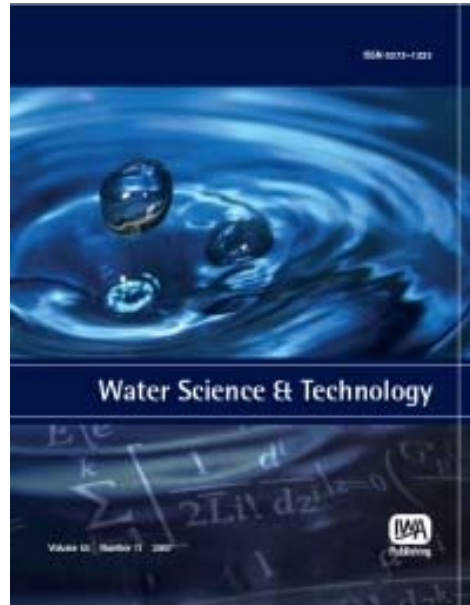


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Nitrification in brackish water recirculating aquaculture system integrated with activated packed bed bioreactor

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ABSTRACT

Recirculation aquaculture systems (RAS) depend on nitrifying biofilters for the maintenance of water quality, increased biosecurity and environmental sustainability. To satisfy these requirements a packed bed bioreactor (PBBR) activated with indigenous nitrifying bacterial consortia has been developed and commercialized for operation under different salinities for instant nitrification in shrimp and prawn hatchery systems. In the present study the nitrification efficiency of the bioreactor was tested in a laboratory level recirculating aquaculture system for the rearing of *Penaeus monodon* for a period of two months under higher feeding rates and no water exchange. Rapid setting up of nitrification was observed during the operation, as the volumetric total ammonia nitrogen removal rates (VTR) increased with total ammonia nitrogen (TAN) production in the system. The average Volumetric TAN Removal Rates (VTR) at the feeding rate of 160 g/day from 54–60th days of culture was 0.1533 ± 0.0045 kg TAN/m³/day. The regression between VTR and TAN explained 86% variability in VTR ($P < 0.001$). The laboratory level RAS demonstrated here showed high performance both in terms of shrimp biomass yield and nitrification and environmental quality maintenance. Fluorescent in-situ Hybridization analysis of the reactor biofilm ensured the presence of autotrophic nitrifier groups such as *Nitrosococcus mobilis* lineage, *Nitrobacter* spp and phylum *Nitrospira*, the constituent members present in the original consortia used for activating the reactors. This showed the stability of the consortia on long term operation.

Key words | fluorescence insitu hybridization analysis, nitrification, packed bed bioreactor, recirculating aquaculture systems, volumetric TAN removal

INTRODUCTION

Over the last two decades, aquaculture has gone through major changes, from small scale homestead-level activities to large scale commercial farming, exceeding landing from capture fisheries in many areas (National Research Council 1992; NACA/FAO 2001). The need to increase aquaculture production drives the industry towards more intensive practices because of limitations in quality and quantity of water, availability and cost of land, limitations on water discharge and environmental impacts. Recirculation-zero water exchange system is the only solution for these issues.

A recirculating aquaculture facility reduces water demands and discharges by reconditioning water to be used over and again (Goldburg *et al.* 2001). Better food conversions are achievable with a recirculating aquaculture system (RAS) which means less waste is generated from the feed (Losordo *et al.* 1998). In recent years, there has been growing concern over the impacts of aquaculture operations (Harache 2002; Cranford *et al.* 2003; Johnson *et al.* 2004) in the surrounding water bodies. Increasing regulatory pressures focusing on discharges to natural water bodies

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will force hatchery operators to adopt methods that are environment friendlier (White *et al.* 2004). RAS technology can reduce the effluent waste stream by a factor of 500–1,000 (Chen *et al.* 1997; Timmons *et al.* 2001) allowing existing operations to upgrade and expand and comply with future regulations.

The applicability of RAS technologies for the production of marine species has been amply demonstrated (Manthe *et al.* 1988; Davis & Arnold 1998) but the commercial production has been limited by a number of factors (Mozes *et al.* 2003). In the saltwater systems, RAS play an important role in the production of healthy, properly sized fingerlings for stock out in net pens or ponds (Fielder & Allan 1997). Recirculating systems are very compatible with the complex nature of reproduction in marine species and the broodstock fecundity of most marine species diminishes the impact of waste processing costs. Biosecurity issues are another important matter for consideration of RAS by the hatchery operators (Otoshi *et al.* 2003; Pruder 2004). Water recirculation dramatically reduces the possibility of pathogen introduction (Davis 1990; Goldberg *et al.* 2001).

The use of external biofilters in RAS was practiced successfully for years, in hatcheries, nurseries, ornamental fish culturing and in some extent in culturing of commodity fish. An efficient biofilter will maintain the water quality preventing the accumulation of toxic metabolites, the most notorious of which are ammonia and nitrite. High levels of ammonia, nitrite, undermine commercial production objectives as the toxic impacts are manifested through impaired growth or chronic diseases (Manthe *et al.* 1985; Svoboda *et al.* 2005). The use of RAS for marine hatchery operations has significant implications for biofiltration. This expanding niche will demand operations under oligotrophic regimes (Malone & de los Reyes 1997) which are rarely demanded by freshwater applications. Marine larval system can demand TAN and nitrite-N levels below 1.0 mg/L (Malone & Beecher 2000). Considering the above factors and aiming 100% closed recirculation system for the tropical hatcheries, a packed bed bioreactor (PBBR) was developed (Patent application no. 828/DEL/2000 of 13-9-2000) with indigenous nitrifying bacterial consortia and successfully demonstrated its performance in brackish water hatchery systems for larval rearing (Kumar *et al.* 2009a). This

technology is now commercialized through M/S Oriental Aquamarine Biotech India Private Ltd, U-140, Kovaipudur, Coimbatore 641 042, India. The present study was undertaken to test the nitrification potential of the PBBR for the rearing of *Penaeus monodon* in a laboratory level RAS for further scaling up. The nitrifying bacterial community of the reactor biofilm was also studied by Fluorescent in-situ Hybridization.

MATERIALS AND METHODS

Packed bed bioreactor

The configuration of the PBBR is detailed by Kumar *et al.* (2009a). Each reactor consists of a shell made of fibre glass with a base of 30 cm² and an overall height of 45 cm. A perforated base plate made of Perspex, carrying 30 cm long and 2 cm diameter 9 PVC pipes (air lift pumps) fixed at 10 cm equidistance, is positioned at the base of the reactor. When air gets passed through, the 10 cm³ area filled with the support medium (polystyrene beads having 5 mm diameter) surrounding each airlift pump, acts as an aeration cell. The base plate is elevated by 5 cm from the bottom supported by 5 cm long PVC pipes having 3 cm diameter. An inlet pipe is fixed at a water discharge height of 35 cm up from the base of the reactor. The outlet pipe, which emerges from the base of the reactor, bends upward at water discharge height of 35 cm from the base to the next reactor.

Activation and integration of the bioreactors into RAS

The reactors were activated with the nitrifying bacterial consortia enriched from brackish water environment (Achuthan *et al.* 2006) and mass produced in a 200 l fermentor (Kumar *et al.* 2009b). The rearing tank was filled with 1,000 L seawater of salinity of 15 g/L. A series of six activated packed bed bioreactors three each in parallel were integrated in to system where in 1,000 L rearing water was recirculated through the reactors making it 100% recirculating (Figure 1). The influent from the rearing tank was pumped in to an overhead tank (282 L) from where water flowed through the two reactors serially by

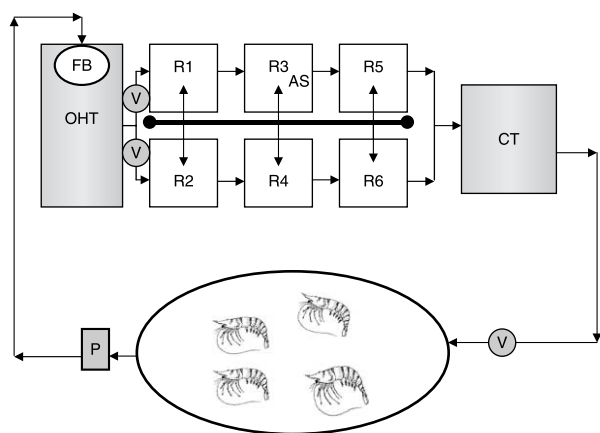


Figure 1 | Packed bed bioreactor (PBBR) integrated into the shrimp rearing tank. FB—Filter Bag; OHT—Over head tank; V—Valves; R1–R6—Reactor 1 to Reactor 6; AS—Aeration supply; CT—Collecting tank; SRT—shrimp rearing tank; P—pump.

gravitation and got collected in a 140 L collection tank, from where the treated water got in to the larval rearing tank. Flow rate of the influent from the overhead tank to the reactors were regulated by valves. Pumping of the influent from the larval rearing tank was controlled by an automated water level controller (V-guard, Kerala, India) fitted inside the overhead tank. The reactors were operated at a flow rate of 4 L/min provided a total recirculation of 5,760 L/day. A bag filter, placed inside the over head tank was used to filter the incoming water from the rearing tanks to remove detritus.

Rearing

Penaeus monodon larvae (Post larvae-20) were tested for White Spot Virus (WSV) by PCR and Monodon Baculo Virus (MBV) by microscopic observation and stocked at a density of 1 larva/L into the rearing tank. The larvae were fed with pelleted feed (Higashimaaru feeds Pvt Ltd, India) of proper size range. The feeding frequency and quantity were based on visual observation. The animals were reared for 60 days and the average body weight and length were calculated every 7 days. The system was well aerated continuously from an air compressor through air spargers. Evaporation loss in the rearing tank was maintained by the addition of freshwater. The detritus generated in the system was degraded by applying a

probiotic 'DetrodigestTM', containing the bacterium *Bacillus* MCCB101 (10^9 – 10^{12} cells/ml) (Singh *et al.* 2004). For controlling the *Vibrio* population an anti *vibrio* probiotic *Micrococcus* MCCB104 (10^9 – 10^{12} cells/ml) (Jayaprakash *et al.* 2005) was applied. Both were added into the system at the rate of 10 ml once in three days. Water from the rearing tanks were analyzed for physico-chemical and bacteriological parameters. Alkalinity destruction which had occurred due to nitrification of the rearing water was corrected by the addition of CaCO_3 . On completion of the study survival of shrimp was determined by manual counting. The process was repeated twice having each cycle for a period of two months.

Analyses

Crude protein content of the feed was calculated by microkjeldhal method (APHA 1998). Water samples from the larval rearing tanks were analyzed daily for TAN (Solórzano 1969), $\text{NO}_2\text{-N}$ (Bendschneider & Robinson 1952) and $\text{NO}_3\text{-N}$ (Strickland & Parsons 1972) and once in three days for alkalinity, hardness, total suspended solids, total dissolved solids and dissolved oxygen (APHA 1998). Heterotrophic bacterial population and *Vibrio* of the rearing water were enumerated once in a week by standard spread plate method employing ZoBell's Marine agar 2216 E and TCBS agar respectively.

TAN production and conversion rates

Since the system was in complete recirculating mode without water exchange, the source of TAN production in the system was only through feed and excretion by the animals reared. In situ nitrification was neglected, and the total daily TAN production (P_{TAN}) based upon the fish feeding rate was taken as the TAN into the system and calculated using the following Equation (Timmons *et al.* 2001):

$$P_{\text{TAN}} = \text{FA} \times \text{PC} \times 0.092/\text{day} \quad (1)$$

Where,

$$P_{\text{TAN}} = \text{Rate of ammonia production (g TAN d}^{-1}\text{)}$$

$$\text{FA} = \text{Amount of feed per feeding (g)}$$

PC = Protein Content of the Feed (%)

0.092 is the fraction of protein nitrogen that is excreted as TAN.

The volumetric total ammonia nitrogen conversion rate (VTR) is used as the principal indicator for evaluation of the filter performance (Colt *et al.* 2006; Pfeiffer & Malone 2006). The VTR was obtained by using

$$VTR = \frac{K_c(TAN_I - TAN_E)Q_R}{V_b} \quad (2)$$

Where,

VTR = gTAN converted per m³ of filter media per day

Q_R = Flow rate through the filter (L/min)

K_c = Unit conversion factor of 1.44

TAN_I and TAN_E = Influent and effluent ammonia (mg/L)

V_b = Volume of filter media (0.023 m³).

The VTR for each TAN concentration was estimated and regression analysis of TAN versus VTR carried out.

The volumetric biomass capacity of the system was estimated by dividing the biomass (g) by V_{media} (m³) (Colt *et al.* 2006).

Fluorescence in situ hybridization (FISH) analysis of the biofilm

After the operation of four months, the diversity of nitrifiers present in the reactor biofilms was analyzed by FISH. Altogether, 25 beads were taken from the reactors and the biofilm was dislodged using a cyclomixer. The biofilm samples were centrifuged and fixed for fluorescent in situ hybridization (FISH) analysis. The FISH analysis of the biofilm was carried out using a Universal bacterial probe (EUB 338) and nitrifiers specific probes, NSO 190 (ammonia-oxidizing β subclass proteobacteria), NEU (Halophilic and halotolerant members of the genus *Nitrosomonas*), NSV 443 (*Nitrosospira* spp.), NmV (*Nitrosococcus mobilis* lineage), NIT2 (*Nitrobacter* sp.), Ntspa 712 (Phylum Nitrospira) and S-Amx-0820-a-A-22 (Anaerobic ammonium oxidizing bacteria) (Kumar *et al.* 2009a).

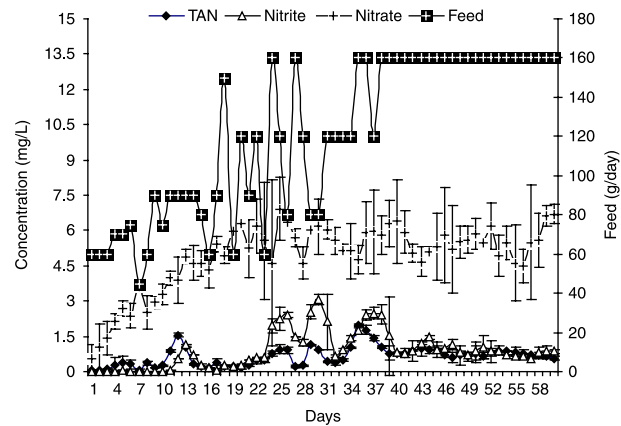


Figure 2 | Progress of nitrification in recirculating *Penaeus monodon* culture system.

RESULTS AND DISCUSSIONS

Nitrification in a laboratory level RAS for *Penaeus monodon*

The progress of nitrification in the RAS with increasing feed rate showed that (Figure 2) TAN, NO₃-N and NO₂-N were within acceptable levels. Dierberg & Kiattisimkul (1996) reported that depending upon prawn stocking density, total pollution loading during an aquaculture production cycle for total phosphorous, nitrogen and suspended solids can be as high as 321, 668 and 215,000 kg/ha per cycle, respectively. In the present study the water quality parameters of the rearing water were maintained without sudden shifts in any of the parameters (Table 1). In a recirculating

Table 1 | Rearing conditions and water quality

Rearing tank volume	1,000 L
Stocking density	1/L ¹
pH	7.5–8.0
Salinity (g/L)	14–15
Water temperature(°C)	28–31
Total suspended solids (mg/L)	10–15
Total dissolved solids (mg/L)	18–21
Dissolved oxygen (mg/L)	5–6
Total alkalinity (mg of CaCO ₃ /L)	64–70
Hardness (mg of CaCO ₃ /L)	2,876–2,900
Total plate count (CFU/ml)	2.51 × 10 ⁵ –4.21 × 10 ⁷
Total <i>Vibrio</i> count (CFU/ml)	75–1,000

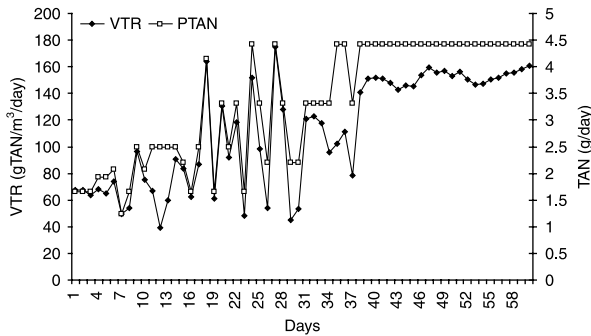


Figure 3 | Volumetric TAN Removal rates and TAN in *Penaeus monodon* recirculation system.

aquaculture system (RAS), the wastes produced have to be removed sufficiently early to avoid deterioration of water quality and to protect cultured species from stressful conditions. The probiotics application was effective in breaking down the detritus indicated by the low TSS and TDS and maintaining the *Vibrio* population below 1,000 cfu/ml. Ammonia, nitrite and nitrate in the culture system were within limits all throughout the experimental period. The TAN concentrations were always below 2 mg/L, where as nitrite concentration recorded a maximum of 3 mg/L on the 30th day of rearing, and were below 1 mg/L in most of the days. The production of $\text{NO}_3\text{-N}$ was found to increase up to a maximum of 6.7 mg/L as observed in our previous experiments (Kumar *et al.* 2009a,c). Nitrification biofilters used in a recirculating aquaculture system (RAS) should be designed based on the ammonia concentration (Wheaton *et al.* 1994). These biofilters must be

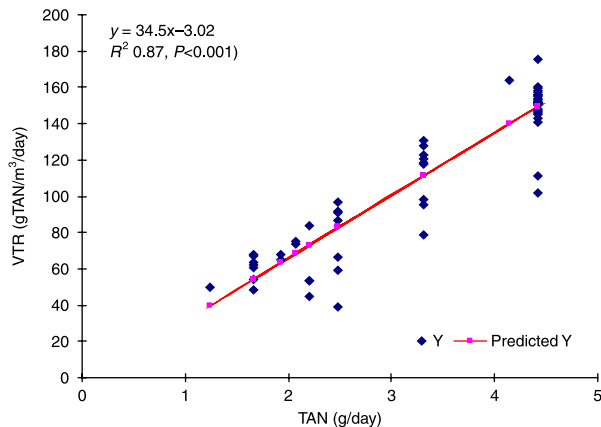


Figure 4 | Regression of Volumetric TAN Removal rates (VTR) versus TAN in *P. monodon* recirculation system- predicted and measured VTR.

able to maintain a high quality of water with sufficiently low ammonium concentration and process TAN at an adequate level to prevent TAN accumulation in the system (Chen *et al.* 2006).

The average Volumetric TAN Removal Rates (VTR) at the feeding rate of 160 g from 54–60th days of culture was 0.1533 ± 0.0045 kg TAN/m³/day (Figure 3). The regression between VTR and TAN explained 87% variability in VTR ($P < 0.001$) (Figure 4). The reported nitrification rates of biofilters varied among systems depending on operating conditions and ammonia loadings. The Volumetric TAN Removal Rates (VTR) is usually used to express the efficiency of the biofilter (Colt *et al.* 2006). Numerous studies have indicated that increasing the TAN concentration in biofilters results in proportional improvements in a filter's conversion ability (Rogers & Klemetson 1985; De Los Reyes & Lawson 1996; Malone *et al.* 1999; Sandu *et al.* 2002). Recommended nitrification rates for fluidized bed filters are 0.7 kg TAN/m³/day for applications in cold water systems and 1.0 kg TAN/m³/day for warm water systems based on a series of pilot scale tests (Timmons *et al.* 2001). However, the nitrification performance of a commercial fluidized sand filter system reported much lower nitrification rates with 0.35–0.49 kg TAN/m³/day in a cold water system (Summerfelt *et al.* 1996) and 0.1 kg TAN/m³/day in a warm water system (Pfeiffer & Malone 2006). The values obtained in the present study are comparable to values suggested by Malone & Beecher (2000). Based on over ten years of floating bead filter research, they (Malone *et al.* 1998; Malone & Beecher 2000) recommended the use of a VTR of 0.035–0.105, 0.07–0.18, and 0.14–0.35 kg TAN/m³/day, for the design of floating bead filters in brood stock, ornamental, and grow-out systems, respectively for warm water systems.

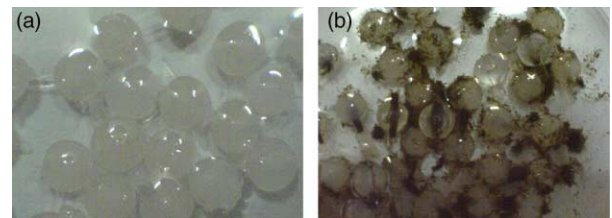


Figure 5 | Polystyrene beads used as support material, (a) without consortia, (b) with biofilms.

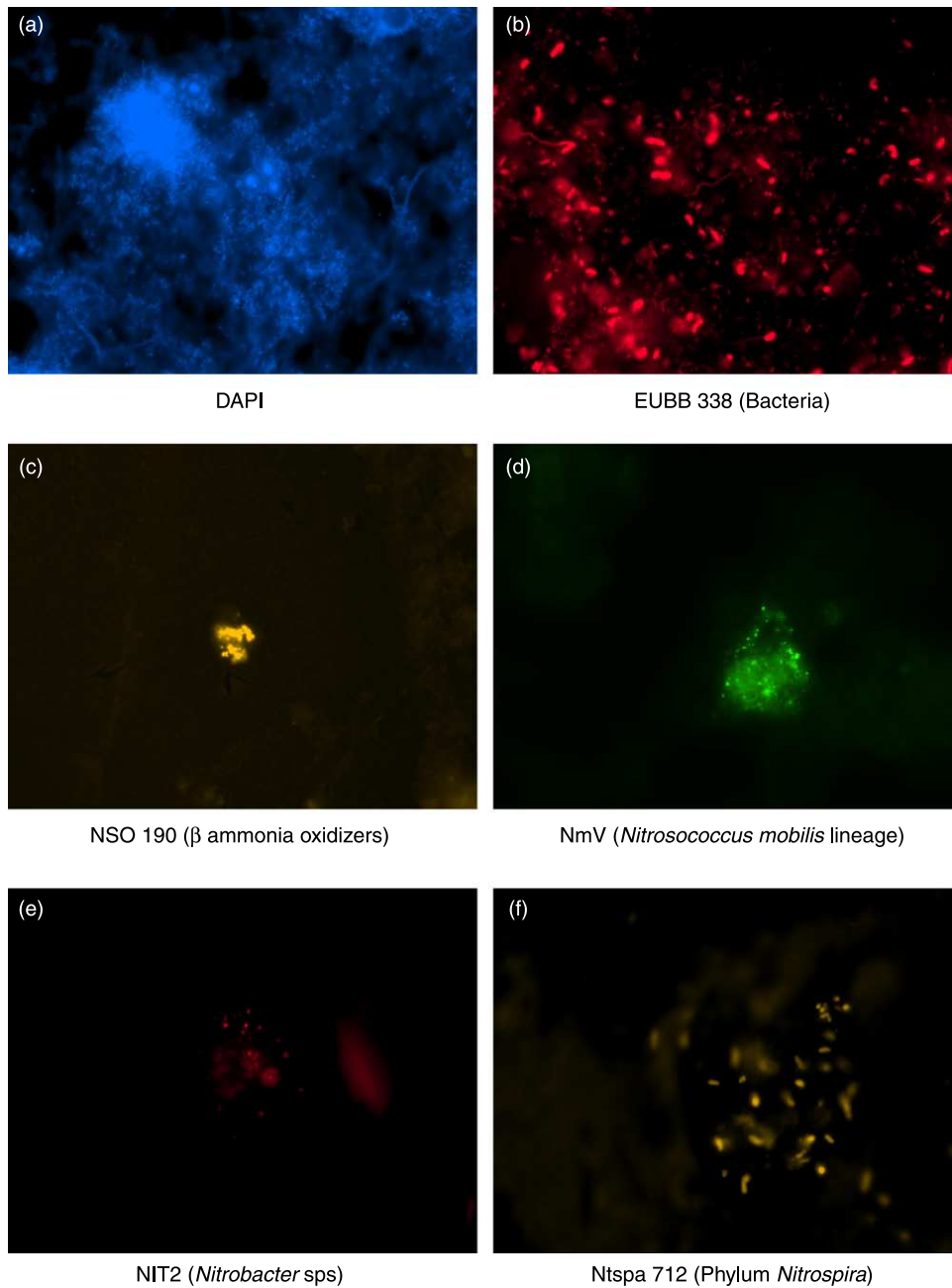


Figure 6 | Fluorescent Insitu Hybridization of a mature biofilm taken from PBBR integrated with *Penaeus monodon* RAS after four months of operation.

The RAS system also supported good biomass with an average yield of 10.3g and average length of the animals was 8cm at the end of 60 day culture, with 60–65% survival. The maximum volumetric biomass capacity of the system was 75g/m³. The main problem observed during the culture period was the cannibalism and the

resultant mortality, therefore, the formulation of a proper diet turns out to be the critical component for RAS. The PBBR developed can form integral parts of organic aquaculture systems as they are specially designed for tropics using indigenous nitrifying bacterial consortia (Singh *et al.* 2004). Currently organic standards are mainly

oriented towards temperate species and it is required to develop organic systems for tropical regions as well.

Fluorescent in situ analysis of the biofilm

Prominent biofilm formation was observed on the beads taken from the reactor after completing an operating period of 4 months (Figure 5a and b). FISH analysis of the biofilms with bacterial probe EUB338 and DAPI staining revealed the diversity of the bacterial cells forming the biofilms (Figure 6a and b). The probes for the β ammonia oxidizers (NSO 190) (Figure 6c), *Nitrosococcus mobilis* lineage (NmV) (Figure 6d), *Nitrobacter* spp (NIT2) (Figure 6e) and for the phylum *Nitrospira* (Ntspa 712) (Figure 6f) have given positive signals from the biofilms. Structure and activity of multiple nitrifying bacterial populations in a biofilm was studied previously by several researchers using FISH probes and microelectrodes (Okabe *et al.* 1999; Schramm *et al.* 2000; Gieseke *et al.* 2003). All the species identified in the biofilm were present in the consortia used for activating the reactors except *Nitrospira*. (Kumar *et al.* 2009a). This proved the usefulness of the activated consortia to establish in to a mature biofilm under real life situation. The *Nitrospira* population observed in the biofilm might have developed from the recirculating water during the time course of operation. This also showed that the plastic beads used as carrier material was well suited for the establishment of nitrifying biofilms in practical sense. Schramm *et al.* (2000) studied the distribution of nitrifying bacteria *Nitrosomonas*, *Nitrosospira*, *Nitrobacter* and *Nitrospira* in a membrane-bound biofilm system with supply of oxygen and ammonium from opposite directions, in which oxic part of the biofilm, which was subjected to high ammonium and nitrite concentration was dominated by *Nitrosomonas europaea* like ammonia oxidizers and by members of the genus *Nitrobacter*, where as *Nitrosospira* and *Nitrospira* were abundant at the oxic-anoxic interface of the biofilm. In the totally anoxic part of the biofilm, cell numbers of all nitrifiers were found relatively low. In the present case the reactor system was operated with O₂ at saturation and a low TAN concentration of maximum of 2 mg/L. Less reports are available for the nitrifying bacterial populations inhabiting the biofilm having a limited supply of the substrates.

Yossi Tal *et al.* (2003) characterized a nitrifying microbial consortium from a moving bed bioreactor (MBB) connected to a marine recirculating aquaculture system using DGGE of amplified 16S rRNA gene fragments. The ammonia oxidizer *Nitrosomonas cryotolerans* and nitrite oxidizer *Nitrospira marina* were found associated with the system as well as a number of heterotrophic bacteria, including *Pseudomonas sp.* and *Sphingomonas sp.* and two *Planctomycetes sp.* were detected in the system suggesting the capability for nitrification, denitrification and annamox in the single system. Similar to this we have also observed a major non-nitrifying population in the biofilm, but the annamox probe gave no positive signals from the biofilm. This may be because of the highly aerated conditions in the system. We also observed a denitrification activity which kept the nitrate concentration always below 10 mg/L.

CONCLUSIONS

In conclusion, the laboratory level RAS exhibited high level performance both in terms of shrimp biomass yield and maintenance of environment quality. The TAN concentration in the system was consistently below 2 and the nitrite below 1 mg/L in most of the days. The substrate removal rate increased linearly with concentration with an average VTR at the feeding rate of 160 g/day was 0.1533 ± 0.0045 kg TAN/m³/day. The regression between VTR and TAN explained 86% variability ($P < 0.001$). The RAS supported relatively higher shrimp biomass with an average yield of 10.3 g and average animal length of 8 cm at the end of 60 day of culture, with an average survival of 60–65%. The maximum volumetric biomass capacity of the system was 75 g/m³. It has been observed that by optimizing the operating conditions such as oxygen, water flow, temperature and nutrient loading the efficiency of the bioreactors could be enhanced for optimal performance. The biofilm on the substrata showed stability in terms of composition of nitrifiers on long term operation.

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