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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

V. J. Rejish Kumar,[a] Valsamma Joseph,[a] R. Vijai, Rosamma Philip[b] and I. S. Bright Singh[a]*

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.

Supporting information may be found in the online version of this article.

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.

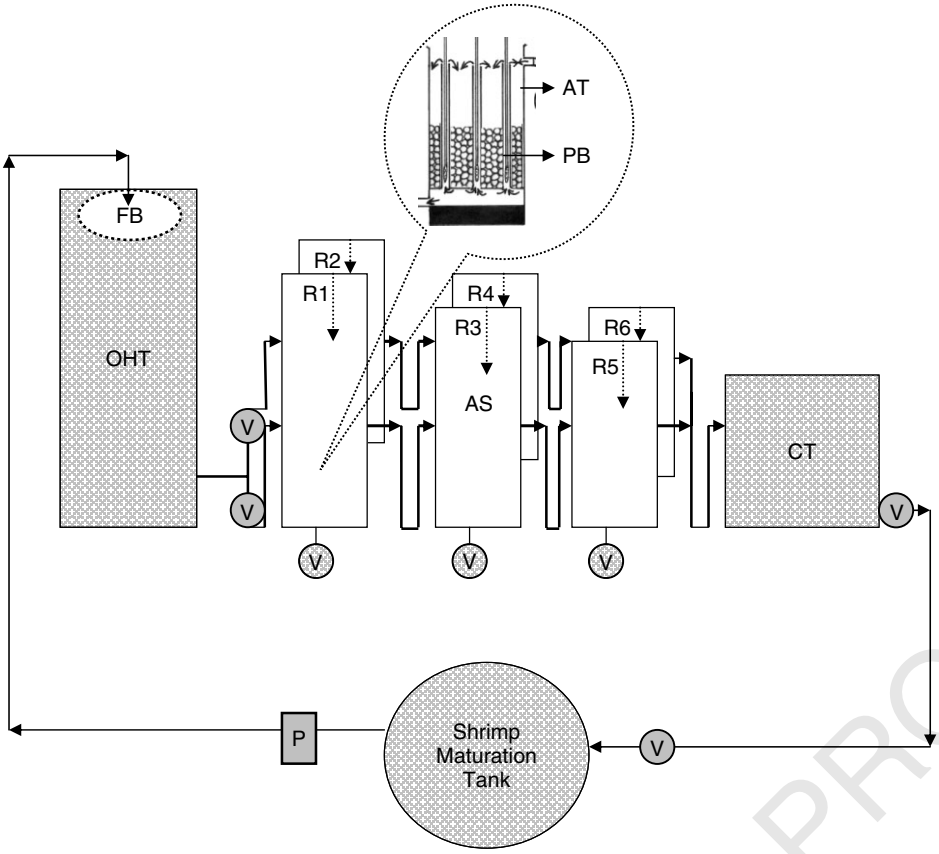

Figure 1. Packed bed bioreactor connected to a shrimp maturation system. AS – aeration supply; AT – aeration tube; CT – collecting tank; FB – filter bags; OHT – Overhead tank; PB – polystyrene beads; P – pump; R1 – R6 – reactor R1 – R6; V – valves.

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

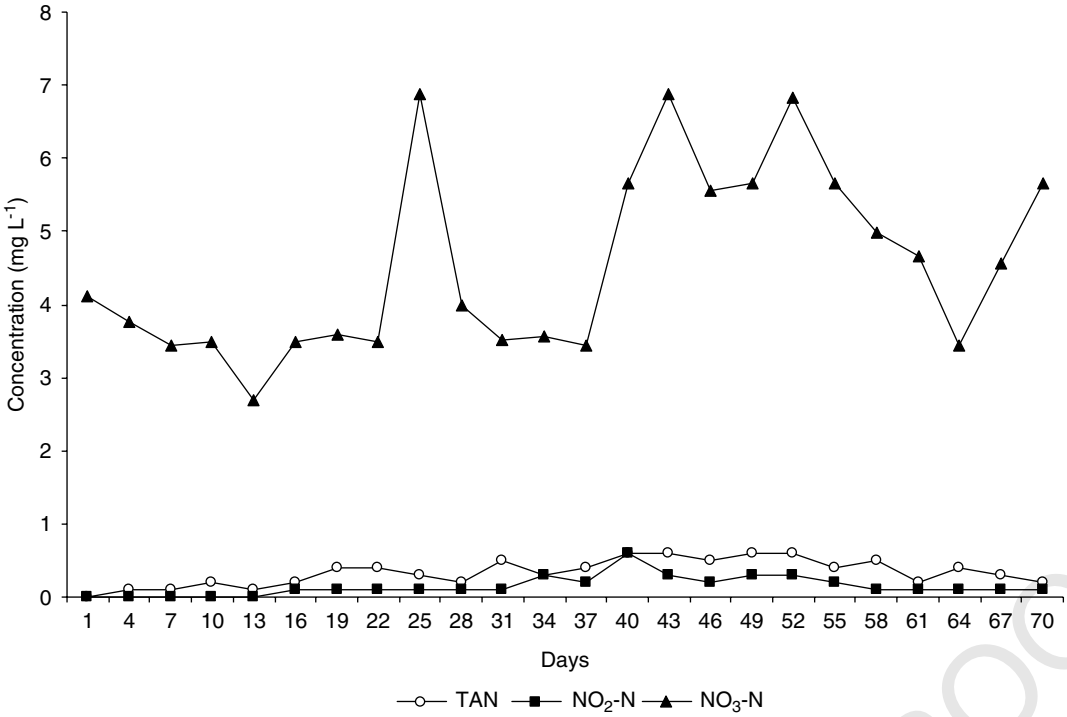

Figure 2. TAN, NO_2-N and NO_3-N concentrations in the rearing water of *P. monodon* maturation system integrated with packed bed bioreactor during 70 days operation.

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

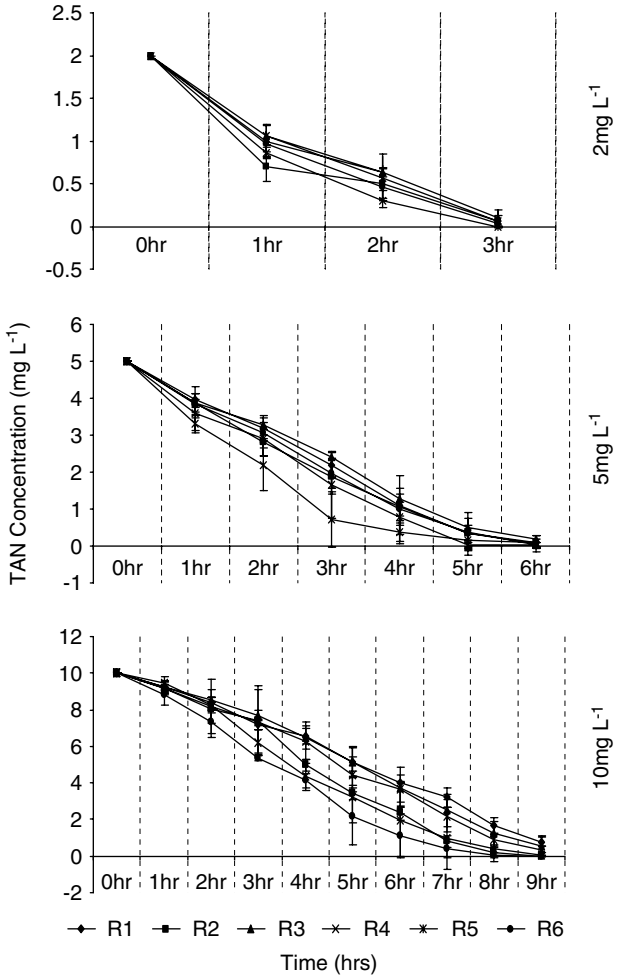

Figure 3. TAN consumption in the reactors fed with TAN concentrations of 2, 5 and 10 mg L^{-1}.

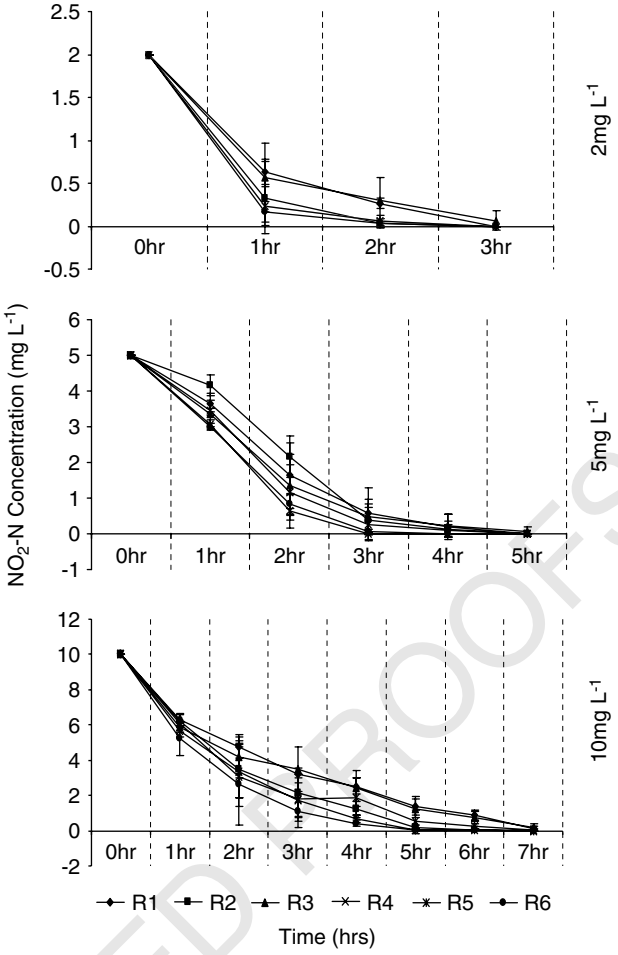

Figure 4. NO_2-N consumption in the reactors fed with NO_2-N concentrations of 2, 5 and 10 mg L^{-1}.

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

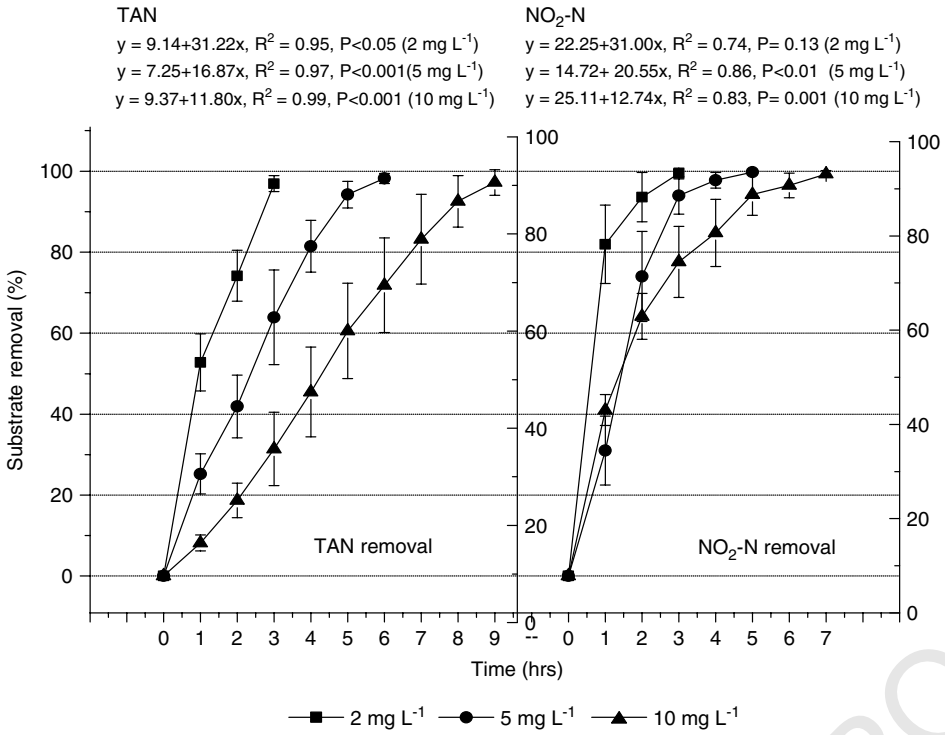

Figure 5. Percentage TAN and NO_2-N removal rates in the reactor system at initial substrate concentrations of 2, 5 and 10 mg L^{-1}.

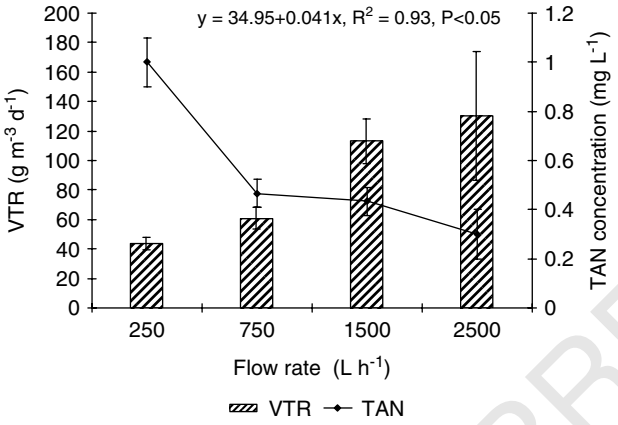

Figure 6. TAN concentrations and volumetric TAN removal rates in the reactor system at different flow rates.

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.

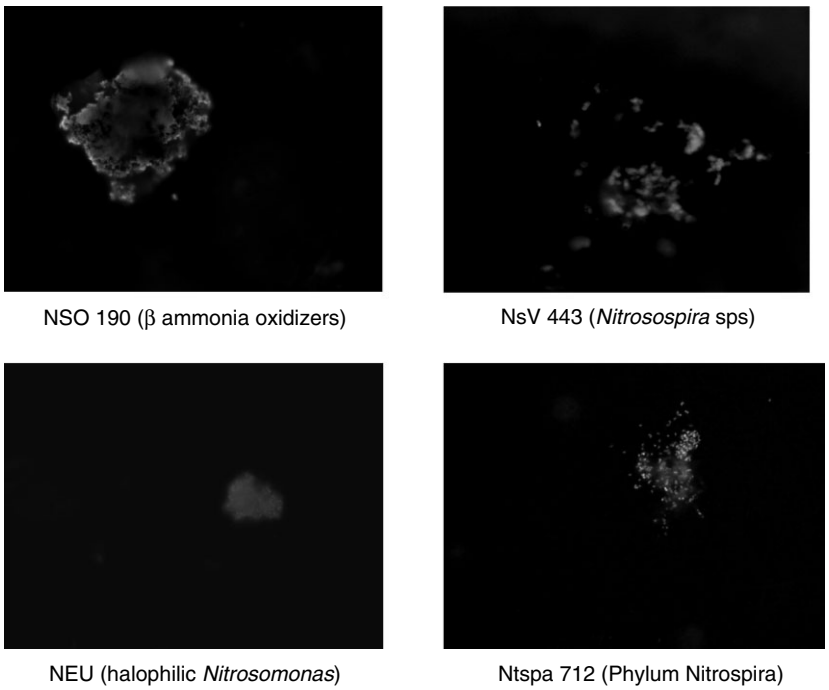

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Figure 7. Fluorescent *in situ* hybridization of the biofilm taken from PBBR integrated with *P. monodon* maturation system after 70 days of operation.

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

Supporting information may be found in the online version of this article.

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