

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 (AMON-PCU-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

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

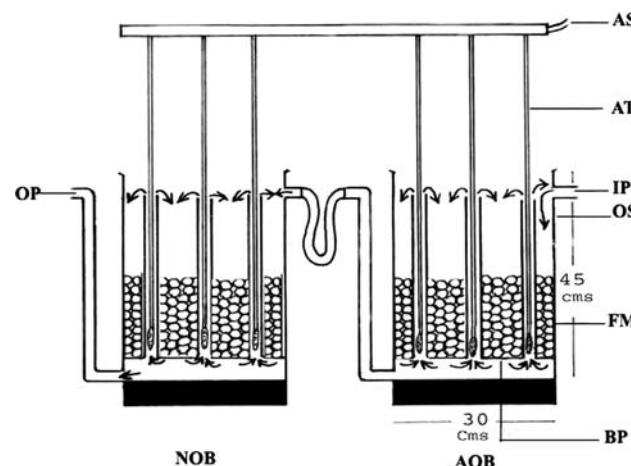


Fig. 1 Cross-sectional view of the bioreactors connected serially (AOB ammonia-oxidizing bioreactor, NOB nitrite-oxidizing bioreactor, BP base plate, FM filter media, OS outer shell, IP inlet pipe, OP outlet pipe, AT aeration tubes, AS air supply)

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.

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 $\text{NH}_4\text{-N}/\text{NO}_2\text{-N}$ and production of $\text{NO}_2\text{-N}/\text{NO}_3\text{-N}$ 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 ($(\text{NH}_4)_2\text{SO}_4/\text{NaNO}_2$), 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 ($(\text{NH}_4)_2\text{SO}_4/\text{NaNO}_2$) 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 ($27 \pm 0.5^\circ\text{C}$) 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 ‘Probibase’ [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-lysine, 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	CGATCCCCCTGCTTTCTCC	CY3	55	20	[41]
NEU	Halophilic and halotolerant members of the genus <i>Nitrosomonas</i>	CCCCTCTGCTGCACCTCTA	CY5	40	56	[66]
NSV443	<i>Nitrosospira</i> spp.	CCGTGACCGTTTCGTTCCG	CY3	30	112	[41]
NmV	<i>Nitrosococcus mobilis</i> lineage	TCCTCAAGAGACTACGCCG	Fluorescein	35	80	[49]
NIT2	<i>Nitrobacter</i> species	CGGGTTAGCGCACCGCCT	Cy5	40	56	[67]
Ntspa712	Phylum <i>Nitrospira</i>	CGCCTTCGCCACCAGGCCTTCC	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 (Enviroeup, 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 O₂ and CO₂ for activation. Optimum culture conditions, as described under “[Nitrifying bacterial consortia](#)”, were maintained in each reactor during the activation period. The substrate concentrations (NH₄-N/NO₂-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)

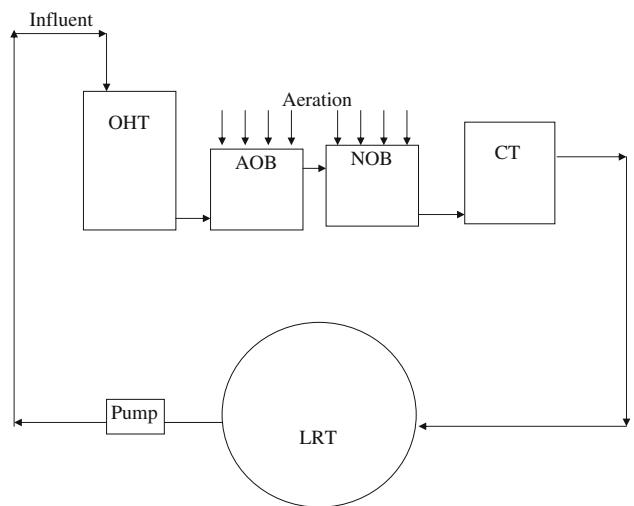


Fig. 2 Process flow diagram of the reactors integrated into the larval rearing tank (*OHT* overhead tank with automatic water level controller, *AOB* ammonia-oxidizing bioreactor, *NOB* nitrite-oxidizing bioreactor, *CT* collection tank for the treated water, *LRT* larval rearing tank)

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]:

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

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 (NO₂-N) [5] and nitrate (NO₃-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

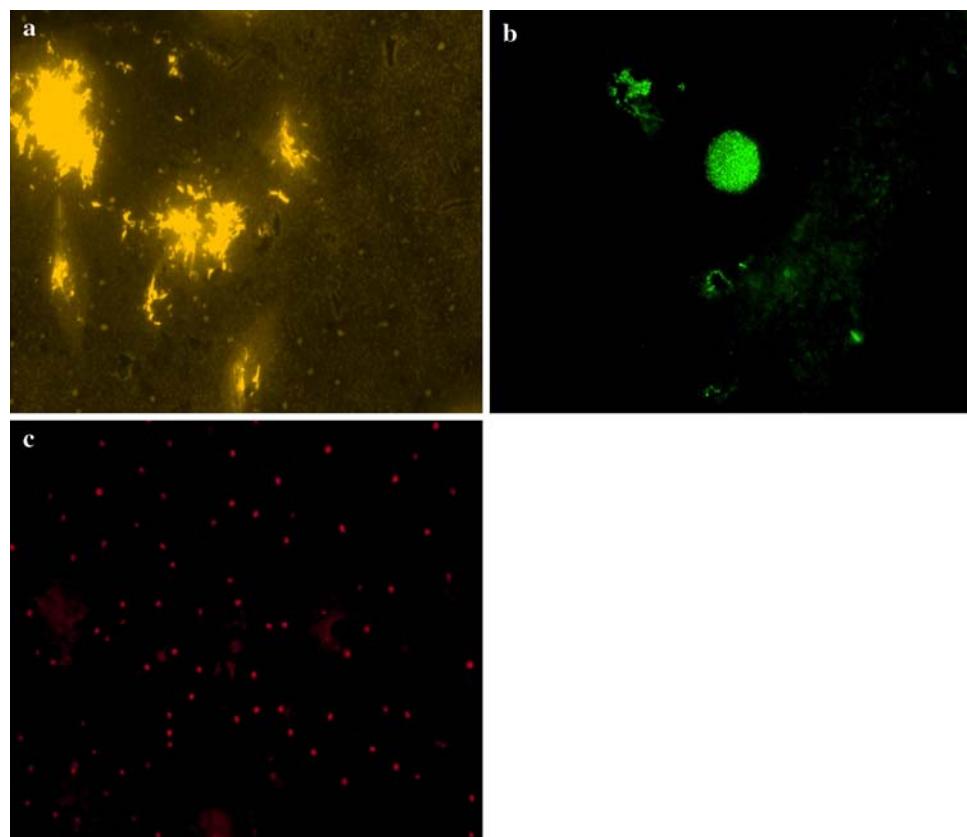
The relationship between removal of ammonia and nitrite and the biomass in suspension during the activation mode was estimated by simple correlation coefficient analysis. The nitrification efficiency and significant percent survival of larvae in the control and reactor integrated tanks were estimated 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 auto-trophic 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 NO₂-N and NO₃-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% NO₂-N removal. There was negative correlation between the percentage removal of TAN ($r = -0.96$, $P < 0.01$), NO₂-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/(m² day) and 45.14 mg NO₂-N/(m² day).

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 NO₂-N after 14 days; however, NO₃-N was never found built up in the system. Meanwhile, there was significant TAN removal ($P < 0.01$) in the experimental tanks with significant ($P < 0.05$) NO₂-N removal. Within 8 days both TAN and NO₂-N concentrations were below detectable levels. NO₃-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, NO₂-N and NO₃-N were lower in the experimental tanks than in those of the controls (fourth day) indicating higher percentage removal of TAN (78%), NO₂-N (79%) and BOD (56%).

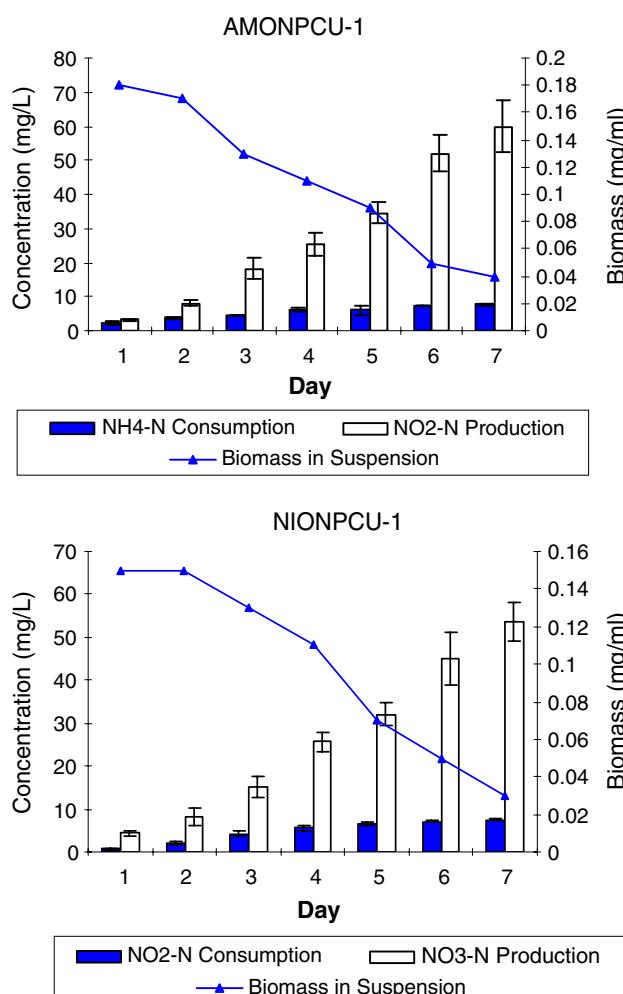


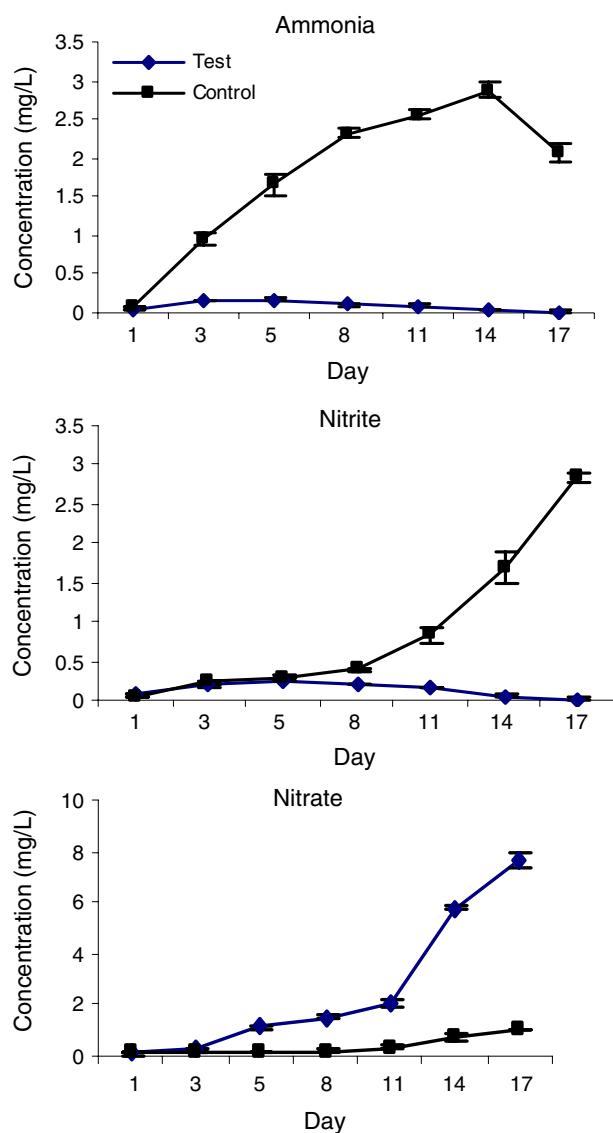
Fig. 4 Activation kinetics of PBBR immobilized with nitrifying consortia developed for *M. rosenbergii* hatchery systems

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 m²/m³. 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	
Control tank	18	18.33 ± 1.53		22.86	Between treatments	1	<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 NO₂-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 NO₂-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 NO₃-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/m³) 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 within 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 bio-security.

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