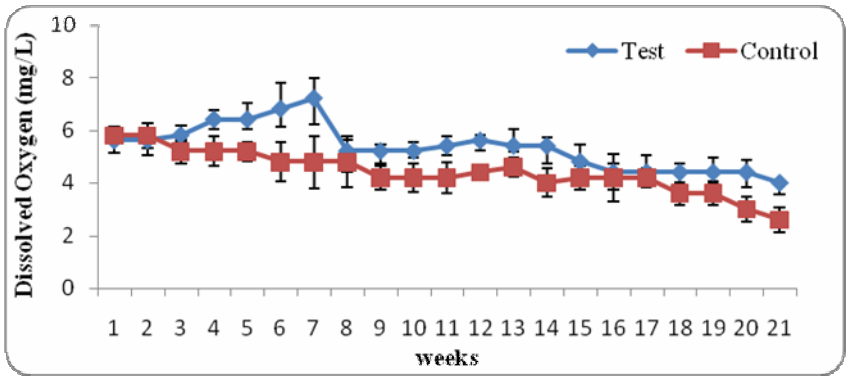

Fig. 4. Variation of dissolved oxygen in the test and control ponds during the culture period

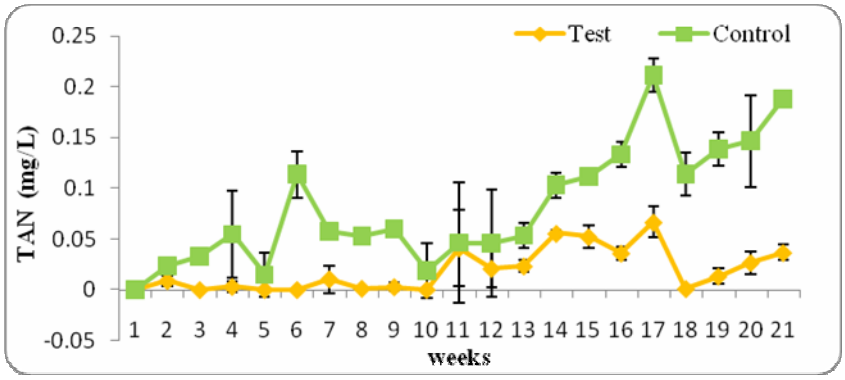

Fig. 5. Variation of TAN in the test and control ponds during the culture period

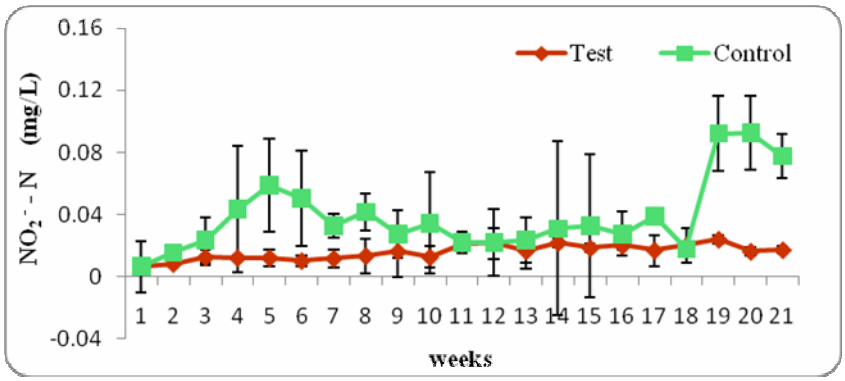

Fig. 6. Variation of NO_2^--N in the test and control ponds during the culture period

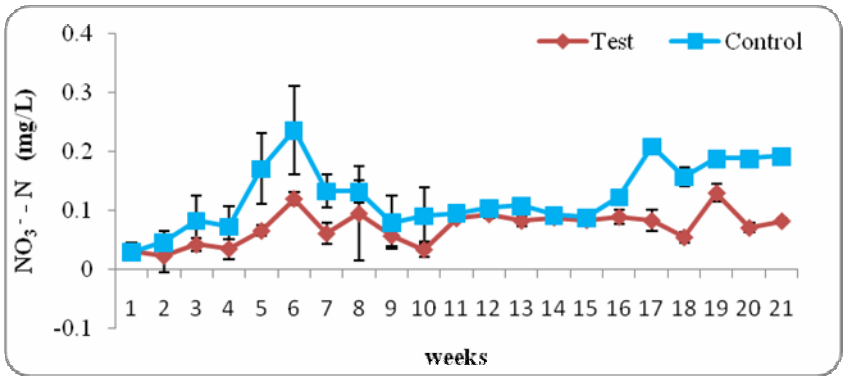

Fig. 7. Variation of NO_3^-- N in the test and control ponds during the culture period

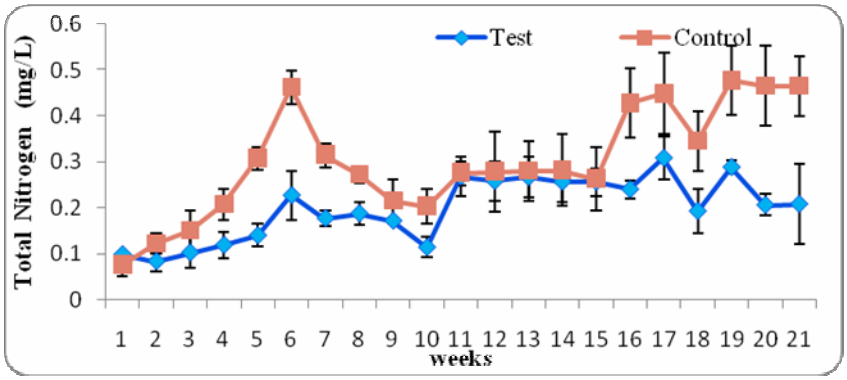

Fig. 8. Variation of total nitrogen in the test and control ponds during the culture period

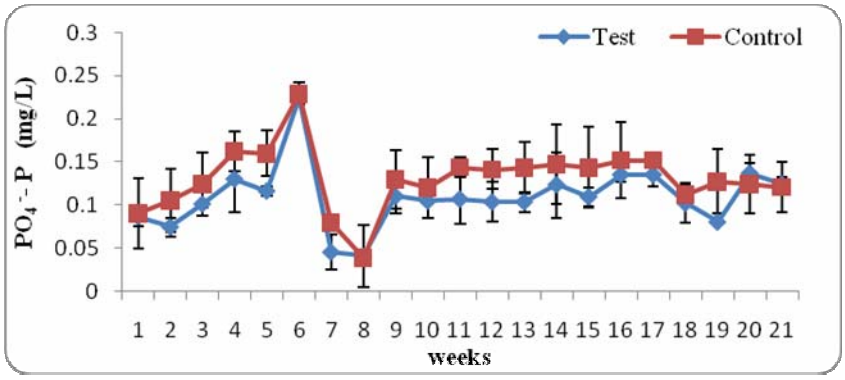

Fig. 9. Variation of inorganic phosphorus in the test and control ponds during the culture period

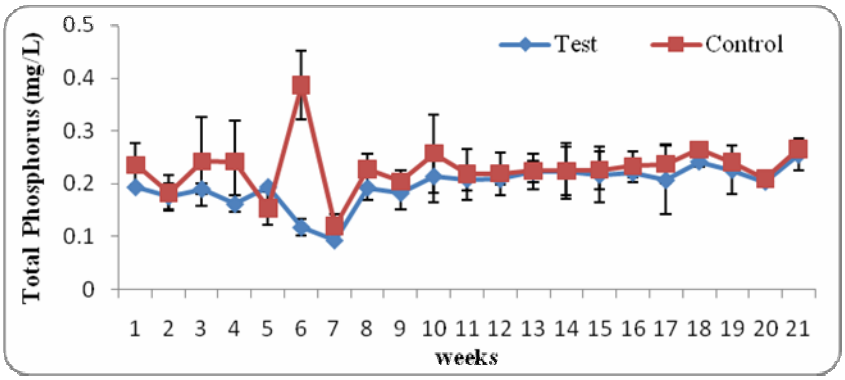

Fig. 10. Variation of total phosphorus in the test and control ponds during the culture period

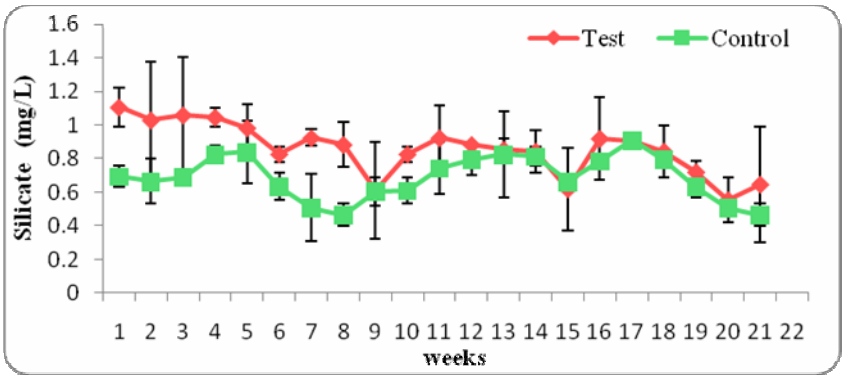

Fig. 11. Variation of silicate concentration in the test and control ponds during the culture period

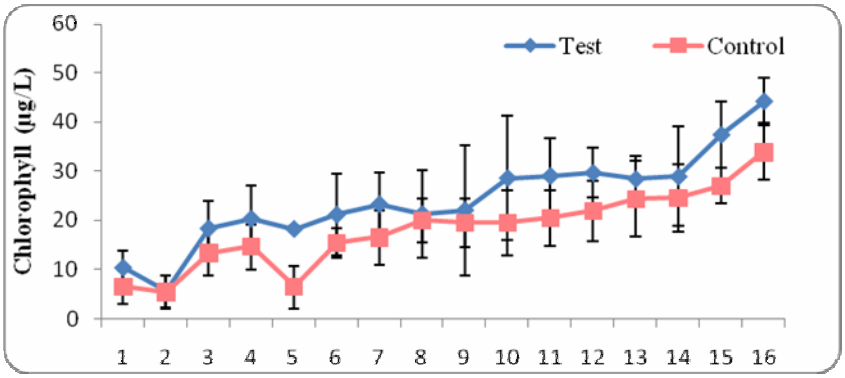

Fig. 12. Variation of chlorophyll in the test and control ponds during the culture period

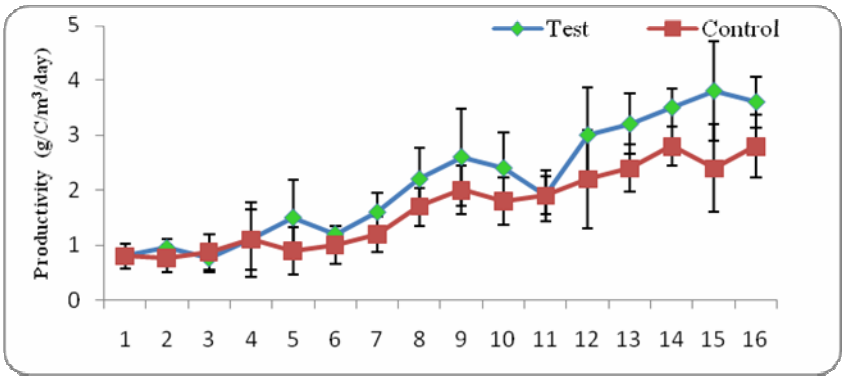

Fig. 13. Variation of primary productivity in the test and control ponds during the culture period

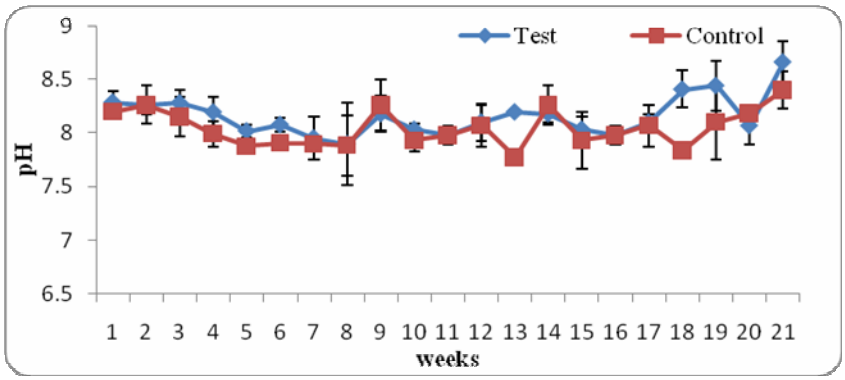

Fig. 14. Variation of soil pH in the test and control ponds during the culture period

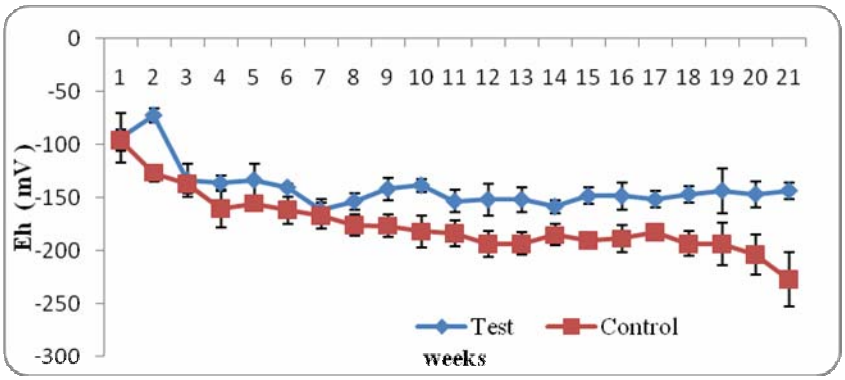

Fig.15. Variation of sediment E*h* in the test and control ponds during the culture period

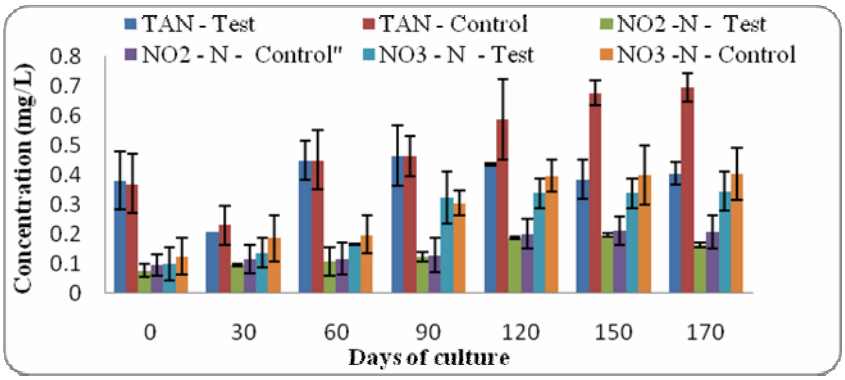

Fig. 16. Variation of sediment TAN, NO_2^-- N and NO_3^-- N in the test and control ponds during the culture period

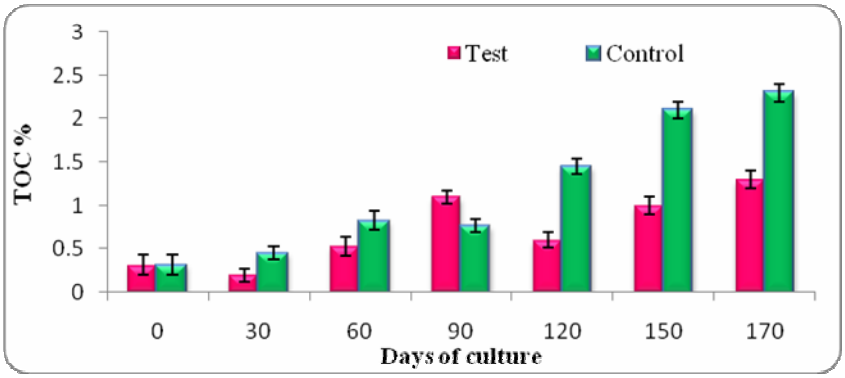

Fig. 17. Sediment total organic carbon concentration in the test and control ponds during the culture period

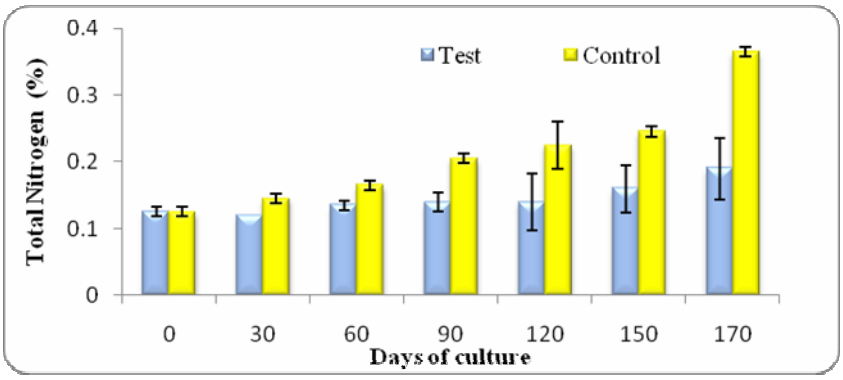

Fig 18. Variation of sediment total nitrogen concentration in the test and control ponds during the culture period

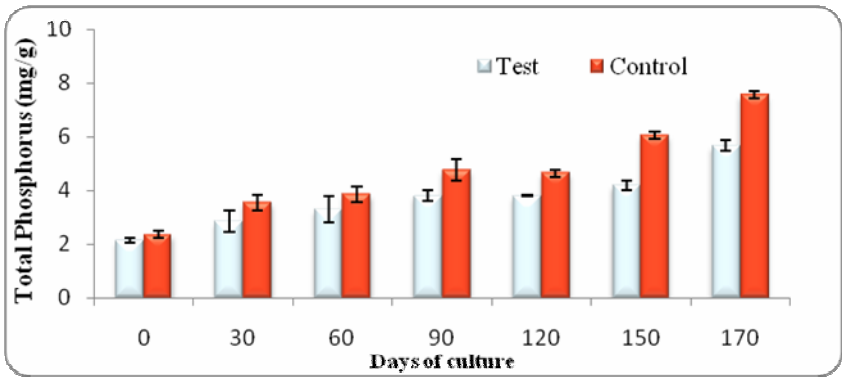

Fig. 19 Variation of sediment total phosphorus concentration in the test and control ponds during the culture period

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Chapter

3

Validation of the zero water exchange technology in different seasons and its comparison with open shrimp grow out systems

3.1 Introduction

Kerala being a maritime state in the south west coast of India has tremendous potentials for aquaculture, among which shrimp farming dominates. Farm reared shrimp from Kerala fetches the best price owing to the overseas demands from countries like Japan and United States. It is estimated that 65,000 ha of brackish water is suitable for shrimp cultivation in India. In Kerala, approximately 7,000 metric tons of shrimps are produced at the rate of 530 kg/ha (Harikumar and Rajendran, 2007). Traditional shrimp farming practices are very popular in the State. However, inadequate management of the whole sector has resulted in frequent disease outbreaks, forcing the farmers to shut down the farms for prolonged periods (Shakir *et al*., 2010).

Zero water exchange shrimp culture potentially eliminates many of the obvious failures of the modern open production systems. It eliminates many of the production risks that are beyond the control of most shrimp farmers such as diseases due to coastal water exchange, natural predators, weather peculiarities, and the side effects or long-term application of medicinal additives such as antibiotics.

Shrimp culture production is frequently determined by the environmental conditions of the pond, especially water and sediment quality. Ultimately, it is the water quality that influences optimal shrimp growth and yield (Case *et al*., 2008). Water quality in the ponds is determined by the initial water quality used for the culture, as well as the organic loadings into the ponds in the form of feeds and fertilizers during the culture cycle. In shrimp culture ponds, usually nutrients such as phosphorus and nitrogen progressively increase as the culture progresses due to excess feed materials and excretory products (Matias *et al*., 2002). Increase of nutrients results in eutrophication characterized by low oxygen, high ammonia, high hydrogen sulphide, and high densities of cyanobacteria (Yusoff *et al*., 2002).

In addition, large amounts of organic matter in the water column finally settles on the pond bottom resulting in an increased layer of anaerobic sludge which constantly releases high concentrations of inorganic phosphorus and ammonia into the water column (Boyd, 1990; Yusoff *et al*., 2001). Studies of Hall *et al*. (1990) and Holmer and Kristensen (1992) revealed that a large amount of organic matter in the pond bottom can also lead to anaerobic processes like sulfate reduction and methanogenesis.

Environmental conditions and stress are important factors that can trigger viral disease outbreaks, causing high mortalities and severe economic loss (Chou *et al*., 1995; Guan *et al*., 2003; Jiravanichpaisal *et al*., 2004). The environmental factors are temperature (Vidal *et al*., 2001; Guan *et al*., 2003), dissolved oxygen (Melena *et al*., 2006), ammonia concentration (Jiang *et al*., 2004) and salinity (Liu *et al*., 2006). On the other hand, it has also been reported that the host and pathogen can coexist with the absence of disease signs if rearing conditions are adequately controlled (Lightner and Redman, 1998).

An alternative approach to waste removal is to increase the capacity of the pond microbial community to remove or transform metabolic waste. This is analogous to the goal of increasing the capacity of soil or aquatic microbial community to biodegrade pollutants through engineered bioremediation (intrinsic bioremediation). It can be accomplished either by improving environmental conditions to stimulate growth and activity of naturally occurring bacteria or by supplementing naturally occurring micro flora with the organism produced in culture (Bioaugmentation).

Bioaugmentation is based on the concept that the rate of particular microbially mediated process may be limited by the abundance of microorganisms carrying out that process. As such, process rates can be increased by introduction of allochthonous microorganisms to the system (Tucker *et al*., 2009). Typically, microorganisms used in bioaugmentation are isolated from nature by means of enrichment techniques and then grown as mass laboratory cultures. Bioaugmentation is most commonly used to remediate soils contaminated with hazardous organic pollutants such as crude oil, petroleum products, or pesticides (Vogel and Walter, 2001).

Bioaugmentation has also been used in wastewater treatment (Van Limbergen *et al*., 1998), in lake and pond management (Boyd *et al*., 1984; Chiayuvareesajja and Boyd, 1993; Quieroz and Boyd, 1998; Duvall and Anderson, 2001; Duvall *et al*., 2001; Lopez *et al*., 2011).

Objective of the study is to evaluate the zero water exchange shrimp (*P.monodon*) culture technology in two prominent seasons such as pre monsoon and post monsoon and compare them with an open system.

3.2 Materials and Methods

3.2.1 Location and description of the ponds

The study was conducted at Nandana Aquaculture Farms Ltd, Kerala as two cultural seasons in two consecutive years (pre monsoon and post monsoon). In each season, two cultures were undertaken. Culture 1 and 3 were from March to June (pre monsoon) and culture 2 and 4 from September to January (post monsoon). Details of the ponds and stocking density are mentioned in Table 1. Pond preparation, fertilization, stocking and management of the ponds were done as per the procedures mentioned in Chapter 2.

As control, two ponds under open system were incorporated in the study. Both the ponds were of 1hectare with sluice gates through which water was allowed to enter during high tide and recede during low tide. The bioaugmentor 'Detrodigest' and the probiotic 'Enterotrophic' were not administered. Even though all physical, chemical and biological parameters were analyzed no steps were taken for any correction. However, the seed stocked was from the same brood stock used for the test ponds, and the same category of feed was given.

Protocols for water and sediment quality analysis and for health assessment have been mentioned in Chapter 2.

3.2.2 Total bacterial count (Epifluorescency)

An aliquot of 2 mL water sample was filtered through 0.2 µm polycarbonate irgalan black stained nucleopore filter paper and stained using acridine orange (1-7 minutes). To get uniform cell distribution, base of the filtration apparatus was wetted prior to placement of the wet polycarbonate membrane. After filtration, nucleopore filters were mounted immediately on a slide using non-fluorescent immersion oil. A minimum of seven filters per field per sample were counted. Bacterial cells fluoresced green taking up acridine orange under blue excitation on an epifluorescence microscope.

Calculation

Bacterial abundance (cells/L) = $C_f R \times 1/fs$

Cf = mean number of cells per field

R = Active area of filters/ area of filed counted

fs = Volume of fluid filtered.

3.2.3 Diagnostic PCR for confirming WSSV

Whenever mortality occurred in any pond the animals were examined for overall signs of diseases and those which showed white spots on the carapace were subjected to diagnostic PCR following Lo *et al.* (1996 b).

Genomic DNA preparation

Apparently healthy *P.monodon* PL/juveniles were used for the DNA extraction (WSSV). The animals were washed in sterile sea water prior to the collection of haemolyph and other tissues. Haemolymph was drawn using

sterile haemolymph collection capillary tube from the rostral sinus and the tissues such as gills and pleopods were removed using sterile forceps and scalpel.

DNA extraction from shrimp tissues

Gill/pleopod/muscle tissue (25-50 mg) and 100 µl of haemolymph were used for DNA extraction. The tissue was homogenized in 200 µl DNAZOL using DNase and RNase free plastic pestle. Further 800 µl DNAZOL was added and mixed by inverting the tubes 10-15 times. The samples when sufficiently solubilised were centrifuged at 11,000 rpm for 20 minutes at 4° C. The supernatant was transferred carefully to a fresh tube containing 500 µl of 100 % distilled ethanol. The pellet was washed twice with 75 % alcohol and air dried at room temperature. The pellet was re-suspended in 200 µl milliQ. The concentration and quality of DNA were determined by measuring absorbance at 260/280 nm using a UV-Visible spectrophotometer (Hitachi, Japan). An aliquot of 5 µl was analyzed by 0.8 % agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

WSSV diagnostic PCR

Genomic DNA extracted from *P. monodon* was used for the nested PCR of WSSV. Briefly, first step PCR was conducted in a 25 µl reaction volume containing 1 µl of 10X buffer, 2.5 mM dNTP (2.0 µl), 1 µl of 10 pmol μl^{-1} of forward (NP 51-F) and reverse primer (NP 51-R) (Table) and 1.0 µl of 0.5U μl^{-1} of Taq DNA polymerase and 1 µl DNA template (75 ng). The PCR programme consisted of heating the mixture to 95 °C for 5 min before addition of Taq DNA polymerase enzyme. The following PCR cycle includes 35 cycles

of 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min with a final extension at 72 °C for 5 min.

The second step PCR (Nested PCR) was conducted in a 25 µl reaction volume containing 1 µl of 10X buffer, 2.5 mM dNTP (2.0 µl), 1 µl of 10 pmol μl^{-1} of forward (NP 52-F) and reverse primer (NP 52-R) (Table) and 1.0 µl of 0.5U μl^{-1} of Taq DNA polymerase and 1 µl PCR template from first step. The PCR programme was the same as in first step PCR. An aliquot of 10 µl (5 µl first step PCR product and 5 µl second step PCR) was analyzed by 1 % agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA). The appropriate positive and negative samples were analyzed for the confirmation of WSSV infection.

Sl.No.	Code No.	Primer Sequence (5'-3')	Tm (°C)	Ampicon size (bp)
1.	NP 51-F	ACTACTAACTTCAGCCTATCTAG	55	1447
	NP 51-R	TAATGCGGGTGTAATGTTCTTACGA		
2.	NP 52-F	GTAACTGCCCCTTCCATCTCCA	55	941
	NP 52-R	TACGGCAGCTGCTGCACCTTGT		

3.3 Results

3.3.1 Physico chemical analysis - Water quality

The average water quality parameters during the two seasons in four cultures are depicted in Table.2.

3.3.1.1 pH

Inter seasonal variation of morning and evening pH did not show significant difference. Evening pH was slightly higher than that of morning in

all the seasons. During the pre monsoon (March to June), two cultures were undertaken during which the morning pH varied from 7.08 to 8.5 and 7.3 to 8.01 respectively. Evening pH stood between the ranges of 7.8 to 9.1 and 7.9 to 9.1 respectively. During post monsoon, (September to January) morning pH varied between 7.5 to 8.4 and 7.6 to 8.9 in the first and second cultures respectively. Evening pH ranged between 7.7 to 9.1 to 7.8 to 9.1 respectively. The overall mean of morning pH was 7.87 ± 0.5 and at evening it was 8.53 ± 0.5 (Table.2).

3.3.1.2 Alkalinity

Overall mean of alkalinity of water was 60.50 ± 4.3 mg/L. It did not show significant difference between the cultures (P >0.05). During the pre monsoon period (March to June) in the two sets of cultures undertaken alkalinity varied from 52 to 74 mg/L and 50 to 78 mg/L respectively. During the post monsoon period (September to January), they were 47 to 70 mg/L and 44 – 77 mg/L in the first and second cultures respectively (Table 2). During the pre monsoon, maximum alkalinity was recorded at the initial period of the culture, and in the post monsoon maximum alkalinity was recorded towards the final stage of the culture.

3.3.1.3 Temperature

Water temperature fluctuated between 25 to 30° C between the seasons.

3.3.1.4 Salinity

Salinity followed a regular annual cycle from as low as 2.5 ± 0.09 g/L (post monsoon) to the highest of 30 ± 0.95 g/L (pre monsoon). During pre monsoon (March to June) salinity ranged between 15 to 30 g/L with the mean

value of 24.8 ± 0.9 g/L and 10 to 28 g/L with the mean of 22.7 ± 0.5 g/L in the first and second cultures respectively. During post monsoon, (September to January), two sets of cultures were undertaken and salinity ranged between 3 to 9 g/ L with a mean of 5.8 ± 0.1 g/L and 2.5 to 12.5 g/L with a mean of 5.2 ± 0.2 g/L respectively (Table 2).

3.3.1.5 Calcium and Magnesium

Calcium and Magnesium showed a similar trend as that of salinity. During pre monsoon (March to June) in both the cultures calcium ranged between 417 to 853 mg/L with the mean value of 734 ± 48.7 mg/L, and 400 to 800 mg/L with the mean value of 665 ± 48 mg/L respectively. During post monsoon, (September to January), both the sets of cultures exhibited calcium concentration between 85 to 165 mg/L with the mean of 135 ± 4.6 mg/L and 75 to 160 mg/L with the mean of 113 ± 21.3 mg/L.

Magnesium concentration stood during pre monsoon between 2183 to 4270 mg/L with the mean of 3683 ± 87 mg/L, and 2000 to 4188 mg/L with the mean of 3481 ± 97 mg/L in the first and second cultures respectively. During post monsoon, magnesium concentration varied between 315 to 550 mg/L with the mean value of 449 ± 11 mg/L and 163 to 885 mg/L with the mean value of 511 ± 17 mg/L respectively.

3.3.1.6 Dissolved oxygen

The concentration of dissolved oxygen (DO) ranged between 3.3 mg/L to 8.3 mg/L during the study period (Table 2). The mean DO concentration was 6.5 ± 1.7 mg/L, greater in the evening hours than the morning as expected because of the greater photosynthesis during the daytime. Concentration of DO did not show significant difference between the seasons. At 12 AM, the

mean concentration of DO used to be 5.4 ± 0.5 mg/L. At 6 AM it was found getting declined as the culture progressed and the least minimum concentration (3.4 mg/L) occurred towards final stage of the culture. At 12 PM and 6 PM, the mean concentration used to be 7.6 ± 0.8 mg/L and 8.15 ± 0.48 mg/L respectively.

3.3.1.7 Secchi disc transparency

Transparency (Secchi depth) is a measure of turbidity and natural productivity of the ponds indicating the presence of phytoplankton and zooplankton in the water body. The mean Secchi disc transparency during the entire culture period was 45.74 ± 6.7 cm. During pre monsoon (March to June) it ranged between 35 to 60 cm with the mean of 43.2 ± 6.7 cm and 30 to 59 cm with the mean of 45.3 ± 6.1 cm in the first and second cultures respectively. During post monsoon which extended from September to January the transparency of the first culture ranged between 32.5 to 65 cm with the mean of 47.1 ± 5.8 cm and in the second culture it ranged between 30 – 64 cm with the mean of 47.2 ± 8.03 cm. The maximum transparency (65 cm) was observed during the first culture of the post monsoon period and minimum (30 cm) during the second culture of the same season (Fig. 1).

3.3.1.8 Total Ammonia – Nitrogen (TAN)

Variations of TAN are given on seasonal basis in Fig. 2 and Table 2. During the pre monsoon period (March to June) two sets of cultures were undertaken in two successive years during which TAN varied from 0 to 0.1mg/L and 0.04 to 0.13 mg/L with the mean values of 0.04 ± 0.009 mg/L and 0.07 ± 0.01 mg/L respectively. During post monsoon, (September to January) in the two cultures undertaken TAN concentrations varied between 0

to 0.08 mg/L and 0 to 0.07 mg/L, with the mean values of 0.02 ± 0.003 mg/L and 0.03 ± 0.007 mg/L respectively. Overall, the mean TAN concentration was only 0.044 ± 0.007 mg/L.

3.3.1.9 Nitrite – Nitrogen

Variations in Nitrite – Nitrogen is given on seasonal basis in Fig. 3 and Table 2. During pre monsoon (March to June), Nitrite - Nitrogen varied from 0.004 to 0.06 mg/L and 0.01 to 0.05 mg/L with the mean values of 0.03 ± 0.005 mg/L and 0.037 ± 0.007 mg/L respectively. During the post monsoon period (September to January) Nitrite – Nitrogen varied between 0.005 to 0.05 mg/L and 0.003 to 0.04 and with the mean values of 0.017 ± 0.008 mg/L and 0.012 ± 0.003 mg/L respectively. Nitrite concentration in the ponds was generally low throughout the culture period with the overall mean of 0.03 ± 0.006 mg/ L. Even though lower in concentration nitrite nitrogen was building up towards the end of the culture.

3.3.1.10 Nitrate – Nitrogen

Variations in Nitrate – Nitrogen is given on seasonal basis in Fig. 4 and Table 2. During the pre monsoon period (March to June) Nitrate - Nitrogen varied from 0.003 to 0.056 mg/L and 0.012 to 0.063 mg/L with the mean values of 0.03 ± 0.007 mg/L and 0.034 ± 0.014 mg/L respectively. During the post monsoon period (September to January) in the two cultures undertaken there was a variation of 0.004 to 0.045 mg/L and 0.002 to 0.059 with the mean values of 0.021 ± 0.009 mg/L and 0.023 ± 0.004 mg/L respectively. Nitrate concentration in the ponds was generally low throughout the culture period and the overall mean was 0.03 ± 0.006 mg/ L. In general, after 7 weeks of culture, the nitrate concentration was building up gradually to 0.06 mg/L.

3.3.1.11 Total Nitrogen

During the pre monsoon period (March to June) total nitrogen varied from 0.037 to 0.18 mg/L and 0.038 to 0.12 mg/L with the mean values of 0.08 ± 0.03 mg/L and 0.08 ± 0.018 mg/L respectively. During the post monsoon period (September to January) in the two cultures undertaken there was a variation of 0.06 to 0.18 mg/L and 0.09 to 0.17 mg/L with the mean values of 0.13 ± 0.03 mg/L and 0.14 ± 0.18 mg/L respectively. In general there was an increasing trend in total nitrogen content from the point of stocking to harvest (Fig. 5 and Table 2) with the mean value of total nitrogen concentration being 0.108 ± 0.065 mg/L.

3.3.1.12 Inorganic phosphorus & Total phosphorus

Both inorganic phosphorus and total phosphorus exhibited an increasing trend from the point of stocking to harvest (Fig. 6 and Table 2) even though there was a drop during the first week of the culture. The mean inorganic phosphate during the experimental period was 0.038 ± 0.01 mg/L. The concentration of total phosphorus during the pre monsoon period varied between 0.06 to 0.21 mg/L with the mean value of 0.11 ± 0.01 mg/L, and 0.077 to 0.26 mg/L with the mean value of 0.14 ± 0.03 mg/L in the first and second cultures respectively. During the post monsoon period in the two cultures there was a variation of total phosphorus from 0.034 to 0.211 mg/L with a mean value of 0.09 ± 0.02 and 0.08 to 0.17 mg/L with the mean of 0.11 ± 0.02 mg/L respectively (Fig.7).

3.3.1.13 Silicates

The concentration of silicate observed a similar pattern in all the seasons. The highest silicate concentration occurred during the initial period of

the culture, and it declined when culture progressed. The overall mean was 1.29 ± 0.08 mg/L and it ranged between 0.32 ± 0.02 mg/L to 2.6 ± 0.06 mg/L (Fig. 8 and Table 2).

3.3.1.14 Hydrogen sulphide

Hydrogen sulphide, a substance that could be toxic at low concentration to shrimp was in the range of 0 to 0.005 mg/L with the mean value of 0.003 mg/L among the cultures (Table 2).

3.3.1.15 Primary productivity and chlorophyll content

There was a positive correlation between primary productivity and chlorophyll a among the culture period in different cultures (R^2 = 0.61, 0.77, 0. 75, 0.91) (Fig. 9 and 10). Primary productivity showed a maximum during the final phase of the culture with a gradual increase after the middle phase. The overall mean of gross primary productivity was 2.61 ± 0.84 g C/m^3/day. The minimum concentration of 1.02 to 1.3 g C/m^3/day was observed during the initial phase with a maximum of around 4.8 to 5.65 g C/m^3/day at the final stage of the culture (Table 2). Net primary productivity ranged between 0.5 ± 0.01 to 3.2 ± 0.45 g C/m^3/day with the mean value of 1.2 ± 0.25 g C/m^3/day.

Ponds usually had dense phytoplankton blooms as evidenced from high chlorophyll *a* concentration. During the pre monsoon period two cultures were undertaken, and the mean chlorophyll *a* content was 26.7 ± 5.6 µg/L and 33.4 ± 6.9 µg/L respectively. During the post monsoon period, the mean concentration was 28.31 ± 4.8 µg/L and 28.1 ± 4.8 µg/L in the first and second cultures respectively. The overall mean of chlorophyll a was 29.1 ± 5.5 µg/L. The maximum chlorophyll *a* content (71.45 ± 7.7 µg/L) was observed during the pre monsoon period (March to June) and the minimum (3.35 ± 0.98 µg/L)

during the initial phase of the culture during the post monsoon period. The overall mean of chlorophyll *b* was 12.58 which varied between 2.35 ± 0.89 to 23.45 ± 1.14 µg/L.

3.3.2 Phytoplankton

The phytoplankton cell diversity is given in Table 3.

Table 3. List of phytoplankton species present in the culture.

Cyanophyceae	Chlorophyceae	Bacillarophyceae	Pyrrophyceae	Euglenophyceae
Anabaena	*Chlorococcus*	*Chaetoceros*	*Gymnodinium*	*Euglena*
Oscillatoria	*Closterium*	*Cyclotella*	*Peridinium*	
Nostoc	*Chlorella*	*Melosira*		
Polycystis	*Spirogyra*	*Navicula*		
Spirulina	*Volvox*	*Pleurosigma*		
Trichodesmium		*Nitzscia*		
		Skeletonema		

3.3.3 Total heterotrophic bacteria (THB) and *Vibrio*

Details of the total heterotrophic bacteria and *Vibrio* counts in the water and sediments are presented in Table 4. In pond water, THB and *Vibrio* counts ranged, respectively, from 2.3×10^5 to 5.6×10^6 CFU/mL and from 5.3×10^1 to 1.53×10^2 CFU/mL. In pond sediment THB ranged from 5.5×10^5 to 3.4×10^7 CFU/g respectively.

3.3.4 Sediment quality

3.3.4.1. Sediment pH and E*h*

The sediment quality parameters are depicted in Table 5. Sediment pH ranged from 6.65 ± 0.48 to 8.3 ± 0.15 during the pre and post monsoon periods. However, the overall pH (based on the average of hydrogen ion

concentration) did not differ significantly (P > 0.0.5) between the ponds and in both the seasons. However, moderate amount of liming material had been applied to the ponds in all seasons, and in all ponds to stabilize the pH.

The mean sediment redox potential during the culture periods was -123.3 ± 16.27 mV. During the pre monsoon period E*h* varied from -124 to -213 mV and -99.5 to -157.5 mV with the mean values of -145.27 ± 19.2 mV and 126.6 ± 13.5 mV respectively. During the post monsoon period it ranged from -70.5 to -144.8 mV with the mean of -109.8 ± 13.3 mV and -85.4 to -145 mV with the mean of -111.8 ± 18.9 mV in the first and second cultures respectively (Fig. 11). The lowest sediment redox potential recorded was -213 mV, on only one occasion during March to June (pre monsoon) and the highest (-70.5 mV) was observed during September to January (Post monsoon). In general, the E*h* values were fluctuating between -150 to -100 during most of the culture period.

3.3.4.2 Total sediment organic carbon and total organic matter

Total organic carbon of the sediment during the entire culture period ranged between 1.32 ± 0.24% and 2.32 ± 0. 24% with the mean of 1.9 ± 0.5 (Fig.12). The total organic matter exhibited the same trend as that of the organic carbon. The highest mean values of total organic matter (3.52 ± 0.45%) occurred during the first culture of the pre monsoon period followed by a decrease in the second culture during the same season (3.23 ± 0.83%), second culture of the post monsoon (3.21 ± 0.8%) and the least during the first culture of the post monsoon (2.88 ± 0.38%) (Fig. 13 and Table 5).

3.3.4.3 Sediment TAN, NO_2-N and NO_3 - N

The concentration of TAN, NO_2-N and NO_3- N did not show significant difference among the seasons. The mean concentrations of TAN during both the seasons in four cultures were 0.14 ± 0.04 mg/L, 0.14 ± 0.05 mg/L, 0.13 ± 0.04 mg/L, and 0.11 ± 0.06 mg/L respectively (Fig.14). The nitrite nitrogen during the entire culture period varied between 0.02 ± 0.002 mg/L to 0.04 ± 0.003 mg/L (Fig.15). The overall mean value of NO_3-N was 0.05 ± 0.02 mg/L with the variation from 0.03 ± 0.005 mg/L to 0.09 ± 0.015 mg/L (Fig. 16, Table 5).

3.3.4.4 Total nitrogen and total phosphorus

The mean concentration of total nitrogen in the sediment among the culture period was 0.34 ± 0.16%. The maximum concentration of total nitrogen was 0.66 ± 0.07% and minimum concentration estimated was 0.16 ± 0.022 considering the cultures altogether (Fig.17) . The mean concentration of total phosphorus during the entire culture period was 0.12 ± 0.04 mg/g which ranged between 0.05 ± 0.01 mg/g to 0.16 ± 0.05 mg/g (Fig. 18. Table 5). A positive linear relationship was observed between total organic carbon and total nitrogen (R^2 = 0.59) and total nitrogen and total phosphorus (R^2 = 0.45).

3.3.5 Animal growth

Details of the health assessment and average body weight, survival and feed conversion ratio are depicted in Table 6 and 7. Shrimp weight did not differ significantly (P > 0. 05) among the cultures between seasons. The maximum body weight (35 g) was observed during the second culture of the post monsoon period and the minimum (30.12 g) during the second culture of the pre monsoon extending a period of 110 - 120 days. Feed efficiency among the culture stood with

in the ranges of 1.3 to 1.5 and did not vary much (P > 0.05). The maximum survival was extending from 62% to 67.25%. Cost benefit analysis is illustrated in Table 8. It denotes that all cultures in all seasons have been commercially viable and profitable.

3.3.6 Principal component analysis of water quality parameters

Principal Component Analysis (PCA) was performed on the normalized data set separately for the two seasons (pre monsoon and post monsoon). PCA of the two data sets yield four PCs for each sites with Eigenvalues >1, explaining 79.5% and 80.5% of the total variance in each water quality data sets. An Eigenvalue gives a measure of the significance for the factor, which with highest Eigenvalue is the most significant. Eigenvalues of 1.0 or greater are considered significant. The factor loadings were classified as 'strong', 'moderate' and 'week', corresponding to absolute loading values of >0.75, 0.75–0.50 and 0.50–0.30, respectively.

For the data set pertaining to pre monsoon among four PCs, PC1, explaining 55.4% of the total variance, has strong positive loadings on evening pH, 6PM DO, NO_3, TP, TN, productivity, chlorophyll, NO_2 and negative loadings on temperature, salinity, calcium, magnesium, transparency and silicate. PC2 explained 9.9% variance and moderate loadings on DO 12 AM and week negative loadings on calcium and magnesium. PC3, explaining 7.1% of the total variance, has strong negative loadings on NH_3 and week positive loading on morning pH. PC4, explaining the lowest variance (6.9%), has strong loadings on alkalinity and a moderate loading on morning pH (Fig. 19 Table 9).

The loadings of the four retained PCs in the post monsoon culture are shown in Table 10 and Fig. 20. PC1, explaining 60.4% of the total variance, the major positive contributor loadings are silicate, transparency, 12 AM and 6AM DO, and the negative loadings are evening pH, temperature, salinity, calcium, magnesium, NO_2, NO_3, PO_4,TP, productivity, chlorophyll . PC2, explaining 6% of the total variance, has moderate loading of morning pH. PC3 and PC4 explained 6.0% and 4.9 % of the total variance respectively.

3.3.7 Comparison of open and zero water exchange shrimp culture systems.

The study on open shrimp culture (with water exchange according to tides) was undertaken in 3 one-hectare ponds.

The water and sediment quality and health parameters during the experimental period are depicted in the Table 11. Most of the water and sediment parameters did not show significant difference except water alkalinity, silicate, chlorophyll, primary productivity and sediment E*h*. Water alkalinity was significantly higher in the ponds with zero water exchange than the open system. The average primary productivity in the open system was 0.95 ± 0.49 and in the closed system, it was 2.73 ± 0.45 g C/m^3/day and significantly different ($P< 0.05$). Average chlorophyll *a* content in the open and closed system was 12.35 ± 4.61 and 30.13 ± 3.21 respectively. In the case of sediment E*h* significantly lower values were recorded in the open system than the systems with zero water exchange. Considering the health parameters the closed system showed a higher survival (64.5%), average body weight of 33.5 g and lower FCR (1.3). In the open systems the culture was terminated on 85th day of culture due to the outbreak of White spot virus disease and subsequent mortality as confirmed by diagnostic PCR (Fig. 21).

3.4 Discussion

This chapter deals with the events that have taken place in four zero water exchange cultures and comparison to open systems.

In the zero water exchange systems evening pH positively correlated with daytime dissolved oxygen (0.57), primary productivity (0.759) and chlorophyll (0.752) during the entire culture seasons. This was attributable to the presence of heavy phytoplankton production throughout the culture period. Primary productivity and chlorophyll were the indicators of phytoplankton production. Water pH among the seasons ranged between 7.08 to 9.1, slightly deviating from the optimum range recommended (7.5 – 8.5) for shrimp culture (Soundarapandian et al., 2009). However, Boyd and Green (2002) reported that pH values within 6.0 – 9.00 are the best for shrimp growth, while a value between 4.0 to 6.0 and 9.0 to 11.0 may lead to slow growth (Hossain *et al*., 2001; Boyd, 2003). Brackish water systems are well buffered against pH changes, where pH seldom falls below 6.5 or rises above 9.0. The minimum pH of 7.27 is not harmful to the cultured species, and mortality does not occur until pH rises to above 9. However, sub – lethal effects may occur at around pH 9, and at high pH greater proportion of ammonium remains as unionized ammonia having much toxicity to shrimp (Boyd and Tucker, 1998). Saha *et al*. (1999) noticed pH of 8.11 to 8.67 in low saline ponds. Daytime peaks in pH observed in this study were related to increase in photosynthetic rate by phytoplankton.

Alkalinity levels in estuarine waters can vary from 10 to 400 mg/L $CaCO_3$ (Campos *et al*., 2009), and in culture of aquatic organisms it should not be less than 20 mg/L $CaCO_3$ because the phosphorus become insoluble under

such a situation (Wurtz, 2002). Boyd (1990) showed that alkalinity below 30 mg/L as $CaCO_3$ limits primary production in well-fertilized ponds, while in unfertilized ponds alkalinity below 120 mg/L can reduce primary production. Alkalinity was maintained during the entire culture period with the addition of dolomite. During pre monsoon period the lowest concentration occurred towards the final stage of culture and during post monsoon the lowest was at the initial stage which might be due to the influence of rain.

The climate is one of the predisposing factors for different diseases and a low atmospheric temperature which affects water temperature is an important risk factor which may pave the way for WSSV out break (Tendencia *et al*., 2010 a). It is also risky to stock shrimp during the cool months as exposure to low water temperature would lead to WSSV infection in *P.monodon* (Tendencia *et al*., 2010 b). This has been attributed to the increase in viral replication and the decrease in immune response of shrimp at low temperature (Vidal *et al*., 2001; Reyes *et al*., 2007).

The wide variation in salinity was depended on rainfall and evaporation. This was in agreement with the study reported by Guerrero – Galvan *et al*. (1999), Mmochi *et al*. (2002), and Everett *et al*. (2007). The optimum salinity suitable for shrimp culture is about 10-35 g/L (Boyd, 1995) which is vital for pond dynamics. The shrimps were grown in salinities between 4 and 26 g/L and it adapted quite well in fresh water conditions also (Chanratchakool *et al*., 1994; Karthikeyan, 1994; Ramakrishnareddy, 2000; Das *et al*., 2001; Collins and Russell, 2003). Culture of *P.monodon* in high salinities of over 30 g / L may cause disease problems, particularly caused by white spot virus, yellow head virus or luminescent bacteria. Varying salinity and pH can trigger disease outbreaks not only by affecting the health and defense mechanism of the host

but also by influencing the virulence of the pathogen. At high salinities, *V.harvei* is more lethal than at high temperatures (Kautsky *et al*., 2000. Salinity is positively correlated with calcium and magnesium in the entire culture seasons (r = 0.989, 0.987).

The suitable range of DO for optimum growth of shrimp is 5.0 – 7.5 mg/L. (Chanratchakool *et al*., 1994; Boyd, 2003). In all seasons DO was elevated because of the lower stocking density and the use of less feed and fertilizer. During the final stage of the culture especially during early morning hour's operation of aerators was required to maintain the required dissolved oxygen well above the critical limit. In systems with reduced water exchange, aerators used to be commonly operated almost continuously to both supply oxygen and circulate water in the ponds (Avnimelech *et al*., 1994; Howerton *et al*., 1993; Avnimelech and Ritvo, 2003). In addition to aerating water column, mechanical aerators could also mix the water column and could be directed to keep part of the pond bottom free of sludge (Losordo and Piedrahita, 1991; Avnimelech and Ritvo, 2003).

There was a positive correlation between evening pH and DO among the seasons (r = 0.58). Diurnal variation of pH and DO were characteristics of aquaculture systems (Johnston *et al*., 2002). Xu *et al*. (2008) concluded that there was a significant positive correlation between pH and DO mainly affected by algal photosynthesis. The study of Zhang *et al.* (2011) in an enclosure aquaculture experiment at Panjiakou Reservoir revealed significant positive linear correlations (r = 0.94 – 0.97) between pH and DO.

Secchi disc transparency is commonly used by aquaculture pond mangers as an indicator of phytoplankton concentration. Secchi Disc visibility

(SDV) measurement may be used in modeling phytoplankton productivity whenever direct phytoplankton measurements are not available (Almazan and Boyd, 1978; Osman *et al*., 2010). In other studies by Koenings and Edmundson (1991), Padmavathy and Prasad (2007) non-phytoplankton components also indicated the Secchi disc transparency. Water transparency showed a declining trend during all the cultural seasons. It may be due to the addition of artificial feed, which contributed to the nutritional requirements of phytoplankton. Rajyalaksmi *et al.* (1988) observed, transparency ranging from 14 to 36 cm in the brackish water ponds of Chilka lake fringe areas. Das *et al*. (2001) suggested that in prawn culture, transparency of 17. 5 cm is quite useful. Higher levels of primary productivity can be influenced by the presence of artificial feed and high feeding rates (Rahman *et al*., 2006 b; Campos *et al*., 2009). Therefore, fertilization rates have to be reduced in order to avoid the nutrient excess in the system.

In all the culture systems during both the seasons TAN concentration was with in the accepted limit and was 0.044 ± 0.007 mg/L considering the overall average. This may be one of the positive sides of the bioaugmentation carried out in these systems. Besides, other reasons could be low stocking density, feeding based on the requirement, relatively higher concentration of DO which might have favored nitrification. Oxidation of ammonia to nitrite and nitrate by nitrifying bacteria is more effective in ponds where dissolved oxygen is not below 3mg/L (Boyd and Tucker, 1998). Regular application of bioaugmentor might have promoted the growth of phytoplankton, which also involved in the uptake of nutrients like ammonia nitrogen produced by the degradation of detritus.

Nitrite, an intermediary compound of nitrification (Hargreaves, 1998), has significant effects on shrimp health (Jayashankar and Muthu, 1983; Lin and Chen, 2003). During a study, Ray *et al.* (2011) observed low survival of shrimp due to the high NO_2^--N in the nursery phase of the culture. The building up of nitrite – nitrogen towards the end of the culture indicates effective nitrification.

Nitrate nitrogen concentration exhibited same pattern as that of nitrite. It did not show a significant difference between the seasons. During the first and second culture of the second season (post monsoon) nitrite and nitrate exhibited significantly high positive correlation (r 0.845 and r 0.858) and the second culture of the first season (pre-monsoon) also showed a positive correlation (r 0.317). However, there was building up of nitrate nitrogen from the mid phase of the culture indicating effective nitrification in the systems. Meanwhile in the studies of Milstein *et al*. (2001) the nitrite and nitrate showed a negative correlation. It indicated that during water exchange the particles carrying nitrifying bacteria were washed away. The higher nitrite and nitrate concentration during the final phase of the culture indicated building up of effective nitrification throughout the culture period in general and towards the end of the culture in particular.

In aquaculture ponds, nitrate nitrogen usually has the concentration of 0.1 to 0.3 mg/L (Boyd, 1995). The major source of nitrate is the oxidation of ammonia – nitrogen and nitrite - nitrogen and ponds have always a low concentration of ammonia nitrogen. In all biological systems nitrate gets reduced to nitrogen gas by denitrifying bacteria under anoxic conditions as in anaerobic sediments (Hargeraves, 1998).

Orthophosphate or soluble reactive phosphorus concentrations were found to be high throughout the cultivation period compared to other aquatic systems. This was higher compared to the one reported by Boyd (1990) which showed that dissolved orthophosphate concentration was in the range of 0.005 – 0.02 mg/L, and seldom exceeded 0.1 mg/L even in highly eutrophic waters. However, this result is in accordance with that of Fast and Lester (1992) who suggested the tolerable concentration for shrimp culture as <3.0 mg/L. Thakur and Lin (2003) also reported that there was a significantly higher concentration of orthophosphate (0.218 – 0.384 mg/L) in shrimp ponds with higher stocking density. In zero-water exchange shrimp ponds, the phosphate concentration was high, which ranged from 0.07 – 1.17 mg/L (Burford *et al*., 2003).

It was found that PO_4 - P and NO_3 - N were correlated with primary productivity and chlorophyll. The correlation between PO_4 – P with productivity and chlorophyll *a* was found to be 0.602, 0.585 and NO_3- N with productivity and chlorophyll was 0.814 and 0.802. It might indicate that phytoplankton biomass was limited by PO_4- P concentrations. This is in accordance with Elser *et al*. (1990), who stated that phosphorus is a major limiting nutrient in most ecosystems. Phosphate can limit productivity because they precipitate rapidly in alkaline aquatic environment (Sonneholzner and Boyd, 2000), where they are then absorbed by the pond soil (Boyd and Munsiri, 1996). Higher amounts of nitrogen and phosphorus compounds enhanced the photosynthesis, explaining the higher phytoplankton production and lower transparency (Milstein and Svirsky, 1996; Moriarty, 1997).

Another probable source of phosphorus other than shrimp feed and shrimp excretion is from the excretion of the zooplankton population (Cremen *et al*., 2007). According to Sullivan and Ritacco (1985) and Buttino (1994), some

zooplankton species can only tolerate the ammonium concentration lower than 0.2 mg/L. Therefore, although the zooplankton population was not quantified in this study, it was assumed that there was an abundance of zooplankton, which in turn contributed to the high phosphate concentration because ammonium concentrations in the present study did not reach 0.2 mg/L. Hence, shrimp pond could have higher reactive phosphorus due to zooplankton excretion.

Total nitrogen and total phosphorus increased with the progress of rearing. Bratvold and Browdy (2001) and Kumar *et al*. (2012) have observed similar gradual increases. In a closed system nutrients kept accumulated within the system over time, this might be an advantage of the closed system as the high nitrogen and phosphorus accumulated within the system could support the growth of natural food organisms contributing ultimately to shrimp growth. In the open shrimp ponds, major output of nutrients was in the discharge water. Briggs and Smith (1994) emphasized that in a culture system with low water exchange water borne loss of nutrients is less important than loss through the sediment, due to the rapid accumulation of sediment in shrimp ponds. Chen *et al*. (1989) reported that sediment plays an important role in the nutrient balance of aquaculture system as it can serve as buffer in water nutrient concentration.

The chlorophyll *a* and primary productivity in all the ponds were low in the initial phase, but steadily increased during the second phase, reaching peaks at the end of the culture period, indicating the effects of increasing nutrient enrichment of the ponds. This study corroborate with the studies of Yusoff *et al.* (2002). The overall average of chlorophyll *a* was 29 µg/L and this value was lower than the reported values in shrimp culture systems (Yusoff *et al*., 2002; Cremen *et al*., 2007)

There was a positive correlation between daytime DO and chlorophyll (0.768), while significantly negative correlation was observed between night DO and chlorophyll (-0.867). In aquaculture systems, changes in DO concentration are affected by algal photosynthesis and the subsequent production of oxygen. On the other hand, changes in DO concentration are affected by aquatic respiration, oxidative decomposition of organic matter and fish waste at the pond bottom. it is likely that the complex interaction between the above processes lead to a more multifaceted link between DO and chlorophyll a. Studies of Ruan and Xu (1998) noticed that a significant positive correlation existed between DO and chlorophyll a in a closed system. Fukushima *et al*. (2004) in an enclosure experiment in Kasumigaura Lake and Xie *et al*. (2009) in an aquaculture experiment with high water exchange found that there was no correlation between chlorophyll and DO.

Phytoplankton count was much higher than those reported by mixed shrimp farming in Mekong delta of Vietnam (8600 ± 800 cells/L) (Johnston *et al*., 2002), Thai intensive shrimp ponds (1822 - 72527 cells/L) (Tookwinas and Songsangjinda, 1999) and semi intensive shrimp ponds in Bangladesh (maximum 211000 cells/L) (Islam *et al*., 2004). The abundance of phytoplankton community suggested a sound base for a sufficient food chain in the zero water exchange shrimp ponds. The phytoplankton genera recorded in our study can be compared to those reported by Rodriques and Paez- Osuna (2003) and Islam *et al*. (2004), who have reported that Bacillarophyceae, Chlorophyceae, Euglenophyceae and Cyanophyceae as the dominant groups in aquaculture systems.

The ranges of THB recorded during the study period are with in the levels reported earlier in India (Sharmila *et al*., 1996; Otta *et al*., 1999) and

South East Asia (Peranginangin *et al*., 1992; Sung *et al*., 2001). Relatively high THB to the tune of 1.2 x 10^8/ g and 2.23 x 10^8 /g sediment of shrimp culture ponds were noticed in Tamil Nadu, India (Prabhu *et al*., 1999).

THB counts in the sediment were higher than that in the water samples in agreement with that reported by Al- Harbi and Uddin (2010). High bacterial abundance is not necessarily a disadvantage if the bacteria are not pathogenic as high bacterial abundance may indicate healthy organic matter recycling and remineralization.

Vibrio counts in the pond water showed a positive correlation with salinity. This was consistent with the findings of Hsieh *et al*. (2007) and Tho *et al*. (2011). Several strains of vibrios are pathogenic to *P. monodon* larvae at a level of 10^3 CFU /mL (Prayitno and Latchford, 1995). The average *Vibrio* concentration during the study period was 1.57 x 10^2 CFU/mL and it was lower than that of the studies of Tho *et al*. (2011) in the mixed shrimp ponds of Mekong delta of Vietnam.

The sediment pH ranged between 6.65 to 8.3 through out the culture periods. The results of the present study are, more or less, in conformity with Chakraborti *et al*. (1986), Abraham and Debasis (2009).

Oxidation – reduction potential (Redox potential) of sediment has been identified as an appropriate indicator of organic accumulation in sediment (Pearson and Black, 2001; Crawfold *et al*., 2002) representing oxygen demand (Teflor and Robinson, 2003). Redox level is strongly correlated with sediment grain size (Winsby *et al*., 1996). In coarse sediments, redox potential used to lower than that in fine sediment because of the higher diffusion rate of oxygen. A positive redox indicates that oxygen is still in the sediment. In undisturbed

sediment, surface E*h* used to be around 300 to 400 mV (Winsby *et al*., 1996). Negative redox potential value is generally indicative of organic matter enriched, fine size grain and or poorly oxygenated, anoxic sediments (Winsby *et al*., 1996). The maximum acceptable range of redox potential in a cage farm of salmon was -150 mV (Shakouri, 2003). E*h* should not be lower than -125 mV at 0 -3 cm depth of sediments (Heining, 2000). The redox potential of mud should not be lower than – 200 mV (Muralidhar *et al*., 2001).

Optimum range of organic carbon in the pond sediments is between 1-3% (Banerjea 1967; Wudtisin and Boyd, 2006). Aquaculture pond sediments seldom contain more than 3% organic carbon (Boyd, 1995). Lower concentration is unfavorable for the growth of benthic organisms that are important food for many species and higher concentrations favor anaerobic conditions at the sediment water interface. Steeby *et al*. (2004) found that channel cat fish ponds in Mississippi had an average organic carbon concentration of 1.77% with a range of 0.71 – 2.89%. In Thailand the greatest organic carbon concentration in tilapia ponds having age ranging from 3 to 39 years was 3.39% with a mean value of 35 ponds as 1.9% (Thunjai *et al*., 2004). Accordingly, range of organic carbon in the present study was essentially the same normally found in aquaculture ponds. The culture pond soils using probiotics contain lower percentage of organic matter and total organic carbon probably due to decomposition of organic materials during the mineralization process (Moriarty, 1997).

The nitrogen content of the sediment is often used as an indicator of sediment enrichment, because this is mostly derived from external inputs (Telfer and Robinson, 2003). Crawford *et al*. (2002) evaluated that high nitrogen is a proper indicator of highly impacted sediment. In our study total

nitrogen of 0.33% corroborated with the studies of Karakassis *et al.* (2000) and Kempf *et al*. (2002), who reported the concentration of total nitrogen less than 0.3%. Reduced sediment nutrient level in our shrimp ponds was in agreement with the previous studies of Wang *et al*. (2005 and 2009) and Matias *et al*. (2002).

The seasonal average of body weight during the time of harvest was 33.7 ± 5.1 g with an average growth rate of 0.31g/day. An analysis of growth reported for the various species of shrimp revealed wide variation. Trimble (1980) reported that *P. vannamei* grew 1.28 g /week at a stocking density of 2.5 shrimp/m^2. Wyban *et al*. (1987) found growth rate to be 1.72 g / week at a stocking density of 5 shrimp/m^2, Gopalakrishnan and Ajay (2005) found 0.22 g/day. Liao (1987) and Sundararajan *et al*. (1979) observed faster growth rates for *P. monodon* (0.32 to 0.39/g/day) in general. The studies of Janakiram *et al*, (2011) in the semi intensive ponds of Andra Pradesh showed the growth rate of 1.6 - 2.4 g/week. The seasonal average survival was found 64 %, and in general survival rates recorded during the present study were higher than those reported by earlier workers from other localities in India and other countries (Eldani and Primavera, 1981; Chakraborty *et al*., 1985, 1997., and Ahamed *et al*., 2000). This seems to be a reflection of the progress achieved in the management practices adopted in culture system. The recorded production in this study was comparable to the earlier work in the shrimp culture system (Gopalakrishnan and Ajay, 2005).

Feed is one of the essential inputs in shrimp production, which determine profit. Feed management should therefore be regulated by feed consumption and demand as shrimp appetite vary with the environmental conditions, i.e., weather, water quality, physical conditions such as moulting,

stress, disease and gut evacuation rate (Dall, 1967). It is generally acknowledged that multiple feeding improves growth and FCR (Sedgewick, 1979) and minimizes accumulation of uneaten feed (Mohanty, 2001). The Average Feed Conversion ratio in our experiment was 1.4. Soundarapandian and Gunalan (2008), Paul Raj (1999), Ramakrishna reddy (2000) and Balakrishnan *et al*. (2011) recorded similar results. More over the studies of Akiyama *et al*. (1991) reported a global average FCR of 2.1 at a stocking density of 10 -15 m^2. The lowest FCR can be attributed to the following reasons. Feed acceptance was checked through feeding trays, and the feed rations were adjusted according to the consumption. This accurate and frequent alteration in feed ration led to a reduction of waste (Smith *et al*., 2002; Chim *et al*., 2008).

During aquaculture production, the growth and survival rate of an individual organism depend primarily on management inputs and type of facilities, and the revenue returns are affected by the level of production, farm price and by the size and quality of products in a competitive seasonal market (Alam *et al*., 2007). According to Shah *et al*. (2000) the major item cost for aquaculture are cost of fingerlings, lease rent, feed and fertilizer and labour cost. In our study the average production cost was Rs. 135 – 145/ Kg (US $ 3/ Kg) and it was comparatively lower than the studies of Alam *et al*. (2007) (US $ 6.81 to 7.54), Ling *et al*. (2001 (US $ 4.07) and Shah *et al*. (2000) (US $ 4.83 .Kg). In our studies in all seasons, the benefit cost ratio indicated that the cultures were economically viable and profitable.

It could be concluded that the adoption of zero water exchange shrimp technology is very well acceptable in all seasons. In the overall process, detritus management is given the greatest emphasis as accumulation of detritus

during the culture is the root cause of the problem, which surfaces as the days of culture advance. At the same time the inherent quality of the soil also does contribute to the success of a culture. This quality has been specifically expressed in quantitative terms as *Eh* and total organic carbon. For successful shrimp culture, *Eh* should not slide down below -150 mV and total organic carbon should not be above 2.0%. Therefore, the technology developed has a powerful bioaugmentor for detritus management, *Bacillus* MCCB 101, produced under the brand name Detrodigest. From the data generated which spread through 16 experimental cultures over a period of two years it is revealed that Detrodigest can bring up *Eh* and bring down organic carbon provided the bioaugmentor is applied regularly. In a zero water exchange system topping up of water is essential to maintain water level. Application of the gut probiotic Enterotrophotic was also essential for the management of intestinal flora to avoid the emergence of vibriosis, a situation often reported. Enterotrophotic a blend of *Bacillus* MCCB 101 and *Micrococcus* MCCB 104 was found suitable and on its application moulting cycle was found to be regulated with the disappearance of vibriosis and Zoothamnium infestation. Feed intake also was found enhanced with the reduction of FCR. In high saline culture *Vibrio* population often goes up and *V.harveyi* happens to be the species dominating. An antagonistic probiotic PS-1 consisting of *Pseudomonas aeuroginosa* MCCB 102 was found suitable to regulate the *Vibrio* population in the water column.

In such zero water exchange shrimp culture system managed exclusively with bioaugmentors and probiotics, depletion of carbonate alkalinity happens to be a reality. This could be managed by the addition of dolomite to regulate and maintain alkalinity above 70 mg/L. On implementing the above protocol,

ammonia and hydrogen sulphide, two toxic compounds in culture system, were found not rising to toxic levels.

On comparing zero water exchange systems with open culture systems significantly higher values of water alkalinity in the zero water exchange culture systems was noticed which might be due to the addition of dolomite or calcium carbonate. The higher silicate value in the system with zero water exchange might be due to the initial fertilization and addition of sodium silicate. The regular application of bioaugmentor 'Detrodigest' in the zero water exchange system lead to high primary productivity and chlorophyll content. The higher sediment *Eh* in this system was due to the regular application of bioaugmentor Detrodigest for the degradation of detritus accumulated in the pond bottom. Hopkins *et al*. (1992) revealed that organic matter was in greater quantity in ponds with limited water exchange compared to ponds that use higher water exchange. Thakur and Lin (2003) found that less nitrogen is excreted per unit of shrimp biomass in closed system. In the zero water exchange culture systems significantly higher survival and average body weight during the time of harvest was due to the adoption of effective management measures from stocking to harvest. Sludge removal, ploughing, liming, complete system dry-out between culture cycles, water filtration through 300 µm mesh screen and phosphorus application through fertilization were reported to reduce risk of WSSV infection (Corsin *et al*., 2001). Sharing the water source with other farms, and water change according to tidal influx were associated with WSSV infection (Tendencia *et al*., 2011). The study revealed that the zero water exchange culture systems with effective bioremediation reduces the risk of wssv and also is effective for the successful

shrimp culture. This can be considered as a package of practice of zero water exchange validated, and sound enough to be transferred to farmers.

Precisely, during pre monsoon and post monsoon periods the zero water exchange culture system could be implemented effectively with out any out break of white spot disease. Accordingly, two successful crops can be obtained in a year leaving the monsoon period the system fallow. On comparing the zero water exchange system to open system, the physico – chemical and biological parameters were more or less stable in the former, a situation much required for shrimp culture, for that matter for any aquaculture practice. This part of the study leads to experimentation with different stocking densities to evaluate the efficacy of the bioremediation protocol in evolving a culture package which is sustainable. This is dealt with in the fourth chapter.

Table 1. Details of culture and stocking density of shrimp grow out ponds during different culture seasons

	Pond No	Area (acre)	Stocking density	Days of culture
First culture Pre monsoon	1	2.25	8	115
	2	2	8	110
	3	1.95	8	115
	4	2.55	9	110
First culture post monsoon	1	2.25	7	115
	2	2	7	115
	3	1.95	7	115
	4	2.55	7	115
Second culture pre monsoon	1	2.25	8	120
	2	2	8	120
	3	1.95	8	125
	4	2.55	8	125
Second culture post monsoon	1	2.25	6	115
	2	2	6	115
	3	1.95	8	110
	4	2.25	8	115

Table 2. Water quality parameters of shrimp grow out ponds during different culture seasons. (Minimum, maximum, mean ± SD)

Parameters	First culture pre monsoon	First culture post monsoon	Second culture pre monsoon	Second culture post monsoon
pH Morn	7.08 – 8.5	7.50 – 8.4	7.3 – 8.01	7.6 – 8.9
	(7.79 ±0.3)	(7.83 ± 0.5)	(7.75 ± 0.51)	(8.09 ± 0.67)
pH Even	7.8 - 9.1	7.7 – 9.1	7.9 - 9.1	7.8 – 9.1
	(8.5 ± 0.4)	(8.5 ± 0.6)	(8.6 ± 0.5)	(8.52 ± 0.3)
Alkalinity	52 - 74	47 - 70	50 -78	44- 77
mg/ L	(60.2 ± 4.6)	(58 ± 2.6)	(63 ± 5.3)	(60.8 ± 4.6
Salinity	15 - 30	3 - 9	10 - 28	2.5 – 12.5
g/L	(24.8 ± 0.9)	(5.8 ± 0.1	(22.7 ± 0.5)	(5.2 ± 0.2)
Calcium	417 - 853	85 - 168	400 - 800	75 - 160
mg/L	(734 ± 48.7)	(135 ± 4.6)	(665 ± 48)	(113 ± 21.3)
Magnesium	2183 - 4270	315 - 550	2000 - 4188	163 -885
mg/L	(3683 ± 87)	(449 ± 11)	(3481 ± 97)	(511 ± 17)
Transparency	35 - 60	32.5 - 65	30 - 59	30 - 64
cm	(43.2 ± 6.7)	(47.1 ± 5.8)	(45.3 ± 6.1)	(47.2 ± 8.03)
DO 12 AM	4.5 – 6.1	4.5 – 6.3	4.5 – 5.5	4.9 – 6.7
mg/L	(5.3 ± 0.6)	(5.7 ± 0.6)	(5 ± 0.4)	(5.42 ± 0.42)
DO 6 AM	3.7 – 5.7	4.8 – 6.6	3.4 – 5.3	3.4 – 5.3
mg/L	(4.6 ± 0.6)	(5.5 ± 0.5)	(4.6 ± 0.6)	(4.5 ± 0.5)
DO 12 PM	5.9 – 8.1	7.03 – 8.8	6.1 – 8.8	6.1 – 8.9
mg/L	(7.3 ± 0.8)	(8.1 ± 0.5)	(7.5 ± 0.8)	(7.5 ± 0.9)
DO 6 PM	7.2 – 9.3	7.8 – 8.8	7.21 – 8.6	7.2 – 9.3
mg/L	(8.01 ± 0.6)	(8.3 ± 0.3)	(8.1 ± 0.4)	(8.2 ± 0.3
TAN (mg/L)	0 – 0.1	0 – 0.08	0.04 – 0.13	0 – 0.07
	(0.04 ± 0.009)	(0.02 ± 0.003)	(0.07 ± 0.01)	(0.03 ± 0.007)
NO_2^- - N	0.004 – 0.06	0.005 – 0.05	0.01 – 0.05	0.003 – 0.04
mg/L	(0.03 ± 0.005)	(0.017 ± 0.008)	(0.037 ± 0.007)	(0.012 ± 0.003)
NO_3^- - N	0.003 – 0.056	0.004 – 0.045	0.012 – 0.063	0.002 – 0.059
mg/L	(0.03 ± 0.007)	(0.021 ± 0.009)	(0.034 ± 0.014)	(0.023 ± 0.004)
Total Nitrogen	0.037 – 0.18	0.06 – 0.18	0.038 – 0.12	0.09 – 0.17
mg/L	(0.08 ± 0.03)	(0.13 ± 0.03)	(0.08 ± 0.018)	(0.14 ± 0.18)
PO_4 - P	0.01 – 0.08	0.01 – 0.08	0.03 – 0.09	0.004 – 0.06
mg/L	(0.04 ± 0.013)	(0.04 ± 0.015)	(0.05 ± 0.003)	(0.023 ± 0.01)
Total phosphorus	0.06 – 0.21	0.034 – 0.211	0.077 – 0.26	0.08 – 0.17
mg/L	(0.11 ± 0.01)	(0.09 ± 0.02)	(0.14 ± 0.03)	(0.11 ± 0.02)
Silicate	0.32 – 2.23	0.33 – 2.1	0. 57 - 2.6	0.54 – 2.3
mg/L	(1.27 ± 0.13)	(1.21 ± 0.06)	(1.2 ± 0.09)	(1.5 ± 0.05)
H_2S	0 – 0.006	0 – 0.007	0 – 0.007	0 – 0.007
mg/L	(0.002 ± 0.001)	(0.003 ± 0.001)	(0.003 ± 0.0009)	(0.003 ± 0.0009)
Primary product	1.13 - 4.16	1.3 – 4.8	1.02 – 5.65	1.15 – 4.8
$g/C/m^3/day$	(2.42 ± 0.98)	(2.75 ± 0.89)	(2.88 ± 0.79)	(2.4 ± 0.69)
Chlorophyll	3.83 – 48.33	6.4 – 53.1	6.42 – 71.45	3.35 – 67.6
µg/L	(26.7 ± 5.6)	(28.31 ± 4.8)	(33.4 ± 6.9)	(28.1 ± 4.8)

Table 4. Changes in the bacterial counts of zero water exchange ponds at different culture seasons

Pond water

DOC	First culture Pre monsoon		First culture Post monsoon		Second culture Pre monsoon		Second culture Post monsoon	
	THC	PVC	THC	PVC	THC	PVC	THC	PVC
0 - 30	2.3 x10^5	5.3 x 10^2	3.2 x 10^5	0.84 x 10^2	4.5 x 10^5	3.4 x 10^2	3.8 x 10^5	0.9 x 10^2
30 -60	4.3 x 10^5	1.9 x 10^2	4.4 x 10^6	0.95 x 10^2	6.6 x 10^5	1x 10^2	4.7 x 10^6	1x 10^2
60 -90	5.6 x 10^6	0.9 x 10^1	7.6x 10^5	1.06 x 10^2	5.6 x 10^6	0.9 x 10^2	6.5 x 10^5	1 x 10^2
90 - 120	7.5 x 10^6	0. 9 x 10^2	8.2 x 10^5	1.43 x 10^2	7.7 x 10^6	0.9 x 10^2	3.5 x 10^6	2.5 x 10^2

Pond Sediment

DOC	First culture Pre monsoon		First culture Post monsoon		Second culture Pre monsoon		Second culture Post monsoon	
	THC	PVC	THC	PVC	THC	PVC	THC	PVC
0 - 30	5.5 x 10^5	1.2 x 10^2	5.8 x 10^5	0.9 x 10^2	6.8 x 10^6	1.2 x 10^2	4.4 x 10^6	1.04 x10^2
30 -60	6.6 x 10^5	0.8 x 10^2	4.5 x 10^6	1.1 x 10^2	7.7 x 10^5	1.3 x10^2	5.5 x 10^6	1.2 x10^2
60 – 90	4.6 x 10^6	1.1 x 10^2	3.3 x 10^7	1.2 x10^2	8.3 x 10^6	1.1 x 10^2	3.4 x 10^7	1.3 x10^2
90- 120	1.3 x 10^7	1.3 x 10^2	7.9 x 10^6	1.3 x 10^2	1.2 x 10^7	1 x 10^2	2.3 x 10^7	1.7 x 10^2

DOC – Days of Culture, THC – Total Heterotrophic Count, PVC – Presumptive Vibrio Count

Table 5. Sediment quality parameters of shrimp grow out ponds at different culture seasons (Minimum, maximum, mean ± SD)

Parameters	First culture pre monsoon	First culture post monsoon	Second culture pre monsoon	Second culture post monsoon
pH	6.65 – 8.04	7.34 – 8.3	7.32 – 7.9	7.42 – 7.53
	(7.17 ± 0.42)	(7.65 ± 0.21)	(7.5 ± 0.18)	(7.4 ± 0.18)
E*h* (mV)	-124 - - 213	-70.5 - -144.8	-99.5 - - 157.5	-85.4 - -145
	(-145. 27 ± 19.2)	(-109.8 ± 13.3)	(-126.6 ± 13.5)	(-111.8 ± 18.9)
TOC (%)	1.89 – 2.32	1.32 – 1.97	1.6 – 2.2	1.4 – 2.3
	(2.04 ± 0.4)	(1.7 ± 0.3)	(1.9 ± 0.4)	(1.9 ± 0.7)
TOM (%)	3.24 – 3.99	2.27 – 3.38	2.82 – 3.77	2.33 – 4
	(3.52 ± 0.45)	(2.88 ± 0.8)	(3.23 ± 0.83)	(3.21 ± 0.8)
TAN	0.124 – 0.16	0.104 – 0.2	0.11 – 0.21	0.1 – 0.21
	(0.14 ± 0.04)	(0.13 ± 0.04)	(0.14 ± 0.05)	(0.11 ± 0.06)
NO_2 -N	0.03 – 0.04	0.03 – 0.04	0.02 – 0.04	0.03 – 0.04
	(0.03 ± 0.005)	(0.031 ± 0.009)	(0.03 ± 0.01)	(0.03 ± 0.01)
NO_3- N	0.04 – 0.05	0.03 – 0.07	0.03 – 0.09	0.04 – 0.06
	(0.04 ± 0.02)	(0.05 ± 0.02)	(0.05 ± 0.02)	(0.06 ± 0.02)
Total nitrogen	0.19 – 0.66	0.16 – 0.48	0.19 – 0.58	0.2 – 0.58
(%)	(0.38 ± 0.15)	(0.32 ± 0.13)	(0.32 ± 0.17)	(0.33 ± 0.17)
Total Phosphorus	0.08 – 0.13	0.08 – 0.15	0.05 – 0.14	– 0.16
(mg/g)	(0.1 ± 0.04)	(0.1 ± 0.04)	(0.09 ± 0.03)	(0.09 ± 0.040

TOC – Total Organic Carbon
TOM – Total Organic Matter

Table 6. Health assessment

DOC	Culture	Colour	Exoskeleton				Antenna	Appendages		Tail			Gills			Intestine		
			Fouling	Necrosis	Melanosis	Physical	cut	Fouling	Colour	Spreadness	Blisters	Fouling	Colour	Swelling	Degeneration	Full gut	Half Gut	Empyt Gut
30	1st Pre monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	90%	10%	0
	1st Post monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	95%	5%	
	2nd Pre monsoon	Normal	Nil	Nil	Nil	Nil	3%	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	88%	10%	2%
	2nd Post monsoon																	
45	1st Pre monsoon	Normal	10%	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	100%	0%	0
	1st Post monsoon	Normal	Nil	Nil	Nil	5%	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	94%	6%	0
	2nd Pre monsoon	Normal																
	2nd Post monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	72%	28%	0
60	1st Pre monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	68%	16%	16%
	1st Post monsoon	Normal	20%	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	64%	32%	4%
	2nd Pre monsoon																	
	2nd Post monsoon	Normal	Nil	Nil	Nil	Nil	5%	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	76%	24%	0
73	1st Pre monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	96%	4%	0
	1st Post monsoon	Normal	Nil	Nil	Nil	5%	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	70%	20%	10%
	2nd Pre monsoon	Normal	25%	Nil	Nil	Nil	4%	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	68%	15%	17%
	2nd Post monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	90%	10%	0
90	1st Pre monsoon	Normal	Nil	Nil	Nil	5%	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	92%	8%	0
	1st Post monsoon																	
	2nd Pre monsoon	Normal	Nil	Nil	Nil	Nil	2%	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	80%	12%	8%
	2nd Post monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	94%	6%	0
110	1st Pre monsoon	Normal	55	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	82%	12%	6%
	1st Post monsoon	Normal	Nil	Nil	Nil	3%	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	76%	20%	4%
	2nd Pre monsoon																	
	2nd Post monsoon	Normal	Nil	Nil	Nil	Nil	Nil	Nil	Normal	Normal	Nil	Nil	Normal	Nil	Nil	80%	12%	8%

Table 7. Health parameters of shrimp grow out ponds at different culture seasons

	Pond No	ABW	SURVIVAL	FCR
First culture pre monsoon	1	34.5 ± 8.5	67	1.7
	2	31 ± 3.5	64	1.2
	3	34 ± 3.6	69	1.1
	4	33.5 ± 5.6	58	1.3
	AVG	33.5 ± 5.3	64.5	1.3
First culture post monsoon	1	31 ± 7.6	63	1.3
	2	35 ± 5.5	70	1.0
	3	33 ± 6.7	62.5	1.54
	4	34.5 ± 5.5	56.5	1.8
	AVG	33.37 ± 6.3	62.9	1.4
Second culture pre monsoon	1	27 ± 5.5	69	1.6
	2	29.5 ± 6.6	72.5	1.3
	3	32 ± 7.6	65.5	1.4
	4	32 ± 6.3	62	1.6
	AVG	30.12 ± 6.5	67.25	1.4
Second culture post monsoon	1	34 ± 5.5	54	1.3
	2	37 ± 6.6	67	1.5
	3	35 ± 7.3	58	1.9
	4	35 ± 3.3	68	1.4
	AVG	35 ± 5.7	62	1.5

Table 8. Cost benefit analysis of shrimp grow out ponds at different culture seasons (1ha)(Rs)

	First culture Pre monsoon	First culture post monsoon	Second culture pre monsoon	Second culture post monsoon
Pond Preparation	11000	10000	15250	15000
Seeds	14400	28000	28750	28250
Feeds	66250	83772	151635	125750
Dolomite	2500	2010	11700	10500
$CaCO_3$			2000	7250
Detrodigest	5750	7094	7750	5200
Enterotrophotic	3500	6175	9680	10280
Vitamin C	450	500	1100	1050
PS 1	720	-	-	-
Labour	15000	25000	30000	46500
Electricity	4000	5000	12500	14000
Harvest	2500	3000	3000	5000
Travel	2000		5000	5000
Total	128070	170549	278365	273783
Yield	206250	232000	374500	435850
Profit	78180	61451	96135	162067

Table 9. Factor loadings of pre monsoon season

PC	Eigenvalues	% variance	Cum. % variance
1	11.7	55.6	55.6
2	2.08	9.9	65.5
3	1.49	7.1	72.6
4	1.45	6.9	79.5
5	0.75	3.6	83.1

	PC1	PC2	PC3	PC4	PC5		PC1	PC2	PC3	PC4	PC5
pH	0.46	0.34	0.48	0.52	0.11	Trans	-0.88	0.21	-0.2	0.1	-0.07
even pH	0.77	0.14	0.08	0.19	0.13	NH3	-0.18	0.13	-0.89	0.12	0.01
temp	-0.93	-0.09	-0.01	0.17	0.08	NO2	0.28	0.54	-0.38	0.45	0.33
salinity	-0.88	-0.37	0.06	-0.08	0.13	NO3	0.87	0.16	-0.29	-0.06	-0.17
calcium	-0.85	-0.47	0.06	0.03	0.11	PO4	0.64	0.43	-0.20	0.04	0.46
Magnesium	-0.89	-0.27	0.02		0.20	TP	0.80	-0.04	0	0.1	0.01
alkalinity	-0.18	-0.45	0.06	0.78	-0.12	TN	0.91	0.12	0.06	0.13	-0.07
DO 12AM	-0.44	0.61	0.27	-0.11	0.35	Silicate	-0.81	-0.21	0.06	0.13	-0.10
6AM	-0.76	0.04	-0.04	-0.43	-0.31	prod	-0.95	0.06	0.08	0.01	-0.05
12PM	0.67	-0.39	-0.13	0.03	0.07	chlor	0.94	0.01	0.02	-0.01	-0.10
6PM	0.76	-0.35	-0.02	0.08	-0.06						

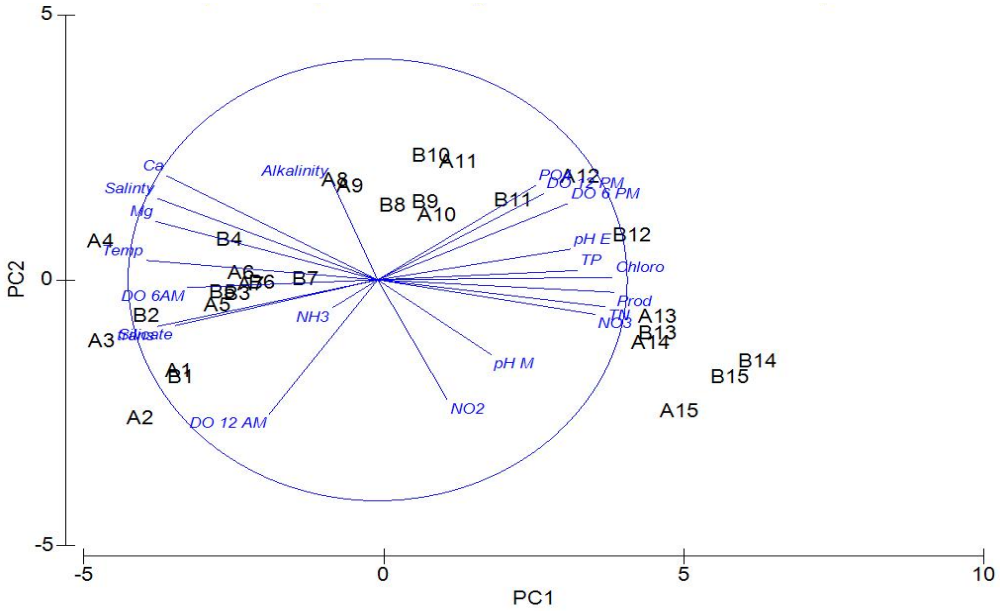

Fig. 15. Principal Component Analysis of Pre monsoon season

Table 10. Factor loadings of post monsoon

PC	Eigenvalues	% variance	Cum. % variance
1	12.7	60.4	60.4
2	1.97	9.2	69.6
3	1.27	6.0	75.6
4	1.04	4.9	80.6
5	0.83	3.9	84.5

	PC1	**PC2**	**PC3**	**PC4**	**PC5**		**PC1**	**PC2**	**PC3**	**PC4**	**PC5**
pH	0.42	0.55	-0.22	0.41	-0.07	Trans	0.97	-0.02	-0.02	0.53	0.06
even pH	-0.78	0.2	0.06	0.36	-0.01	NH3	-0.62	0.33	0.31	0.05	0.30
temp	-0.79	0.32	-0.05	0.11	0.17	NO2	-0.83	-0.11	0.46	-0.06	0.34
salinity	-0.89	-0.34	0.09	-0.03	-0.13	NO3	0.9	-0.05	0.14	-0.04	-0.02
calcium	-0.83	-0.44	0.05	-0.08	0.08	PO4	-0.81	-0.15	0.05	-0.01	0.11
Magnesium	-0.83	-0.12	-0.26	-0.03	-0.22	TP	-0.58	0.32	0.44	-0.21	0.02
alkalinity	-0.72	0.17	-0.51	0.08	-0.06	TN	-0.39	0.56	0.29	0.19	0.07
DO 12AM	-0.63	-0.23	-0.32	-0.03	-0.32	Silicate	0.91	0.16	0.04	0.15	0.12
6AM	0.53	-0.59	0.14	0.08	0.18	prod	-0.97	-0.06	0.01	0.04	-0.02
12PM	-0.69	-0.33	-0.12	0.43	-0.1	chlor	-0.97	-0.5	-0.11	0.23	0.11
6PM	-0.85	0.01	-0.35	0.27	-0.1						

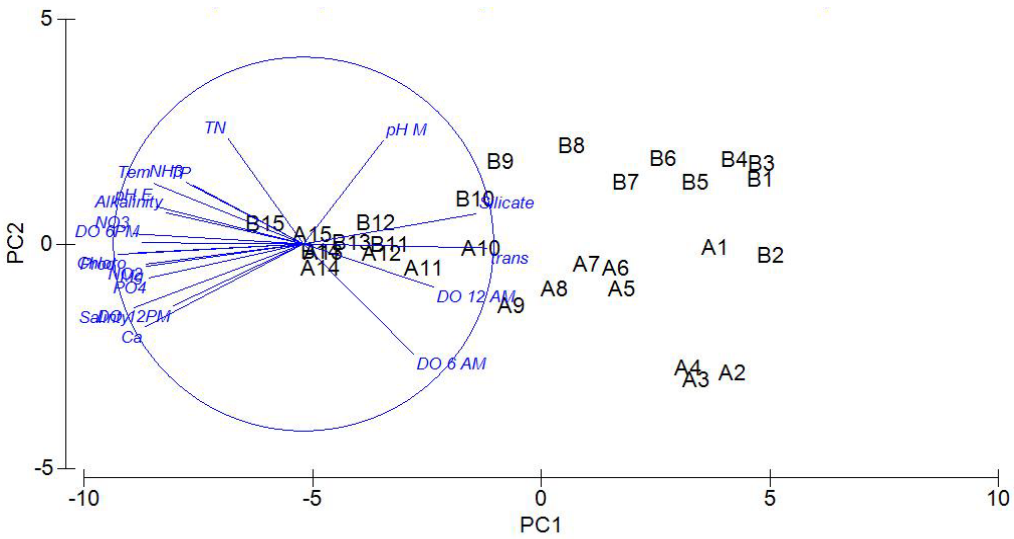

Table.16. Principal Component Analysis and Factor loadings of post monsoon

Table 11. Mean ± SD of water, sediment and health parameters of pen and zero water exchange shrimp culture systems

Parameters	Open System	Zero water exchange
pH	6.25 ± 0.17	7.87 ± 0.155
Alkalinity (mg/L)	18.39 ± 7.81	63.6 ± 11.4
Dissolved oxygen	4.2 ± 0.75	4.63 ± 0.63
TAN (mg/L)	0.04 ± 0.019	0.04 ± 0.024
NO_2^-- N (mg/L)	0.029 ± 0.026	0.021 ± 0.007
NO_3^- –N (mg/L)	0.025 ± 0.021	0.021 ± 0.009
PO_4 –P (mg/L)	0.021 ± 0.014	0.049 ± 0.019
Total Phosphorus (mg/L)	0.062 ± 0.068	0.142 ± 0.051
Silicate (mg/L)	0.053 ± 0.025	0.709 ± 0.59
Total Nitrogen (mg/L)	0.13 ± 0.37	0.134 ± 0.05
H_2S (mg/L)	0.04 ± 0.02	0.006 ± 0.001
Primary productivity	0.95 ± 0.49	2.73 ± 0.45
Chlorophyll (µg/L)	12.35 ± 4.61	30.13 ± 3.21
Sediment pH	6.81 ± 0.59	7.17 ± 0.22
Sediment E*h* (mV)	-193.2 ± 24.5	-133.2 ±13.03
TOC (%)	2.15 ± 0.56	1.83 ± 0.21
Animal growth (g)	20	33.5
Days of culture	85 (Mortality)	120
Survival (%)	25	70
FCR		1.3

Table.3.11 Pearson correlation of water quality parameters

	pH	EpH	temp	Sa	Ca	Mg	alk	DO12	6AM	12PM	6PM	Trans	NH3	NO2	NO3	PO4	TP	TN	Sili	prod
pH	1																			
EpH	0.149	1																		
temp	-0.383	-0.03	1																	
Sa	-0.389	-0.013	0.674	1																
Ca	-0.385	-0.05	0.666	**0.989**	1															
Mg	-0.345	-0.055	0.665	**0.987**	**0.991**	1														
al	-0.076	0.303	0.48	0.226	0.214	0.21	1													
DO12	0.193	-0.383	-0.201	-0.335	-0.327	-0.301	-0.403	1												
6AM	-0.021	-0.525	-0.038	-0.227	-0.18	-0.199	-0.231	0.515	1											
12PM	-0.008	**0.526**	-0.254	-0.25	-0.3	-0.322	0.268	-0.181	-0.148	1										
6PM	0.032	**0.578**	-0.151	-0.2	-0.253	-0.28	0.406	-0.492	-0.441	0.619	1									
Trans	0.033	-0.732	0.096	0.011	0.068	0.089	-0.401	0.478	0.613	-0.632	-0.734	1								
NH3	-0.36	0.148	0.48	0.555	0.515	0.541	0.292	-0.31	-0.33	-0.045	-0.024	-0.147	1							
NO2	-0.114	0.379	0.341	0.487	0.425	0.455	0.183	-0.295	-0.335	0.091	0.155	-0.329	0.597	1						
NO3	-0.11	0.61	-0.053	0.152	0.083	0.084	0.239	-0.561	-0.658	0.42	0.577	**-0.766**	0.438	**0.6**	1					
PO4	-0.194	0.667	0.101	0.231	0.192	0.162	0.263	-0.515	-0.476	0.358	0.575	**-0.751**	0.329	0.568	0.719	1				
TP	0.148	0.661	-0.086	0.035	6E-04	-0.008	0.106	-0.439	-0.525	0.321	0.465	**-0.658**	0.232	0.522	0.708	0.581	1			
TN	0.411	0.361	-0.527	-0.705	-0.736	-0.738	0.003	-0.06	-0.181	0.444	0.482	-0.445	-0.273	-0.104	0.301	0.002	0.34	1		
Si	0.21	-0.613	0.043	-0.036	0.015	0.04	-0.318	0.56	0.483	-0.534	-0.678	0.795	-0.17	-0.393	-0.751	-0.576	-0.466	-0.357	1	
prod	0.033	**0.759**	-0.15	-0.073	-0.135	-0.153	0.294	-0.459	**-0.549**	**0.582**	**0.73**	**-0.879**	0.149	0.463	**0.814**	**0.602**	**0.677**	0.474	-0.799	1
chlor	0.012	**0.752**	-0.14	-0.03	-0.091	-0.1	0.284	-0.431	**-0.6**	**0.607**	**0.698**	**-0.884**	0.226	0.485	**0.802**	**0.585**	**0.689**	0.442	**-0.788**	**0.92**

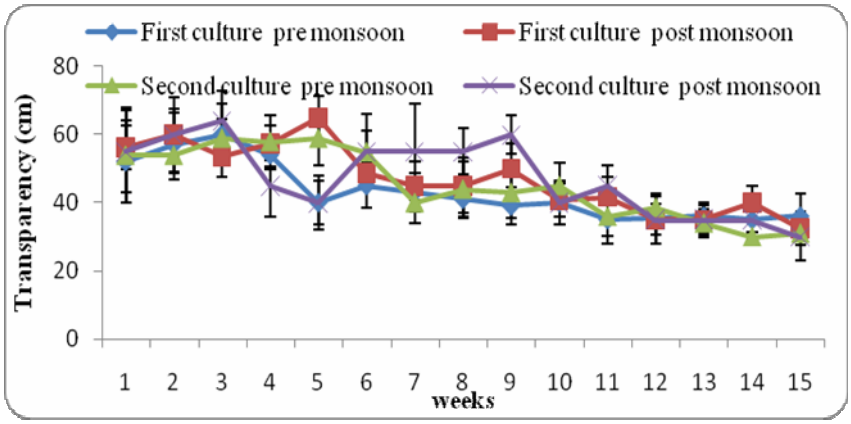

Fig. 1. Variation of secchi disc transparency during the culture period

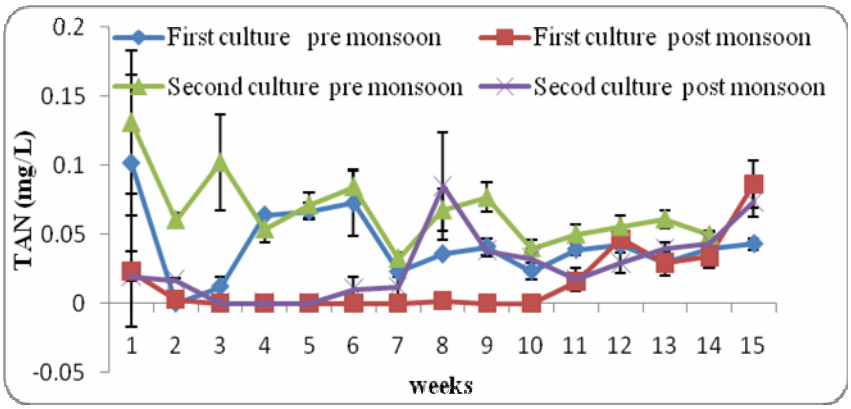

Fig. 2. Variation of TAN during the culture period at different seasons

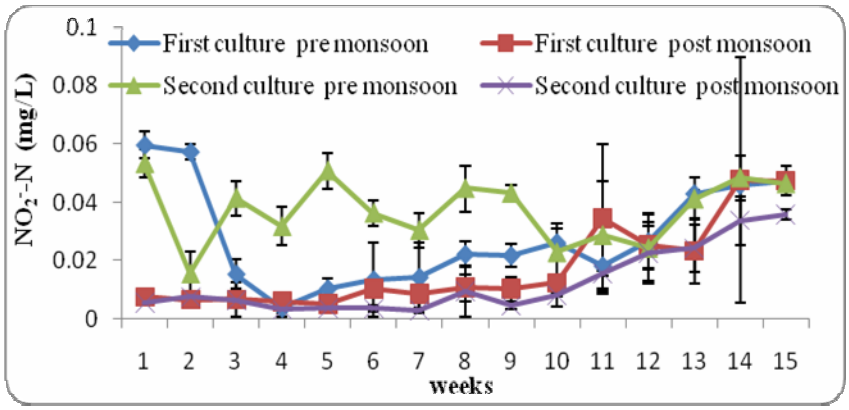

Fig. 3. Variation of NO_2^--N during the cultrue period at different seasons

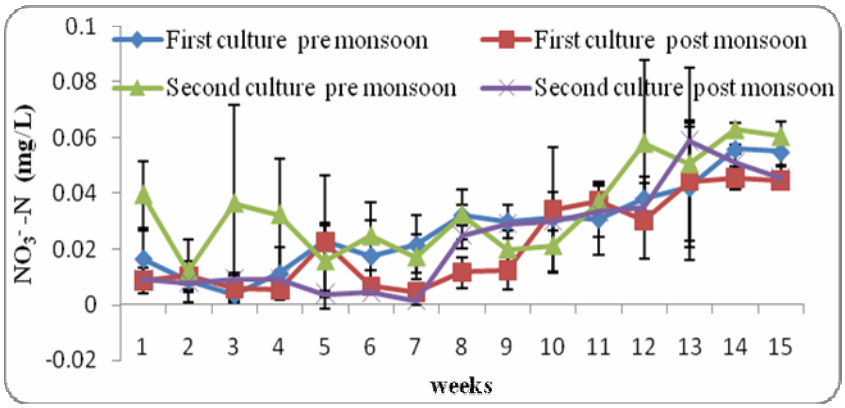

Fig. 4. Variation of NO_3^-- N during the culture period at different seasons

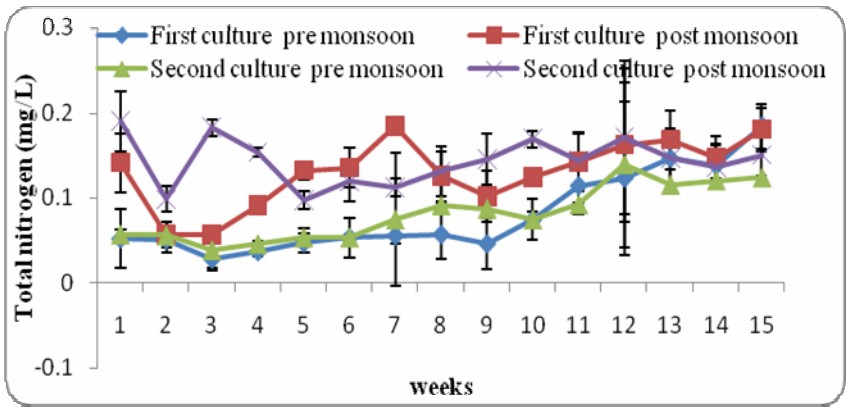

Fig. 5. Variation of total nitrogen during the culture period at different seasons

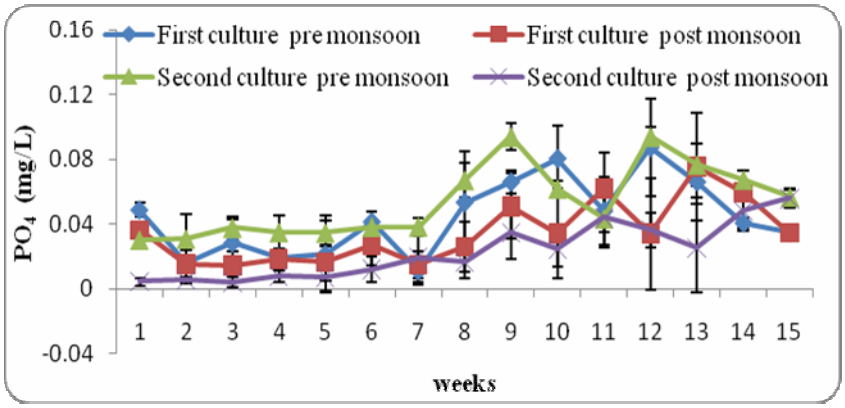

Fig. 6. Variation of PO_4-P during the culture period at different seasons

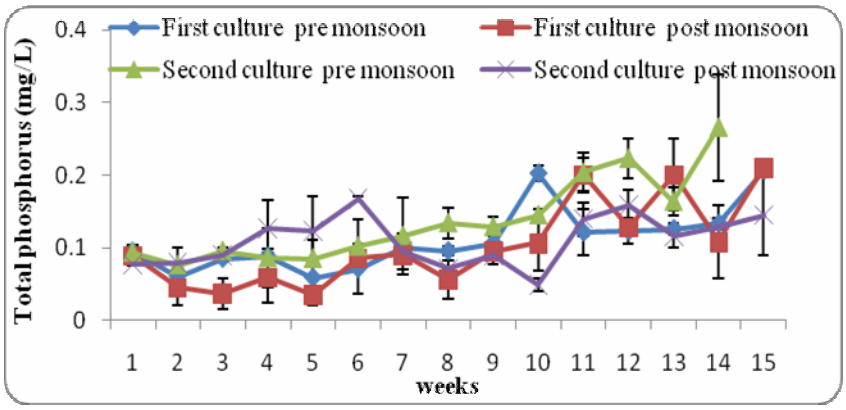

Fig. 7. Variation of total phosphorus during the culture period at different seasons

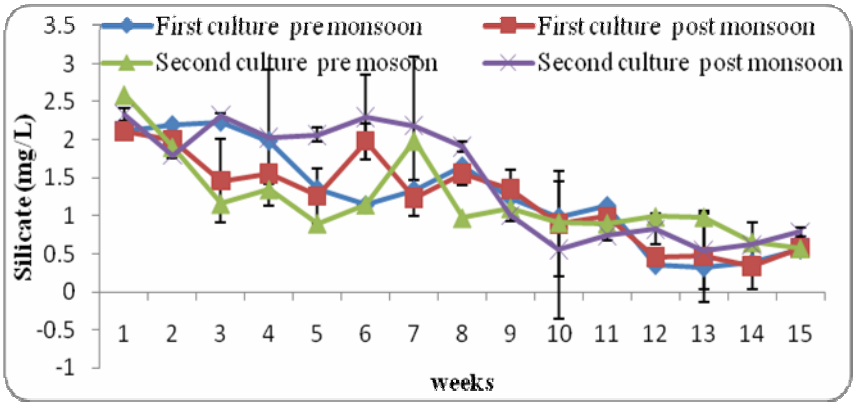

Fig. 8. Variation of silicate concentration during the culture period

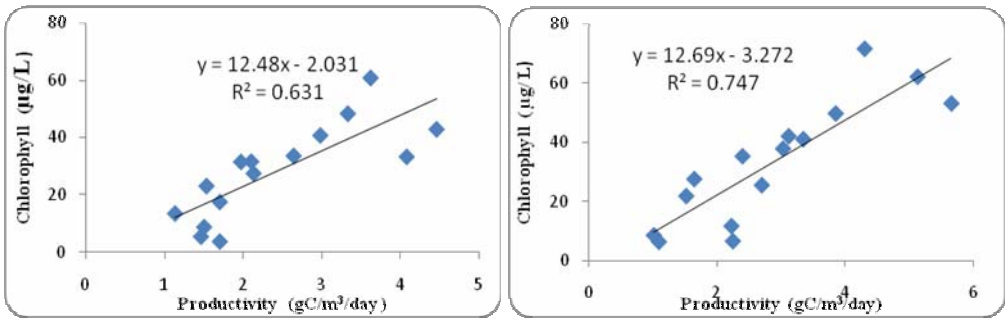

Fig. 9. Relation between Primary productivity and chlorophyll during the culture (First and Second culture – pre monsoon)

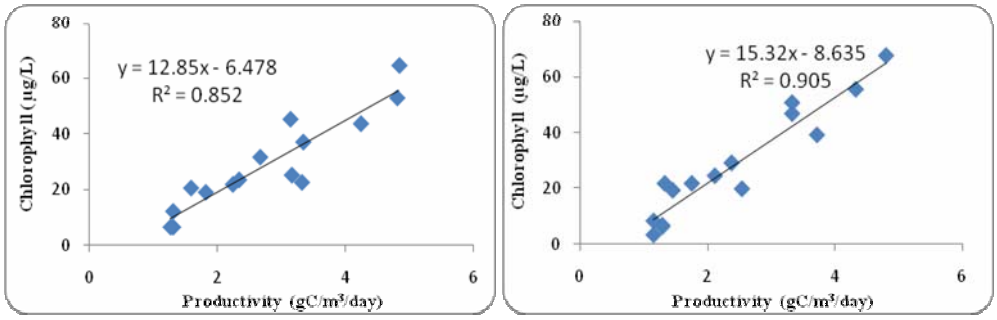

Fig. 10. Relation between Primary productivity and chlorophyll during the culture (First and Second culture – post monsoon)

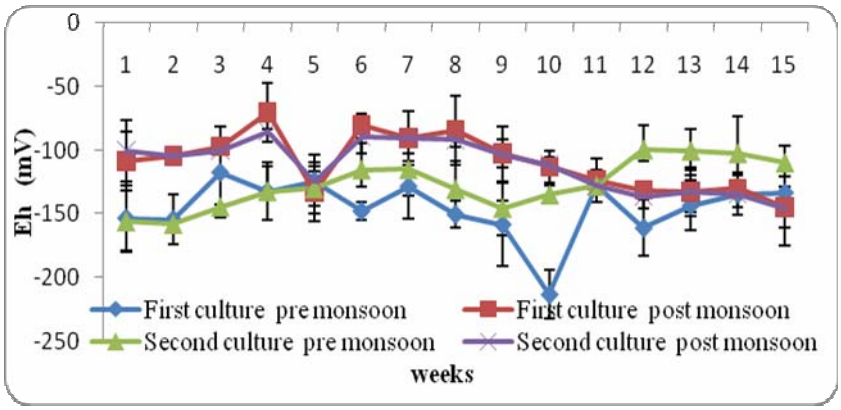

Fig. 11. Variation of sediment *Eh* during the culture period at different seasons

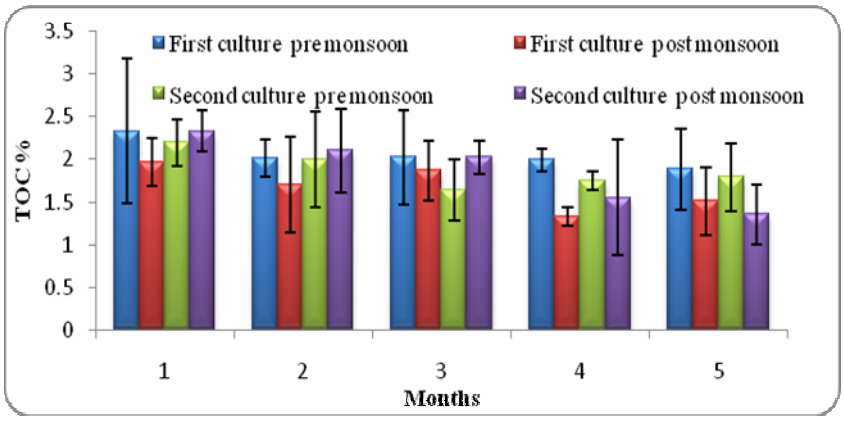

Fig. 12. Variation of sediment organic carbon during the culture period

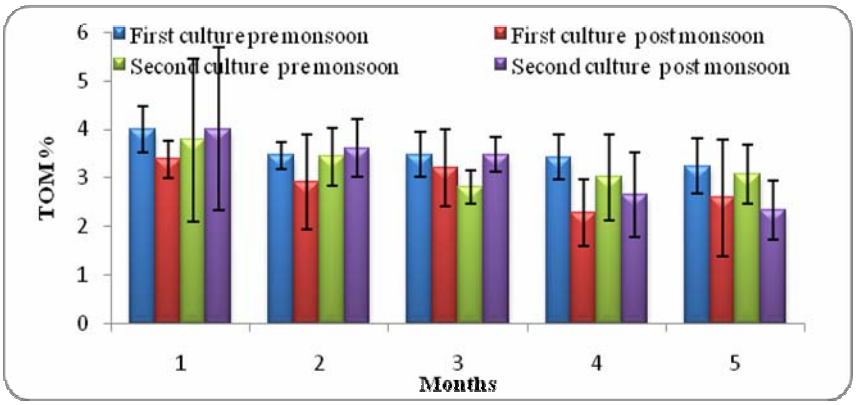

Fig. 13. Variation of sediment organic matter during the culture period

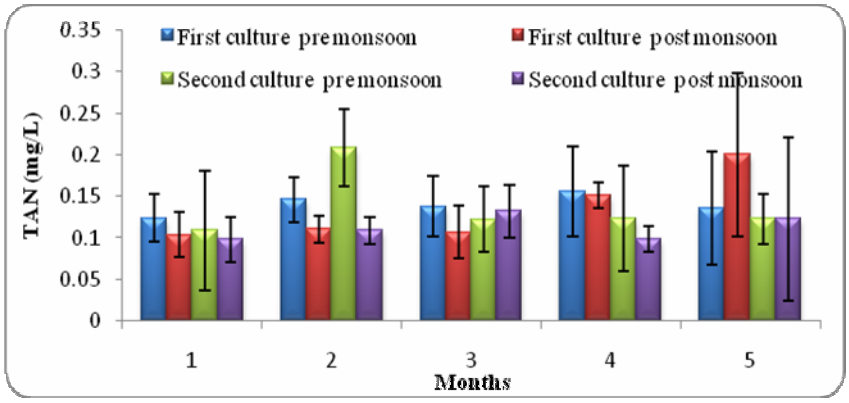

Fig.14. Variation of Sediment TAN during the culture period

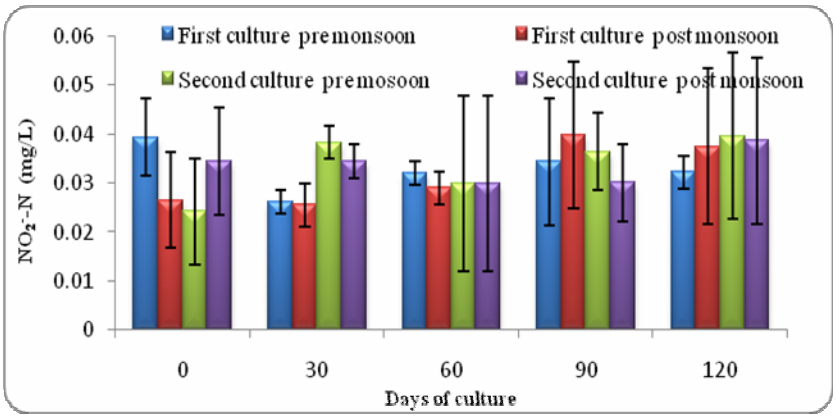

Fig. 15. Variation of sediment NO_2-N during the culture period

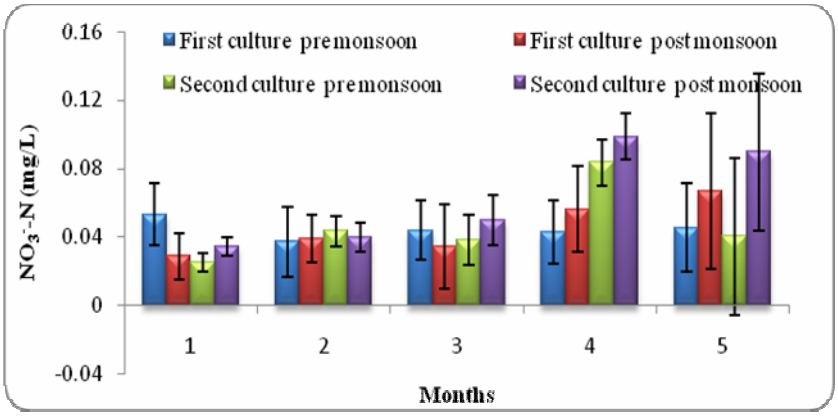

Fig. 16. Variation of sediment NO_3-N during the culture period

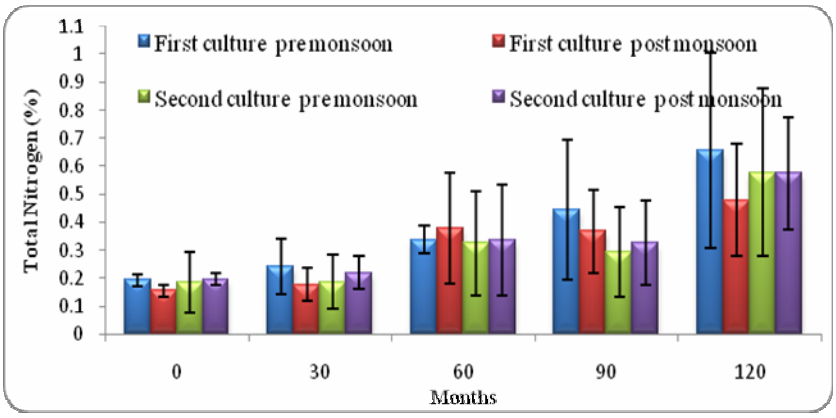

Fig. 17. Variation of sediment total nitrogen during the culture period

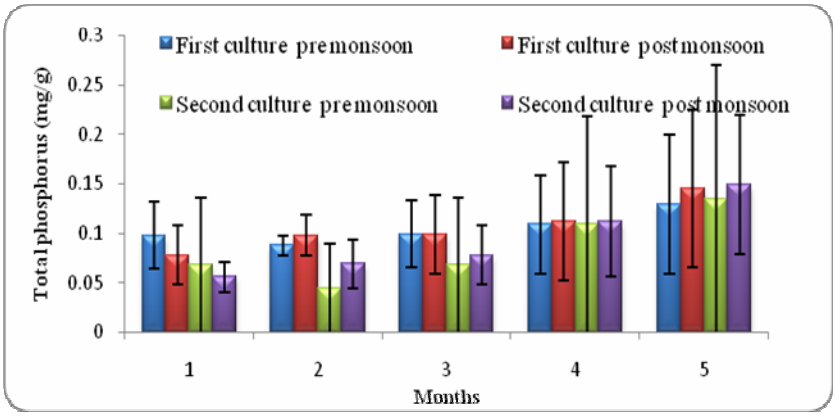

Fig.18. Variation of sediment total phosphorus during the culture period

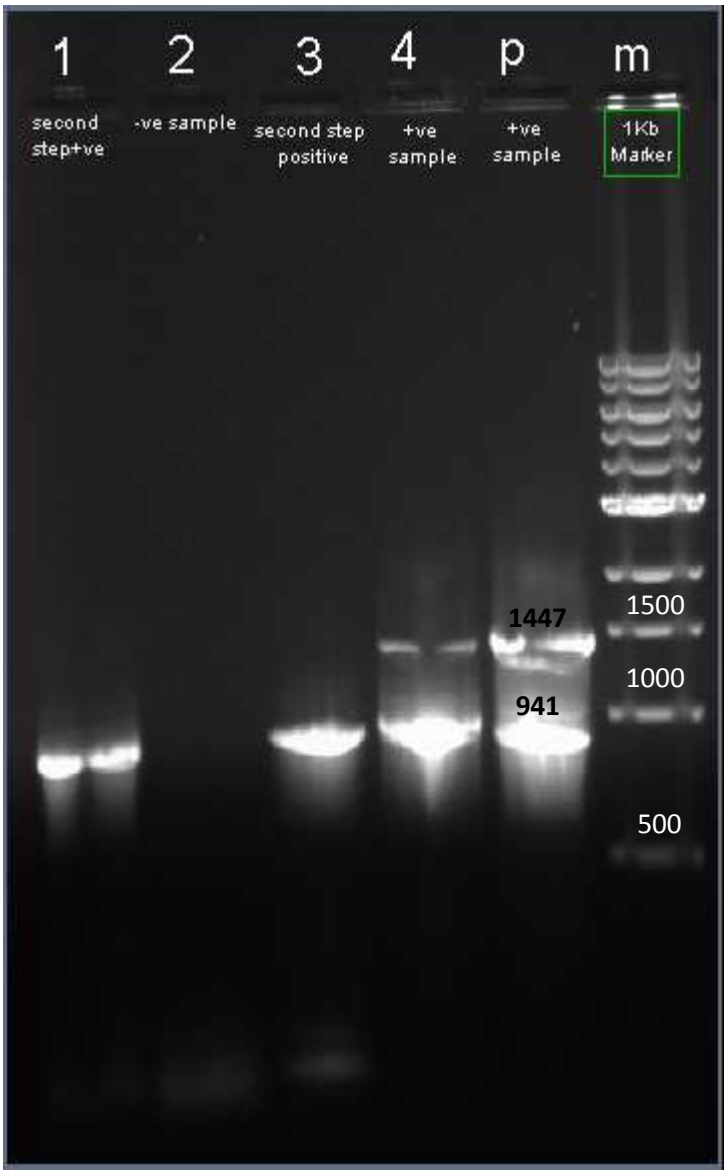

Fig. 21. Gel picture of diagnostic PCR of samples of shrimp, which exhibited clinical signs of white spot virus disease and subsequent mortality in the control ponds.

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Chapter 4

Effects of stocking density on water and sediment quality, and on growth of *Penaeus monodon* cultured in earthen ponds under zero water exchange

4.1 Introduction

Stocking density is a major factor, which affects growth, survival and yields of shrimp in particular and for that matter any aquatic animal. Generally, increase in stocking density results in decrease in growth and survival (Ahmed *et al*., 2000: Arnold *et al*., 2006; Soundarapandian and Gunalan, 2008; Khoi and Fotedar, 2010) and deterioration of water quality (Thakur and Lin, 2003) in a culture system. Recently efforts have been made to culture shrimp under biosecured zero-water exchange systems in order to reduce the input of water and waterborne pathogens, and subsequently to reduce the release of nutrient rich effluent to the surrounding environment (Tacon *et al*., 2002). The associated higher construction and operating costs with these systems are generally offset by increased stocking density.

However, the high stocking density is often associated with increased input of organic matter into the pond in the form of uneaten feed and fecal matter, which result in poorer growth and survival rates. Apart from these impediments, there is lower contribution from natural biota as food to the overall nutritional budget of the cultured stock (Tacon, 1996; Martin *et al*., 1998).

For a long period, the average stocking density of post larvae (PL) of penaeid was approximately 7- 10 per m^2, but under high stocking density mode it got increased, over the last two decades, to about 50-100 PL. This is mainly due to the availability of sufficient shrimp seed from hatcheries and an inclination of farmers to produce more from limited space where management becomes comparatively easier.

However, higher stocking density has been met with several negative impacts. Primarily, more shrimp per unit area translates to 'crowding' effect, which induces high level of stress on the animals. This in turn lowers their resilience to pathogens and makes them more susceptible to diseases. Secondly, higher stocking density produces more waste per unit area and per unit volume which, if not managed properly, could become toxic to the animals through the production of anoxic conditions and subsequent generation of H_2S (Al–Ameeri and Cruz, 2006). The deteriorating environment also produces stress on the animals increasing their vulnerability to pathogens. In general, growth rate of shrimp is negatively related to stocking density and there are several reports on the density – dependent growth in culture or experimental systems (Calliout *et al*., 1976; Sedgewick, 1979; Magire and Leedow, 1983; Kautsky *et al*., 2000: Arnold *et al*., 2006; Khoi and Fotedar, 2010). However, the degree of the density dependence rests

on other factors like over all water quality, water exchange rate and feeding pattern.

Having the above at the background, this work was undertaken to evaluate the efficacy of two distinct stocking densities of *Penaeus monodon* on water and sediment quality, health of the reared stock and overall productivity while being cultured in earthen ponds.

4.2 Materials and Methods

The experiments were conducted in two 1 hectare ponds each at a stocking density of 7 per m^2 (Low stocking density - LSD) and 14 per m^2 (High Stocking density - HSD) at Nandana Aquaculture farms, Kodungallor, Kerala, India. The culture methods adopted and analysis performed were as mentioned in Chapter 2.

4.3 Results

4.3.1 Water quality

Summary of the water quality parameters measured during the culture period is given in Table 1. pH, temperature, salinity and alkalinity did not show significant difference between the ponds with LSD and HSD. Dissolved oxygen was high throughout the culture period in both the systems. The TAN was significantly lower ($P<0.03$) in the pond with LSD compared to the ones with HSD (Fig.1). The TAN content which was not detectable during the initial period of culture gradually increased to a maximum of 0.28 mg / L at the end of the culture period in the ponds with HSD. However, in the pond with LSD it ranged from 0 to 0.12 mg/ L. NO_2^- - N concentrations in the ponds were generally low through out the culture period ranging from 0.01 mg/ L to 0.04 mg/ L in the ponds with LSD and 0.03 mg/ L to 0.08 mg/ L in those with

HSD (P<0.006) (Fig. 2). There was a significantly lower value of NO_3^- - N in the ponds with LSD with a mean value of 0.03 mg/ L compared to the ones with HSD with a mean value of 0.05 mg/ L (P<0.04) (Fig.3). The mean total nitrogen in the ponds with LSD was 0.18 mg/ L and the ones with HSD 0.25 mg/ L and the difference was significant (P<0.003) (Fig.4). Inorganic phosphorus levels were not differing significantly between the two category of ponds (LSD and HSD) ranging between 0.04 to 0.13 mg / L and 0.05 to 0.15 mg/ L respectively. However, the total phosphorus level was significantly higher in the ponds with HSD (P<0.05) (Fig.5). There was no significant difference between silicate levels in both the category of ponds (Fig. 6).

Primary productivity and chlorophyll content did not show significant differences (Fig.7). The mean values of primary productivity in both the systems (LSD and HSD) were 2.93 g C/m^3/day. Mean value of Chlorophyll *a* in ponds with HSD was 34.2 µg/L and in the ponds with LSD it was 38.74 µg/L (Fig. 8 Table 1). Total bacterial count in the pond water of HSD ranged between 2.1 x 10^4 to 2.8 x 10^5 CFU/mL and the ones with LSD 2.7 x 10^4 to 3.3 x 10^5 CFU/Ml.

4.3.2 Soil Quality

Soil pH ranged between 6.9 to 8.45 in both the category of ponds, there was no significant difference between the ponds, and almost steady state was maintained through out the culture period (Table 1). However, there was a significant difference between soil E*h* values between the two categories of ponds and it ranged between -95 to -195 mV in the ponds with HSD and -76 to -142.5 mV in the one with LSD (Fig. 9). In the ponds with high stocking E*h* decreased as the culture progressed. Mean soil organic carbon in the ponds

with LSD was 1.86% and the one with HSD 2.04 % and the organic matter was 3.3 and 3. 5% respectively with no significant difference between the two categories of ponds (Fig. 10). Total bacterial count in the sediments of ponds with LSD and HSD ranged between $1x10^{5}$ to $1x10^{6}$ CFU/g.

4.3.3 Growth and feed efficiency

Results of overall production of shrimp is summarized in Table.2. The production was significantly higher in the ponds with LSD (2150 Kg/ha) than the ones with HSD (1850 Kg/ha) when harvested after 120 days of culture. Meanwhile, the average weight of shrimp at the time of harvest was 28 g in both the systems. The major difference observed was in the rate of survival, 50% in those with HSD and 78% with LSD. The FCR was better in the pond with low stocking (1.1) than the ones with high stocking (1.6).

4.3.4 Relationship between water quality and survival

The multiple regression analysis of survival and water quality parameters such as NH_4^+-N, NO_2^--N and NO_3^--N showed relative significance with respect to LSD and not with HSD. Under LSD the fitted multiple regression was $Y= -56.78 X_1 +502.74X_2 + 370.5X_3$ ($P=0.006$, $r^2 = 0.70$) and under HSD it was $Y = -45.86X_1 +288.94X_2 +110.34X_3$ ($P =0.06$, $r^2 = 0.52$) where Y = growth rate, $X_1 = NH_4^+$-N, $X_2 = NO_2^-$- N and $X_3 = NO_3^-$- N.

4.4 Discussion

Water quality is an important factor that influences the growth of cultured shrimp. During the study period, the parameters like pH, alkalinity, salinity and temperature did not show significant differences between the ponds with LSD and HSD. pH of water in general varied between 6.9 to 8.7.

Ramanthan *et al*. (2005) pointed out that for maximum growth a pH range of 6.8 to 8.7 was required for shrimp culture. Alkalinity ranged between 52 to 70 mg/ L and dolomite was added to maintain it wherever it was necessary. Water temperature ranged between 32-35°C and salinity between 8-28 g/L. Temperature was at the optimal range, which had a considerable effect on the overall activity, food consumption and growth (Fabio *et al*., 2007). At high salinity, the shrimps grow slowly but appear healthy and resistant to diseases. At lower salinity the shell turns out to be weak and the animals are thereby prone to diseases. The lower salinity observed during the initial period of the culture was due to the south west monsoon in Kerala.

Dissolved oxygen levels in the ponds could be maintained at optimum level through out the culture period. It was higher in the ponds with low stocking during morning hours; however, during evening hours there was no significant difference in oxygen level between the ponds. Usually in aquaculture systems, morning oxygen level happens to be comparatively lower than that during the evening hours due to respiration during night. But, in the LSD ponds, biomass and organic loading were comparatively lesser and consequently utilization of oxygen was also low. These results agreed with the studies of Hopkins *et al*. (1996) and Thakur *et al*. (2003).

Transparency ranged between 27- 46 cm in both the category ponds and this decreasing trend was due to the input of nutrients leading to the increase of phytoplankton biomass. Decreasing trend of transparency was caused by high density of phytoplankton as evident from the higher chlorophyll-concentrations. Weekly decline in Secchi disc transparency with a corresponding increase in phytoplankton and chlorophyll *a* also suggested an increase in nutrients in the ponds

However, the critical parameters like TAN, NO_2^--N, NO_3^--N were significantly lower in the ponds with LSD compared to the ones with HSD. Nitrogen plays an important role in the zero exchange aquaculture systems due to its dual role, as a nutrient and toxicant (Burford and Lorenzen, 2004). Nitrogen in the form of NH_4^+- N and NO_2^-–N are toxic to shrimp, however, the toxicity depends on various factors including species tolerance and water quality parameters such as pH, temperature, salinity, dissolved oxygen and duration of exposure (Hargreaves, 1998; Barajas *et al*., 2006). During the study period, TAN level in the ponds with LSD was significantly lower compared to the one in the ponds with HSD. Implementation of high stocking densities to maximize production might impair water quality because of excess nitrogen residues from excretion (Jobling, 1994). NO_2^- -N and NO_3^-- N level in the ponds with LSD was also lower than the ones with HSD. This is true with regard to total nitrogen values also.

There was no significant difference in PO_4^--P level between the two categories of ponds; however, it increased from around 0.04 mg/ L during the beginning of the culture to 0.15 mg/ L, towards its termination. This result agrees with the study of Matias *et al*. (2002) and Burford *et al*. (2003) in a closed system, where phosphate concentration was between 0.07 – 1.17 mg/ L and was lower than that reported by Thakur *et al*. (2003) and Shariff *et al*. (2001). However, Boyd (1990) observed that dissolved orthophosphate concentrations in ponds were usually not greater than 5-20 μg/ L and seldom exceeded 100 μg/L even in highly eutrophic waters. In the present study, nutrient concentrations in the water column were linearly correlated with the cumulative feed input. In an experimental study by Thakur and Lin (2003) the total nitrogen and total phosphorus concentrations in water were found going

up with the progress of culture, and within the closed system they supported growth of natural food organisms, thereby contributing positively to shrimp growth. Aquaculture ponds receiving feeds often have total phosphorus concentration above 0.5 mg/L and total nitrogen 2 or 3 mg/L (Boyd and Tucker, 1992). In the present study, the regression of phosphate and feed supplementation in the ponds with LSD is $Y = 0.0013x + 0.0545$, $R^2 = 0.68$, $P = 0.0002$ and in the one with HSD is $Y = 0.0016 x + 0.059$, $R^2 = 0.75$ $P = 0.0001$. Inorganic phosphorus and total phosphorus positively correlated with chlorophyll and primary productivity in both the category ponds, correlation coefficients in HSD was 0.849, 0.909, 0.986, and 0.940 and in the LSD it was 0.897, 0.756, 0.891 and 0.765. Silicate concentration in the pond water was relatively constant throughout the culture cycle, indicating lack of shortage of silica in the water column for diatom growth.

Primary productivity and chlorophyll did not show any significant difference between the two treatments. This result is lower than the one reported by Hepher and Pruginin (1981) and Ali (1986), however, higher than the reported values of Cowan *et al*. (1999) at lower intensive systems.

Soil pH ranged between 6.9 to 8.45 in both the types of culture systems, which were within the accepted level of soil pH in aquaculture. Most soil microorganisms and especially soil bacteria function best at pH 7 to 8 and maximum availability of soil phosphorus usually occurs at about pH 7. As for sediment pH, a value of 7 is generally considered as the lowest without adverse effects on reared organisms (Clifford, 1992). Soil E*h* in the ponds with LSD was significantly higher (-114 mV) compared to the one with HSD (-140 mV). During the end of the culture, redox potential in the pond with HSD was in a decreasing trend contrary to the one with LSD. This is probably due to the

accumulation of comparatively higher load of organic matter at the pond bottom. Reduction of sulphates to the potentially toxic sulphides takes place at a redox potential below -150 mV.

Total organic carbon in the ponds with HSD was comparatively higher (2.04 %) than the ones with LSD (1.85 %). Meanwhile, the level of organic matter in the LSD was 3.3% and in the HSD 3.5%. The study of Yuvanatemya (2007) indicated that organic matter lower than 3% affected pond productivity. According to Boyd (1995) bottom organic carbon concentration seldom exceeds 5%.

The range of total heterotrophic bacterial count recorded in this study in the water column were within the levels reported earlier in India (10^4 to 10^5) and in South-East Asia (Sharmila *et al.*, 1996; Otta *et al.*, 1999). In general, the bacterial load present in sediment was higher than that in the water sample. The total aerobic count in water ranged from 1x 10^4–1x 10^5 CFU/ mL while it was 1x 10^5–1x 10^6 CFU / g in sediment. The high bacterial load in the sediment could be attributed to the high content of organic matter.

The striking observation of this study was the lower level of survival and subsequent lower production in the pond with high stocking. On regression analysis the survival was found significantly related inversely to the concentration of TAN, NO_2^- -N and NO_3^--N in the ponds with LSD, and in the ones with HSD it was not significant. If so which were the factors responsible for the low survival obtained in the ponds with HSD? An answer could not be found out for the low survival with the available data and more investigations were required in this direction. Supporting the finding it could be observed that the FCR was higher in the ponds with HSD than the one with LSD. Due to

this reason the cost benefit ratio showed that HSD was not profitable, in spite of the fact that there was no significant difference between shrimp growth rate in both the category ponds and this growth rate was higher than the one reported by Sandifer *et al*., (1990) who reported growth rate of 0.94 g per shrimp per week in super intensive pond production system. The production in both the category ponds was higher compared to the one attained so far in zero-water exchange farms in the Chilka region (Balasubramanyan *et al*., 2005). The FCR value could be reasonably compared with the previous reports on *P. monodon* culture (Chen *et al*., 1989; Lumare *et al*., 1993). This study showed the potential of closed system in producing shrimp with low FCR, as nutrients and organic matter released to the system could be accounted well for the production of natural food organisms. In the present study, the survival rate of 50% (HSD) and 78% (LSD) is in agreement with Krantz & Norris (1975) who stated that survival rates of 60 to 80% are to be expected for *P. monodon* under suitable rearing conditions. Avnimelech *et al*. (1994) also emphasized that in ponds operated with high water exchange rate, a large fraction of the feed and the organic matter accumulating in the pond is drained off and often wasted.

The study points to the fact that shrimp can be effectively grown under zero water exchange mode coupled with bioremediation and application of probiotics. Water and sediment quality could be maintained enhancing primary productivity in such systems maintaining biosecurity. In the study undertaken, the culture could be completed with out any disease problems during a period of widespread white spot syndrome virus out breaks in the region. Out of the two modes of stocking one with 7 animals per m^2 and the other with 14 animals per m^2, the system with LSD performed well yielding (2150 Kg/ha) than the ones with high stocking density (1850 Kg/ha). In the system with

LSD the critical parameters such as TAN, NO_2^--N, NO_3^--N were comparatively and significantly lower and soil E*h* was significantly higher. The study suggested that under zero water exchange system lower stocking density provided better yield with low FCR. Detrodigest was found to be a good bioaugmentor and Enterotrophotic as a good gut probiotics.

Routine sampling of the animals from both the systems were carried out, however, gross disease signs could not be observed during any occasion. That was the reason why data could not be generated on health issues. We were also not able to point out the period during which mortality occurred. The information which could be gathered was that a semi-intensive system with a stocking density of seven should be a better option under zero water exchange mode for sustainable culture of *P. monodon*.

All the preceding chapters including this one were dealing with zero water exchange systems supported with bioremediation of detritus which lead to high E*h* of sediment and improvement of general water and sediment quality. Having all the systems brought under study been with low and moderate stocking densities, ammonia toxicity was not experienced in any of the systems. However, undoubtedly, one of the toxicants to be addressed in shrimp culture, especially in intensive systems, is unionized ammonia. To address this issue a process of immobilizing nitrifying bacterial consortia on wood powder had been developed by Manju *et al*. (2009) to facilitate their easy and effective application in culture systems when ever there was higher ammonia out put. While doing so large quantities of immobilized nitrifiers have to be generated and their efficacy also has to be studied in bioassay systems prior to undertaking the study in grow out ponds. This process is dealt with in the next chapter (Chapter 5)

Table 1. Water and sediment quality parameters of low and high stocking ponds (Minimum, maximum, means ± SD)

Parameters	Low stocking ponds	High stocking ponds
pH (Morning)	7. 2 – 8.6 (7.72 ± 0.06)	6.9 – 8.6 (7.5 ± 0.2)
pH (Evening)	7.8 – 9.1 (8.4 ± 0.3)	7.9 – 9.1 (8.5 ± 0.5)
Alkalinity (mg/L)	52.5 – 70 (61.13 ± 4.43)	52.5 – 63.5 (58.6 ± 5.09)
Salinity (g/L)	10 – 28 (22.7 ± 0.5)	10 – 28 (21.5 ± 0.8)
Calcium (mg/L)	417 – 853 (734 ± 48.7)	400 – 800 (665 ± 48)
Magnesium (mg/L)	2183 – 4270 (3683 ± 87)	2000 – 4183 (3481 ± 97)
Transparency (cm)	27 - 46 (34.5 ± 3.56)	27 – 42 (33.5 ± 4.56)
DO (Morning)	3.9 – 5.15 (4.7 ± 0.31)	3.1 – 4.9 (4 ± 0.24)
DO (Evening)	6.4 – 8.8 (7.9 ± 0.21)	5.8 – 9.15 (8.2 ± 0.31)
TAN (mg/L)*	0 – 0.12 (0.06 ± 0.02)	0.005 – 0.28 (0.112 ± 0.02)
NO_2^- –N (mg/L)*	0.014 – 0.04 (0.03 ± 0.007)	0.03 – 0.08 (0.05 ± 0.01)
NO_3^- – N (mg/L)*	0.015 – 0.07 (0.03 ± 0.016	0.02 – 0.09 (0.05 ±0.011
PO_4 – P (mg/L)	0.04 – 0.13 (0.09 ± 0.04)	0.05 – 0.15 (0.11 ± 0.02)
Total phosphorus (mg/L)	0.07 – 0.24 (0.16 ± 0.02)	0.13 – 0.3 (0.2 ± 0.04)
Silicate (mg/L)	0.25 – 0.69 (0.5 ± 0.1)	0.31 – 0.71 (0.52 ± 0.1)
Total Nitrogen (mg/L)	0.13 – 0.25 (0.18 ± 0.05)	0.18 – 0.39 (0.25 ± 0.1)
Primary productivity	1.4 – 4.5 (2.93 ± 1.2)	1.2 – 4.3 (2.93 ± 0.9)
Chlorophyll (µg/L)	9.8 – 70.34 (38.7 ± 6.5)	5.5 – 65.36 (34.2 ± 5.6)
Sediment pH	6.9 – 8.4	7.3 – 8.5
Sediment E*h* (mV)	-76 - ¯142.5 (-114 ± 7.1)	-95 - ¯195 (-140.3 ± 6.1)
TOC (%)	1.4 – 2 (1.85 ± 0.21)	1.95 – 2.3 (2.04 ± 0.3)
TOM (%)	2.4 – 3.9 (3.3 ± 0.5)	3.3 – 3.8 (3.5 ± 0.4)

* $P<0.05$

Table 2. Average harvest details and economics of low and high stocking ponds

Parameters	Low stocking ponds	High stocking ponds
Pond area (m^2)	10300	10300
Stocking density	80000	150000
Initial stock/m^2	7	14
Culture period (days)	114	114
Total production (Kg)	2150	1850
Size of Harvest (g)	28	28
Survival rate (%)	78	50
Feed intake (Kg)	2628	2884
FCR	1.1	1.6
Income (Rs)	4,15,000.00	3,34.000.00
Total seed cost (Rs)	26,000.00	31,500.00
Total feed cost (Rs)	1,44,650.00	1,58,620.00
Other expenses (pond prep, probiotics etc) (Rs)	1,25,355.00	1,47,105.00
Net profit (Rs)	1,18995.00	-3225.00

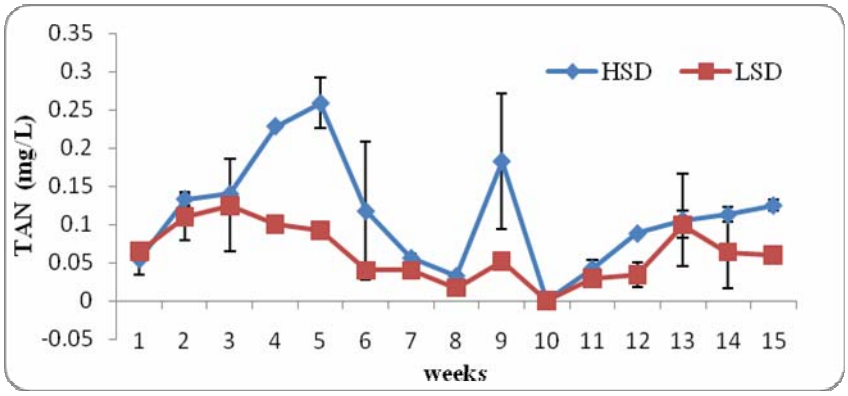


Fig. 1. Variation of TAN in the ponds with low stocking and high stocking (LSD and HSD)

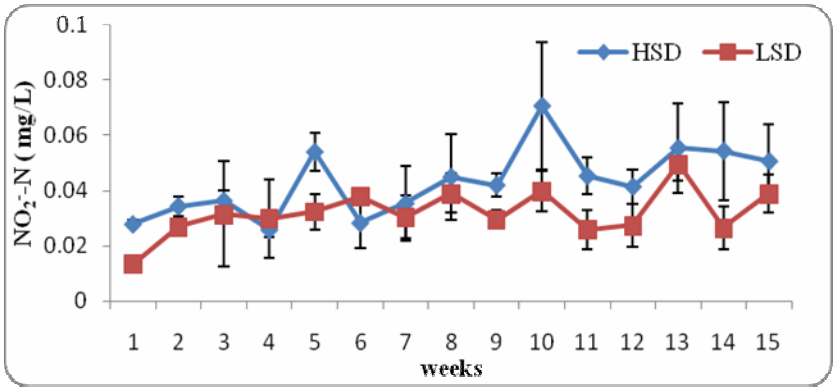


Fig. 2. Variation of NO_2-N in the ponds with low stocking and high stocking (LSD and HSD)

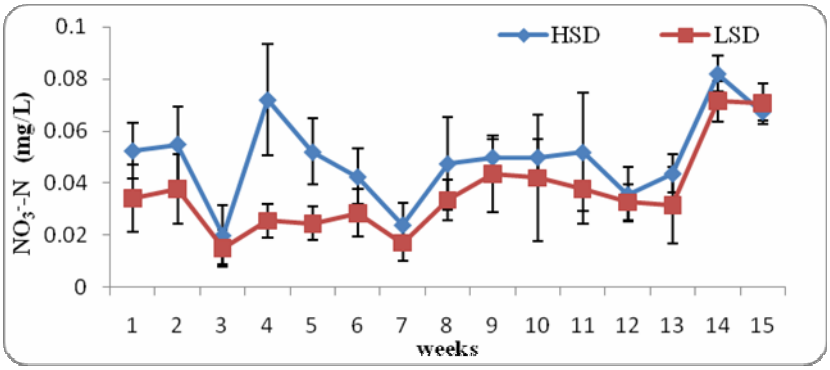


Fig. 3. Variation of NO_3- N in the ponds with low stocking and high stocking (LSD and HSD)

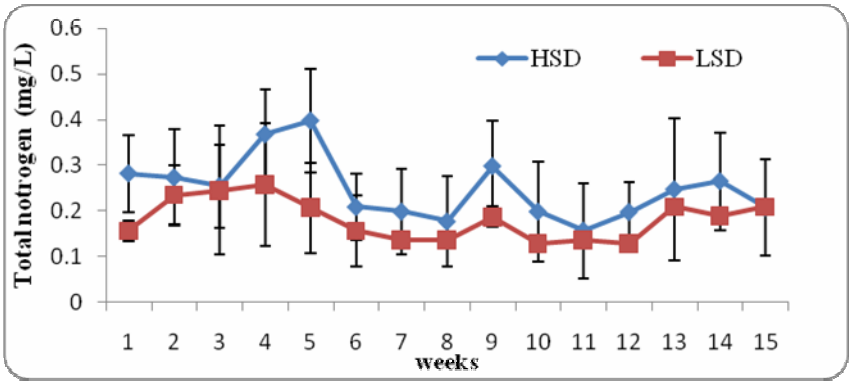


Fig. 4. Variation of total nitrogen in the ponds with low and high stocking (LSD and HSD)

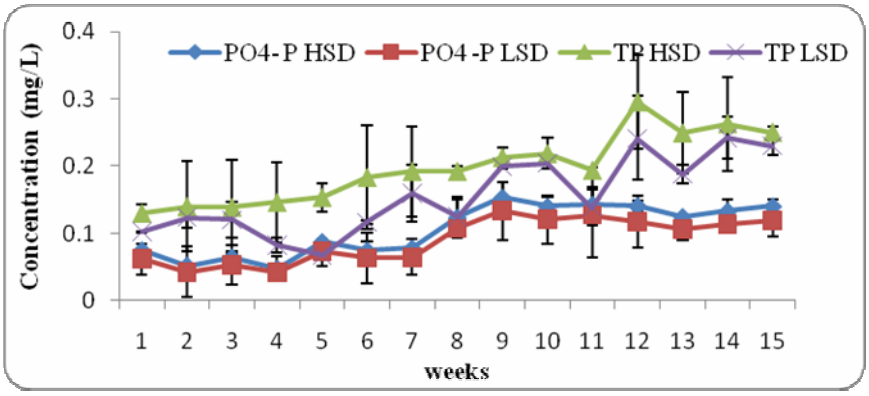


Fig .5. Variation of PO_4-P and total phosphorus in the ponds with low stocking and high stocking (LSD and HSD)

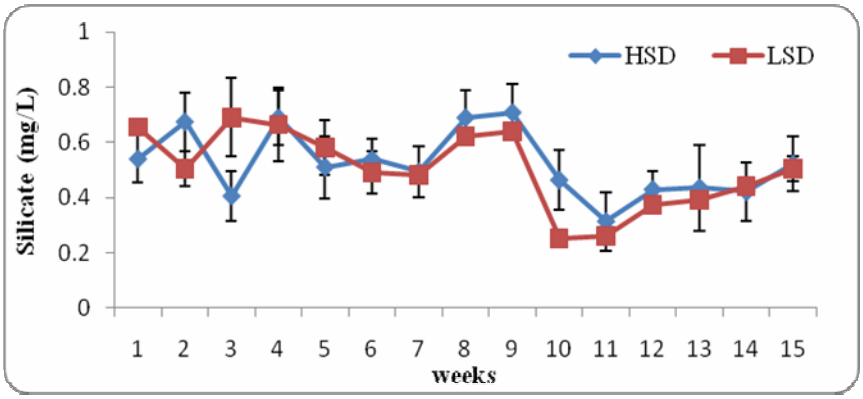
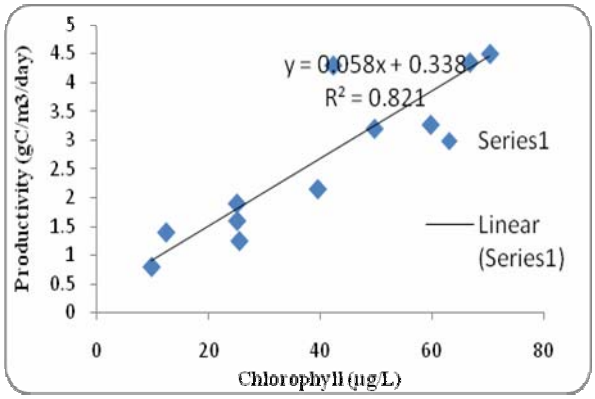


Fig. 6. Variation of Silicate in the ponds with low stocking and high stocking (LSD and HSD)



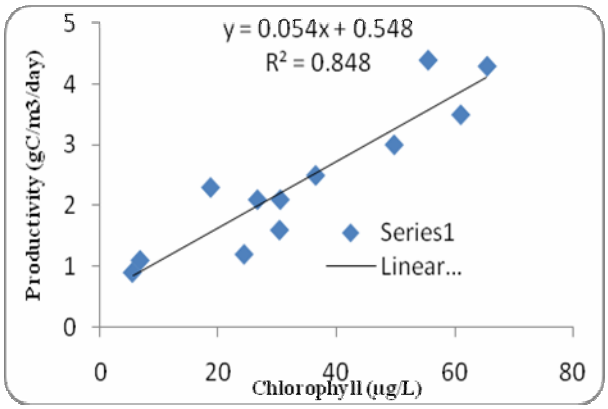


Fig. 7 & 8. Realtionship between chlorophyll and productivity in the ponds with low stocking and high stocking (LSD and HSD)

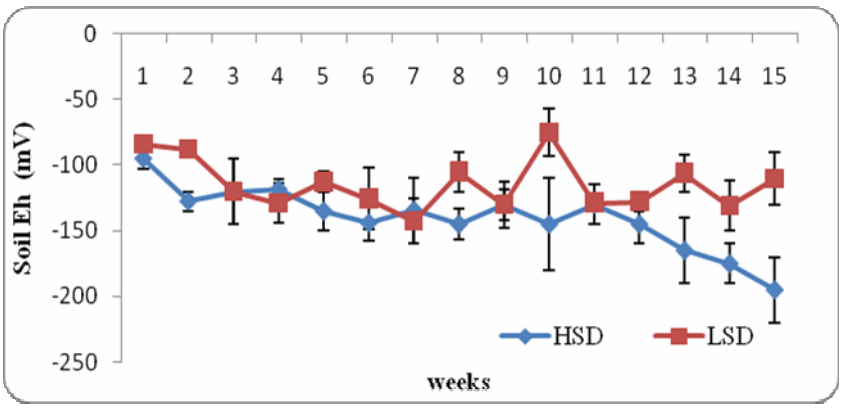


Fig. 9. Variation of sediment E*h* in the ponds with low and high stocking (LSD &HSD)

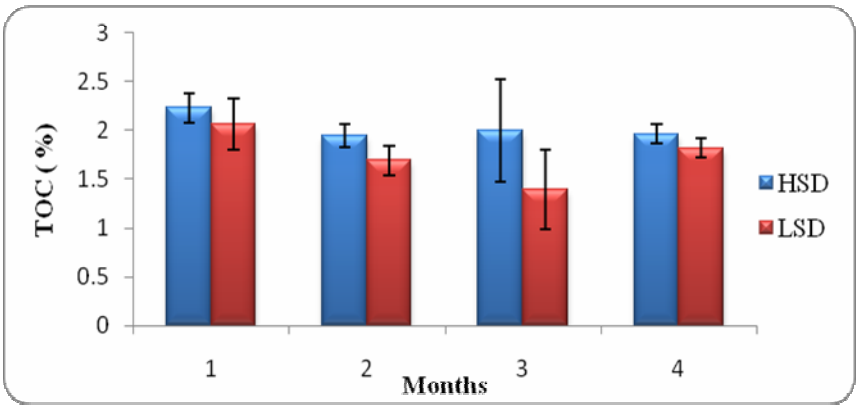


Fig. 10. Total organic carbon of sediment in the ponds with low and high stocking (LSD & HSD)

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Chapter

5

Mass production of immobilized nitrifying bacterial consortia on wood powder and validation

5.1 Introduction

In zero water exchange high stocking density shrimp culture systems ammonia is toxic even at low concentrations and it originates from animal excreta and decomposing organic solids such as uneaten feed (Krishnani et *al*., 2009). One of the main problems after low dissolved oxygen in intensive shrimp culture systems is the accumulation of dissolved nitrogen, especially ammonia (Ebeling *et al*., 2006; Kuhn *et al*., 2010). High TAN accumulation in pond water can be deleterious to the cultured organisms. Optimum shrimp growth demands less than 0.1 mg/L unionized ammonia in culture ponds (1.33 to 1.53 mg/L TAN at pH 8 and 28°–30°C temperature) (Shan Obbard, 2001). These metabolites increase blood pH, reduce oxygen content in the blood, affect gills, create stress, and result in reduced feeding and disposition to diseases.

One of the common practices for ammonia removal is water exchange, which leads to large-scale consumption of water, loss of bio-security and consequent disease outbreaks. In this context, the most widely used method for addressing ammonia toxicity in aquaculture is the establishment of biological nitrification using biological filters, biofilm reactors and nitrifying bacteria as such. Nitrification is the oxidation of ammonia to nitrite and nitrite to nitrate by autotrophic bacteria (Ebeling *et al.,* 2006). Nitrifying bacteria are autotrophic microorganisms that obtain energy from the oxidation of reduced nitrogen. The biological process of nitrification involves conversion of toxic ammonia (NH_4) to non-toxic nitrate (NO_3) through the action of autotrophic nitrifying bacteria.

$$NH_4^{+} + 1.5\ O_2 \rightarrow 2H^{+} + H_2O + O_2 \text{ ---------- } (5.1)$$

$$NO_2^{-} + 0.5\ O_2 \rightarrow NO_3 \text{ ---------- } (5.2)$$

Combining equation 1 and 2

$$NH_4^{+} + 2O_2 \rightarrow NO_3^{-} + 2H + H_2O \text{ ---------- } (5.3)$$

On adding the cell growth to the equation for *Nitrosomonas,* when NH_4^{+} is the basis

$$NH_4^{+} + 2.45\ O_2 + 6.716\ HCO_3^{-} \rightarrow 0.114 + C_5H_7O_2N$$
$$+ 2.509\ NO_2^{-} + 1.056\ H_2O + 6.153\ H_2CO_3 \text{ ---------- } (5.4)$$

And for *Nitrobacter*, NO_2 is the basis.

$$NO_2^{-} + 0.001\ NH_4^{+} + 0.014\ H_2CO_3 + 0.003\ HCO_3^{-}$$
$$+ 0.339\ O_2 \rightarrow 0.006\ C_5H_72N + 0.003\ H_2O + 1.348\ NO_3^{-} \text{ - } (5.5)$$

Combining these two equations the over all stochiometry is

$$NH_4^+ + 3.300\ O_2 + 6.708\ HCO_3^- \rightarrow 0.129\ C_5H_7O_2N$$
$$+3.373\ NO_3^- + 1.041\ H_2O + 6.463\ H_2CO_3 \text{ ---------- } (5.6)$$

According to the equation large amount of alkalinity is destroyed during the oxidation of ammonia to nitrate, equivalent to 8.62 mg HCO_3^- /mg NH_4 - N removed. The vast majority of alkalinity utilization is associated with the neutralization of hydrogen ion released during the oxidation of ammonia. Only a small part of the alkalinity is incorporated in to the cell material. The equation also tell us that considerable oxygen is required for nitrification: which is equivalent to 4.33 mg O_2/ mg of NH_4^+-N oxidized to NO_2-N. Of the amount, 3.22 mg O_2 will be used by *Nitrosomonas* and 1.11 will be used by *Nitrobacter*. The oxygen requirement of nitrifying bacteria can have a significant impact on the total amount of oxygen required by a biochemical operation. It can be seen that relatively little biomass will be formed, reflecting low yields associated with autotrophic growth. For every mg of NH_4^+-N removed only 0.129 mg of biomass will be formed, which is equivalent to 0.166 mg biomass/ mg NH_4^+ - N removed. Most of that (0.146 mg biomass/ mg NH_4^+-N) removed will be due to the growth of *Nitrosomonas* and only 0.020 mg biomass/ mg NH_4^+- N removed will be due to *Nitrobacter.* Over all, the growth of nitrifying bacteria will have little impact on the quantity of biomass in a biochemical operation, but will have a large impact on the oxygen and alkalinity requirements (Grady *et al*., 1999).

The natural colonization of nitrifying bacteria in a system take relatively long time (4 to 8 weeks) to establish healthy and viable population of both ammonia oxidizing and nitrite oxidizing bacteria (Kuhn *et al.,* 2010). The

nitrifying bacterial population can also be sensitive to chemical and physical stresses such as salinity or temperature changes (Malone and Pfeiffer, 2006; Emparanza, 2009). Such stresses can inhibit nitrification rates resulting in spikes in either ammonia and or nitrite. Commercial nitrifying preparations are least studied on their ability to control TAN loadings under continuous TAN production.

The importance of immobilization of effective commercial preparations of nitrifying bacteria has resulted in considerable research for industrial applications. Immobilization technology has been used extensively in commercial bioreactor fermentations (Chen *et al.,* 2000; Nakano *et al*., 2004; Zala *et al*., 2004, Li and Logan, 2004). Immobilized *Nitrosomonas europaea* (ATCC 19718) biofilters have been successfully applied to the removal of NH_3 alone (Chung and Huang, 1998), for the treatment of mixtures of H_2S and NH_3 with a two-stage biofilter (Chung *et al*., 2007) and immobilized *Nitrosomonas europaea* in a biotrickling filter packed with polyurethane foam (Ramirez *et al.,* 2009). Several natural materials (agar, agarose, collagen, alginates and chitosan, microbial cellulose) and synthetic polymer materials (polyacrylamide, polyurethane, polyethylene glycol and polyvinyl alcohol) have been used as the substrata for immobilization (Jianlong *et al.,* 1998; Fang *et al*., 2004; Rezaee *et al.,* 2008; Boonpauk *et al.,* 2011; Peirong and Wei, 2011).

To satisfy the requirement National Centre for Aquatic Animal Health developed an economically viable and user friendly technology based on nitrifying bacterial consortia (Achuthan *et al*., 2006) immobilized on wood powder as biodegradable carrier, which would not leave any residue on degradation (Manju *et al.,* 2009). Wood powder from the plant species

Ailanthus altissima was used for the purpose. Immobilization is a general term that describes many different forms of cells attached or entrapped, including flocculation on surfaces, covalent bonding to carriers, cross linking of cells, encapsulation in a polymer gel, and entrapment in a matrix. Immobilized microbial enzymes, organelles, and cells have been used in a variety of scientific and industrial applications.

The cultivation of nitrifying bacteria is especially challenging because of their slow growth rates and the frequent occurrence of heterotrophic bacteria. There are not many reports on the mass production of autotrophic nitrifying bacteria. Chapman *et al.* (2006) developed improved methods for the cultivation of the chemolithoautotrophic *Nitrosomonas europaea* in fed batch and continuous cultures at laboratory level. Continuous culture in bioreactors with cell cycle is a relatively new method to produce concentrated cultures of actively growing microbial species that have low growth rates and yields. This technique has been used previously to maintain resting nitrifying bacteria in pure and mixed culture with complete biomass retention (Zart and Bock, 1998).

The determination of immobilized bacterial biomass is difficult compared to other bacterial biomass determinations. Measurement of ATP concentration is one of the best options in biomass estimation (Karl, 1980). It is widely used as an index for bacterial biomass determination in clinical microbiology, food quality control and environmental analysis (Stemler *et al.,* 1987; Tue *et al.,* 2000; Ishida *et al*., 2002; Anwar *et al.,* 2006; Velten *et al*., 2007., He *et al*., 2009). It is based on the assumption that there is relatively uniform concentration of ATP in the protoplasm of all microbial cells. It is ubiquitous in all living cells, has a relatively short half-life following cell

death and autolysis, and is present as a fairly constant intracellular concentration regardless of nutritional mode.

ATP measurement using bioluminescence based on firefly luciferin is easy, rapid and sensitive. In the presence of luciferase and the substrate luciferin, extracted from the firefly *Photinus pyralis*, ATP-dependent oxidation of the substrate luciferin in the presence of luciferase produces oxyluciferin, carbon dioxide, AMP, inorganic phosphate, and light (Mazer *et al.*, 1987). The amount of light emitted is directly correlated with the amount of ATP present (Chen and Cushion, 1994).

The objective of the work undertaken was the mass production of immobilized NBC (Nitrifying Bacterial Consortia) on wood powder and validation of the efficacy of the product for the removal of TAN in shrimp culture systems.

5.2 Materials and Methods

5.2.1 Nitrifying Bacterial Consortia (NBC)

The nitrifying bacterial consortia (AMOPCU – 1 Ammonia oxidizing bacteria for Penaeid shrimp culture system) were generated in the nitrifying bacterial consortia production unit (NBCPU) (Kumar *et al.*, 2009) maintained at NCAAH.

5.2.2 Wood powder

5.2.2.1 Processing of the carrier material

Wood powder of the plant species *Ailanthus altissima* was collected from local timber industry. They were dried, crushed and sieved to get particle size 300-500 µm. As lignin in wood powder might interfere with the process, it was delignified.

For delignification, the method proposed by Wood and Saddler (1988) was employed as follows: One g crushed wood powder was immersed in 50 mL tap water containing 1% V/V H_2O_2, with 0.1 N NaOH, pH of the suspension adjusted to 11.5. The suspension was stirred gently at 25° C for 3 - 5 hours on magnetic stirrer, with hourly correction of pH to 11.5 as per requirement. The suspension was filtered and the insoluble residue collected, washed, till pH dropped to neutrality. Delignified wood residue was dried at 100°C before storage. Presence of lignin residue was checked by treatment with hot sodium sulphite. To 0.5 g delignified material 5 mL aqueous hot sodium sulphite (5g/ 500 mL) were added. Release of magenta coloured liquor indicated delignification.

5.2.3 Quantification of nitrifying bacterial consortia (AMOPCU) by epifluorescency

An aliquot of 75 ml consortium was drawn from the Nitrifying Bacterial Consortia Production Unit (NBCPU), sonicated for 4 min at 25% amplitude. After sonication, 1.5 mL samples were filtered through 0.2µm polycarbonate irgalan black stained nucleopore filter paper and stained using acridine orange (1-7 minutes). Base of the filtration apparatus was wetted prior to placement of the wet polycarbonate membrane to get uniform cell distribution. After filtration, nucleopore filters were mounted

immediately on a slide using non-fluorescent immersion oil. A minimum of seven fields per filter per sample were counted. Bacterial cell fluoresced green taking up acridine orange under blue excitation on an epifluorescence microscope.

Calculation

Bacterial abundance (cells/liter) = $C_f RX1/fs$

C_f = mean number of cells per field.

R = Active area of filter/ area of field counted.

fs = Volume of fluid filtered.

5.2.4 Immobilization of nitrifying bacterial consortia (AMOPCU -1) on wood powder

5.2.4.1 Device for immobilization

A culture device was designed based on the requirements for obtaining maximum biomass with in the shortest duration possible and fabricated with locally available materials. The 50 L capacity cylindrical immobilization device with conical tapering bottom was fitted with a stirrer assembly and an air diffuser at the bottom. Seawater (40 L) was chlorinated using 200 ppm sodium hypochlorite and after several hours de-chlorinated using 15 g sodium thiosulphate. The vessel was aerated for 2 days through a cartridge filter (0.2 µm) and plated out on to ZoBell's agar prepared in aged seawater to record the presence of total viable bacterial population, prior to inoculating with the nitrifiers. As nutrients, 10 mg/L NH_4^+-N and 2 mg/L PO_4-P (as NH_4Cl and KH_2PO_4) were added, pH adjusted to 7.5 using Na_2CO_3. The inoculum was introduced with final concentration of 4 L (10^5cells/mL). A quantity of 800 g crushed, sieved and delignified wood powder from *Ailanthus altissima*

(Pongalayam) as the substratum was added to the immobilization tank and the aeration set at 6L/min. Samples were analysed daily for pH, TAN, NO_2-N and NO_3-N. The pH was maintained using aqueous 10% sodium carbonate and the temperature was regulated at 28^0 C by a thermo circulator. As the consumption of NH_3^+ - N progressed; it was supplemented with the aliquots of fresh substrate at an exponential rate. This process was continued until the culture attained stationary phase with daily monitoring of NH_4^+-N consumption and NO_2-N and NO_3-N production.

During the process, samples (immobilized nitrifiers on wood powder) were drawn, washed gently in sterile medium and transferred to fresh medium for analyzing nitrifying potency.

5.2.5 Determination of immobilized nitrifying bacterial biomass

The determination was based on the 'ATP bioluminescent method'. The enzyme Luciferase (from fire fly *Photinus pyralis*) catalyses oxidation of D-luciferin using ATP as energy source, there by reducing adenosine triphosphate to adenosine diphosphate and releasing the free energy as light.

$$\text{ATP} + \text{Luciferin} \rightleftharpoons \text{Adenyl} - \text{luciferin} + \text{PPi} \quad \text{----------} (1.6)$$

$$\text{Adenyl} - \text{luciferin} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + CO_2 + \text{light} \quad \text{----} (1.7)$$

ATP Extraction

An aliquot of 1 g sample was dropped in to a test tube containing 5 mL boiling Tris buffer (pH 7.75, 0.1 M). The content was boiled for a further 60 s, then the tube was cooled and after centrifuging (1000 g) the supernatant was used for ATP determination.

ATP estimation

Standardization

A primary ATP standard was prepared by dissolving 10 mg of high purity ATP (sodium salt) dissolved in 10 mL distilled water. The solution was diluted to 1/10 of the primary standard. Placed a portion of the solution in to a 1 cm quartz cuvette and measured the absorbance at 259 nm. The exact concentration of ATP was calculated as follows

$$A = Elc$$

Where A = absorbance at 259 nm

E = ATP molar extinction coefficient (15.4×10^3)

l = path length of cuvette (1 cm)

c = concentration of ATP in moles/ L.

1 µg/mL ATP solution was prepared by the dilution of required quantity of the stock in to 0.02 M tris buffer (pH 7.74). Working standards of 10, 30, 50, 70 and 100 ng were prepared.

An aliquot of 30 µL sample was added to an optical sensing cell and 270 µL Luciferase – Luciferin reagent was added subsequently. The luminescent intensity was measured using Luminometer (Turner Bio systems). The ATP standards were also analyzed the same way to draw a calibration curve to obtain the factor value.

Luciferase – Luciferin reagent

39 µg/mL Luciferase

78 µg/mL Luciferin

1.1 mmol/L EDTA 2 Na

11 mmol/L magnesium acetate tetrahydrate

1.1 mg/mL BSA

0.6 mmol/L DTT

25 mmol/L Tris – acetate pH 7.8

5.2.6 Determination of nitrification potential of immobilized NBC on wood powder

The nitrifying potential of immobilized nitrifiers on wood powder was determined by the sample was filtered using tea filter, dried over blotting paper and maintained in a desiccator under vacuum, without vacuum and also with out desiccation, all at room temperature. After drying the content was transferred to 100 mL Watson medium (1965) (composed of sea water (salinity 15 or 30g/L) with NH_4-N (10 mg/L), PO_4 -P (2 mg/L) and pH 8.0) and maintained on a shaker (Remi, India) at 100 rpm and the activity was determined by measuring TAN consumption and NO_2^--N/NO_3^--N production.

5.2.7 Determination of shelf life of immobilized NBC

The shelf life of the nitrification potential of immobilized nitrifiers after storage was determined by the sample was filtered using tea filter, dried over blotting paper and 1 g was aseptically transferred to polyethylene bags, sealed and maintained in a box at room temperature. Once in seven days 1 g each was transferred to 100 mL Watson medium (1965) and maintained on shaker at 100 rpm and the activity was determined by measuring TAN consumption and NO_2^--N /NO_3^--N production.

5.2.8 Determination of the quantity of immobilized nitrifiers required for treating unit volume of water

Activated immobilized nitrifiers of 0.1 to 1 g were administrated to 1 L seawater based media in triplicates with aeration. Nitrification over a period of

three days was monitored and measured in terms of TAN consumption and NO_2^--N/ NO_3^--N production.

5.2.9 Evaluation of the nitrifying potency of immobilized NBC (AMOPCU-1) in a low stocking density shrimp culture system.

The experimental design consisted of six tanks each holding 24 L 15 g/L salinity seawater maintained under aeration at a rate of 2 L/minute. Each set consisted of control and test tanks in triplicate. The experiment was conducted having 6 shrimps/m^2 with an average weight of 15 g (*P.monodon*) maintained without water exchange and fed with commercial pelleted feed (CP Feed, Chennai, India) at a rate of 4% of body weight. After 8 days when TAN loadings were 5 mg/L, 3 g immobilized nitrifying consortia (AMOPCU-1) were applied per tank. Water quality parameters such as, TAN, NO_2^--N, NO_3^--N, alkalinity and pH were monitored daily. Extent of degradation of wood powder used as substratum was also evaluated daily by physical examination.

5.2.10 Evaluation of immobilized NBC (AMOPCU- 1) in high stocking density shrimp culture system.

The experiment was conducted in 100 L capacity fiber glass tanks. The experimental design consisted of six tanks, three each for control and test with 15 g/L seawater. The shrimps were stocked at the rate of 24/m^2 with an average body weight of 8 – 10 g maintained without water exchange and fed with commercial pelleted feed (CP Feed, Chennai, India) at a rate of 4% of the body weight with a frequency of twice a day. After one week when the TAN level became 5 mg/L, 15 g immobilized nitrifying consortia were added to the test tanks. Water quality parameters such as, TAN, NO_2^--N, NO_3^--N, alkalinity and pH were monitored daily.

5.3 Results

5.3.1 Immobilization of NBC (30 ppt) on wood powder

The substrate consumption and product formation during the mass immobilization is depicted in Fig 1. The system which started with 10 mg/L residual NH_4^+ - N could consume 666.6 mg/L NH_4^+-N over a period of 71 days with a total corresponding out put of 471.33 mg/L NO_3^--N. From the growth curve, it could be inferred that until 8 days of incubation there was a progressive build up of NO_2^--N, and subsequently it rapidly declined and nitrate began to accumulate. After that, no residual nitrite could be detected and oxidation of NH_4^+– N and NO_2^- -N was found to take place simultaneously to form nitrate. The TAN removal rate of 1 g immobilized NBC (wet weight) introduced in to fresh mineral base medium is given in Table 1.

5.3.2 Immobilization of NBC (15 ppt) on wood powder

The substrate consumption and product formation during the mass immobilization of NBC meant for 15 g/L salinity culture system is illustrated in Fig 2**.** The system which started with 10 mg/L residual NH_4^+ -N consumed 583.6 mg/L NH_4^+- N over a period of 75 days with a total corresponding output of 415.6 mg/L NO_3^-- N. The growth curve showed that there was a progressive build up of NO_2^-- N until seven days and subsequently it rapidly declined and NO_3^-- N began to accumulate. The TAN removal rate of immobilized NBC introduced in to fresh medium is shown in Table 2.

5.3.3 Determination of immobilized nitrifying bacterial biomass

The immobilized nitrifying bacterial biomass estimated at stationary phase of the culture was 4.24 x 10^7 CFU /g. This result was based on the relationship 1.61 log CFU/g = 3.18 log fg/g ATP (Ukuku *et al*., 2005).

5.3.4 Determination of nitrifying potential of immobilized NBC on wood powder

The immobilized NBC were dried under different conditions, such as desiccation under vacuum and with out vacuum, and at room temperature (RT). One gram wood powder with immobilized NBC (wet weight) was incubated in 100 mL Watson medium (1965) and kept on a shaker at 100 rpm. To test the nitrifying capability, analyzed for TAN removal and NO_2^--N/ NO_3^--N production.

The results showed that TAN removal got reduced within a day in the experimental system inoculated with immobilized NBC. In the system inoculated with NBC dried in vacuum desiccator the TAN removal and NO_2^- -N production were 1.07 and 0.25 mg/L/day respectively. In the system inoculated with NBC dried in desiccator with out vacuum the removal and production were 7.09 and 3.7 mg/L/day respectively. However, in the system inoculated with NBC dried in room temperature, TAN removal was 8.9 and NO_2^- -N production was 4.18 mg/ L/day. On day 2nd in the systems inoculated with NBC dried under vacuum and dried at room temperature no residual TAN was detected, however, the one inoculated with vacuum desiccated NBC the TAN removal was negligible. Following a similar trend, highest NO_2^--N production was recorded in the systems inoculated with NBC dried in desiccator with out vacuum and the one dried under room temperature (Table 3).

5.3.5 Determination of shelf life of immobilized NBC

In this experiment, total ammonia nitrogen removal (10 mg/L) was achieved within 48 h with respect to the immobilized NBC stored for three weeks. The immobilized NBC, which were stored for 8 weeks, could consume

NH_3^+-N (10 mg/L) within 72 h, and others having a storage period up to 12 weeks took 96 hours for the same (Table 4).

Considering the rate of ammonia consumption by the immobilized consortia stored over a period of time, the one, which was stored for a week, could consume ammonia nitrogen up to 110 mg/L with in 288 h (Fig. 4). Meanwhile, the samples stored for prolonged period were showing reduced rate of consumption during the same period.

5.3.6 Determination of the quantity of immobilized nitrifiers required for treating unit volume of water

To accomplish the above, varying quantities of immobilized NBC on wood powder were incubated in 1L seawater having 15 g/L salinity. Initially TAN was maintained at 10 mg/L. Nitrification was monitored in terms of per day TAN removal and NO_2^--N production. On day 1, the TAN removal rates in the NBC of salinity 15 g/L inoculated with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1g immobilized nitrifying bacterial consortium on wood powder were 1.65, 3.98, 4.11, 4.2, 4.3, 4.4, 4.62, 4.39, 4.9 mg/L respectively, whereas in the control it was 0.8 mg/L. The nitrite production in the system inoculated with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1g immobilized nitrifying bacterial consortium on wood powder was 0.03, 0.98, 1.83, 2.08, 2.34, 2.01, 2.38, 2.18, 2.09, 2.99 respectively, whereas in the control it was 0.098 mg/L (Table 5). TAN removal per day and quantity of immobilized NBC showed a positive correlation (0.895). Compared to the control tanks 0.1 g immobilized NBC inoculated system did not show significant difference ($P < 0.05$). The results indicate that the higher the quantity of immobilized NBC the greater will be the nitrification in the treated system, however, ANOVA with a set of different quantity of the NBC did not show significant differences in the nitrification.

The results showed that a concentration of 10 mg TAN in 1 L sea water could be completely removed with in three days with a minimum quantity of 0.2 g immobilized NBC.

5.3.7 Evaluation of nitrifying potency of immobilized nitrifying bacterial consortia in a low stocking density culture system.

TAN removal was obvious from second day onwards (Fig 5.). After two days, the entire TAN (4.99 mg/L) was removed in the test tanks. Average lowering of 2.75 mg/L/day of TAN removal was observed in the test tanks, whereas in the corresponding control a lowering of 0.76 mg/L/day, three fold lower than the test tanks was observed. On the second day, the NO_2^--N production in the test tank was 2.26 mg/L and a maximum concentration 3.9 mg/L was observed on the third day, which declined in the subsequent days as NO_3^- -N production had started on the 4th day onwards with a corresponding decrease of NO_2^--N (Fig. 6). A negative correlation was observed between TAN and NO_3^--N in the test (r -0.59) indicating effective nitrification, whereas in the control tanks elevated levels of ammonia and nitrite – nitrogen could be experienced. TAN removal and drop in alkalinity (Fig 7) in the test tanks were positively correlated (r = 0.838).

5.3.8 Evaluation of nitrification potency of immobilized nitrifying bacterial consortia in a high stocking density experimental culture system

In the high density culture system, the entire (9.98 mg/L) quantity of total ammonia nitrogen in the experimental tanks was removed within five days (Fig. 8). Average lowering of 2.002 mg/ L/ day TAN was observed in the test tanks, whereas in the corresponding control tanks it was 0.58 mg/L/ day. In the test NO_2^--N registered maximum value on the 14th day (6.71 mg/L),

which declined corresponding to the decline of TAN concentration (Fig. 9). Mean NO_3^- -N level increased from 0.1 to 6.39 mg/L in the test tanks, and remained at 0.005 to 1.89 mg/L in the control system. TAN removal and drop in alkalinity were positively correlated (r 0.769) (Fig. 10).

5.4 Discussion

High density zero water exchange shrimp grow out systems are specialised and highly dynamic aquauclture production systems where bioremediation of ammonia and nitrite are the vital factors for the success of the culture. Ammonia originates from the animal excreta, uneaten feed and decomposing organic solids. Unionized ammonia (NH_3) is the most toxic species and its percentage depends upon the variation of pH and temperature. At high pH and temperature the NH_3 concentrations turns out to be higher. In nature nitrifying bacteria cause the oxidation of ammonia to nitrite and to comapartively innocuous nitrate, the process termed nitrification. In biological ammonia removal systems, nitrifying activity of suspended bacteria has been reported to be extremely low, due to slow growth rate and inhibition of nitrification by free ammonia and nitrite under the alkaline conditions of seawater (Bower and Turner, 1981; Furukwa *et al.*, 1993). With out the addition of nitrifiers in the start up culture, 2-3 months are needed to establish nitrification in marine system (Manthe and Malone, 1987) and 2-3 weeks in the fresh water (Masser *et al.*, 1992). There is an agreement, among researchers and between laboratory research and commercial application, on the fact that salt water systems need a much longer start up period. Under such situation, immobilization techniques have been found useful to overcome the delay in the initiation of nitrification (Sung Koo *et al.*, 2000). The immobilization of bacteria has been studied extensively and found to have

numerous benefits over free cells for treatment of domestic waste water including increased conversion rates, decreased growth rates, higher cell concentrations, no need for cell separation and elimination of wash out possibility (Cao *et al.*, 2002; Yang *et al.*, 2002; Hill and Khan, 2008)

For such application of start up cultures, the nitrifying bacteria have to be generated in large quantity. An important consideration for the design of the mass production is the cost – effectiveness. The medium optimized here was seawater based and required only the addition of substrate (ammonia – nitrogen), and carbonate to maintain optimum pH. For the design of 50 L, conical tapering fermentation tank locally available fiber glass was used. An electrically operated stirrer/agitator was used to accomplish agitation and mixing up of the carrier material and nitrifying bacteria. The fermentation tanks have been made opaque and placed well protected from sunlight, because the visible and UV light rays are lethal to nitrifying organisms (Johnstone and Jone, 1988; Diab and Shilo, 1988). The carrier material, wood powder (*Ailanthus altissima*), used for the immobilization was locally available, inexpensive and with low lignin content. Initially the seawater was sterilized by chlorination with 300 mg/L sodium hypochlorite and aerated until the chlorine was removed. This helped to remove all the unwanted microorganisms. Finally, sodium thiosulphate was added to ensure the complete neutralization of chlorine content. The carrier material was sterilized by autoclaving at 15 lbs/ 15 m. Nitrifying bacterial consortia were taken from the nitrifying bacterial production unit maintained at NCAAH (Kumar *et al.,* 2009).

The activated 4 L nitrifying bacterial consortia (10^5 cells/mL) along with 800 g wood powder were used for immobilization on the solid substratum.

The device used for mass production of immobilized NBC was designed and fabricated for successful immobilization of nitrifying bacterial consortia meant for 15 and 30 g/L salinity regimen. It is an accepted fact that when population of nitrifying bacteria gets established under steady state conditions residual nitrite shall be too low to be detected with progressive building up of nitrate. Similar observations were also made by Achuthan *et al.* (2006) during the enrichment nitrifying culture from shrimp ponds and by Kumar *et al.* (2009) during mass production. It has also been established that nitrite oxidation to nitrate was more rapid than the preceding step (Stesel and Barnad, 1992). This was also true in this experiment as nitrite was below detectable level after seven to nine days of enrichment.

The time period required for immobilization of NBC was determined by the inoculation of immobilized NBC into fresh medium and analyzing the TAN removal rate. The TAN removal rates were 4.53, 6.33, 6.7 and 6.5 mg/L/day at the salinity 30 g/L on the 7th, 8th, 9th and 10th day, whereas at 15 g/L salinity it was 5.1, 5.57,6.6 and 6.5 mg/L/day respectively. The results showed that on the seventh day onwards, the immobilization had started and there was not much difference in the subsequent days.

A simple technique for the processing of immobilized NBC after harvest with out loss of its nitrifying potency was developed. Two methods could be evolved, one method was drying at room temperature and another was drying in a desiccator with out vacuum. NBC processed by both the methods exhibited significant TAN removal compared to the one processed under vacuum ($P < 0.05$). Reduction in the nitrifying activity of immobilized NBC processed in a vacuum desiccator might be due to the excessive loss of

moisture content from the preparation. Among all methods, the ones identified were less expensive compared to the other methods.

The shelf life of the bacterial products happens to be a major issue in all commercial applications. During this experiment 1g immobilized NBC was stored in sealed polythene bags at room temperature for the period of one week to twelve weeks. The results showed that storage of the immobilized nitrifies over a period of twelve weeks under ambient conditions did not affect much of the nitrifying potency. However, instantaneous TAN removal rates had been slightly reduced in the sample stored for more than one month indicating that the nitrification potential was not lost from the consortium at the immobilized state on long storage instead there happened prolongation of the lag phase during the storage or there might have happened partial loss of organisms essential for their functioning

The minimum quantity of immobilized NBC required for treating unit volume of water was determined and it was found that within the range of 0.2 to 1g immobilized NBC per litre, there was progressive increase in nitrification in 15 ppt sea water suggesting that the quantity of NBC correlated with the rate of nitrification, however it did not show statistically significant difference. This offers much flexibility to the choice of the quantity of immobilized NBC for application.

Evaluation of immobilized NBC in the low stocking density and high stocking density culture systems showed a remarkable reduction in the total ammonia concentration in the tests. The total ammonia concentration in the test tank of the low stocking density system was 4.99 when immobilized NBC was applied, and within two days, the concentration could be brought down to

0 mg/L. Meanwhile in the high stocking density system 9.98 mg/L TAN was brought down to 0 mg/L within five days. Nitrite also showed a lowering after a slight increase in both the cases. The nitrate ranged between 4 to 6 mg/L. In these systems, the ammonia oxidation was established within a day, but the nitrite oxidation took 4 days. The delay in nitrite oxidation could have been the requirement of a certain level of nitrite accumulation for activating nitrite oxidizers in the consortium until a steady state equilibrium was reached. (Sharma and Ahler, 1977; Smith *et al*., 1997; Vadivelu *et al*., 2007). As the system is already with ammonia – nitrogen, ammonia oxidizing bacteria get activated initially producing nitrite sufficient enough for the nitrite – oxidizing bacteria (NOB) to get activated. TAN removal and drop in alkalinity showed a positive correlation in these systems. The conversion of ammonia to nitrate consumes alkalinity in the form of bicarbonate supplemented. Alkalinity in the form of bicarbonate and carbonate become one of the carbon sources apart from Carbon dioxide for nitrifying bacteria (Chen *et al*., 2006). Alkalinity is normally consumed at approximately 7.14 g/g N oxidised during nitrification (Villaverde *et al*., 1997; Timmonas *et al*., 2002). At the end of the experiment of the intensive culture system, the percentage survival of shrimp in the test tanks was 83.3 ± 8.9% and in the control tanks 45.5 ± 9.9%.

Nitrate levels were higher in the test tanks compared to control tanks. Nitrate in the control system never appeared to increase. This is another evidence of incomplete nitrification in the controls (Sandu *et al*., 2002).

Based on the experiment conducted in the high stocking density culture system the TAN removal rate was 2.002 mg/L/day. In literature, TAN removal rates per unit medium surface area, in submerged flow biofilters (Abeysinghe *et al*., 1996), high-rate linear-path trickling nitrification filters (Twarowska *et*

al., 1997) and bench-scale fluidized bed bioreactors using activated carbon granules as the substratum (Ng *et al.,* 1996) were 1.05, 0.33, and 0.33 g/m^2 per day, respectively. However, the systems were not comparable.

Overall, it could be concluded that the effective control of TAN in shrimp pond water could be achieved through the application of immobilized NBC. From the investment and operational aspects, the proposed system is superior as it requires lesser initial cost and composed of locally available materials. Moreover, the carrier material, wood powder, is locally available and economically viable.

Table 1. TAN removal of immobilized NBC (30 ppt) on introduction to a fresh medium

Days	TAN removal mg/L/day
Day 7	4.93 ± 0.306
Day 8	6.33 ± 0.47
Day 9	6.7 ± 0.44
Day 10	6.5 ± 0.82

Table 2. TAN removal of immobilized NBC (15 ppt) on introduction to a fresh medium

Days	TAN removal mg/L/day
Day 7	5.1 ± 0.44
Day 8	5.57 ± 0.75
Day 9	6.6 ± 0.8
Day 10	6.5 ± 0.98

Table 3. TAN removal and NO_2^--N production of immobilized NBC dried in different methods.

TAN removal by NBC (mg/L)		Day 1	Day 2	Day 3
Dried in Vacuum desiccator	10.86 ±0.04	9.79 ± 0.37	10.14 ± 0.19	9.57 ± 0.41
Dried in desiccator without vacuum	10. 54 ± 0.155	3.45 ± 0.445	0	0
Dried at room temperature.	10.55 ± 0.014	1.65 ± 0.17	0	0
NO_2^-- N Production (mg/L)				
Dried in Vacuum desiccator	0.003 ± 0.04	0.25 ± 0.04	0.05 ± 0.003	0.07 ± 0.002
Dried in desiccator without vacuum	0.009 ± 0.01	3.7 ± 0.62	3.8 ± 0.98	2.67 ± 0.83
Dried at room temperature.	0.005 ± 0.001	4.18 ± 1.05	3.55 ±1.04	2.77 ± 0.15

Table 4. TAN removal rate (mg/L) of immobilized NBC (15 ppt AMOPCU - 1) after storage

Period of storage	0 hour	24 hour	48 hour	72 hour	96 hour
week 1	10.98 ± 1.28	5.77 ± 0.98	0	0	
week 2	10.66 ± 0.89	6.42 ± 0.87	0	0	
week 3	10.09 ± 1.09	5.67 ± 0.98	0	0	
week 4	10.57 ± 2.34	6.73 ± 0.88	5.10 ± 0.35	0.471 ± 0.12	0
week 5	10.09 ± 2.09	7.46 ± 0.98	2.3 ± 0.66	0.11 ± 0.03	0
week 6	10.99 ± 2.99	7.39 ± 1.45	3.63 ± 1.09	1.08 ± 0.098	0
week 7	9.98 ± 1.98	6.76 ± 0.88	4.29 ± 0.89	0.53 ± 0.08	0
week 8	10.36 ± 2.09	7.02 ± 1.32	6.47 ± 1.34	0.36 ± 0.04	0
week 9	10.35 ± 1.09	7.93 ± 0.57	5.85 ± 0.99	1.86 ± 0.06	0
week 10	9.9 ± 1.95	6.17 ± 1.49	4.38 ± 1.29	0.45 ± 0.56	0
week 11	10.67 ± 2.78	7.38 ± 0.76	5.39 ± 0.34	4.19 ± 0.567	0
week 12	10.03 ± 1.98	5.86 ± 0.98	4.66 ± 1.89	0.84 ± 0.04	0

Table 5. TAN removal and NO_2^--N production by different quantity of immobilized nitrifying bacterial consortia.

Immobilized NBC	TAN Removal (mg/L)		NO_2^--N Production (mg/L)	
	Day 1	Day 2	Day 1	Day 2
0.1 g	1.05 ± 0.34	1.85 ± 0.25	0.03 ± 0.004	0.98 ± 0.05
0.2 g	3.98 ± 0.56	3.3 ± 0.66	0.98 ± 0.03	1.98 ± 0.09
0.3 g	4.11 ± 0.37	3.37 ± 0.72	1.83 ± 0.98	2.45 ± 0.08
0.4 g	4.2 ± 0.87	2.88 ± 0.55	2.08 ± 0.88	3.55 ± 0.09
0.5 g	4.2 ± 0.68	3.03 ± 0.77	2.34 ± 0.78	2.65 ± 0.98
0.6 g	4.3 ± 0. 78	3.13 ± 0.78	2.01 ± 0.56	2.87 ± 0.87
0.7 g	4.45 ± 0.99	2.99 ± 0.55	2.38 ± 0.87	3.45 ± 0.99
0.8 g	4.62 ± 0.88	3.3 ± 0.78	2.18 ± 0.55	3.38 ± 0.65
0.9 g	4.39 ± 1.22	3.9 ± 1.09	2.09 ± 0.08	3.78 ± 0.9
1.0 g	4.98 ± 1.05	3.56 ± 1.45	2.99 ± 0.09	3.98 ± 0.98
Control	0.8 ± 0.005	1.02 ± 0.99	0.098± 0.008	0.98 ± 0.008

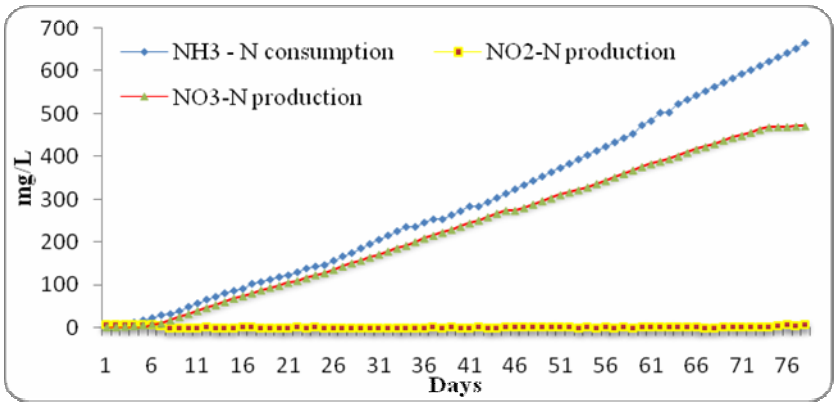

Fig. 1. TAN consumption and NO_2^--N / NO_3^--N production recorded in NBC production unit maintained at 30 ppt salinity.

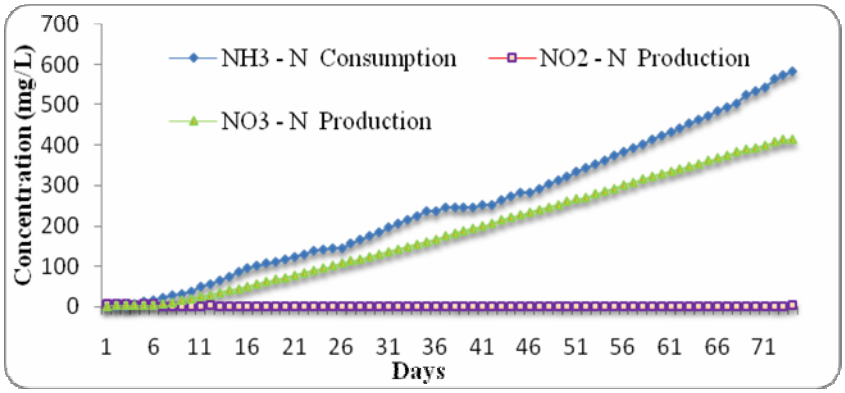

Fig. 2. TAN consumption and NO_2^--N / NO_3^--N production recorded in NBC production unit maintained at 15 ppt salinity.

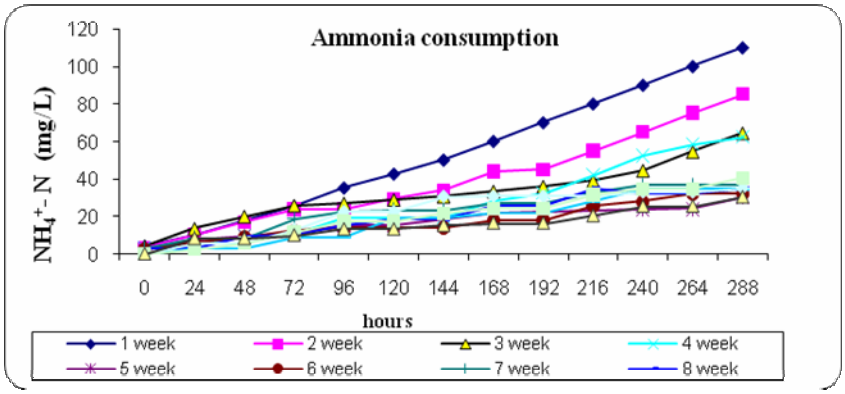

Fig. 4. Consumption of NH_4^+ -N - Shelf life experiment

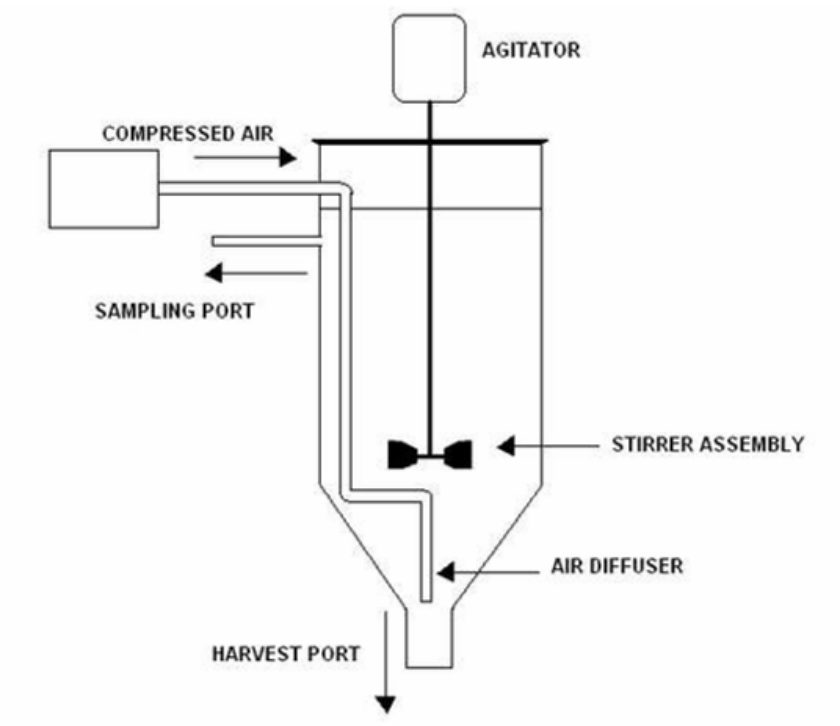

Fig. 3. Immobilized NBC Production unit

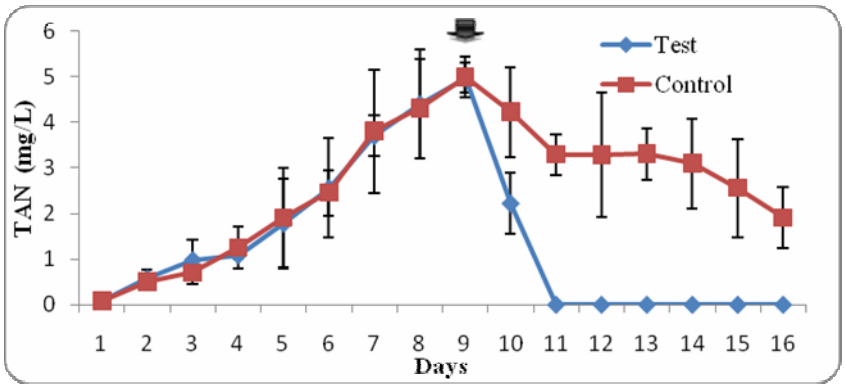

Fig. 5. Decrease in average TAN in the test and control tanks (arrow denotes the point of application of immobilized NBC.

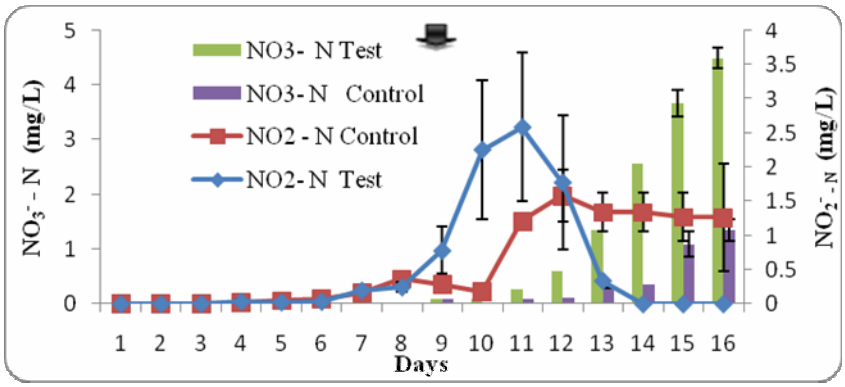

Fig. 6. NO_2^--N and NO_3^- –N production in the test and control tanks (arrow denotes the point of application of immobilized NBC)

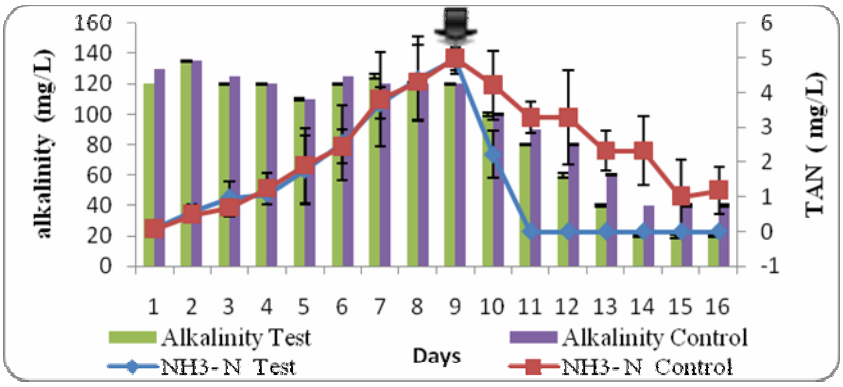

Fig. 7. TAN removal and drop in alkalinity in the test and control tanks (arrow denotes the point of application of immobilized NBC).

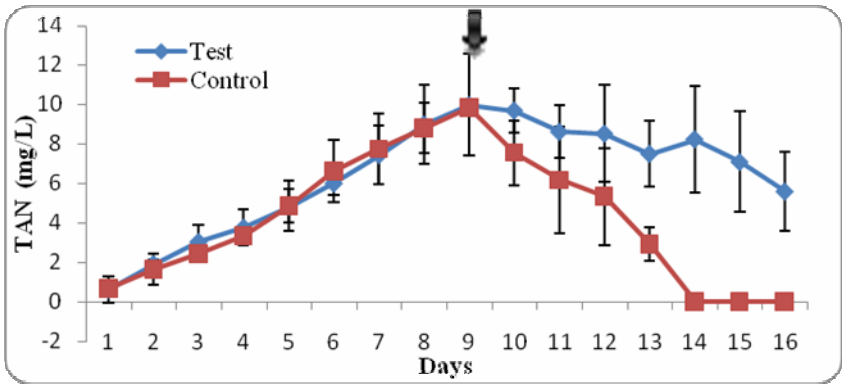

Fig. 8. Decrease in average TAN in the test and control tanks (arrow denotes the point of application of immobilized NBC).

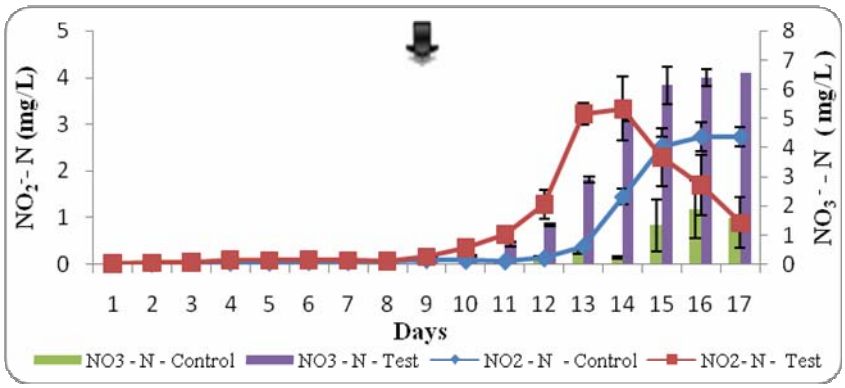

Fig. 9. NO_2^-–N and NO_3^-–N production in the test and control tanks (arrow denotes the point of application of immobilized NBC).

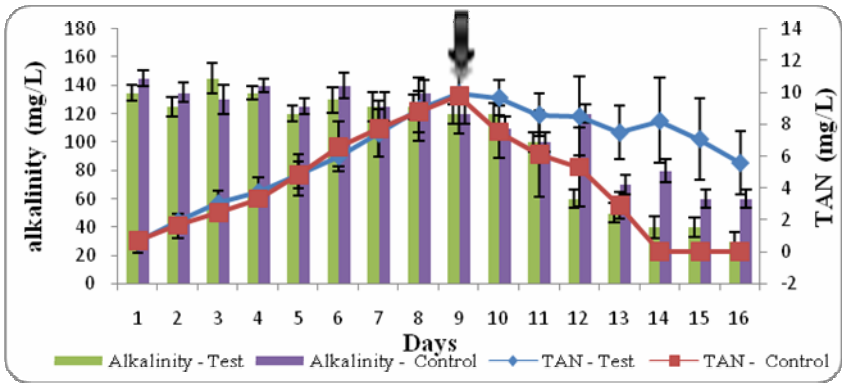

Fig. 10. TAN removal and drop in alkalinity in the test and control tanks (arrow denotes the point of application of immobilized NBC).

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Chapter 6

Summary and Conclusions

Aquaculture is one of the fastest growing food sectors in the world. Amongst the various branches of aquaculture, shrimp culture has expanded rapidly across the globe because of its faster growth rate, short culture period, high export value and demand in the International market. Indian shrimp farming has experienced phenomenal development over the decades due to its excellent commercial viability. Farmers have adopted a number of innovative technologies to improve the production and to maximize the returns per unit area. The culture methods adopted can be classified in to extensive, modified extensive and semi intensive based on the management strategies adopted in terms of pond size, stocking density, feeding and environmental control. In all these systems water exchanges through the natural tidal effects, or pump fed either from creek or from estuaries is a common practice. In all the cases, the systems are prone to epizootics due to the pathogen introduction through the incoming water, either brought by vectors, reservoir hosts, infected tissue debris and free pathogens themselves. In this scenario, measures to prevent the introduction of pathogen have become a necessity to protect the crop from the onslaught of diseases as well as to prevent the discharge of waste water in to the culture environment.

Zero water exchange shrimp culture system is one of the alternatives to reduce the introduction of infectious diseases and better control in the environmental problems. It also helps introduction of effluent quality management measures and reduction in the requirement of space for culturing animals. In the absence of effective bioremediation, zero water exchange system has experienced several problems, such as water quality alterations, NH_4^+-N, NO_2^--N and H_2S toxicity, reduction in soil redox potential less than -150 mV, reduction in feed consumption after 80-120 days of culture, moulting problems, zoothamnium infestation, black spot on the shells, vibriosis and subsequent mortality. To overcome these problems a protocol has been developed in zero water exchange shrimp culture system integrated with appropriate bioremediation of detritus and ammonia nitrogen to attain sustainability and economic viability.

The salient features of the protocol are as follows:

Pond preparation – Ponds were eradicated of the weed fishes by applying lime (1275 kg/ha) and ammonium sulphate (255 kg/ ha) at a ratio of 5:1 and tea seed powder at the rate 100 kg/ha after maintaining the water level to 10 cm. Maintained pH and *Eh* of the sediment to 7.0 to 8.5 and -100 to -125 mV respectively and total organic carbon below 2%.

Fertilization - Based on the nutrient status, inorganic fertilizers were supplemented as per requirements to initiate bloom. A combination of nutrients such as Nutrimix, 650 g (NCAAH, CUSAT), triple super phosphate, 15 kg/ha (FACT, Kerala) and 100 L cow dung extract prepared from 50 kg cow dung, were applied to the ponds during morning hours. In addition, another nutrient preparation 'Micromix' containing ferric chloride and magnesium chloride at a ratio of 1:1 was also applied at a rate of 300 mL/ ha.

Stocking - Before stocking soil pH was maintained between 7–8.5, *Eh* between -100 to -125 mV and alkalinity between 70 -120 mg/L.

Management of the ponds – All physical, chemical and biological parameters of the water and sediment were monitored regularly. If the phytoplankton blooms did not remain stable, it indicated the nutrient deficiency. It was replenished either by the addition of Detrodigest^{TM} and/or by the application of nutrients. Whenever dissolved oxygen was detected below 2 ppm, it was resorted through paddle wheel aeration. Whenever, bloom production was in excess, feeding was reduced. Whenever pH rose to above 9 in the evening hours it was maintained by regulating alkalinity to above 70 mg/L by the addition of Dolomite at the rate of 50 Kg per hectare. After one month of culture, body weight was assessed and feeding regulated along with health assessment. Detrodigest^{TM} application was continued once in a week. Application of Enterotrophotic^{TM}, a gut probiotics, was commenced from the 30th day onwards at the dosage of 50 mL/10000 animals/day by coating on to pelleted feed for the first 70 days and 100mL/10000 animals/day during rest of the period.

The protocol was implemented in three experimental ponds and three control ponds at Pancham Aquaculture farms Ltd., Mumbai.

- During the experimental period, ammonia and nitrite were with in the safe level in the test ponds through out the culture and in the control ponds they were significantly higher and fluctuating.
- Dissolved oxygen stood above 3 mg/L except towards the terminal part of the culture even during night hours in the test ponds.

- Chlorophyll and productivity were significantly higher in the test ponds compared to control ponds.
- Soil E*h* in the test ponds ranged from -72 to -166 mV and in the control ponds, it was -96 to -226 mV.
- The average final body weight of animal in the test ponds was 37 g with in 160 days and in the control ponds it was 33.4 g with in 182 days.
- FCR was 1.9 and survival 74% in the test ponds and in the controls 2.7 and 54% respectively.

Validation of the protocol

- Validation of the protocol was undertaken in two different seasons such as pre - monsoon (March to June) and post - monsoon (September to January).
- During the validation period ammonia and nitrite concentrations were with in the permissible range indicating efficient nitrification.
- Soluble iron detected was very low suggesting that anaerobic condition had not been set in the system.
- Chlorophyll content reached 71.45 µg/mL and primary productivity ranged from 1.02 to 5.65 g C/m^3/day indicating higher productivity.
- Period of the culture ranged from 110 to 120 days only.
- Average body weight at the time of harvest was 30 to 35 g and FCR was 1.3 to 1.5, better than the National average

- Average soil E*h* was -123 mV and total organic carbon content less than 3%.
- All cultures were economically viable and profitable.
- Comparison of zero water exchange system with open culture system showed that there were significant differences between the parameters such as alkalinity, primary productivity, chlorophyll of water and E*h* of sediment.
- In the open system the cultures did not prolong more than 80 days due to mortality with the average body weight reaching to 20 g having 25% survival.
- In the zero water exchange system average body weight was 32.5 g having the survival of 62.5%.

Experiment with two stocking densities

- On experimenting with two stocking densities, the stocking density of $7/m^2$ was found better than $14/m^2$.
- Concentration of TAN, NO_2^--N and NO_3^--N of the pond water was significantly lower in the ponds with low density stocking.
- Soil E*h* was significantly lower in the ponds with higher stocking density.
- In the ponds with high stocking density significantly lower average body weight with higher feed conversion ratio could be observed

Mass production technology of immobilized nitrifying bacterial consortia (NBC)

- For bioaugmenting nitrification, mass production technology of immobilized nitrifying bacterial consortia (NBC) on wood powder was standardized.
- The nitrifying bacteria were quantified by epifluorescence and 10^5 cells/mL were used for immobilization.
- Immobilized nitrifying bacterial biomass was determined by ATP estimation and the results revealed that it contained 4.24 x 10^7 CFU/g.
- Activity of immobilized NBC under different process conditions such as drying at room temperature, desiccation with and with out vacuum was determined. The results showed that maximum TAN removal took place with respect to the samples dried at room temperature and the one desiccated with out vacuum.
- Storage of the immobilized nitrifiers over a period of twelve weeks under ambient conditions did not affect much of the nitrifying potency except the marginal reduction in the TAN removal rates with the samples stored for more than one month.
- It indicated that the nitrification potential had not been lost of the consortium due to storage under immobilized condition instead what happened was only prolongation of lag phase on activation.
- The minimum quantity of immobilized NBC required for treating unit volume of water was within the range of 0.2 to 1 g/L

- The evaluation of the immobilized product in the semi intensive and intensive culture systems under laboratory conditions showed remarkable reduction in the total ammonia concentration in the test tanks.
- On experimentation the total ammonia concentration of 4.99 mg/L in the semi intensive systems under laboratory conditions could be brought down to undetectable level on administration of the immobilized NBC.
- In the intensive culture system 9.98 mg/L TAN could be brought down to undetectable level within five days.
- Nitrite removal could be initiated on application of NBC in both semi intensive and intensive systems under laboratory conditions.
- Through out the study with nitrification, nitrate concentration stood between 4 to 6 mg/L indicating efficient denitrification.

Major findings of this study

- A bioremediation technology for zero water exchange shrimp culture has been developed/standardized.
- Detritus management has been given the greatest emphasis in the zero water exchange system, as for successful shrimp culture E*h* should not slide down below -150 mV and total organic carbon should not rise above 2.5%.
- The technology developed has a powerful bioaugmentor for detritus management, *Bacillus* MCCB 101, produced and supplied under the brand name Detrodigest^{TM}.

- From the data generated through out the experimental period it could be revealed that Detrodigest™ could bring up E*h* and bring down total organic carbon provided the bioaugmentor was applied regularly.
- In the zero water exchange system only topping up of water was adopted.
- A gut probiotic for the management of intestinal flora was found essential.
- The gut probiotics, Enterotrophotic™ , a blend of *Bacillus* MCCB 101 and *Arthrobac*ter MCCB 104, was found suitable to streamline moult cycle, protection from vibriosis and *Zoothamnium* infestation, enhancement of feed intake and reduction in FCR.
- An antagonistic probiotic PS-1 consisting of *Pseudomonas aeruginosa* MCCB 103 was found suitable to regulate the *Vibrio* population of the culture system.
- Over all, it could be concluded that the zero water exchange shrimp culture technology is ripe enough for extensive application in the Indian shrimp culture industry.
- The proposed system helps to reduce the emergence of diseases and environmental degradation.
- Considering from the point of investment and operational aspects, the proposed system is user friendly and economically viable with no additional capital investment.

- The immobilized NBC is effective in the control of TAN in high density shrimp culture system. The carrier material is wood powder locally available and less expensive and biodegradable.

Integration of zero water exchange with bioremediation

When zero water exchange shrimp culture is practiced there happens degradation of water and sediment quality leading to emergence of diseases and consequent death of the rearing stock. It is in this context a bioremediation technology has been designed along with application of gut probiotics to attain sustainability. The key issues addressed are prevention of detritus accumulation and maintenance of required water/sediment quality using a bioaugmentor preparation 'Detrodigest' and regulation of gut microbial flora favourable to the animal using a gut probiotic preparation 'Enterotrophotic'. After standardization of the technology it was validated in different seasons and under different stocking densities in systems deemed as semi-intensive, and found suitable to be adopted. It has been adopted as a package recommended by the National Centre for Aquatic Animal Health, Cochin University of Science and Technology to farmers of Kerala and Maharastra, especially Mumbai to be practiced. There has been good response from farmers in these regions and those who have practiced it adhering to the protocol strictly have had good crop amidst outbreak of white spot disease in the region.

Areas which require further research

1) Adaptive trials implementing the practice in different regions in the coastal zone of all maritime states having varying physical, chemical and biological properties.

2) Investigations in to the response of the microbial community of the culture system to the 'Detrodigest' and variations in the bio-geo-chemical cycles.

3) Investigations in to the response of the gut microbial flora to the probiotic preparation 'Enterotrophotic' and the floral changes brought in favour of the animal.

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