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Activated packed bed bioreactor for rapid nitrification in brackish water hatchery systems

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Abstract A packed bed bioreactor (PBBR) was developed for rapid establishment of nitrification in brackish water hatchery systems in the tropics. The reactors were activated by immobilizing ammonia-oxidizing (AMONPCU-1) and nitrite-oxidizing (NIONPCU-1) bacterial consortia on polystyrene and low-density polyethylene beads, respectively. Fluorescence in situ hybridization demonstrated the presence of autotrophic nitrifiers belong to *Nitrosococcus mobilis*, lineage of β ammonia oxidizers and nitrite oxidizer *Nitrobacter* sp. in the consortia. The activated reactors upon integration to the hatchery system resulted in significant ammonia removal ($P < 0.01$) culminating to its undetectable levels. Consequently, a significantly higher percent survival of larvae was observed in the larval production systems. With spent water the reactors could establish nitrification with high percentage removal of ammonia (78%), nitrite (79%) and BOD (56%) within 7 days of initiation of the process. PBBR is configured in such a way to minimize the energy requirements for continuous operation by limiting the energy inputs to a single stage pumping of water and aeration to the aeration cells. The PBBR shall enable hatchery systems to operate under closed recirculating mode and pave the way for better water management in the aquaculture industry.

Keywords Nitrifying bioreactors · Nitrifying consortia · Immobilization · Nitrification · Closed system shrimp hatchery

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Introduction

On assuming the dimensions of an industry, aquaculture systems are bound to operate under strict environmental safety standards. With high land and water costs, the systems are destined to maintain high biological carrying capacity in relatively little space with minimal water exchange. These requirements led to the advent of recirculating aquaculture systems (RASs) which allowed companies to (1) be competitive in both domestic and world commodity markets by locating production closer to markets, (2) improve environmental control, (3) reduce catastrophic losses due to diseases, (4) avoid violation of environmental regulations on effluent discharge, (5) reduce management and labor costs, and (6) improve product quality and consistency [29].

Driven by the above demands, several attempts have been made to develop and optimize RAS focusing on total ammonia nitrogen (TAN) as the key limiting water quality parameter [18, 31, 33]. The toxic effects of ammonia have been demonstrated for several cultured crustaceans [26, 27, 72] and found more pronounced in early developmental stages. Nitrite is also harmful to larvae as it causes reduction of hemolymph oxyhemocyanin (in *Penaeus monodon*) with concomitant increase in the partial pressure of oxygen (pO_2) in hemolymph and reduced oxygen affinity (P_{50}) [9]. However, it is less toxic than ammonia

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[3], and only under conditions of long-term exposure the toxicity is manifested [69] in the reared animals. Likewise, ammonia and nitrite toxicity in *Macrobrachium rosenbergii* adults and larvae have been investigated by various researchers [7, 8, 43, 65] and felt the need for their regulation for successful larval production. However, nitrate is relatively harmless to the cultured aquatic organisms [62].

In biological ammonia removal systems nitrifying activity of bacteria suspended in seawater has been reported to be extremely low primarily due to their slow growth rate and inhibition of nitrification by free ammonia and nitrite [19]. However, immobilization techniques have been useful to overcome the situation [61] and accordingly, fixed film nitrification biofilters are commonly used for TAN removal in RAS [56, 57, 70]. In such installations attached growth as biofilm offers several advantages over suspended culture-based systems, such as handling convenience, increased process stability to shock loading and prevention of the bacterial population from being washed off [17, 44]. In the light of the emergence of various types of biofilters, a performance rating strategy as well as standards for reporting the performance have been brought out to benefit the customers to choose the most appropriate one [10, 14, 38]. In spite of following such protocols, at least in a few cases, the immobilized nitrifiers in RAS have exhibited low performance, besides demanding too long a start-up period imposing operational difficulties [23, 61]. Therefore, instead of selecting a nitrification system from market it became imperative for the tropics to develop a user-friendly and economically viable technology having the advantages of short start-up time and easiness to integrate to the existing hatchery designs without modifications. Accordingly, a specialized nitrifying packed bed bioreactor (PBBR) (Patent application no. 828/DEL/2000 of 13 September 2000) was developed with indigenous nitrifying bacterial consortia (NBC) and tested for its potential for nitrification in a *M. rosenbergii* seed production system in support of the industry.

Materials and methods

Fabrication of the PBBR

Cross-sectional view of the nitrifying bioreactors (ammonia oxidizing and nitrite oxidizing) connected serially is given in Fig. 1. Both the reactors have the same configuration consisting of shell made of fiberglass with a base of 30 cm^2 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 (airlift pumps) fixed at 10 cm equidistance, is positioned at the base of the reactor. When air gets passed through, the 10 cm^3 area filled with the support medium 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.

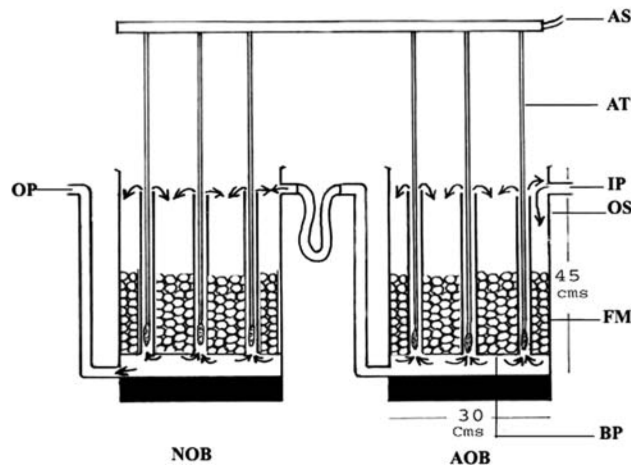

Fig. 1 Cross-sectional view of the bioreactors connected serially (*AOB* ammonia-oxidizing bioreactor, *NOB* nitrite-oxidizing bioreactor, *BP* base plate, *FM* filter media, *OS* outer shell, *IP* inlet pipe, *OP* outlet pipe, *AT* aeration tubes, *AS* air supply)

Based on a previous study [1], polystyrene (PS) and low-density polyethylene (LDPE) were selected as suitable support materials for immobilizing ammonia-oxidizing and nitrite-oxidizing consortia, respectively. This selection was based on percentage consumption of NH_4–N/NO_2–N and production of NO_2–N/NO_3–N by the immobilized nitrifiers on the beads, cost of the raw material and easiness to mold into beads. The beads were having 5 mm diameter and a surface area of 0.785 cm^2 with spikes on the surface. The reactors have been packed with the respective support material; the characteristics are described in Table 1.

Nitrifying bacterial consortia

Two types of NBC, ammonia-oxidizing non-penaeid culture-1 (AMONPCU-1) and nitrite-oxidizing non-penaeid culture-1 (NIONPCU-1), developed by enrichment technique from brackish water systems under perpetual salinity regimes around 15 g/L were used after getting optimized growth and culture conditions [2]. This consisted of simple seawater-based medium having 15 g/L salinity supplemented with 10 mg/L substrate ($(NH_4)_2 \cdot SO_4/NaNO_2$), 2 mg/L KH_2PO_4 at an optimum temperature of 28°C and pH 8.5 for ammonia and 7.5 for nitrite oxidizers. After harvesting, the cultures were maintained at 4°C with

Table 1 Filter and media characteristics of the PBBR

Filter height (m)	0.45
Water height (m)	0.40
Water discharge height (m)	0.35
Filter volume (L)	40
Total surface area of media (m^2)	4.71
Total media volume (m^3)	0.023
Specific surface area (m^2/m^3)	205

periodic addition of the substrate ($(NH_4)_2 \cdot SO_4/NaNO_2$) and adjustment of pH (using 1% Na_2CO_3) to the optimum. For generating sufficient biomass in order to facilitate their immobilization in the reactors, both the consortia were acclimated to room temperature (27 ± 0.5°C) in 250 mL conical flasks on a shaker for 7 days, amplified in a 2 L baby fermentor for 1 month and subsequently mass produced in an indigenous NBC production unit (NBCPU) under optimum pH, temperature and salinity. The NBCPU consists of a 200 L fermentor vessel made of polyethylene, fixed with a central 0.5 HP AC/DC agitator (500 W, 0–500 rpm). Provisions have been given for (a) temperature regulation employing a thermo-circulator, (b) pH probe insertion, (c) addition of medium, (d) supply of filter sterilized air and (e) harvesting matured consortium [28].

Fluorescence in situ hybridization (FISH) of the consortia

As a preliminary characterization, FISH analysis of the consortia was carried out using seven different nitrifier-specific 16S rRNA-targeted oligonucleotide probes labeled with Cy3, Cy5 or fluorescein (Table 2). The fluorescent oligonucleotide probes were purchased from Thermo Electron Corp. (Germany). The specificity and the hybridization conditions were confirmed with 'Probebase' [32]. Actively growing consortia, harvested by centrifugation, were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) containing KH_2PO_4 and NaCl, prepared in diluted seawater having salinity 15 g/L. The samples were stored at −20°C in a 1:1 mixture of PBS:ethanol until further processing. Hybridizations were performed on six-well Teflon-coated slides (Electron Microscopy Sciences, USA). Prior to the hybridization, the slides were coated with poly-L-lysin, 10 μL of the fixed consortia were spread on to the well, dried at 46°C for 10 min, and dehydrated by successive passage through 50, 80 and 98% ethanol (3 min each). Working solutions of the probes were prepared to obtain a final concentration 5 pmol/μL for CY3/5 and 8.3 pmol/μL for fluorescein-labeled probes. Hybridization buffer (2 mL) containing 360 μL 5 M NaCl, 40 μL 1 M Tris–HCl (pH 8.0), 4 μL 10% SDS and formamide were added according to the probe used (Table 2). For hybridization, 10 μL hybridization buffer was dispensed into the wells, and then 1 μL probe stock solution was added. A hybridization tube was prepared by folding a tissue paper into a 50 mL falcon tube into which the remainder of the hybridization buffer was dispensed. After the addition of probes, the slides were immediately transferred into the hybridization tube and incubated for 1.5 h at 46°C in a hybridization oven (Thermo Electron Corp.). Washing buffer containing 1 M Tris/HCl, 5 M NaCl and 0.5 M EDTA at pH 8 was prepared as per the formamide concentration in the hybridization buffer in a separate 50 mL Falcon tube and made up to 50 mL by adding MilliQ. Finally, 50 μL of 10% (w/v) SDS was added and the washing buffer was preheated at 48°C in a water bath. On elapse of the

Table 2 Oligonucleotide probes and hybridization conditions applied in this study

Probe	Target organisms	Probe sequence (5′–3′)	Fluorescent dye used for labeling	FA[a] (%)	NaCl[b] (mM)	Reference
NSO190	Ammonia-oxidizing β subclass *proteobacteria*	CGATCCCCTGCTTTTCTCC	CY3	55	20	[41]
NEU	Halophilic and halotolerant members of the genus *Nitrosomonas*	CCCCTCTGCTGCACTCTA	CY5	40	56	[66]
NSV443	*Nitrosospira* spp.	CCGTGACCGTTTCGTTCCG	CY3	30	112	[41]
NmV	*Nitrosococcus mobilis* lineage	TCCTCAGAGACTACGCGG	Fluorescein	35	80	[49]
NIT2	*Nitrobacter* species	CGGGTTAGCGCACCGCCT	Cy5	40	56	[67]
Ntspa712	Phylum *Nitrospira*	CGCCTTCGCCACCGGCCTTCC	CY3	50	28	[11]
S-Amx-0820-a-A-22	Anaerobic ammonium-oxidizing bacteria	AAAACCCCTCTACTTAGTGCCC	Fluorescein	40	56	[54]

[a] Percentage of formamide in the hybridization buffer

[b] Concentration of the sodium chloride in the washing buffer

incubation period, the hybridization slides were taken out and rinsed and transferred to the washing buffer, where the slides were incubated for 10–20 min at 48°C. After the incubation the slides were rinsed with MilliQ water and dried. The cells were counter stained with DAPI having the final concentration of 0.2 µg/mL for 1 min, washed, dried and added an anti-fading mounting fluid (Vectashield, Vector Laboratories Inc., Burlingame, CA). The slides were observed under Olympus BX 51 epifluorescent microscope equipped with a monochromatic camera (Evolution VF, Media Cybernetics Inc., MD, USA). Images were processed using the "Image pro-express" software (Media Cybernetics Inc., MD, USA).

Activation of the reactors with NBC

The beads (substratum) were immersed in 0.1 N HCl for 3 h, washed with 10% Extran (Enviroeuip, Sydney, Australia), rinsed with tap water followed by distilled water and air dried. The reactor 1 was filled with 60,000 PS beads and the reactor 2 with the same number of LDPE. The ammonia-oxidizing and the nitrite-oxidizing consortia (20 L each) were introduced into the reactors 1 and 2, respectively, and airlift pumps operated by supplying 1 L/min to effect adequate circulation of the culture through the beads and to assure supply of O_2 and CO_2 for activation. Optimum culture conditions, as described under "Nitrifying bacterial consortia", were maintained in each reactor during the activation period. The substrate concentrations (NH_4–N/NO_2–N) in both the reactors were made up to 10 mg/L daily by the addition of aqueous ammonium sulfate or sodium nitrite. Evaporation loss was made up by adding distilled water daily.

Integration of the bioreactors into *M. rosenbergii* seed production system

The facility used consisted of two larval rearing tanks of 5,000 L capacity, one integrated with the activated reactors and the other without any, used as control. Chlorinated–dechlorinated seawater (salinity 15 g/L) was used for all the experiments. The tanks were initially filled with 2,000 L seawater, freshly hatched mysis of *M. rosenbergii*, dipped in 0.025 mg/L formalin (SRL, Mumbai, India) for 20 s, 0.03 mg/L iodophore (Growel Formulations, Hyderabad, India) for 20 s and washed in running seawater, were introduced into the tanks at a stocking density of 0.2 million per tank.

The process flow diagram of the experimental system is given in Fig. 2. The ammonia-oxidizing and nitrite-oxidizing reactors were connected serially. The influent from the rearing tank was pumped into an overhead tank (282 L)

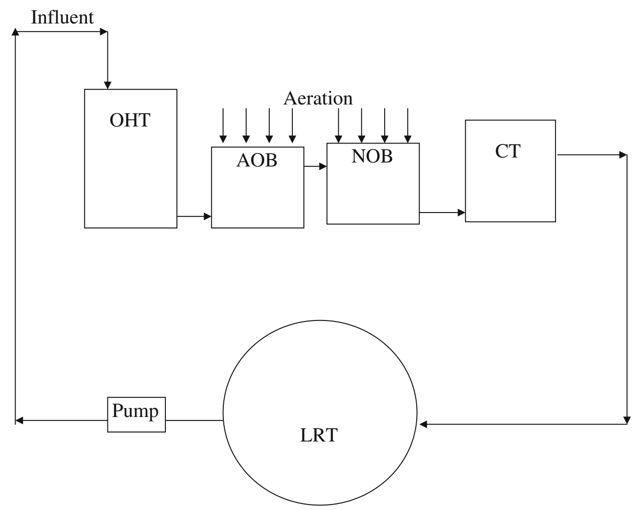

Fig. 2 Process flow diagram of the reactors integrated into the larval rearing tank (*OHT* overhead tank with automatic water level controller, *AOB* ammonia-oxidizing bioreactor, *NOB* nitrite-oxidizing bioreactor, *CT* collection tank for the treated water, *LRT* larval rearing tank)

from where water flowed through the two reactors serially by gravitation and got collected in a 140 L collection tank, from where the treated water got into the larval rearing tank. 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. A regulator valve was connected to the overhead tank to maintain a flow rate of 4 L/min to the system attaining a total circulation of 5,760 L/day.

During the experiment, the rearing water was supplemented with 1 mg/L EDTA (Matrix Formulations, Hyderabad, India), 5 mg/L sulfated vitamin C (Matrix Formulations, Hyderabad, India) and 1 mg/L treflan (Growel Formulations, Hyderabad, India). The larvae were fed with freshly hatched *Artemia* nauplii up to stage 9 (when pleopods with setae appear) and with both *Artemia* nauplii and egg custard subsequently. The experiment was continued for 17 days till the larvae metamorphosed to post-larvae, and repeated three times for concurrent results. At the end of the experiment the survival was estimated by counting the larvae manually and the relative percentage survival (R.P.S.) was estimated as the following equation [21]:

$$\text{R.P.S.} = [1 - (\%\,\text{mortality in the test tank}/\ \%\,\text{mortality in the control tank})] \times 100$$

In another experiment, the reactor was tested for its nitrification potential in spent water after the larval culture. Water from the larval rearing tanks, subsequent to harvest of post-larvae, was collected and stored in a 5,000 L capacity storage tank. This was subsequently circulated through the bioreactor assembly at a rate of 2 L/min. Meanwhile, another system without integration of the reactor was kept as the control. The experiment was repeated three times.

Analyses

When the reactors were in the activation mode, substrate/product levels were determined daily by estimating ammonia (TAN) [59], nitrite (NO_2–N) [5] and nitrate (NO_3–N) [60]. The nitrifying biomass was determined gravimetrically by passing 10 mL bacterial suspension from the reactors through pre-weighed cellulose acetate syringe filters of 0.22 µm porosity with a diameter of 13 mm. Water samples from the larval rearing tanks were analyzed once in 3 days for alkalinity, hardness [4], ammonia, nitrite and nitrate as above. The heterotrophic bacterial community of the rearing water was determined once in a week by standard spread plate method employing ZoBell's Marine Agar 2216 E prepared in diluted seawater of salinity 15 g/L.

In spent water nitrification experiments, water quality parameters such as phosphate, sulfate, iron, chloride, dissolved oxygen, BOD [4], ammonia, nitrite and nitrate as above were estimated for 8 days.

Statistical analyses

The relationship between removal of ammonia and nitrite and the biomass in suspension during the activation mode was estimated by simple correlation coefficient analysis. The nitrification efficiency and significant percent survival of larvae in the control and reactor integrated tanks were estimated by one-way analysis of variance. Least significant difference (LSD) at 0.1% level was calculated for delineation of the two treatments.

Results

FISH of the consortia

Fluorescence in situ hybridization analysis of the two NBC with seven nitrifying bacterial specific probes confirmed the presence of autotrophic nitrifiers (Fig. 3). Most of the nitrifiers observed were in the form of aggregates. FISH of AMONPCU-1 revealed presence of the autotrophic ammonia oxidizer, *Nitrosococcus mobilis*, lineage of β ammonia oxidizers, and that of NIONPCU-1 the autotrophic nitrite oxidizer, *Nitrobacter* sp. However, *Nitrosomonas*, *Nitrosospira*, anaerobic ammonia oxidizers

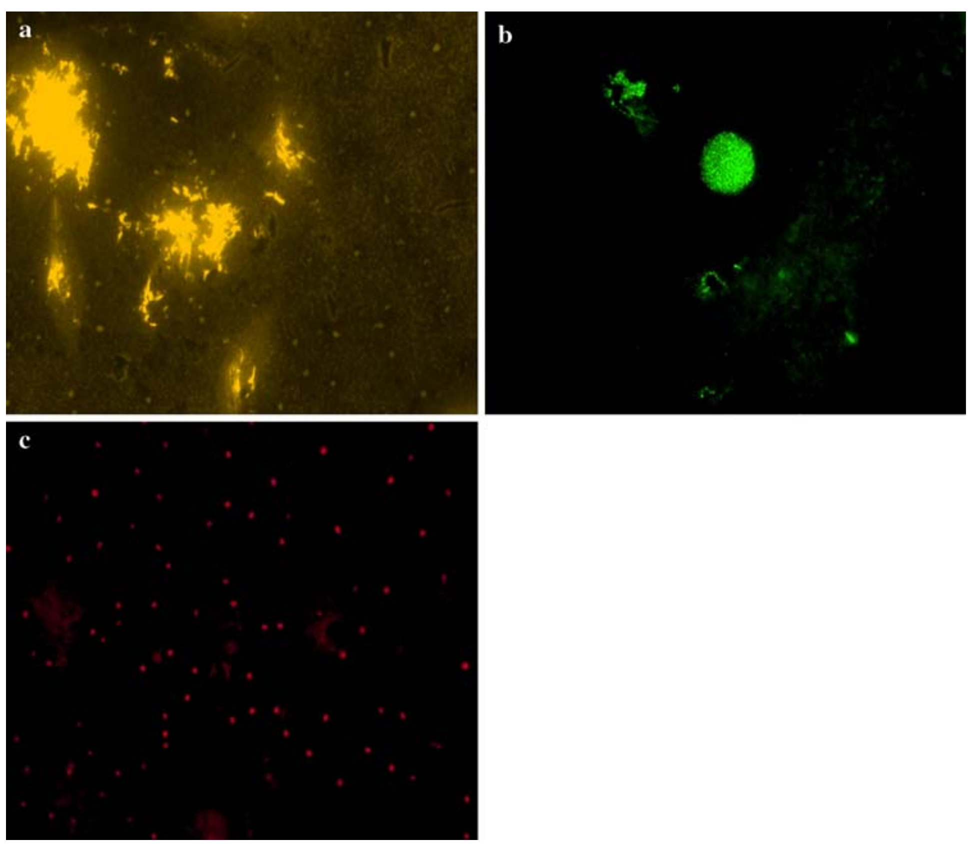

Fig. 3 Fluorescence in situ hybridization of the nitrifying bacterial consortia (**a** epifluorescent image of AMONPCU-1 with CY3-labeled probe NSO190 targeting β ammonia oxidizers; **b** epifluorescent image of AMONPCU-1 with fluorescein-labeled probe NmV targeting *Nitrosococcus mobilis* lineage; **c** epifluorescent image of NIONPCU-1 with CY5-labeled probe NIT2 targeting *Nitrobacter* sp.)

and nitrite oxidizers belonging to phylum *Nitrospira* were not detected in both the consortia.

Activation of the reactors

Activation kinetics of PBBR during the period of immobilization of the consortia, AMONPCU-1 and NIONPCU-1, are presented in Fig. 4. In both the reactors nitrification could be established within 24 h of initiation of the process and there was progressive reduction in the suspended biomass and increase in NO_2–N and NO_3–N, respectively. The system was monitored for 7 days, during which there was reduction of more than 90% of the bacterial biomass from the activation medium with 78% TAN and 75.3% NO_2–N removal. There was negative correlation between the percentage removal of TAN ($r = -0.96$, $P < 0.01$), NO_2–N ($r = -0.93$, $P < 0.01$) and the suspended biomass. Average ammonia and nitrite removal rates in the reactor at the end of the activation period were 46.82 mg TAN/(m^2 day) and 45.14 mg NO_2–N/(m^2 day).

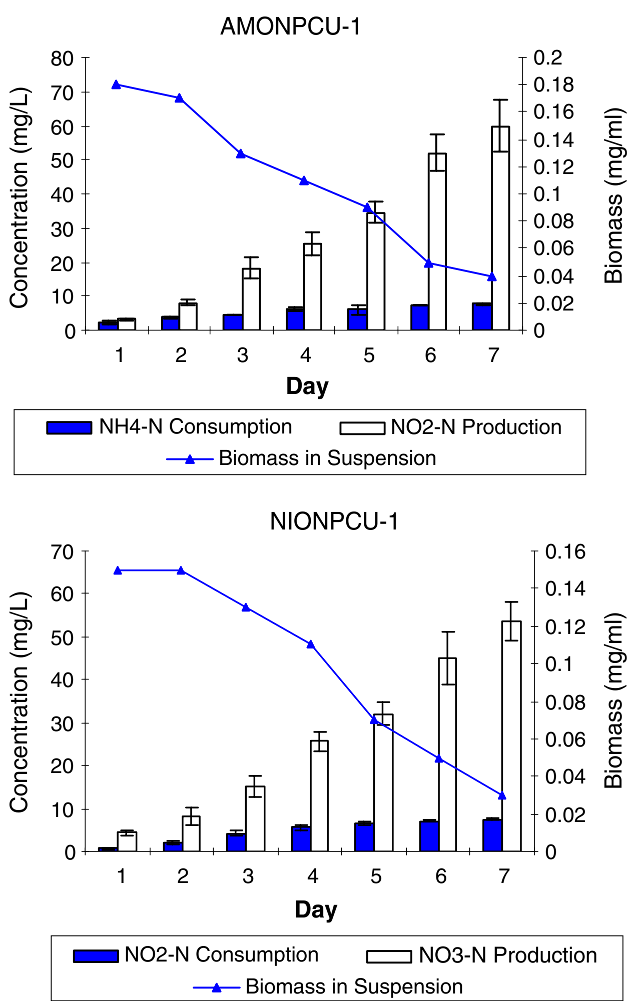

Fig. 4 Activation kinetics of PBBR immobilized with nitrifying consortia developed for *M. rosenbergii* hatchery systems

Integration of the reactors into the hatchery system

The minimum and maximum values of pH, temperature, salinity, alkalinity, hardness and total bacterial count in the rearing water of the experimental and control tanks during each treatment are summarized in Table 3. Heterotrophic bacterial community expressed as colony forming units (CFUs) in ZoBell's Marine Agar in the control tank increased substantially and there was no remarkable difference in the other water quality parameters between the tanks. The extent of nitrification during the period is presented in Fig. 5. In the control tanks TAN exhibited progressive increase with its subsequent decline and concomitant increase of NO_2–N after 14 days; however, NO_3–N was never found built up in the system. Meanwhile, there was significant TAN removal ($P < 0.01$) in the experimental tanks with significant ($P < 0.05$) NO_2–N removal. Within 8 days both TAN and NO_2–N concentrations were below detectable levels. NO_3–N exhibited progressive increase to 7.6 mg/L within 17 days of the experiment.

The overall percent survival of larvae in the control and test tanks was estimated and presented in Table 4. The tank with the reactor exhibited significantly higher ($P < 0.001$) percentage survival (LSD at 0.1% = 15.19) with an average R.P.S. of 22.86%.

The average water quality parameters of the spent water are given in Table 5. TAN, NO_2–N and NO_3–N were lower in the experimental tanks than in those of the controls (fourth day) indicating higher percentage removal of TAN (78%), NO_2–N (79%) and BOD (56%).

Discussion

Proper selection and sizing of biofilters are critical to the technical and economic viability of RAS [38]. In saltwater systems RAS plays an important role in the production of healthy and properly sized fingerlings [16] and has significant implications in maintaining the required water quality as the system demands operations under oligotrophic conditions. The PBBR described here are packed with plastic media having specific surface area of 205 m^2/m^3. This is comparable to those in trickling filters used in aquaculture [25]. The plastic beads with spikes on the surface provide high void ratios that avoid clogging [15] increase the aeration within the system; poor aeration reduces nitrification capacity of the biofilter [71]. As a matter of fact most of the biofilters on recirculation systems have been focusing on aerobic fixed films [30, 52, 58] and in several systems plastic media used to be the substrata for

Table 3 Physico-chemical and microbial quality of rearing water in the hatchery during the experiment

Water quality parameters	Test tank	Control tank
pH	7.5–8.0	7.5–8.0
Salinity (g/L)	14–15	14–15
Temperature (°C)	28–31	28–31
Alkalinity (mg $CaCO_3$/L)	64–70	66–70
Hardness (mg $CaCO_3$/L)	2,876–2,900	2,987–2,900
Total heterotrophic community in ZoBell's Marine Agar (CFU/mL)	2.51×10^5 to 4.21×10^7	1.91×10^5 to 1.14×10^9

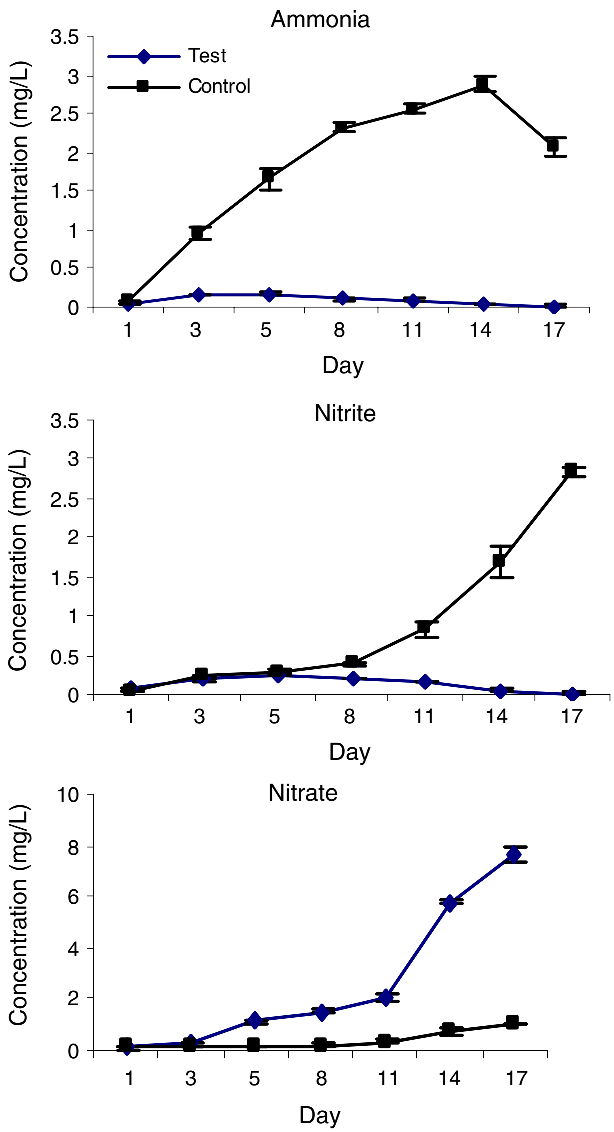

Fig. 5 Nitrification in *M. rosenbergii* hatchery system integrated with PBBR

immobilization [22, 52, 56]. In the present case plastic beads have been used due to its reusability and inertness besides the cost factor and preferential acceptability by the NBC for attachment and growth.

The NBC used here originated from a brackish water environment by enrichment with confirmed nitrification potential and designated as AMONPCU-1 and NIONPCU-1 [2]. Transmission electron microscopic observations demonstrated characteristic cyst formation and intracytoplasmic membranes similar to autotrophic nitrifiers [28]. Using FISH the consortia could be partially characterized, demonstrating the presence of *N. mobilis*, lineage of β ammonia oxidizers and *Nitrobacter* sp. in AMONPCU-1 and NIONPCU-1, respectively. Where as, other nitrifiers such as *Nitrosomonas*, *Nitrosospira*, phylum *Nitrospira*, and anaerobic ammonia oxidizers were not observed. In literature oligonucleotide probe-based FISH, targeting signature regions of the 16S rRNA of ammonia and nitrite-oxidizing bacteria, has been successfully applied for phylogenetic identification in environmental and engineered systems [24, 41, 45, 55, 68]. Rowan et al. [51] studied the composition and diversity of ammonia-oxidizing bacterial communities in a biological aerated filter (BAF) and a trickling filter and all the samples analyzed appeared to be dominated by AOB most closely related to *N. mobilis*.

As a general principle low concentration of nutrients in aquaculture systems [48] results in slow growth of nitrifiers and low bacterial yield to form effective biofilm by natural process. This necessitates activated bioreactors with high attached bacterial density for optimal performance. Under such situations the time required for activating the reactors becomes a crucial factor for their successful and timely starting up and operation. To satisfy this requirement, NBC were used for activating the reactors by which nitrification could be established within 24 h of initiation and attained 78% ammonia and 75.3% nitrite removal by the seventh day. Attachment of the NBC and formation of biofilm were irreversible, which demonstrated the soundness of the technology. Wherever such activations had not been carried out, 2–3 months were reported for the establishment of nitrification in marine [39] and 2–3 weeks in freshwater systems [40].

On integrating the PBBR to the larval rearing system, ammonia oxidation was established within a day and it took 8 days for nitrite oxidation. Meanwhile, in the control larval rearing systems 14 days were required for the

Table 4 Larval survival after the integration of PBBR into the *M. rosenbergii* hatchery system

Treatment	% survival	Average % survival	Relative % survival	ANOVA			
				Source of variation	*df*	Mean sum of squares	*P*
Control tank	18	18.33 ± 1.53	22.86	Between treatments	1	522.67	<0.001
	17						
	20						
Test tank	36	37 ± 2.65		Within treatment	4	4.67	
	35						
	40						

Table 5 Mean water quality parameters of the spent water from hatchery during the experiment

Parameter	Control tank	Test tank
Salinity (g/L)	15.33 ± 0.47	15 ± 0.41
pH	8.09 ± 0.07	8.36 ± 0.12
Eh	110 ± 0.82	104.33 ± 16.46
Ammonia (mg/L)	3.71 ± 0.43	0.83 ± 1.46
Nitrite (mg/L)	2 ± 0.23	0.43 ± 0.55
Nitrate (mg/L)	0.3 ± 0.09	13.28 ± 6.57
Phosphate (mg/L)	0.03 ± 0.04	0.02 ± 0.01
Sulfate (mg/L)	15.93 ± 1.23	13.35 ± 0.37
Alkalinity (mg $CaCO_3$/L)	79.33 ± 0.94	79.83 ± 5.34
Hardness (mg $CaCO_3$/L)	2,483.33 ± 107.81	2,672.83 ± 134.64
Chloride (mg/L)	10,273.513 ± 295.31	10,505.83 ± 504.00
DO (mg/LO_2)	5.73 ± 0.39	5.81 ± 0.64
BOD (mg/LO_2)	0.71 ± 0.018	0.31 ± 0.29

initiation of nitrification. The delay in establishing active nitrite oxidation in the reactor integrated system suggests a consequence of lower multiplication rate of nitrite oxidizers compared to that of ammonia oxidizers [47]. Under the 'nitrifying bioreactor integrated mode' the maximum average TAN and NO_2–N concentrations in the larval rearing tanks were 0.18 and 0.25 mg/L, respectively, the values typical of any marine system. It has to be emphasized that marine larval rearing systems demand TAN and NO_2–N levels below 0.1 mg/L well below the maximum limit (0.3 mg N/L) under the oligotrophic category [37]. During the progression of the experiment the NO_3–N concentrations increased progressively up to 7.6 mg/L, however, it remained well below the toxic levels for *M. rosenbergii* larval culture [34]. Management of ammonia in the larval rearing systems of *M. rosenbergii* is important as significantly lower survival rates (0–20%) of larvae were noticed at total ammonia concentrations ranging from 1 to 8 mg/L with 0.43–3.41 mg/L non-ionic ammonia at pH 9 [35]. The higher relative percent survival (22.86%) obtained in the reactor integrated experimental system proved the impact of the technology in enhancing the larval survival.

Under oligotrophic conditions ammonia diffuses into a relatively thin vertically homogenous biofilm that is dominated by autotrophs, principally due to low BOD (<5 g/m^3) of the culture water [36]. Such a situation could be observed here where organic loading to the system was as low as 0.31 mg/L BOD. On the basis of the above BOD–nitrification relationship, it may be inferred that there has been minimal heterotrophic inhibition of nitrification [53, 73] in the reactors as also evidenced by the progressive increase in the rate of nitrification from the day of initiation. Since nitrification reactions occur in the biofilm and not in the bulk fluid [42], the substrate utilization rate depends on local substrate concentrations within the biofilm. At such local reaction sites, reactant concentrations are depressed and products elevated [6]. This warranted circulation of water through the cartridge with the biofilm, and the rate of TAN removal could be theoretically proportional to the rate of circulation. That was why the reactors were designed to have both vertical and horizontal flow of water through the aeration cells for providing oxic conditions. Zhu and Chen [74] established that the turbulence caused by diffused air substantially improved the nitrification efficiency of fixed film biofilters. Tschui et al. [63] ascertained that nitrification rates could be increased with increased water velocities along with increased airflow. This sort of stronger turbulence in the reactor cartridge decreases the laminar boundary layer and simultaneously enhances diffusion. Such highly aerated systems reduce the chances for the generation of anaerobic pockets with in the reactor.

RAS adoption for larvae, fry and fingerling production is driven by bio-security issues [46, 50] and water recirculation dramatically reduces the possibility of pathogen introduction [12, 20]. In this context integration of PBBR for nitrification of hatchery spent water with high percentage removal of ammonia (78%), and nitrite (79%) by fourth day strengthens the possibility of reuse of water with limited discharge and reduced intake paving the way for bio-security.

Packed bed biofilter systems have been utilized in a variety of formats for recirculating shrimp production systems because of their economic feasibility [13, 64]. The PBBR designed and evaluated here was configured in such a way that the flow of water could be maintained by gravitational force and the energy needed could be restricted to pumping water to the reservoir tank and to operate an air pump to effect aeration. If nitrification is not completed during a single circulation, there is provision to recirculate it through the treatment system over and again. However, such requirements might be overcome by increasing the biomass of the nitrifying consortia used for activation of the reactors or by enhancing the hydraulic retention time. Another specialty of the package is upgradeability of the system with different types of filters for removal of particulate matter and UV disinfection equipment for elimination of pathogens which might enter the system accidentally.

The PBBR evaluated here shall enable hatchery systems to operate as closed recirculation systems, maintaining water quality during the operation and minimizing discharge of spent water. Collectively the technology shall pave way for better water management in the aquaculture industry. Besides, by integrating the reactors during larval production significantly high percentage larval survival also could be obtained. The PBBR designed here is flexible as it is interchangeable between prawn (salinity 15 g/L) and shrimp (salinity 30 g/L) larval rearing systems by replacing the NBC depending on the salinity [2]. A modification of the system can be used for shrimp maturation facility too as recirculation is one of its prime requirements for maturation in the perspectives of water quality and biosecurity.

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Stringed bed suspended bioreactors (SBSBR) for in situ nitrification in penaeid and non-penaeid hatchery systems

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Abstract For establishing nitrification in prawn (non-penaeid, salinity 10–15 ppt) and shrimp (penaeid, salinity 30–35 ppt) larval production systems, a stringed bed suspended bioreactor (SBSBR) was designed, fabricated, and validated. It was fabricated with 5 mm polystyrene and low density polyethylene beads as the substrata for ammonia and nitrite oxidizing bacterial consortia, respectively, with an overall surface area of 684 cm^2. The reactors were activated in a prototype activator and were transported in polythene bags to the site of testing. Performance of the reactors activated with the nitrifying bacterial consortia AMONPCU-1 (ammonia oxidizers for non-penaeid culture) and NIONPCU-1 (nitrite oxidizers for non-penaeid culture) was evaluated in a *Macrobrachium rosenbergii* larval rearing system and those activated with AMOPCU-1 (ammonia oxidizers for penaeid culture) and NIOPCU-1 (nitrite oxidizers for penaeid culture) in a *Penaeus monodon* seed production system. Rapid setting up of nitrification could be observed in both the static systems which resulted in a higher relative per cent survival of larvae.

Keywords Closed system shrimp hatchery · Immobilization · Nitrification · Nitrifying bioreactors · Nitrifying consortia · Shrimp/prawn larval production

Abbreviations

AMONPCU-1	Ammonia oxidizers for non-penaeid culture
AMOPCU-1	Ammonia oxidizers for penaeid culture
IHHNV	Infectious hypodermal and hematopoietic necrosis virus

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NBCPU	Nitrifying bacterial consortia production unit
NIONPCU-1	Nitrite oxidizers for non-penaeid culture
NIOPCU-1	Nitrite oxidizers for penaeid culture
RAS	Recirculating aquaculture systems
RPS	Relative percent survival
SBSBR	Stringed bed suspended bioreactor
TAN	Total ammonia nitrogen
TNN	Total nitrite nitrogen
WSSV	White spot syndrome virus
YHV	Yellow head virus

Introduction

High levels of ammonia and nitrite undermine commercial production objectives in the aquaculture industry as the toxic impacts are manifested through impaired growth or chronic diseases (Manthe et al. 1985; Cheng et al. 2004). The toxic effects of ammonia are more pronounced during the early developmental stages (Wajsbrot et al. 1993; Guillen et al. 1994) as it has been demonstrated that the tolerance of larval *Penaeus monodon* to ammonia increases as the larvae metamorphose to postlarvae. A conservative estimate of the "safe level" of ammonia for rearing larval *P. monodon* on the basis of an estimated 96 h LC_{50} was 0.01 mg l^{-1} NH_3–N (Chin and Chen 1987). Nitrite is reported to cause reduction of hemolymph oxyhemocyanin in *P. monodon* with a concomitant increase of pO_2 and P_{50}, indicating reduced affinity to oxygen (Cheng and Chen 1999). Likewise, ammonia and nitrite toxicity in *Macrobrachium rosenbergii* adults and larvae have been investigated by various researchers (Chen and Lee 1997; Cavalli et al. 2000; Wang et al. 2004; Naqvi et al. 2007) who felt the need for their regulation for successful larval production. Other than water exchange this can be achieved only through biological nitrification, for which it becomes essential to establish a stable nitrifying bacterial system for converting ammonia to nitrate as a mitigation measure of ammonia and nitrite toxicity. Nitrate is relatively harmless to finfish and shellfish (Russo and Thruston 1991).

In aquaculture systems, the serious issue of ammonia and nitrite toxicity can be addressed using rotating biological contactors, trickling biofilters, and fluidized bed bioreactors in RAS mode (Jewell and Cummings 1990; Nijhof and Bonverdeur 1990; Yang et al. 2001). Among them, fixed film biofilters are commonly used for TAN removal (Greiner and Timmons 1998; Singh et al. 1999). Recently, they have been reviewed (Eding et al. 2006; Colt 2006; Gutierrez-Wing and Malone 2006) and rating standards developed (Colt et al. 2006; Malone and Pfeiffer 2006). In such systems, polystyrene micro-bead packed beds are successfully used by several commercial growers (Timmons et al. 2006).

However, for establishing instantaneous nitrification in static larval production systems, a specialized nitrifying bioreactor other than the above was found to be required. This paved the way for the development of Stringed Bed Suspended Bioreactor (SBSBR) (PCT Patent application no. 828/DEL/2000/India), a technology commercialized through M/s Oriental Aquamarine Biotech India (P) Ltd., Coimbatore, India. In this device, specially designed polystyrene and polyethylene beads have been used as the substratum for immobilizing nitrifying bacterial consortia. The reactors were activated under laboratory conditions, transported to the prawn/shrimp hatchery systems, and tested and validated for their potential for rapid setting up of nitrification.

Materials and methods

Configuration of stringed bed suspended bioreactor

The reactor has four components: (1) an outer shell of 10 cm^3 with conical bottom; (2) an inner cartridge comprising a solid frame work and beads on strings with filter plates both at its top and bottom; (3) an airlift pump at the center of the filter plates; and (4) a black lid with perforations on top. Based on a previous study by Achuthan (2000), polystyrene and low density polyethylene beads of 5 mm diameter and a surface area of 0.785 cm^2 with spikes on the surface had been selected as the substrata for immobilizing ammonia and nitrite oxidizing consortia, respectively. On full assembly (Fig. 1), the cartridge with beads was inserted into the outer shell and the black lid was placed on top. The beads stringed in the reactor cartridge provided an overall surface area of >684 cm^2 to support the nitrifying biofilm. This is in addition to the inner surface of the shell and cartridge framework.

The nitrifying bacterial consortia

Two ammonia oxidizing consortia, namely AMOPCU-1 (ammonia oxidizers for penaeid culture) and AMONPCU-1 (ammonia oxidizers for non-penaeid culture), and two nitrite oxidizing consortia, namely NIOPCU-1 (nitrite oxidizers for penaeid culture) and NIONPCU-1 (nitrite oxidizers for non-penaeid culture) (Achuthan et al. 2006) were used

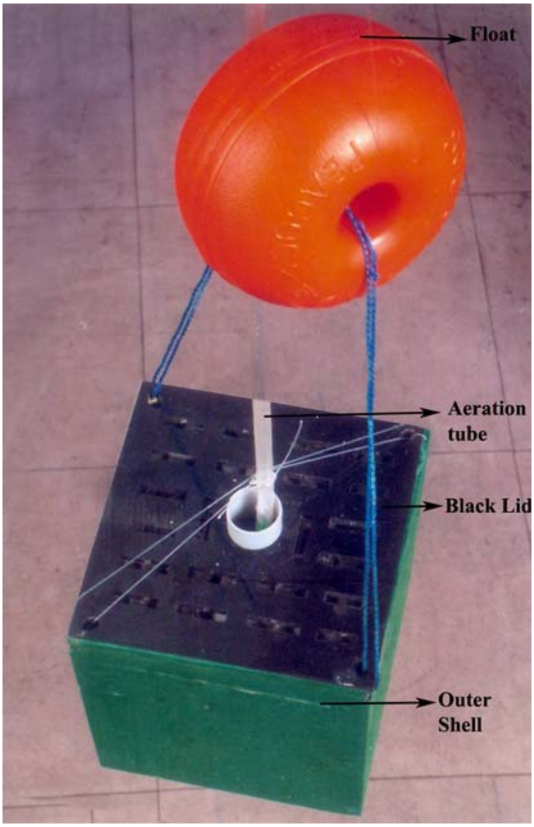


Fig. 1 Stringed bed suspended bioreactor on full assembly

for activating the reactors. The consortia were acclimated to room temperature (27 ± 0.5°C) from refrigeration (4°C) in 250-ml conical flasks on a rotary shaker for 7 days, and subsequently amplified in a 2-l baby fermentor (New Brunswick, USA) at 28°C for 1 month under optimum pH (8.5 and 7.5) for ammonia and nitrite oxidizers, respectively. This was used as the inoculum for mass production in an indigenous 200-l nitrifying bacterial consortia production unit (NBCPU) under the same conditions (unpublished). A simple seawater base medium (100 ml) supplemented with 10 mg l^{-1} substrate ($(NH_4)_2 \cdot SO_4/NaNO_2$) and 2 mg l^{-1} KH_2PO_4 was used. The reactors for penaeid hatchery systems were activated maintaining salinity optima of 25–30 ppt, and non-penaeid systems under salinity of 10–15 ppt.

Activation of the reactors with nitrifying bacterial consortia

For activation, the lid of the reactor was removed and the void volume filled with an aliquot of 750 ml of the respective nitrifying bacterial consortium, and clamped in place of a thermostatically controlled serological water-bath as the activator. The consortia were circulated through the cartridge by operating the airlift pump at a rate of 1 l min^{-1}. For avoiding the escape of aerosols during the operation, the top of the reactor was covered with 'aerosol arrestor', a cup-shaped device made of perspex having a central hole for extending aeration tubing to the airlift pump. The media composition and culture conditions were the same as above. During activation of the reactors, substrate uptake and product formation were monitored depending on the consortia used. When the reactors were under activation with ammonia oxidizers, NH_4^+–N and NO_2^-–N were determined daily as the substrate and product, respectively (Solorzano 1969; Bendschneider and Robinson 1952). NO_3–N was not determined during the period as the consortia used were without the potency to oxidize NO_2^-–N. When the reactors were under activation with nitrite oxidizers, NO_2^-–N and NO_3–N were determined as the substrate and product, respectively, following Bendschneider and Robinson (1952) and Strickland and Parsons (1968). During activation, the substrates were adjusted to the optimum 10 mg l^{-1} (Achuthan et al. 2006). Substrate consumption and product formation, and the disappearance of the nitrifying bacterial biomass from bulk volume (gravimetric determination) were plotted against days of activation. To confirm attachment of the nitrifiers to the substratum and the reactor activation, the fluid in the reactor was drained off and replaced with sea water of the required salinity, supplemented with the substrates, and monitored for nitrification as described above.

Transportation

Prior to transportation of the activated reactors, the aerosol arrestors were removed, the black lid on top of the reactor replaced, and the medium drained off leaving about 250 ml to maintain moisture inside, then tied securely in a polythene bag. On reaching the site, the reactors were suspended from a float through a string 30 cm below the water level. Two tanks of 2,000-l capacity each were maintained, one with reactors and the other as control without the reactors.

Demonstration and validation of the reactors

Performance of the reactors activated with AMOPCU-1 and NIOPCU-1 were evaluated in the *P. monodon* hatchery system of Matsyafed, Ponnani, Kerala, India, and the ones with

AMONPCU-1 and NIONPCU-1 in the *M. rosenbergii* hatchery of M/s Rosen Fisheries, Trichur, Kerala, India.

The reactors were deployed in the larval rearing tanks connected to the air supply of the hatcheries with air flow regulated using an airflow meter to 1 l min^{-1}. In this operational mode, water entered the reactor through the perforations on top of the black lid, passed through the cartridge, and came out through the airlift pump.

The control and test tanks (2000 l) of *M. rosenbergii* larval production system with 250 l filtered diluted seawater having a salinity of 15 ppt were stocked with 0.10 million mysis individually. The reactors (2 ammonia-oxidizing and 2 nitrite-oxidizing) were deployed, and physical, chemical, and biological parameters were quantified once every 3 days. Every day for 10 days, addition of 200 l filtered, chlorinated–dechlorinated seawater having salinity 15 ppt brought the water level to the maximum capacity of 2000 l. During this period, the larvae were fed with freshly hatched *Artemia* nauplii. It took 30 days for larvae to metamorphose to post larvae.

In the *P. monodon* hatchery, the same operation was adopted except for the use of seawater having 30 ppt salinity. Larvae were fed with *Chaetoceros*, *Spirulina*, and commercial Zoea/Mysis feed, and post larvae with newly hatched *Artemia* nauplii. During the period of larval rearing, there was no water exchange in either system. The experiment was repeated three times.

Physical and chemical parameters measured during the experiment other than the substrates and products of nitrification were salinity (Refractometer, Erma-Japan), pH (pH probe, Euteck-Singapore), alkalinity, and hardness (APHA 1998). Heterotrophic bacterial population was estimated following standard spread plate method employing ZoBell's agar 2216 E prepared in seawater of corresponding salinity. On completion of the experiment (when the post larvae of *M. rosenbergii* attained the stage PL 5 and *P. monodon* PL 15), overall survivals in both the control and experimental sets of tanks were estimated numerically. Relative per cent survival was calculated according to the equation (Gram et al. 1999):

$$\begin{aligned} &\mathrm{R.P.S} \\ &= \{1 - (\%\,\text{mortality in the tank with the reactor}/\%\,\text{mortality in the control tank without} \\ &\qquad \text{the reactor})\} \times 100. \end{aligned}$$

The validation experiments were repeated three times and the significance of variation in nitrification and larval survival rates between the control and test systems were analyzed using one-way analysis of variance.

Results

Activation of the reactors

Progress of nitrification during activation of the reactors is presented in Fig. 2. Nitrification could be established in all reactors within 24 h of initiation of the process, and there was progressive reduction in the suspended biomass and increase in NO_2^-–N and NO_3–N in the reactors activated with ammonia and nitrite oxidizers, respectively. By the 7th day, more than 90% of the bacterial biomass had disappeared from the activation medium with a removal of 80.7 and 78.1% ammonia and 76.2 and 75.3% nitrite in the reactors meant for *M. rosenbergii* and *P. monodon* larval production systems, respectively. The average

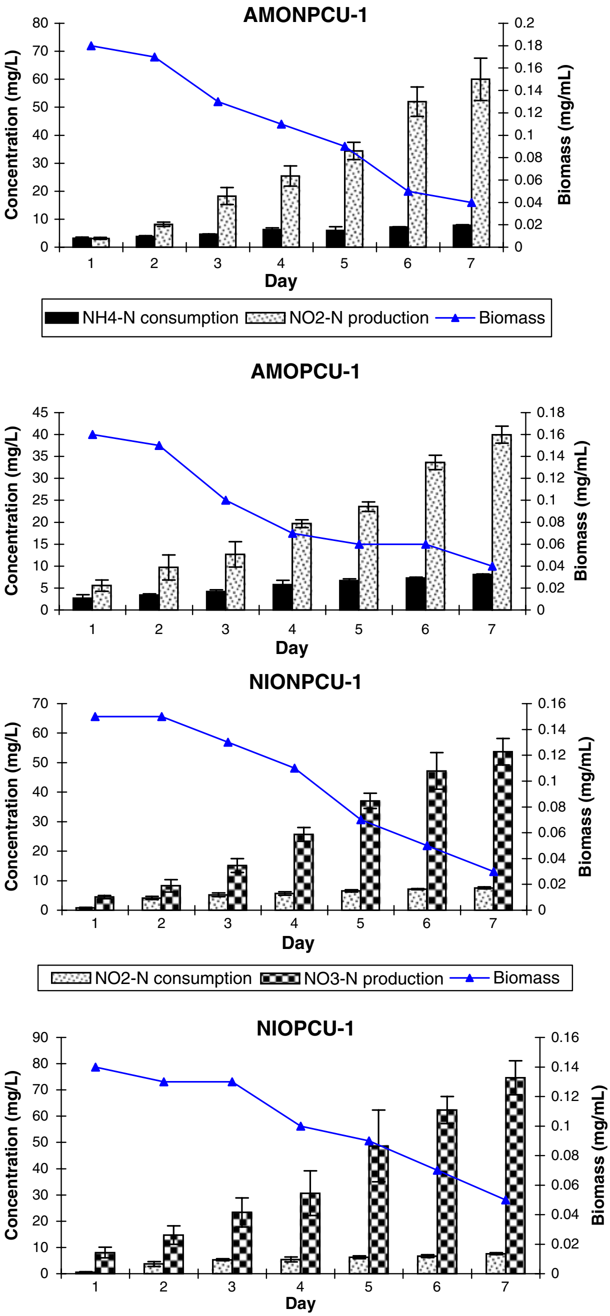


Fig. 2 Activation kinetics of SBSBR immobilized with nitrifying consortia developed for *Macrobrachium rosenbergii* and *Penaeus monodon* hatchery systems

ammonia and nitrite removal rates at the end of the activation period were 0.88 and 0.86 g TAN m^{-2} day^{-1} and 0.84 and 0.83 g TNN m^{-2} day^{-1}, respectively.

The activated reactors subsequent to deployment in the experimental larval rearing tanks their performance in establishing nitrification instantaneously was evaluated. The water quality parameters measured during the process in the experimental and control tanks are summarized in Table 1. During validation of the reactors, nitrification was found established in the experimental tanks instantly and progressed rapidly from the 3rd day onwards (Figs. 3, 4). There was significant ammonia ($P < 0.001$) and nitrite ($P < 0.05$) removal in both the larval production systems compared to that in the control, where ammonia oxidation was found to have set in only after 8 days of commencement of the experiment with no nitrite oxidation. Meanwhile, in the larval rearing tanks with the reactors, NH_4^+–N and NO_2^-–N were not found to have built up; instead, there was a steady increase of NO_3–N. This proved establishment of the two-step nitrification process in larval rearing tanks deployed with the reactors. During the experiment, ammonia and nitrite concentrations in the control tanks were found to have gone up above 2 and 1.6 mg l^{-1}, respectively, whereas in the test tanks both were always below 0.25 mg l^{-1}. In both the systems, there were no significant differences in the other parameters between the control and test tanks except a slight decrease in alkalinity in the experimental tanks and the moderately higher total bacterial population in the control tanks (Table 1).

On terminating the experiment at PL 5 in the case of *M. rosenbergii* and at PL 15 in the case of *P. monodon*, the relative per cent survival was found to be significantly higher ($P < 0.01$) in the experimental tanks with the activated bioreactors (Table 2).

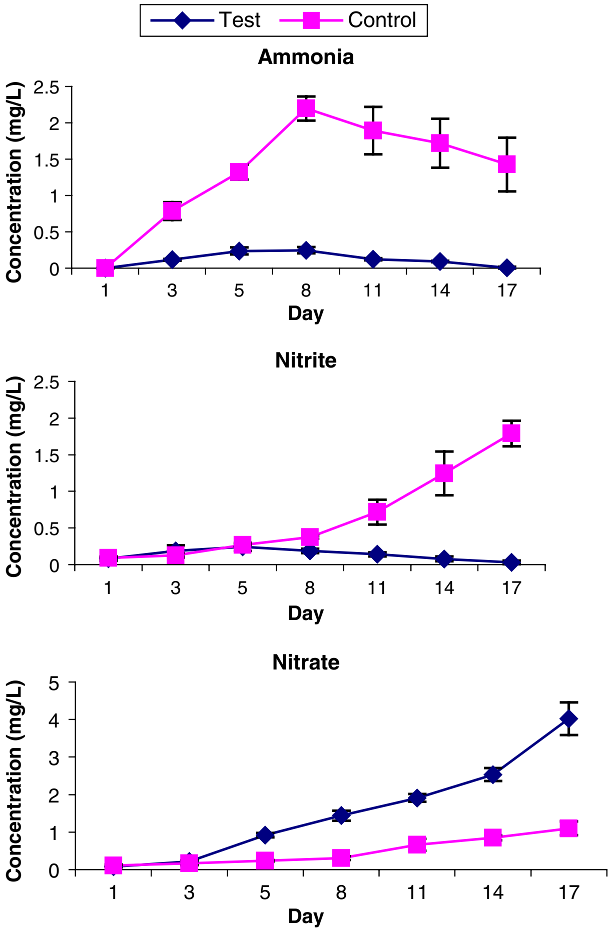
Discussion

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; Furukawa et al. 1993). Without the addition of nitrifiers as start-up cultures, 2–3 months are needed to establish nitrification in marine systems (Manthe and Malone 1987) and 2–3 weeks in freshwater (Masser et al. 1999). There is an agreement among researchers and between laboratory research and commercial applications that saltwater systems need a much longer start-up period. Under such situations, immobilization techniques have been found useful to overcome the delay in the initiation of nitrification (Sung-Koo et al. 2000). Integration of activated SBSBRs to prawn and shrimp hatcheries was found to be an

Table 1 Physico-chemical and microbial quality of rearing water in the hatchery during the experiment ($n = 3$)

Rearing water quality	*Penaeus monodon* system		*Macrobrachium rosenbergii* system	
	Test tank	Control tank	Test tank	Control tank
pH	7.5–8	7.5–8	7.5–8	7.5–8
Salinity (ppt)	30–32	30–32	14–16	14–16
Temperature (°C)	27–29	27–29	28–31	28–31
Alkalinity (mg of $CaCO_3$ l^{-1})	60–69	72–78	62–70	65–70
Hardness (mg of $CaCO_3$ l^{-1})	5,790–5,820	5,790–5,950	2,190–2,260	2,100–2,277
TPC (CFU ml^{-1})	1.43×10^6–1.82×10^8	1.59×10^6–3.78×10^9	4.51×10^5–2.27×10^7	1.73×10^5–1.03×10^9

Fig. 3 Performance of SBSBR in *M. rosenbergii* hatchery systems



important means of overcoming this difficulty in systems which are static where rapid setting up of nitrification is required.

In terms of TAN removal rates per unit of media surface area, Westerman et al. (1993) reported 0.25 g TAN removal m^{-2} day^{-1} for the rotating biological contactors and 0.1–0.15 g TAN m^{-2} day^{-1} for up-flow sand bed filters. For a trickling filter, Van Rijn and Rivera (1990) reported a removal rate of 0.43 g TAN m^{-2} day^{-1}. The average TAN removal rates (g TAN m^{-2} $day^{-1)}$ reported for frequently used biofilters in aquaculture systems are rotating biological contactors 0.19–0.79, trickling filters 0.24–0.64, bead filters 0.30–0.60, and fluidized sand filter 0.24 (Crab et al. 2007). The average TAN and TNN removal rates of SBSBRs during the activation period were higher than the reported values.

Maintenance of ammonia and nitrite during larval rearing is crucial as they cause lethal and sublethal toxicity and plays an important role in the production of healthy and properly sized fingerlings (Fielder and Allan 1997). Marine larval systems can demand TAN and TNN levels below 1.0 mg l^{-1} well below the maximum set for the oligotrophic category (0.3 mg l^{-1} N) (Malone and Beecher 2000). During the validation of the reactors, TAN and TNN could be maintained below 0.25 mg l^{-1}.

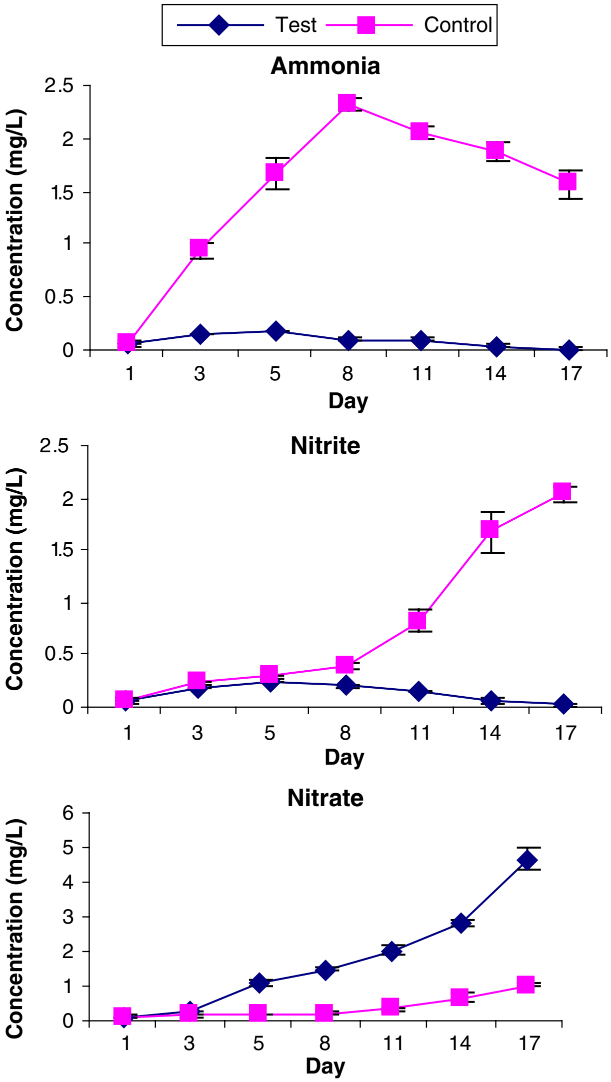


Fig. 4 Performance of SBSBR in *P. monodon* hatchery systems

Table 2 Impact of SBSBR in larval survival on integration in to hatchery systems ($n = 3$)

Hatchery system	Treatment	Survival (%)	*P*	Relative survival (%)
P. monodon	Control tank	16.33 ± 1.53	<0.01	17.67
	Test tank	31 ± 1.73		
M. rosenbergii	Control tank	19.67 ± 1.53	<0.01	20.67
	Test tank	36.33 ± 3.51		

The SBSBRs described here are designed for setting up nitrification in shrimp/prawn larval rearing tanks during the operation without either water exchange or under RAS mode. The technology is relatively user friendly in the sense that they can easily be lifted out of water during disinfection and can also be shifted from one rearing tank to another. Loss of beads encountered in many biofilter systems (Timmons et al. 2006) are not experienced in the case of SBSBRs as the beads are strung together within the reactors. Moreover, the operational costs of the reactors are minimal and no energy costs are added up to the overall production cost as the aeration system already available in the hatchery are used for operating the airlift pumps. Especially, SBSBRs are meant for small-scale larval rearing systems rather than bigger systems where handling of huge quantity of water is warranted. SBSBRs can also find application during the live transportation of spawners as their survival is diminished mainly due to the ammonia they produce (Babu and Marian 1998). The reactors are sufficiently small enough to be incorporated in the transportation containers.

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ORIGINAL PAPER

Mass production of nitrifying bacterial consortia for the rapid establishment of nitrification in saline recirculating aquaculture systems

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Abstract Two distinct nitrifying bacterial consortia, namely an ammonia oxidizing non-penaeid culture (AMO NPCU-1) and an ammonia oxidizing penaeid culture (AMOPCU-1), have been mass produced in a nitrifying bacterial consortia production unit (NBCPU). The consortia, maintained at 4°C were activated and cultured in a 2 l fermentor initially. At this stage the net biomass (0.105 and 0.112 g/l), maximum specific growth rate (0.112 and 0.105/h) and yield coefficients (1.315 and 2.08) were calculated respectively, for AMONPCU-1 and AMOPCU-1 on attaining stationary growth phase. Subsequently on mass production in a 200 l NBCPU under optimized culture conditions, the total amounts of NH_4^+–N removed by AMONPCU-1 and AMOPCU-1 were 1.948 and 1.242 g/l within 160 and 270 days, respectively. Total alkalinity reduction of 11.7–14.4 and 7.5–9.1 g/l were observed which led to the consumption of 78 and 62 g Na_2CO_3. The yield coefficient and biomass of AMONPCU-1 were 0.67 and 125.3 g/l and those of AMOPCU-1 were 1.23 and 165 g/l. The higher yield coefficient and growth rate of AMOPCU-1 suggest better energy conversion efficiency and higher CO_2 fixation potential. Both of the consortia were dominated by *Nitrosomonas*-like organisms. The consortia may find application in the establishment of nitrification within marine and brackish water culture systems.

Keywords Ammonia oxidizing consortia · Nitrifying bacteria · Mass production · Maximum specific growth rate · Nitrification · Yield coefficient

Introduction

The most prominent requirement of any recirculating aquaculture system (RAS) is an efficient biofilter to prevent the accumulation of toxic metabolites such as ammonia and nitrite. High levels of ammonia and nitrite undermine commercial production objectives, as their toxic impacts are manifested through impaired growth or chronic diseases (Cheng et al. 2004; Svobodova et al. 2005). This is especially true in shrimp/prawn hatcheries where the daily specific excretion of ammonia by larvae and post-larvae is five fold higher than that of adults. To address this issue, fixed film biofilters are commonly employed for total ammonia nitrogen (TAN) removal (Seo et al. 2001; Shnel et al. 2002). Experience has shown that the biofilters in RAS require activation to achieve high bacterial growth (Valenti and Daniels 2000) due to the habitually low concentrations of nutrients in the system (Piedrahita 2003), where natural bacterial yield is too low, thus delaying colonization and biofilm formation. To remediate this situation and to establish nitrification rapidly in RAS, mass production of nitrifiers has become essential. However, this has been a challenge, owing to their slow growth rate (Voytek and Ward 1995).

Nitrification involves the simultaneous action of ammonia oxidizing bacteria that oxidize ammonia to nitrite

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(nitritation, Eq. 1); and the nitrite oxidizing bacteria that oxidize nitrite to nitrate (nitratation, Eq. 2) (Wiesmann 1994).

$$NH_4^+ + 1.5\ O_2 \rightarrow\ NO_2^- + H_2O + 2H^+ \quad (1)$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (2)$$

In nitritation, the oxidization of one mol of ammonia produces two protons (Eq. 1) and by means of the stoichiometric equations as shown in Eqs. 3–5, the rate of ammonia oxidation can be evaluated indirectly by monitoring the rate at which the protons are neutralized by the addition of a base, such as carbonate (Gernaey et al. 1998).

$$CO_3^{2-} + H^+ \leftrightarrow HCO_3^- \quad (3)$$

$$HCO_3^- + H^+ \leftrightarrow\ CO_2 + H_2O \quad (4)$$

$$NH_4^+ + 1.5\ O_2 + CO_3^{2-} \rightarrow NO_2^- + 2\ H_2O + CO_2 \quad (5)$$

Based on the above principle, Singh et al. (2007) developed two types of nitrifying bioreactors named Stringed Bed Suspended Bioreactors (SBSBRs) and Packed Bed Bioreactors (PBBRs) for application in shrimp/prawn hatcheries. These reactors function using nitrifying biofilms immobilized on plastic beads assembled in the form of cartridges. To generate the biofilm, two ammonia oxidizing consortia, AMOPCU-1 and AMONPCU-1, were developed by enrichment from shrimp culture systems (Achuthan et al. 2006) and immobilized on the substrata. However, to accomplish commercialization of the technology, the consortia need to be generated in larger quantities. In this study, an indigenous nitrifying bacterial culture production unit (NBCPU) was designed, fabricated, and applied for mass production of the consortia under optimized culture conditions. The consortia thus generated should find application in a variety of biofilters/bioreactors besides SBSBR and PBBR in RAS and in brackish and marine culture systems.

Materials and methods

Ammonia oxidizing bacterial consortia

Two nitrifying bacterial consortia, AMONPCU-1 and AMOPCU-1 developed by enrichment technique from shrimp culture systems under perpetual salinity regimes of 15 and 30 parts per thousand (ppt) respectively, were used in this study following optimization of culture conditions (Achuthan et al. 2006). The growth medium was prepared in seawater (salinity 15/30 ppt), supplemented with 10 mg/l substrate $(NH_4)_2\ SO_4$, 2 mg/l KH_2PO4 (Watson 1965) and incubated at an optimum temperature of 28°C and at pH 8.5. Prior to transferring the cultures to NBCPU, a preliminary characterization was accomplished by transmission electron microscopy (TEM). Aliquots of 10 ml each of the consortia were centrifuged at 6000*g* in a refrigerated centrifuge for 15 min. The pellets were washed with seawater of respective salinity and fixed in 2.5% glutaraldehyde prepared in seawater at 4°C overnight, post-fixed in 2% osmium tetroxide at 4°C for 2 h, washed repeatedly with seawater and dehydrated through an acetone series of 70–100%, embedded in epoxy resin, sectioned and stained with lead citrate and uranyl acetate and examined under an electron microscope (Morgagni 268-D, Netherlands).

Activation of stored consortia

The nitrifying bacterial consortia maintained under refrigeration (4°C) were brought to room temperature (26–28°C) and required aliquots were mixed with equal volumes of their respective growth media and kept on a rotary shaker operated at 100 rpm. The quantity of NH_4^+–N consumed (Solorzano 1969) and NO_2^-–N formed (Bendschneider and Robinson 1952) were monitored daily. The pH was maintained by supplementing sodium carbonate and evaporation loss was corrected with sterile distilled water. The incubation continued until the cultures actively started nitrification.

Culturing of the nitrifying consortia in 2 l fermentor

A 2 l capacity fermentor (New Brunswick, USA, Bioflow 2000) was used for scaling up the two ammonia oxidizing consortia. The detachable fermentor vessel with the respective growth medium was autoclaved at 10 psi for 10 min and reassembled. Optimum pH and temperature were auto-adjusted and the agitation was set at 200 rpm with an airflow rate of 1.0 l/min. Inocula were taken from the cultures that were activated on a rotary shaker. The fermentor vessel was kept covered, to prevent photoinactivation of monoxygenase. The NH_4^+–N consumed and NO_2^-–N formed were assayed once every 24 h. The pH was adjusted to optima by addition of 10% $Na_2\ CO_3$ through the base port. As consumption of NH_4^+–N progressed it was supplemented with fresh aliquots maintaining 10 μg/ml as the final concentration. Upon attaining stationary phase (characterized by the decline in the production of NO_2^-–N), the cultures were harvested and used for inoculating the 200 l fermentor, prior to which the biomass generated were determined gravimetrically. This was accomplished by passing a known quantity of the culture through a pre-weighed membrane (0.22 μm), drying at 80°C overnight, stabilizing in a desiccator and determining the difference as the biomass by dry weight. Subsequently, μ^{max} for each of the consortia at 28°C was estimated using a mathematical model of the quadratic form fitted into the experimental data on log NO_2^-–N formed versus time by the method of least squares, using the statistical software SPSS (SPSS Inc., USA).

Mass production of ammonia oxidizing bacterial consortia

Designing and fabrication of the NBCPU

The NBCPU (Fig. 1) was designed based on the requirements for obtaining maximum biomass within the shortest duration possible and fabricated with locally available materials. A 200 l wide-mouthed polyethylene carboy fixed with a central 0.5 H.P AC/DC agitator motor (500 W, 0–500 rpm), served as the fermenter vessel. Inside the vessel, a coiled stainless steel pipe circulated water at constant temperature of 28°C from a thermocirculator (2500 W) to maintain the optimum temperature. A temperature probe and portals for addition of medium and inoculum were supplied. Aeration was provided through a stainless steel pipe, which delivered filtered, sterile air to the bottom centre through an air sparger. A drain line was placed at the base of the tank. The medium was subjected to the boiling temperature (100°C) for 30 min in a 100 l boiler (5000 W, stainless steel), and was passed through a sterile (autoclaved, 15 psi for 15 min) pipe to the fermentor vessel.

Before use, the fermentor vessel was rinsed with 70% ethanol (1 l) and after 30 min, washed with sterile tap water and drained off. The vessel was filled with the respective medium and aerated for 2 days through a cartridge filter (0.22 μm) and plated out on to ZoBell's agar and Saboraud dextrose agar prepared in aged seawater to record the presence of total viable bacterial and fungal populations, prior to inoculating with the nitrifiers. The inocula (stationary phase cultures) were introduced to a final concentration of 1% (v/v) and incubated under agitation at a rate of 100 rpm and with continuous aeration at 2 l/min, maintaining optimum culture conditions. The pH was maintained using aqueous 10% sodium carbonate and the temperature was regulated at 28°C by a thermocirculator. As the consumption of NH_4^+–N progressed it was supplemented with aliquots of fresh substrates at an exponential rate. This process was continued until the cultures attained stationary phase with daily monitoring of NH_4^+–N consumption and NO_2^-–N and NO_3–N production. Nitrate was estimated following the method of hydrazine sulfate reduction (Strickland and Parsons 1972). The initial and final biomass was measured gravimetrically. Based on the data generated, growth pattern, substrate consumption and product formation were determined. Alkalinity reduction due to hydroxylamine production was calculated based on the relationship 6.0–7.4 mg alkalinity

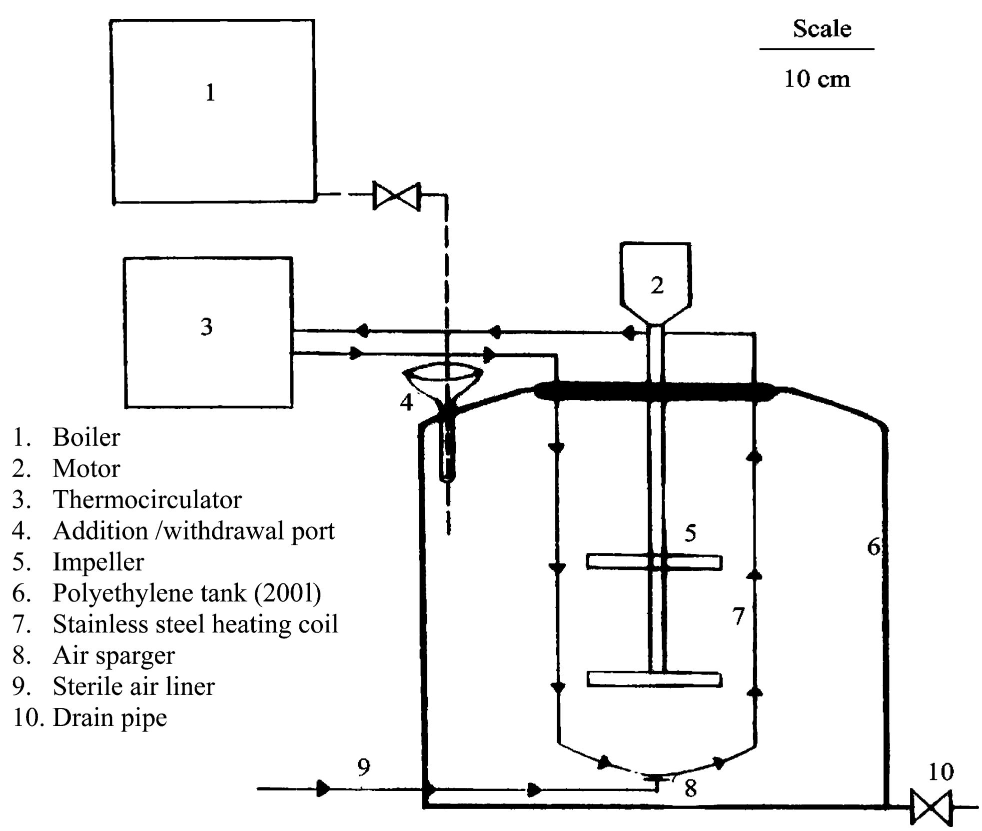

Fig. 1 Nitrifying Bacterial consortia Production Unit (NBCPU)

got consumed per milligram NH_4^+–N oxidized to NO_2^-–N (EPA 1975).

Yield coefficient/cell yield 'Y' of the consortia

The yield coefficient, 'Y' was determined as the ratio of weight of cells, or biomass generated, to the weight of substrate oxidized, or the ratio of the quantity of product formed to the quantity of substrate utilized (Sharma and Ahlert 1977). Cell yield or biomass generated was determined gravimetrically as described earlier.

Results

Characterization, activation and culturing of the consortia in 2 l fermentor

Transmission electron microscopy of the consortia as shown in Fig. 2 demonstrated characteristic cyst formation and cytomembranes resembling autotrophic nitrifies. Both the consortia were dominated by *Nitrosomonas*—like forms (0.55 × 0.43 µm), having intracytoplasmic membranes as flattened vesicles in the peripheral region of the cytoplasm besides cytoplasmic inclusion bodies resembling carboxysomes. This observation was supported by fluorescent in situ hybridization (FISH) employing fluorescent probes, specific for *Nitrosomonas* (unpublished). About 4–6 days were required for the cultures to become active after refrigeration for 5 years (Fig. 3). In both the consortia, the quantity of the product (NO_2^-–N and NO_3–N) formed was higher than that of the substrate (NH_4^+–N and NO_2^-–N) consumed. On amplification in a 2 l fermentor the product formation was still higher in magnitude than the substrate consumption (Fig. 4a, b). After 20–25 days culture, the consortia began to enter stationary phase. The net biomass yield, maximum specific growth rates and

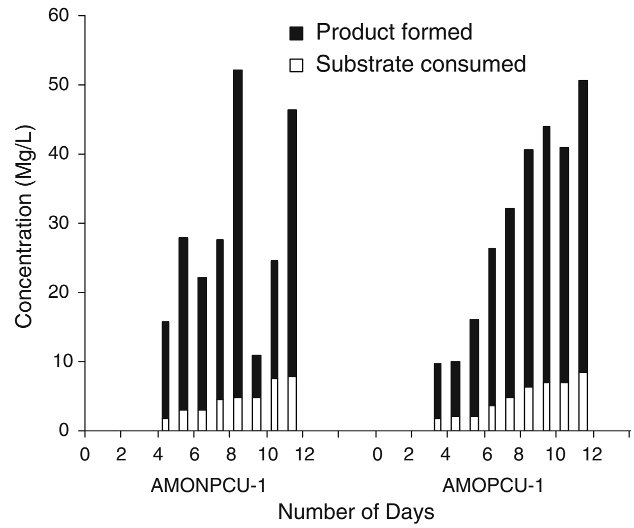

Fig. 3 The pattern of substrate consumption and product formation during activation of stored (4°C) nitrifying consortia at room temperature

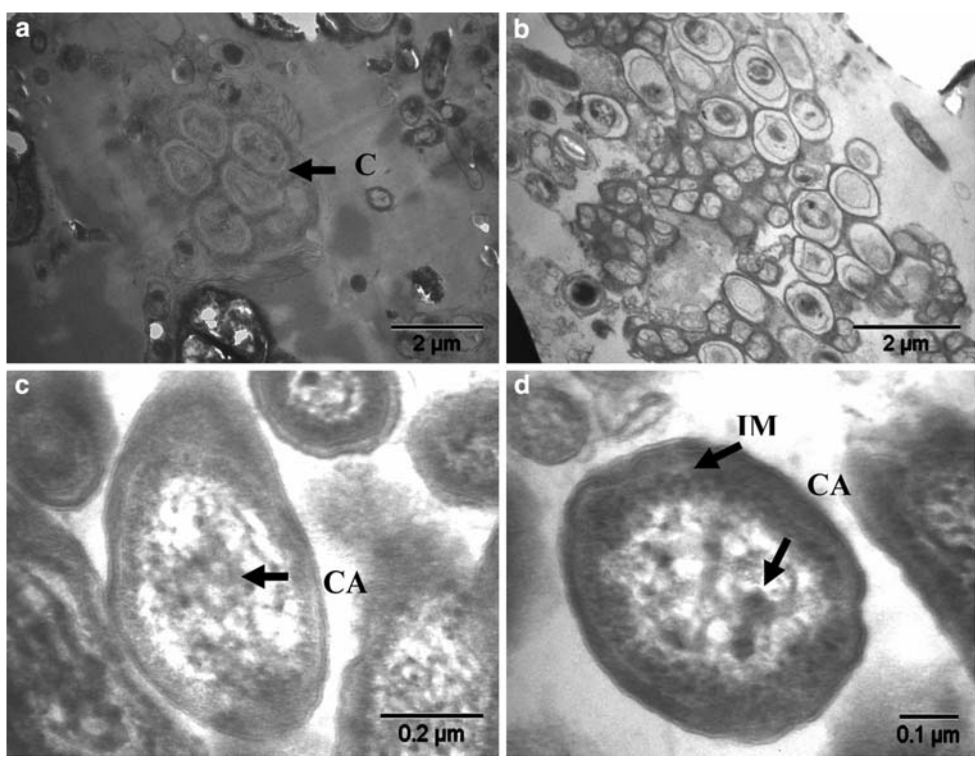

Fig. 2 Transmission electron micrographs of the nitrifying bacterial consortia. **a** and **b** Cysts in AMOPCU-1 and AMONPCU-1, **c** and **d** *Nitrosomonas*-like forms in AMOPCU-1 and AMONPCU-1). *G* Glycocalyx, *C* Cyst, *Ca* Carboxysomes, *IM* Intracytoplasmic membranes

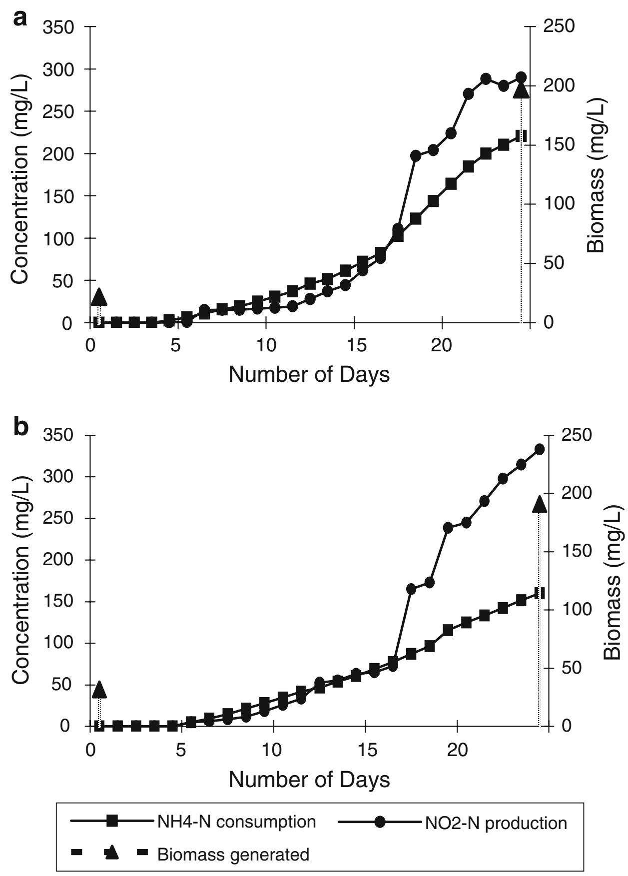

Fig. 4 a–b Growth curves of nitrifying consortia in 2 l fermentor

yield coefficients of the consortia during growth in 2 l fermentor are summarized in Table 1.

Mass production of the consortia in 200 l fermentor

On examining the total viable heterotrophic bacterial and fungal count prior to inoculation of the 200 l fermentor, around 15–20 CFU ml^{-1} of the former could alone be recovered. The substrate consumption and product formation during the growth of AMOPCU-1 and AMONPCU-1

Table 1 Growth and yield of ammonia oxidizing consortia, AMOPCU-1 and AMONPCU-1, during activation in 2 l fermentor and on mass production in 200 l fermentor

Nitrifying consortia	Biomass (g dry wt l^{-1}) at the day of harvest	μ_{max} (h^{-1})	Yield Coefficient (g product formed to g substrate utilized)
AMONPCU-1			
2 l	175.0 (25 d)	0.112	1.315
200 l	125.3 (160 d)		0.667
AMOPCU-1			
2 l	159.2 (25 d)	0.105	2.08
200 l	165.0 (260 d)		1.23

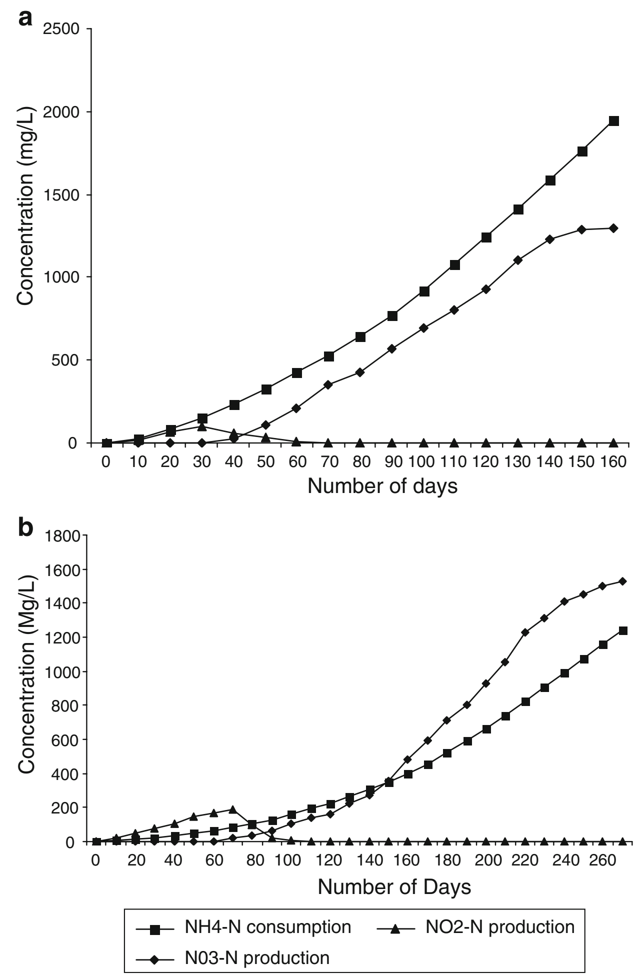

Fig. 5 a–b. Growth of nitrifying bacterial consortia in 200 l fermentor

in 200 l NBCPU are shown in Fig. 5a, b. The systems which started with 0.01 g/l residual NH_4^+–N consumed 1.24 and 1.95 g NH_4^+–N/l over a period of 270 and 160 days respectively, with a total corresponding output of 1.53 and 1.30 g NO_3–N/l. From the growth curve of AMOPCU-1 it could be inferred that until 70 days of incubation there was progressive build up of NO_2^-–N. Subsequently within another 20 days it rapidly declined and nitrate began to accumulate. After 90 days no residual nitrite could be detected and NH_4^+–N was found to be oxidized directly to NO_3–N. In a similar pattern, AMONPCU-1 exhibited a progressive build up of NO_2^-–N during the first 30 days, and subsequently within another 20 days it rapidly declined alongside the build up of NO_3^-–N, with no residual NO_2^-–N.

While the total NH_4^+–N removed by AMOPCU-1 and AMONPCU-1 was 1.24 and 1.95 g/l, respectively, for the culture period of 270 and 160 days, the total alkalinity reduction was by 7.45–9.19 and 11.69–14.42 g/l, respectively (Table 2). To compensate the alkalinity reduction,

Table 2 Alkalinity destroyed and carbonate used during mass production of ammonia oxidizing consortia AMOPCU-1 and AMONPCU-1

Consortia	Salinity (ppt)	pH	Total NH_4-N removed (g/l)	Total alkalinity destroyed (g)	Na_2CO_3 used (g)
AMPOCU-1 (Penaeid)	30	7.5	1.242	7.452–9.191	62.0
AMONPCU-1 (Non-penaeid)	15	8.5	1.948	11.688–14.415	78.0

the consortia were supplied with 62 and 78 g Na_2CO_3, respectively, during the period of culture.

Yield coefficient and biomass on mass production

Since the consortia attained equilibrium in the two step process of nitrification by converting NH_4^+–N directly to NO_3–N, without permitting residual NO_2^-–N to reach detectable levels, the yield coefficient was calculated from the quantity of NO_3–N produced. On attaining stationary phase, the yield coefficient of AMOPCU-1 was found to be 1.23 and that of AMONPCU-1 0.67. The net biomass yield of AMONPCU-1 at the end 160 days culture in 200 l fermentor was 125.3 g/l, while that of AMOPCU-1 at the end of 270 days was 165 g/l.

Discussion

Mass production of nitrifying bacterial cultures has always been a challenge due to their slow growth (Nejidat and Abeliovich 1994; Voytek and Ward 1995), and the difficulty in turbidometric growth determinations. The ultrastructure and FISH analysis of the consortia revealed the dominance of *Nitrosomonas* - like organisms, an important ammonia oxidizing bacteria (AOB) commonly found in biological wastewater treatment plants (Wagner et al. 1996). Although there are a few reports on the cultivation of pure cultures of nitrifiers (Tappe et al. 1996; Zart and Bock 1998; Chapman et al. 2006), attempts have never been made to scale up the process to large volumes. An important consideration for the design of mass production systems has been cost-effectiveness and accordingly, the medium optimized in this report was in seawater with addition of substrates, and carbonate to maintain optimum pH. Additionally, while fabricating the 200 l fermentor, locally available materials were used to reduce costs. As a specific requirement the fermentor vessel was kept opaque and well protected from sunlight, as visible and UV light have been reported to be lethal to some nitrifying organisms (Johnstone and Jones 1988; Diab and Shilo 1988). The overall heat treatment given to the medium could bring down the heterotrophic bacteria substantially facilitating cultivation of the nitrifying bacterial consortia.

While 4 to 6 days were required for activation of the consortia, culturing in a 2 l fermentor required 20–25 days. In both of these processes the quantity of the product formed was considerably higher than the substrate consumed as observed in our previous studies (Ramachandran 1998). The maximum specific growth rate of the ammonia oxidizers at a salinity of 30 and 15 ppt were 0.112 and 0.105/h, higher than the reported values of 0.014/h (Lawrence and McCarty 1970) and 0.092/h (Sharma and Ahlert 1977) for *Nitrosomonas.* In contrast, 0.032/h was considered the typical maximum specific growth rate at 20°C (Rittmann and Snoeyink 1984). Temperature has a strong effect on growth of nitrifiers, with the maximum growth rate at 30°C, three times higher than that at 20°C (USEPA 1993). In our studies the consortia were cultured at 28°C, and the possible reason for their higher specific growth rate values compared to the reported ones might be the comparatively higher temperature of incubation. Nevertheless, the values reported in the literature for ammonia oxidizers at higher temperatures had always been lower (0.043/ h) even at 30°C (Vadivelu et al. 2006), and (0.042/h) at 35°C (Van Hulle et al. 2004). The large variations in between the reported values and those of the present study might be due to the differences in the cultures used and the growth conditions provided as pointed out by Vadivelu et al. (2006) while investigating stoichiometric and kinetic characterization of *Nitrosomonas* sp. in mixed cultures.

The nitrifying bacterial consortia production unit (NBCPU) was successfully used for mass production of ammonia oxidizing consortia at salinities of 30 and 15 ppt where the two-step nitrification process could be established. It is an accepted principle that when populations of nitrifying bacteria are stabilized under constant conditions, residual nitrites will be at undetectable levels, with progressively increasing nitrate concentrations (Gray 1990). Similar observations were also made by Achuthan et al. (2006) during enrichment of the consortia from shrimp ponds. It has also been established that oxidation of nitrite to nitrate is more rapid than the preceding step (Stensel and Barnard 1992). This was especially true when the consortia AMOPCU-1 and AMONPCU-1 were grown in NBCPU, as no residual nitrite could be detected after 90 days of culturing, establishing the existence of population of nitritifiers and nitratifiers under equilibrium. This is in agreement with Bovendeur (1989) who demonstrated that nitratification (nitrite to nitrate) is slightly delayed and is subsequent to the development of nitritification (ammonia to nitrite) capability. The successful mass production of

AMOPCU-1 or AMONPCU-1 having nitritifiers and nitratifiers in equilibrium is advantageous in the practical sense that activation of the nitrifying bioreactors/biofilteres and establishment of rapid nitrification in RAS can be achieved with a single consortium.

During this production process the cultures AMOPCU-1 and AMONPCU-1 consumed comparatively less Na_2CO_3 for compensating the alkalinity reduction, adding little to the cost of production. There are reports that carbonate-based pH control yielded less cell density of *Nitrosomonas europea* (Chapman et al. 2006) as compared to bicarbonate-fed batch cultures. However, in the present study the addition of carbonate was sufficient to yield high biomass. Yield coefficients of AMOPCU-1 (1.23) and AMONPCU-1 (0.67) on mass production were comparatively high when compared to the estimated value (0.2) based on the thermodynamics of growth of ammonia oxidizers. In the practical sense there also have been reports of very low yield coefficient for ammonia (0.03–0.13) and nitrite (0.02–0.08) oxidizers (Sharma and Ahlert 1977). This is indicative of improved energy conversion efficiency and higher carbon dioxide fixation potentials of AMOPCU-1 and AMONPCU-1, primarily as the characteristics of tropical organisms, and secondarily may be due to their mixed culture configuration. While experimenting with nitrifying consortia developed from sewage in India, Ramachandran (1998) experienced a similar comparatively higher yield coefficient (0.1864–0.1939) for ammonia oxidizers and (0.1745–0.1986) nitrite oxidizers.

In the cultivation of *Nitrosomonas europea* pure cultures, batch-fed culturing with bicarbonate buffering yielded a culture density of 30 mg dry wt/l, whereas continuous culture under energy-limited growth conditions in a bench-scale bioreactor using a microfiltration membrane for high cell recycling produced final culture densities greater than 350 mg dry wt/l (Chapman et al. 2006). Conversely AMONPCU-1 yielded 125.3 g/l with in a period of 160 days and AMOPCU-1 yielded 165 g/l during 270 days of incubation. The characterization of the consortia showed dominance of autotrophic ntirifiers, while the higher yield coefficients suggested better energy conversion and higher CO_2 fixation potential of the consortia. The mass production process accomplished in these studies yielded sufficiently larger active biomass for immobilization in the bioreactors SBSBR and PBBR, with possible applications in marine and brackish water aquaculture systems, to establish instantaneous nitrification.

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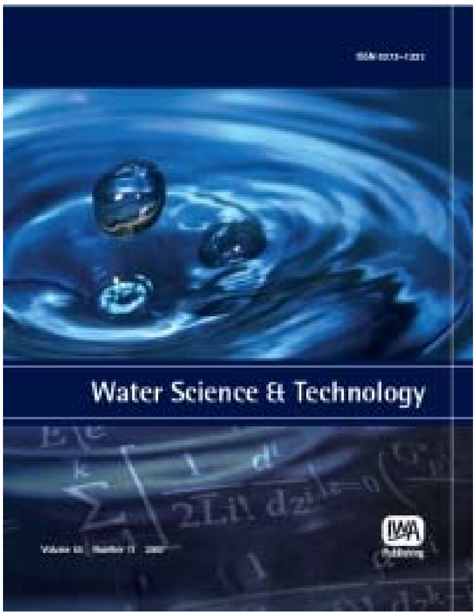

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Nitrification in brackish water recirculating aquaculture system integrated with activated packed bed bioreactor

V. J. Rejish Kumar, Valsamma Joseph, Rosamma Philip and I. S. Bright Singh

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/m^3/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

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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; Goldburg *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.* 2009*a*). 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.* (2009*a*). Each reactor consists of a shell made of fibre glass with a base of 30 cm^2 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 cm^3 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.* 2009*b*). 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

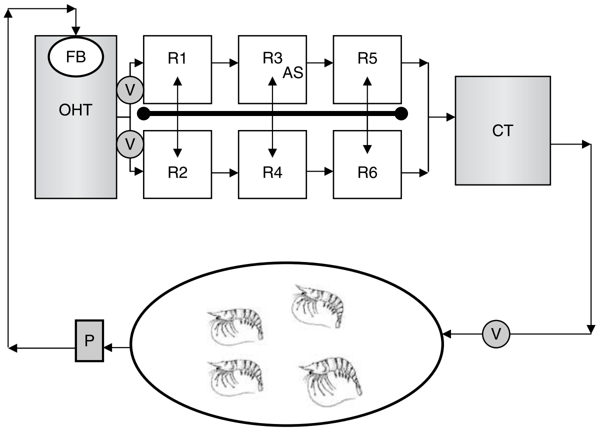

Figure 1 | Packed bed bioreactor (PBBR) integrated into the shrimp rearing tank. FB—Filter Bag; OHT—Over head tank; V—Valves; R1–R6—Reactor 1 to Reactor 6; AS—Aeration supply; CT—Collecting tank; SRT—shrimp rearing tank; P—pump.

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 lavae-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 'Detrodigest™' 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), NO_2-N (Bendschneider & Robinson 1952) and NO_3-N (Strickland & Parsons 1972) and once in three days for alkalinity, hardness, total suspend 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):

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

Where,

P_{TAN} = Rate of ammonia production ($g\,TAN\,d^{-1}$)
FA = 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 = g TAN converted per m^3 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 m^3).

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} (m^3) (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.* 2009*a*).

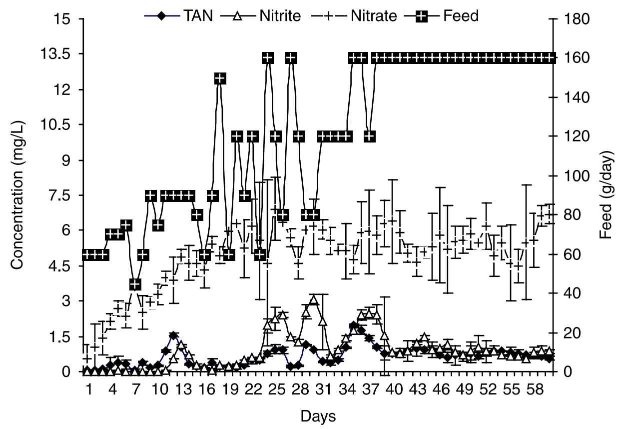

Figure 2 | Progress of nitrification in recirculating *Penaeus monodon* culture system.

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, NO_3-N and NO_2-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 *Vibrio* count (CFU/ml)	75–1,000

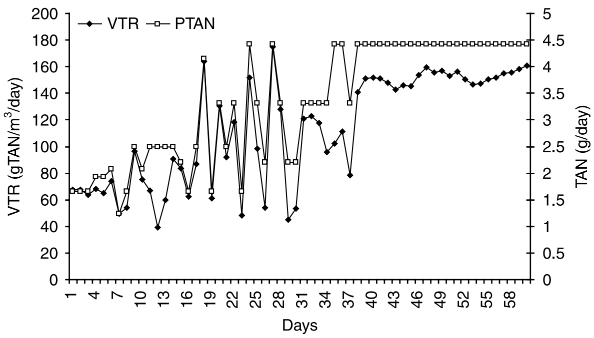

Figure 3 | Volumetric TAN Removal rates and TAN in *Penaeus monodon* recirculation system.

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 bellow 1 mg/L in most of the days. The production of NO_3-N was found to increase up to a maximum of 6.7 mg/L as observed in our previous experiments (Kumar *et al.* 2009*a*,*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 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/m^3/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/m^3/day for applications in cold water systems and 1.0 kg TAN/m^3/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/m^3/day in a cold water system (Summerfelt *et al.* 1996) and 0.1 kg TAN/m^3/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/m^3/day, for the design of floating bead filters in brood stock, ornamental, and grow-out systems, respectively for warm water systems.

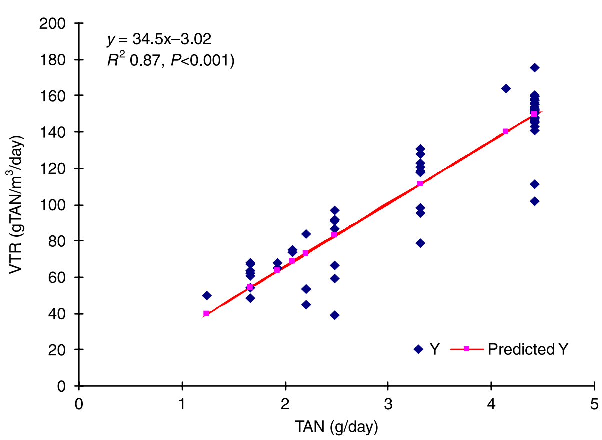

Figure 4 | Regression of Volumetric TAN Removal rates (VTR) versus TAN in *P. monodon* recirculation system- predicted and measured VTR.

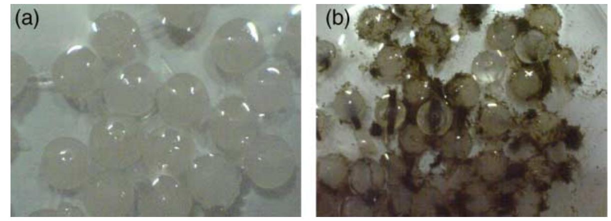

Figure 5 | Polystyrene beads used as support material, (a) without consortia, (b) with biofilms.

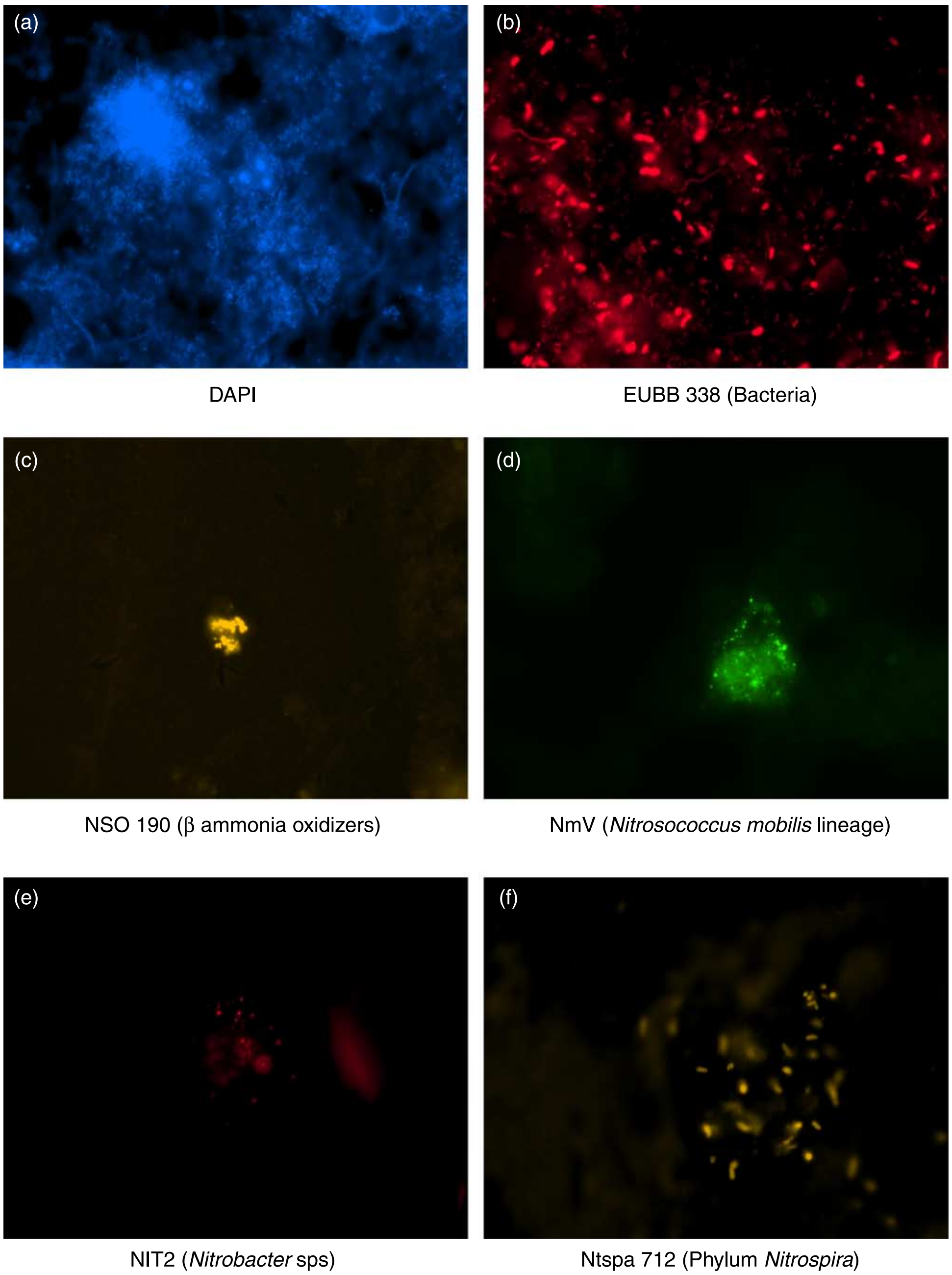

Figure 6 | Fluorescent Insitu Hybridization of a mature biofilm taken from PBBR integrated with *Penaeus monodon* RAS after four months of operation.

The RAS system also supported good biomass with an average yield of 10.3 g and average length of the animals was 8 cm at the end of 60 day culture, with 60–65% survival. The maximum volumetric biomass capacity of the system was 75 g/m^3. 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.* 2009*a*). 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 O_2 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 bellow 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/m^3/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/m^3. 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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Research Article

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Nitrification in a packed bed bioreactor integrated into a marine recirculating maturation system under different substrate concentrations and flow rates

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Abstract

BACKGROUND: A packed bed bioreactor (PBBR) activated with an indigenous nitrifying bacterial consortia was developed and commercialized for rapid establishment of nitrification in brackish water and marine hatchery systems in the tropics. The present study evaluated nitrification in PBBR integrated into a *Penaeus monodon* recirculating maturation system under different substrate concentrations and flow rates.

RESULTS: Instant nitrification was observed after integration of PBBR into the maturation system. TAN and NO_2-N concentrations were always maintained below 0.5 mg L^{-1} during operation. The TAN and NO_2-N removal was significant ($P < 0.001$) in all the six reactor compartments of the PBBR having the substrates at initial concentrations of 2, 5 and 10 mg L^{-1}. The average volumetric TAN removal rates increased with flow rates from 43.51 (250 L h^{-1}) to 130.44 (2500 L h^{-1}) gTAN m^{-3} day^{-1} ($P < 0.05$). FISH analysis of the biofilms after 70 days of operation gave positive results with probes NSO 190 ((β ammonia oxidizers), NsV 443 (*Nitrosospira* spp.) NEU (halophilic *Nitrosomonas*), Ntspa 712 (Phylum Nitrospira) indicating stability of the consortia.

CONCLUSION: The PBBR integrated into the *P. monodon* maturation system exhibited significant nitrification upon operation for 70 days as well as at different substrate concentrations and flow rates. This system can easily be integrated into marine and brackish water aquaculture systems, to establish instantaneous nitrification.
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Keywords: packed bed bioreactor; nitrification; recirculating aquaculture systems; total ammonia nitrogen; flow rate

INTRODUCTION

In recent years, there has been growing concern over the environmental impacts of aquaculture operations,[1–3] and recirculating aquaculture systems (RAS) have emerged as the major environmentally sustainable solution to combat these impacts. A recirculating aquaculture facility reduces water demands and discharges by reconditioning water to be recycled, and increases food conversions resulting in less waste generation from feed.[4,5] The RAS technologies are highly applicable to the production of marine species[6–8] as reliable supply of fingerlings is a bottleneck for their commercial production.[9,10] Biosecurity is another important matter for consideration in the use of RAS by the hatchery operators[11,12] as the water recirculation dramatically reduces the possibility of pathogen introduction.[5,13]

The most prominent characteristic of any RAS is a nitrifying biofilter to prevent accumulation of metabolites like ammonia and nitrite, which at high levels undermine commercial production objectives as their toxic impacts are manifested through impaired growth or chronic diseases.[14–16] However, nitrate is relatively harmless to the aquatic organisms.[17] Fixed film biofilters are commonly used for total ammonia nitrogen (TAN) removal in RAS,[18–20] where attached growth as biofilm offers several advantages as handling convenience, increased process stability to shock loading and prevention of the bacterial population from being washed off.[21,22] However, at least in a few cases, the immobilized nitrifiers in RAS have exhibited low performance, besides demanding too long a start-up period imposing operational difficulties.[23,24] Considering these drawbacks, we developed a specialized nitrifying packed bed bioreactor (PBBR) (Indian Patent no. 241 648) immobilized with an indigenous nitrifying bacterial

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consortia and having the advantages of short start-up time and ease of integration into the existing hatchery designs without modifications.[25] The PBBR will enable hatchery systems to operate as closed recirculating systems, maintaining water quality during the operation and minimizing discharge of spent water.[42]

Many studies have provided details of system design, operation and performance evaluations on fluidized bed reactors, floating bead filters, trickling filters and moving bead filters for their application in aquaculture systems.[26–29] However, information on process mechanism and kinetics relative to nitrification biofilters applied to aquaculture systems is still insufficient. In general, nitrification kinetics of fixed film reactors used in RAS was found to be affected mainly by water quality parameters.[30] The TAN concentrations, especially the minimum concentration that a biofilter can maintain and the relationship between nitrification rate and TAN concentrations are very important in the performance of a nitrifying biofilter. The substrate limitation rather than substrate inhibition is often the major concern for biofilter designs in RAS due to the low ammonia concentration in these systems.[18] Within the TAN concentration range that is common to RAS, the nitrification rate is proportional to the substrate concentration.[30] The flow rate into the bioreactor is another important criterion affecting the turbulence and thus has great impact on the mass transfer flux into biofilm as well as the nitrification rate. Stoodley *et al*.[31] investigated the relationship between local mass transfer coefficients and fluid velocity in heterogeneous biofilms and found that the effects of biofilm heterogeneity on mass transport were strongly dependent upon the average flow velocity. Ling and Chen[32] also reported higher nitrification rates in biofilters with high turbulence levels, suggesting that the nitrification rate may be significantly improved through increasing the turbulence. In the present study we have analyzed the nitrification performance of PBBR integrated into a marine *Penaeus monodon* maturation system for 70 days during which the animals showed signs of maturation. The nitrification efficiency of the system was subsequently evaluated under different input TAN concentrations and at increasing flow rates and it is hypothesized that there will be increase in the yield (nitrification) with increase in flow rates. Fluorescent *in situ* hybridization (FISH) was performed to identify the nitrifying bacterial community present in the biofilm of the reactor after an operating period of 70 days.

EXPERIMENTAL PROCEDURES

Packed bed bioreactor

The configuration of the PBBR detailed by Kumar *et al*.[25] was used with slight modifications. The PBBR was integrated into a *Penaeus monodon* maturation system as shown in Fig. 1. The influent from the maturation tank was pumped into an overhead tank (282 L) from where water flowed through the reactors connected serially by gravitation and was collected in a 140 L collection tank, from where the treated water flowed into the maturation tank. Pumping was controlled by an automated water level controller (V-guard, Kerala, India) fitted inside the overhead tank. A regulator valve was connected to the overhead tank to maintain the influent flow through the reactors. All six reactors (R1–R6) have the same configuration consisting of shell made of fiberglass with a base of 30 cm^2 and an overall height of 90 cm for R1 and R2, 75 cm for R3 and R4 and 45 cm for the R5 and R6 with effective volume of 20 L each. A perforated base plate made of Perspex, carrying nine 30 cm long and 2 cm diameter PVC pipes (airlift pumps) fixed at 10 cm equidistances, is positioned at the base of the reactor. When air is passed through, the 10 cm^3 area filled with the support medium surrounding each airlift pump acts as an aeration cell. The baseplate 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. Polystyrene beads having 5 mm diameter and a surface area of 0.785 cm^2 with spikes on the surface were used as substrata for immobilization. Each reactor was packed with 60 000 polystyrene beads. The bottom of all six reactors was fitted with a valve for periodical backwashing.

Activation and integration of the PBBR to the maturation system

The reactors were activated with nitrifying bacterial consortia enriched from a brackish water environment[33] and mass produced in a 200 L fermentor.[34] For the activation, each reactor was supplied with a 5 L consortium having a cell density of $3-4 \times 10^5$ cells mL^{-1}, quantified by epifluorescence microscopy. After introduction of the consortium into the reactors the airlift pump was operated, supplying air at a rate of 1 L min^{-1} to ensure adequate circulation of the culture through the beads and to assure supply of O_2 and CO_2 for activation. Optimum conditions for activation were provided as reported in Kumar *et al*.[25] After 1 week of activation the reactors were connected to a maturation tank in which 50 specimens of *Penaeus monodon* adults (average weight 120 ± 10 g) were reared in 6 m^3 of 30 gL^{-1} salinity seawater. The animals were fed three times a day with 300 g natural feed containing cooked meat mixture of clam, squid and crab. The reactors were operated at a flow rate of 400 L h^{-1} with a hydraulic residence time (HRT) of 1.34 h, which provided a total recirculation of 9600 L d^{-1}. A bag filter placed inside the overhead tank was used to filter the incoming water from the rearing tanks to remove detritus. The reactors were operated for 70 days and the evaporation loss was made up through the addition of fresh water to the system. The water samples were collected from the rearing tank and analyzed for TAN,[35] NO_2-N[36] and NO_3-N[37] concentrations once in every 3 days for 70 days.

Nitrification at different substrate concentrations

The effect of higher substrate concentrations on PBBR was tested after 70 days of operation. Prior to analysis, circulation through the reactors was stopped and kept for 10 min to remove the residual ammonia from the reactors. Upon reaching zero TAN concentration, NH_4Cl stock solution having a strength of 10 000 mg L^{-1} was added to each of the reactor compartments to give a concentration of 2 mg L^{-1} each, and the pH was adjusted to the optimum of 8.00. Subsequently, the TAN removal for each reactor was measured independently on an hourly basis by analyzing the water samples drawn through each compartment's drain pipe until complete consumption of the substrate was recorded. The experiments were repeated with 5 and 10 mg L^{-1} TAN concentrations in each reactor as described above after removing the residual ammonia, and all the experiments were repeated twice. NO_2-N removal efficiency of the reactors was measured at concentrations of 2, 5 and 10 mg L^{-1} as above, using 10 000 mg L^{-1} $NaNO_2$ as the stock solution. The average percentage TAN and NO_2-N removal rates of the reactor system at each substrate concentration over a period were calculated.

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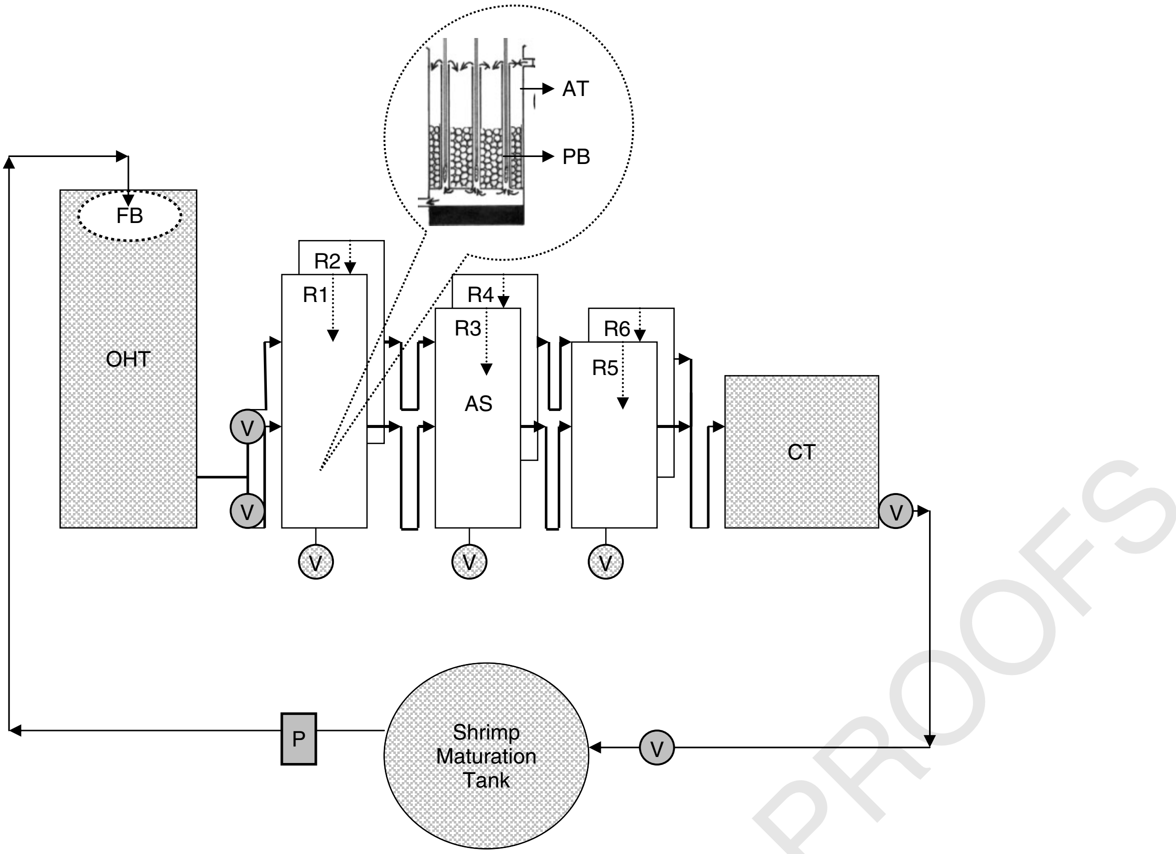

Figure 1. Packed bed bioreactor connected to a shrimp maturation system. AS–aeration supply; AT–aeration tube; CT–collecting tank; FB–filter bags; OHT–Overhead tank; PB–polystyrene beads; P–pump; R1–R6 – reactor R1–R6; V–valves.

TAN removal at different flow rates

TAN removal efficiency of the reactor system was measured at different flow rates. An 8 m^3 concrete tank was filled with 2 m^3 of 30 g L^{-1} salinity seawater and NH_4Cl solution of 10 000 mg L^{-1} concentration was added to the tank to get a final TAN concentration of 2 mg L^{-1}. pH of the seawater was adjusted to the optimum of 8 using 10% Na_2CO_3.This medium from the tank was circulated through the reactors under different flow rates of 250, 750, 1500 and 2500 L h^{-1}. Flow rates through the reactors were adjusted using the valve connecting the overhead tank to the reactors and calculated by measuring the outgoing water from the reactors using measuring cylinder. TAN removal was calculated by the analysis of outcoming water from the reactors and, by using these values, volumetric TAN conversion rate (VTR) of the reactors under different flow rates was calculated following Colt *et al.*[38]

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

where VTR is the g TAN converted per m^3 of filter media per day,
Q_R the flow rate through the filter (L min^{-1}),
K_c the unit conversion factor of 1.44 to convert mg min^{-1} to g day^{-1},
TAN_I and TAN_E the influent and effluent total ammonia nitrogen (mg L^{-1}), and
V_b is the volume of filter media (0.023 m^3).

The experiment was repeated twice.

Fluorescence *in situ* hybridization (FISH) analyses of the biofilm

After 70 days of operation, 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 at 10 000*g* and fixed for fluorescent *in situ* hybridization (FISH) analyses. The FISH analyses of the biofilm was carried out using a universal bacterial probe (EUB 338) and nitrifier-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).[25]

Statistical analyses

The significance of TAN and NO_2-N removal over time in the maturation system during 70 days of operation and at different substrate concentrations were tested by one-way ANOVA. The percentage TAN and NO_2-N removal rates over time were analyzed by simple regression analyses. The relationship between flow rate and VTR was also estimated by simple regression analysis.

RESULTS AND DISCUSSIONS

Nitrification in the PBBR integrated *Penaeus monodon* maturation system

Instant nitrification was observed after integration of the PBBR into the maturation system (Fig. 2). During 70 days of rearing, TAN

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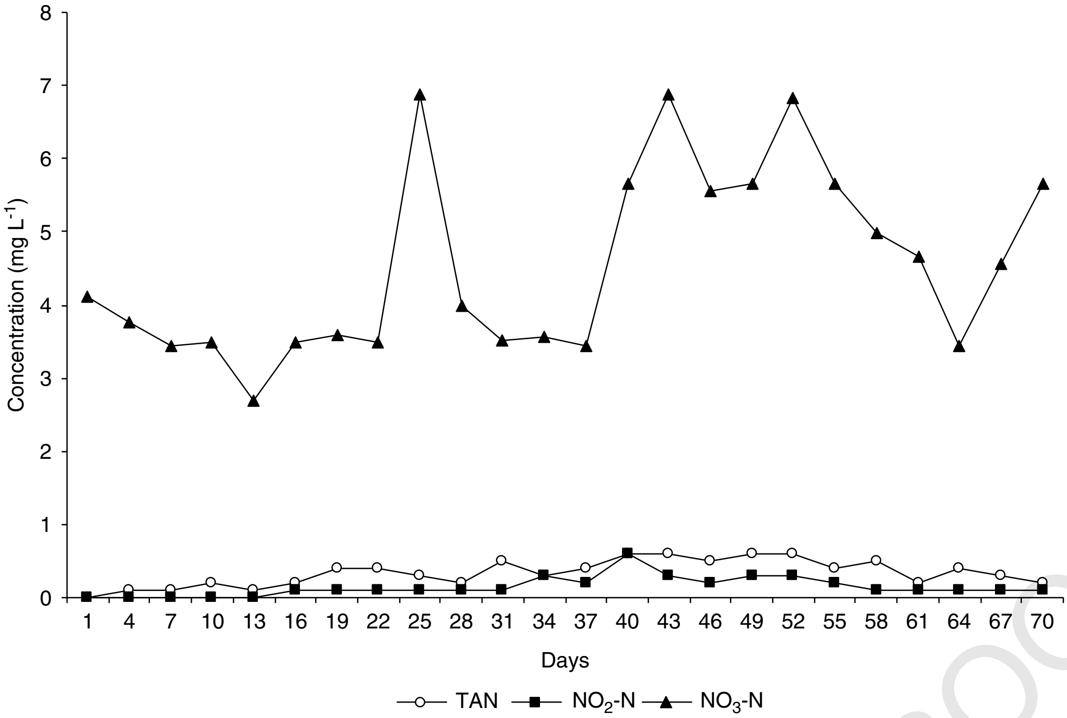

Figure 2. TAN, NO_2-N and NO_3-N concentrations in the rearing water of *P. monodon* maturation system integrated with packed bed bioreactor during 70 days operation.

and NO_2-N concentrations were always near to zero while the NO_3-N observed a maximum of 7 mg L^{-1}. Even though the TAN and NO_2-N concentrations of the incoming water from the rearing tank was above 0.5 mg L^{-1}, the outcoming water from the PBBR maintained a concentration below 0.1 mg L^{-1} and the extent of removal was highly significant ($P < 0.001$). The water quality maintained through the reactors was ideal for the maturation of the *P. monodon* as indicated by the development of mature ovaries.

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.[39] Without the addition of nitrifiers as start-up cultures, 2–3 months are needed to establish nitrification in marine systems and 2–3 weeks in fresh water and there is an agreement that saltwater systems need a much longer start-up period.[40] Under such situations, immobilization techniques have been found useful to overcome the delay in the initiation of nitrification.[41] It was interesting to note that even after 70 days of operation of the reactor the residual NO_3-N level in the system was not going above 7 mg L^{-1} suggesting an active denitrification process in the system. Earlier studies showed that the PBBR was potent in establishing nitrification in brackish water recirculating larval rearing system,[25,42] resulting in enhanced larval survival. In the present study the PBBRs performed efficiently in the maturation systems supporting higher biomass.

Nitrification at different substrate concentrations

The initial TAN concentrations in all the reactors (2, 5 and 10 mg L^{-1}) decreased significantly ($P < 0.001$) over time (Fig. 3). With the increasing TAN concentrations (2, 5 and 10 mg L^{-1}) the time for substrate removal was found increased, respectively, to 3, 6 and 9 h. NO_2-N concentrations in the reactors as substrate concentrations of 2, 5 and 10 mg L^{-1} also decreased over a period significantly ($P < 0.001$) (Fig. 4). The percentage TAN removal rates in the reactor system were found to increase over the period (Fig. 5) and regression analyses showed significant removal rates. The regression equation for NO_2-N at 2 mg L^{-1} was not significant as 82% of the substrate was removed in 1 h after which there was no substantial reduction. Since substrate limitation is a major concern for aquaculture biofilter designs,[18] blind comparison of data from traditional wastewater treatment processes to the design of aquaculture biofilters looks inappropriate as nitrification conditions in aquaculture systems differs substantially from domestic and industrial wastewater. Compared with domestic wastewater,[43] aquaculture wastewater has a relatively low concentration of pollutants[44] having total ammonia nitrogen (TAN) ranging from 1 and 3 mg L^{-1} in rainbow trout and catfish aquaculture systems, respectively,[45] whereas in domestic it ranges from 20–50 mg L^{-1}.[43]

To date very limited attempts have been made to investigate nitrification kinetics of aquaculture biofilters. Bovendeur *et al.*[46] investigated nitrification kinetics of a trickling filter in a warm water system and found that the biofilter nitrification rate followed half-order kinetics for a TAN concentration of less than 2 mg L^{-1}, while zero-order kinetics was applied to a TAN concentration of 2 to 10 mg L^{-1}. Tseng and Wu[47] studied the effects of temperature, ammonia, and suspended solids on biofilter ammonia removal efficiency and developed a regression model to provide operating guidelines for biofilter backwash frequency. Many of the biofilter nitrification rates obtained for aquaculture systems used synthetic substrate solutions; however, under field conditions it might differ as the aquaculture wastewater contains organic matter. Moreover, salinity is also an important factor affecting nitrification in aquaculture systems, as many of these systems are operating under different salinities.[30] The maximum nitrification capacity

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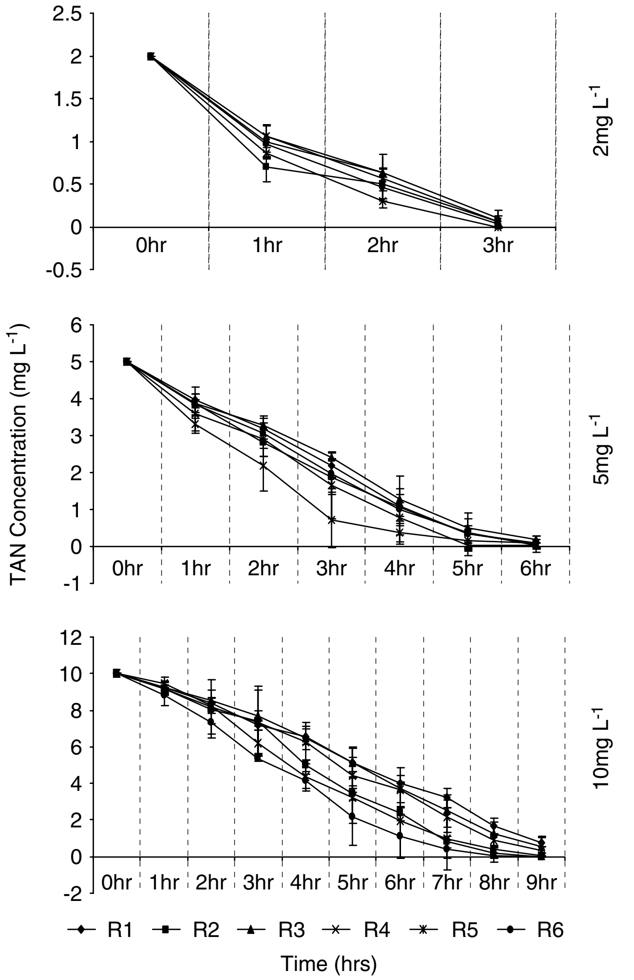

Figure 3. TAN consumption in the reactors fed with TAN concentrations of 2, 5 and 10 mg L^{-1}.

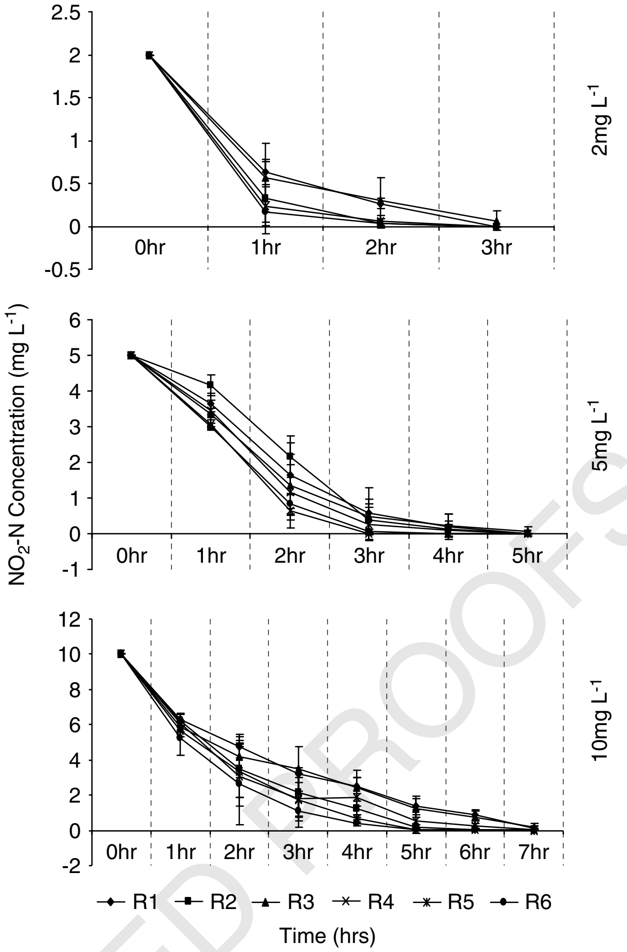

Figure 4. NO_2-N consumption in the reactors fed with NO_2-N concentrations of 2, 5 and 10 mg L^{-1}.

in saltwater systems was found to be considerably lower than in freshwater systems,[48] although Saucier[49] was able to obtain a sufficient nitrification rate comparable with that reported for freshwater systems. Unlike the above observations, in typical salt water systems investigated here there was no delay in the initiation of nitrification as the reactors could be activated with potent nitrifying bacterial consortia having the optimum salinity 30 g L^{-1}. Many earlier studies reported that nitrification rates increase linearly with the increase of TAN substrate concentration.[32,50–52] In the present study there was significant nitrification at all the substrate concentrations tested.

TAN removal at different flow rates

The average volumetric TAN removal rates increased with flow rates from 43.51 (250 L h^{-1}) to 130.44 (2500 L h^{-1}) g TAN m^{-3} d^{-1} ($P < 0.05$) and there was a decreasing TAN concentration with increased flow rates (Fig. 6). The increase in the average TAN removal rates is due to the increased turbulence and subsequent mass transfer of substrate into the biofilms during the high flow rate. In an earlier study of PBBR integrated into a brackish water recirculation system the average volumetric TAN removal rates (VTR) at the feed rate of 160 g day^{-1} and flow rate of 240 L h^{-1} from days 54–60 of culture was 153.3±0.4.5 g TAN m^{-3} d^{-1}.[42] However, in the present study the increase in the flow rate coincided with the decreasing hydraulic retention time (HRT) as the experimental duration lasted until the overall total substrate consumption. Stoodley *et al.*[31] investigated the relationship between local mass transfer coefficients and fluid velocity in heterogeneous biofilms using microelectrodes and confocal scanning laser microscopy and found that the effects of biofilm heterogeneity on mass transport were strongly dependent upon the average flow velocity. de Beer *et al.*[53] measured DO concentration profiles on heterogeneous biofilm and found that the thickness of the mass transfer boundary layer on the film decreased exponentially with increasing flow velocity. Zhu and Chen[54] investigated the relationship between total ammonia nitrogen removal rate and the Reynolds number (Re) in a steady-state nitrification-fixed biofilm and observed that the Reynolds number of the flow had a significant impact on the ammonia removal rate demonstrating that the hydraulic condition of the biofilm surface was a major factor affecting TAN removal rate. In another study by Zhu and Chen[55] it was shown that the turbulence caused by air mixing had a significant impact on nitrification rate in the fixed film biofilters suggesting that increasing turbulent air flow could be an effective method to improve the nitrification efficiency of fixed film biofilters.

The nitrification rate can be improved significantly through increasing the turbulence as nutrient mass flux determines the efficiency of a fixed film biofilter. Turbulence affects the thickness of the water film and subsequently the transfer resistance of

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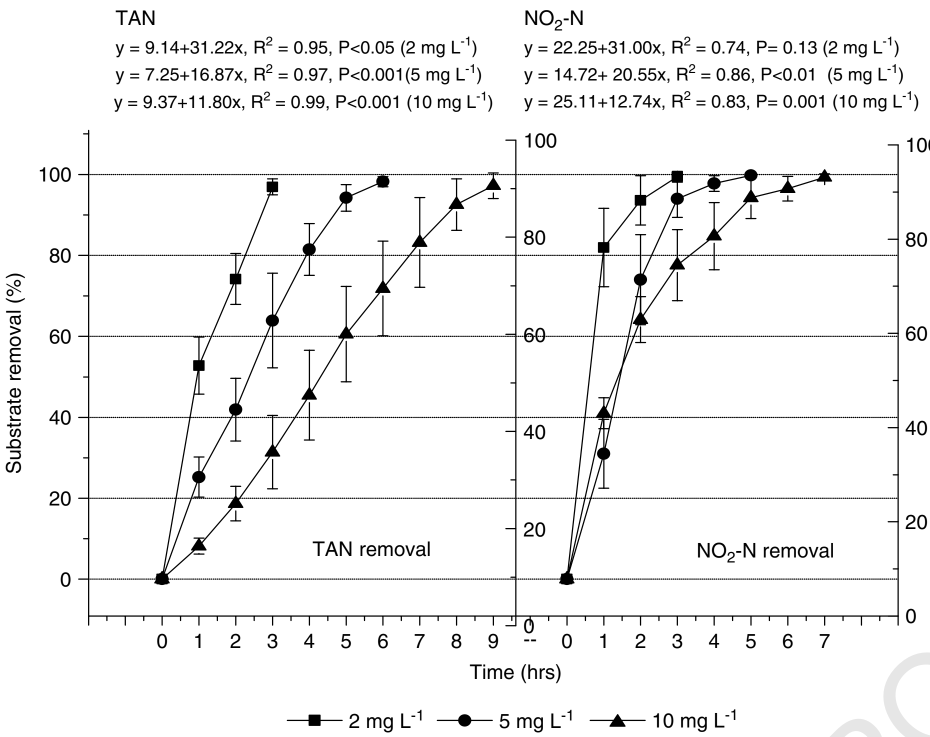

Figure 5. Percentage TAN and NO_2-N removal rates in the reactor system at initial substrate concentrations of 2, 5 and 10 mg L^{-1}.

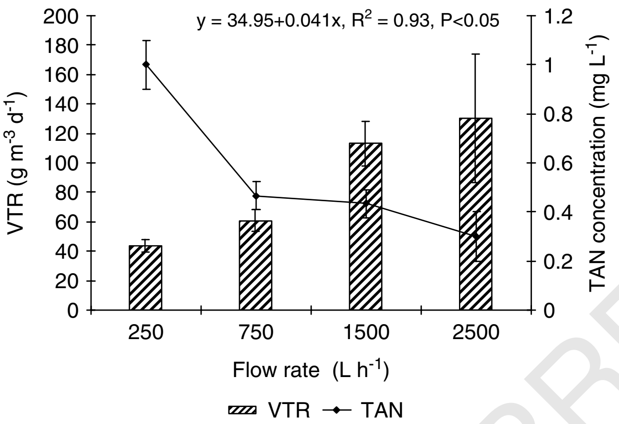

Figure 6. TAN concentrations and volumetric TAN removal rates in the reactor system at different flow rates.

substrate from bulk liquid into the biofilm. The transport of substrate in moving liquid is governed by molecular diffusion and advection.[56] Experimental studies have shown that the turbulence caused by air mixing had a significant impact on nitrification rate in the fixed film biofilters.[30,55] Hsu *et al.*[57] examined the kinetic behaviors of nitrogen compounds in biofilm channeling under laminar and turbulent flow conditions and found that the flow velocity significantly influenced the nitrification and denitrification conversion rates.

Fluorescence *in situ* hybridization (FISH) analysis of the biofilm

Prominent biofilm formation was observed on the beads taken from the reactor after completing an operating period of 70 days (Fig. 7). FISH analysis of the biofilms with probes NSO 190 (β ammonia oxidizers), NsV 443 (*Nitrosospira* sps) NEU (halophilic *Nitrosomonas*), Ntspa 712 (Phylum Nitrospira) 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 the FISH probes and microelectrodes.[58–60] In FISH analysis of the mature biofilm from the PBBR after 4 months operation at 15 g L^{-1} salinity, Kumar *et al.*[42] reported positive signals from probes for the β ammonia oxidizers (NSO 190), *Nitrosococcus* mobilis lineage (NmV), *Nitrobacter* spp (NIT2), and for the phylum Nitrospira (Ntspa 712). This proved the usefulness of the activated consortia to establish mature biofilm in real life situations. 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 were well suited for the establishment of nitrifying biofilms in practical sense. Schramm *et al.*[59] 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,* whereas *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 O_2 at saturation and TAN at low concentrations where the biofilm was dominated by autotrophic nitrifiers. However, denitrifiers could also be expected based on the evidence that NO_3-N was stabilized between 2.5 and 7 mg L^{-1}. Fewer reports are available for the nitrifying bacterial populations inhabiting the biofilm having a limited supply of the substrates especially in aquaculture systems.

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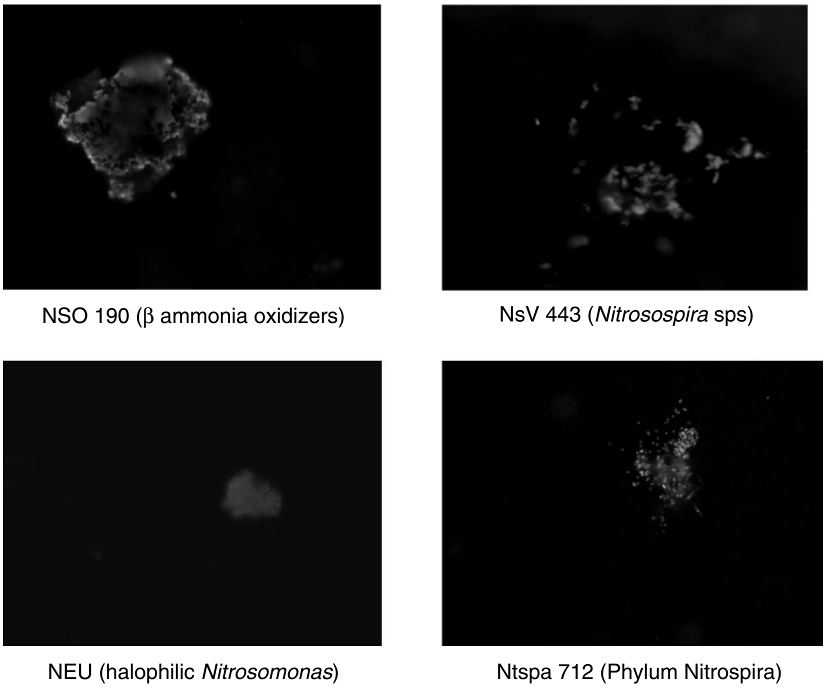

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Figure 7. Fluorescent *in situ* hybridization of the biofilm taken from PBBR integrated with *P. monodon* maturation system after 70 days of operation.

CONCLUSIONS

In conclusion, the PBBR integrated into the *P.monodon* maturation system exhibited significant nitrification ($P < 0.001$) during operation for 70 days as well as at different substrate concentrations and flow rates. The TAN concentration in the system consistently reduced significantly during normal operation maintaining the animals safe as observed in our earlier studies on PBBR. In all substrate concentrations tested, the nitrification was instant and there was significant removal over time. The average volumetric TAN removal rates increased with flow rates due to the increased turbulence and subsequent mass transfer. The FISH analyses of biofilm on the substrata showed stability in terms of composition of nitrifiers on long-term operation as observed in our earlier studies. All these observations suggest that the PBBRs can be integrated into marine and brackish water aquaculture systems making them closed recirculation systems for maintaining biosecurity and environmental quality.

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Supporting information

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