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Research paper

Immobilization of nitrifying bacterial consortia on wood particles for bioaugmenting nitrification in shrimp culture systems

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ABSTRACT

Shrimp grow out systems under zero water exchange mode demand constant remediation of total ammonia nitrogen (TAN) and NO_2^- – N to protect the crop. To address this issue, an inexpensive and user-friendly technology using immobilized nitrifying bacterial consortia (NBC) as bioaugmentors has been developed and proposed for adoption in shrimp culture systems. Indigenous NBC stored at 4 °C were activated at room temperature (28 °C) and cultured in a 2 L bench top fermentor. The consortia, after enumeration by epifluorescence microscopy, were immobilized on delignified wood particles of a soft wood tree *Ailantus altissima* (300–1500 μm) having a surface area of 1.87 $m^2\ g^{-1}$. Selection of wood particle as substratum was based on adsorption of NBC on to the particles, biofilm formation, and their subsequent nitrification potential. The immobilization could be achieved within 72 h with an initial cell density of 1×10^5 cells mL^{-1}. On experimenting with the lowest dosage of 0.2 g (wet weight) immobilized NBC in 20 L seawater, a TAN removal rate of 2.4 mg L^{-1} within three days was observed. An NBC immobilization device could be developed for on site generation of the bioaugmentor preparation as per requirement. The product of immobilization never exhibited lag phase when transferred to fresh medium. The extent of nitrification in a simulated system was two times the rate observed in the control systems suggesting the efficacy in real life situations. The products of nitrification in all experiments were undetectable due to denitrifying potency, which made the NBC an ideal option for biological nitrogen removal. The immobilized NBC thus generated has been named TANOX (Total Ammonia Nitrogen Oxidizer).

1. Introduction

Expansion and intensification of aquaculture have been taken up as means for attaining food security in several tropical countries triggered by the rapid decline in ocean fisheries. However, this intensification has led to increased organic loading culminating in deterioration of water and sediment quality especially with high total ammonia nitrogen (TAN) (Shan and Obbard, 2001). As ammonia build up in aquaculture systems is deleterious to the rearing stock, their mitigation is of primary concern (Paungfoo et al., 2007) for sustainability. Optimum shrimp growth demands less than 0.1 ppm unionized ammonia (1.33 to 1.53 mg L^{-1} TAN at pH 8.0 and 28–30 °C) (Shan and Obbard, 2001), which otherwise leads to poor feed intake, retarded growth, poor survival and high susceptibility to diseases. Such situations are negotiated by water exchange up to 40% intermittently to facilitate removal of the toxic waste metabolites (Deb, 1998). While doing so a higher concentration of ammonia is discharged into the receiving waters (Jones et al., 2001), a situation often correlated with eutrophication (Shan and Obbard, 2001). Therefore, management of water quality in shrimp culture systems is an essential prerequisite for maximizing productivity and minimizing the impacts of shrimp culture effluent discharged into the surrounding environment.

Sequestering ammonia from culture systems has been achieved by chemical (Gräslund and Bengtsson, 2001) and biological filters (Malone and Pfeiffer, 2006) and through *in situ* application of microbial amendments (Rombaut et al., 2003). In small scale shrimp grow out systems, zero or limited water exchange system based on chemical and biological filtrations and recirculating aquaculture systems is not practical, and economically not viable (Schryver et al., 2008). Main constraints are high capital cost, and technical problems related to their establishment and operation (Shan and Obbard, 2001). An alternative for this is the bio-flocs technology (BFT) (Avnimelech, 2006; Crab et al., 2007), where heterotrophic bacteria and algae are grown into flocs under controlled conditions within the culture ponds. The intensive growth of heterotrophic bacteria immobilizes inorganic nitrogen depending on the C/N ratio. However, factors responsible for their dynamics and their effects on growth and survival of cultured species warrant further investigation to exploit the merits of BFT (Crab et al., 2007). Under such situations use of nitrifying bacteria as bioaugmentors has been found to be a better option, and biological nitrification can be sustained by maintaining optimal conditions for their proliferation. Both the groups of nitrifiers, ammonia and nitrite oxidizers, involved in nitrification are obligate autotrophs, and slow growers, and have different levels of sensitivities to environmental factors.

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However, immobilization techniques help overcome these limitations to a large extent (Seo et al., 2001), and while doing so, maintenance of a high cell density of viable culture of nitrifying bacteria in the active growth phase turns out to be a key factor in providing an effective *in situ* treatment for aquaculture (Shan and Obbard, 2001). Such studies in shrimp grow out systems are limited.

This prompted us to take up the study with an objective to develop an immobilized nitrifying bioaugmentor preparation for *in situ* treatment of TAN in shrimp culture systems. Four indigenous nitrifying bacterial consortia (NBC) developed for two salinity regimes in aquaculture (Achuthan et al., 2006) were used in this process. This study is also aimed at ascertaining the utility of immobilized nitrifying bioaugmentor as a viable option for *in situ* bioremediation of TAN and NO_2^- – N in zero water exchange shrimp grow out systems.

2. Materials and methods

2.1. Nitrifying bacterial consortia (NBC)

Ammonia oxidizing bacterial consortia and nitrite oxidizing bacterial consortia for non-penaeid and penaeid culture systems (AMONPCU-1, AMOPCU-1 and NIONPCU-1, NIOPCU-1) respectively (Achuthan et al., 2006) were used for the study. The NBC were cultured in a 2 L fermentor (Bioflo 2000, New Brunswick Scientific, USA) using seawater (salinity 15 or 30) based Watson's medium (1965) containing NH_4^+–N or NO_2^-–N (10 mg L^{-1}), PO_4^-–P (2 mg L^{-1}) and pH adjusted to 8.0.

The NBC stored at 4 °C were activated by incubating 50 mL culture with 50 mL Watson's (1965) medium on a rotary shaker at room temperature (28 ± 0.5 °C). Consumption of NH_4^+–N and production of NO_2^-–N in the case of ammonia oxidizers, and consumption of NO_2^-–N and production of NO_3^-–N in the case of nitrite oxidizers were treated as indications of activation. The activated cultures were grown in a 2 L fermentor under obscurity, substrate consumption (NH_4^+–N / NO_2^- –N) and product formation (NO_2^-–N / NO_3^-–N) monitored, and drop in pH, if any, was adjusted to the optimum (7.0 to 8.0) using 0.1 M sodium carbonate. Evaporation loss was compensated by the addition of sterile distilled water. As the substrate consumption progressed, cultures were supplemented with fresh aliquots of NH_4^+–N / NO_2^-–N to maintain the concentration at 10 mg L^{-1}.

Since activated NBC had larger aggregates of cell biomass as biofilm and flocs, their disaggregation was essential for enumeration. Ultrasonication, a suitable technique for non-destructive disaggregation of bacterial flocs (Salhani and Deffur, 1998), was employed in the present study. Aliquots of NBC equivalent to 0.01 g wet weight were subjected to ultrasonic treatments (Vibra cell, Sonics, USA) at different power outputs of 100, 125 and 150 W lasting for 1, 2, 3, 4 and 5 min. Cell dispersion was assessed by epifluorescence microscopy. Sonicated NBC were stained with acridine orange and filtered through 0.2 μm Irgalan black stained polycarbonate filters (Millipore GTBP011300). They were mounted immediately on a slide using non-fluorescent immersion oil and a minimum of seven fields per filter per sample was counted using an epifluorescence microscope (Olympus CX-41, Olympus Opticals Co., Japan).

2.2. Selection of carrier materials

To satisfy the requirements of an environment friendly and economically viable growth support medium with large specific surface area for immobilizing NBC, wood powder from *Ailanthus altissima*, *Macaranga peltata*, *Hevea braziliensis*, *Mangifera indica*, and chitin flakes, supplied by M/s India Seafoods Private limited, Cochin, India were chosen for initial screening. Samples were dried, crushed and sieved to get particles in the size ranges of 300–500 μm, 500–710 μm, 710–2000 μm, 2000–2500 μm, 2500–3000 μm and 300–1500 μm.

As lignin present in the wood particles might interfere with the nitrification potency of NBC, all samples were subjected for delignification following the method of Wood and Saddler (1988). Accordingly, crushed wood powder (2% w/v) was treated in tap water containing 1% (v/v) H_2O_2 at pH, 11.5. The suspension was gently stirred on a magnetic stirrer at 25 °C for 3–5 h, with hourly correction of pH to 11.5 on demand. The suspension was filtered and wood particles washed with tap water to neutral pH. Delignified wood particles were dried in hot air oven at 80 °C.

Surface profile of the support material of particle size in the range of 300–500 and 500–710 μm was determined by scanning electron microscopy (SEM). The particles were dried in critical point drying apparatus and spread on electron microscope stubs, gold coated and observed under SEM (Leo 435 VP SEM, UK). Two dimensional areas of the particles were calculated by image analysis using Soft Imaging Viewer, Soft Imaging System GmbH, version 3.1, build 507.

2.3. Initial experiment: Immobilization of the NBC

Delignified wood particles of the size ranges 300–500 μm and 500–710 μm drawn from *A. altissima*, *M. peltata*, *H. braziliensis*, *M. indica* and Chitin flakes were weighed to get an equal surface area (calculated based on the 2D image analysis) and transferred to test tubes containing 25 mL Watson's medium (1965). The tubes were inoculated with AMONPCU-1, NIONPCU-1, AMOPCU-1 and NIOPCU-1 having 1×10^6 to 1×10^7 cells mL^{-1}. They were incubated under obscurity with constant supply of filtered air at the rate of 0.25 L min^{-1}. Nitrification was monitored in terms of substrate consumption and product formation, and after 10 days of incubation the carrier material was gently washed in the growth medium and transferred to fresh aliquots to determine nitrifying potency of the immobilized NBC.

To confirm and document the extend of adhesion of nitrifying bacterial consortia to wood and chitin particles, scanning electron microscopy was performed by the following procedure: The growth support medium with immobilized nitrifiers was washed with sterile seawater (salinity 15 or 30) and fixed in 2.5% gluteraldehyde prepared in seawater (salinity 15 or 30) at 4 °C overnight. The pellets were washed and post fixed in 2% osmium tetroxide at 4 °C for 2 h. Subsequently, they were washed repeatedly with seawater and dehydrated through an acetone series of 70–100%, dried in critical point drying apparatus, spread on SEM stubs, gold coated and observed under SEM (Leo 435 VP SEM, UK).

2.4. Effect of particle size of the carrier material on nitrification

Based on the results of initial screening, *A. altissima* was selected as the source of support material for further experiments. Wood powders of particle size 500–710 μm, 710–2000 μm, 2000–2500 μm, 2500–3000 μm and 300–1500 μm were subjected for NBC immobilization to select the most appropriate size range which could sustain the highest nitrification rate. The experiment was conducted by using two sets of particles, one with equal surface area and the other with equal weight. Wood particles were weighed, sterilized and transferred to test tubes with 25 mL growth medium and inoculated with 1 mL NBC (AMONPCU-1, NIONPCU-1, AMOPCU-1 and NIOPCU-1 having 1×10^6 to 1×10^7 cells mL^{-1}). Tubes were incubated under obscurity with constant aeration (1 L min^{-1}) and nitrification monitored daily. The preparation was transferred to fresh medium to evaluate nitrification of the immobilized consortia as described above. Wood particles having the specific size range, which supported the highest nitrification rate, were selected for further experiments. Specific surface area of the wood powder selected was analyzed by Brunauer–Emmett–Teller (BET) method using BET analyzer (NOVA automated gas sorption system, Quantachrome instruments).

2.5. Nitrification potential of NBC immobilized wood powder on storage

Aliquots of the preparations (0.1 g wet weight) were examined initially for TAN and NO_2^-–N removal by incubating in 50 mL Watson's medium (1965) in 250 mL conical flask under obscurity and continuous aeration (1 L min^{-1}). Subsequently, the preparations were stored in

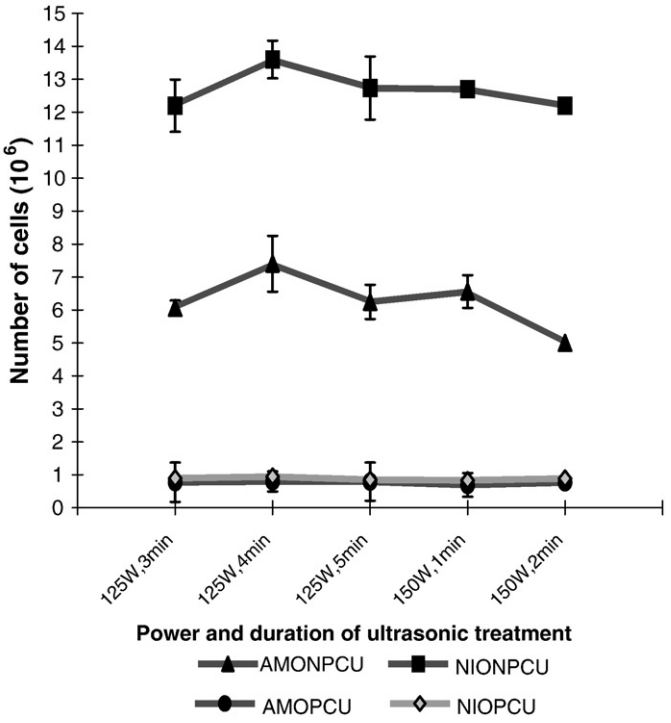

Fig. 1. Efficacy of ultrasonication on enumerating constituent cells in NBC. Number of replicates (n) = 3.

sealed polystyrene bottles at room temperature (28 ± 0.5 °C). After one month, 0.1 g aliquots (wet weight) were transferred to fresh medium and incubated under aerated and non-aerated conditions. TAN and NO_2^-–N removal rates were determined and the retention of nitrification potential assessed.

2.6. Time course of immobilization

Sterile wood powder (1 g) of *A. altissima* of particle size 300–1500 μm was suspended in 100 mL NBC (1×10^7 to 1×10^{10} cells mL^{-1}) in 250 mL conical flasks, supplemented with NH_4^+–N / NO_2^-–N to a final concentration 10 mg L^{-1} each. The flasks were incubated under obscurity at room temperature (28 ± 0.5 °C) with constant supply of filtered air at the rate of 1 L min^{-1}. Nitrifying activity was monitored and wood powder samples were retrieved at 6th, 12th, 24th and 72nd h of incubation and fixed for scanning electron microscopy.

2.7. Optimum cell count of NBC required for immobilization

Experiments were conducted to determine the optimum cell count of NBC required for immobilizing 1 g wood powder (Particle size 300 – 1500 μm). NBC were inoculated so as to get 10^5, 10^6, 10^7 and 10^8 cells mL^{-1} in 100 mL Watson's medium (1965) containing 1 g substratum. The system was kept under aeration at the rate 1 L min^{-1} for 3 days under obscurity. The supernatant was discarded, the wood powder re-suspended in fresh medium and nitrification potential determined. The lowest cell count, which exhibited the highest activity, was chosen for further immobilization experiments.

2.8. Quantity of NBC immobilized wood powder required for treating unit volume of seawater

Activated NBC (AMONPCU-1 and AMOPCU-1) having 1×10^6 to 1×10^7 cells mL^{-1} in 500 mL aliquots were aerated with sterile wood powder (10 g) with periodic pH correction and substrate addition. After three days, 0.2, 0.4, 0.6, 0.8, and 1.0 g (wet weight) each of the substratum was administered to fiber reinforced plastic (FRP) tanks, holding 20 L seawater (salinity 15/30) supplemented with 10 mg L^{-1} NH_4^+–N. Control tanks were maintained with seawater administered with sterile wood powder alone. Each treatment consisted of two

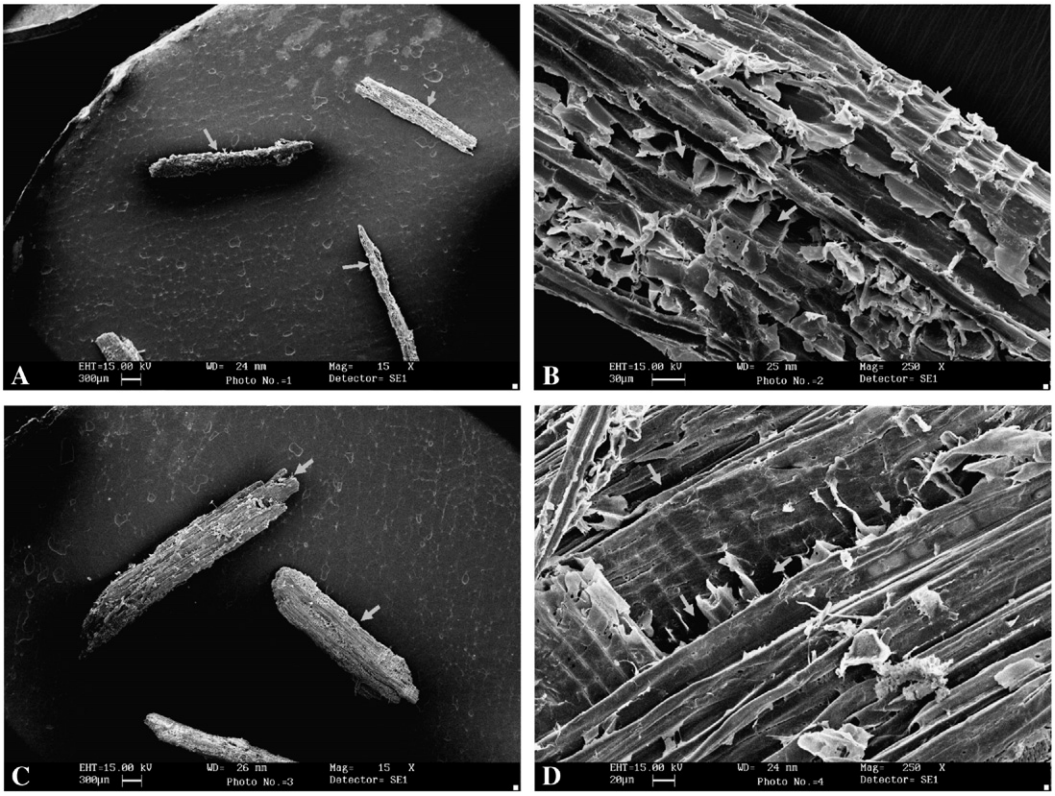

Fig. 2. Scanning electron micrograph of delignified wood particles derived from *A. altissima*, the selected support material for immobilization. (A and B: Particle size, 300–500 μm. C and D: Particle size, 500–710 μm).

Table 1
Consistency in TAN and NO_2^-–N removal observed with the nitrifying bacterial consortia during immobilization onto wood powder drawn from different plant species and subsequent suspension in fresh medium.

Carrier material derived from and the particle size	Coefficient of variance in TAN and NO_2^-–N removal (%)	
	During immobilization	In fresh medium
A. altissima (300–500 μm)	18.50	26.60
A. altissima (500–710 μm)	7.03[a]	5.80[a]
M. peltata (300–500 μm)	13.88	15.70
M. peltata (500–710 μm)	7.90	18.20
H. braziliensis (300–500 μm)	18.90	25.20
H. braziliensis (500–710 μm)	22.60	8.23
M. indica (300–500 μm)	13.70	20.60
M. indica (500–710 μm)	14.10	3.82
Chitin flakes (300–500 μm)	30.40	15.90
Chitin flakes (300–500 μm)	17.80	20.90

[a] Lowest coefficient of variance.

replicates, maintained with constant aeration at the rate of 2 L min^{-1} and nitrification over a period of 3 days was monitored and measured.

2.9. Evaluation of the nitrifying potency of immobilized NBC in bioassay system

The experiment was conducted in the salinity regimes of 10, 20 and 32. The consortium AMONPCU-1 was evaluated at salinity 10 and 20 and AMOPCU-1 at 32. The experimental design consisted of 12 tanks, each holding 24 L seawater, maintained under aeration at a rate of 2 L min^{-1}. Each set consisted of 3 replicates. The experiment was conducted with 3 shrimps (*P. monodon* of average weight 15 g) in each tank maintained without water exchange, and fed with commercial shrimp pelleted feed (Higashimaru Pvt. Ltd.) at a rate of 4% of body weight/day. After 8 days when TAN loadings were high (10–20 mg L^{-1}), 3 g (wet weight) immobilized nitrifying consortia were applied per tank. Water quality parameters such as, TAN, NO_2^-–N, NO_3^-–N, alkalinity, pH, BOD, turbidity, salinity and temperature were monitored daily up to 16th day of experiment. The extent of degradation of wood particles was also evaluated daily by physical examination, and BOD measurements were made on 30th and 60th day of experiment.

2.10. Mass immobilization of AMOPCU-1 on wood particles

For mass immobilization of NBC a simple device was designed and fabricated in FRP. The cylindrical device (70 cm total height, diameter 34 cm) with conical tapering bottom (20 cm height) has a capacity of 50 L and fitted with a stirrer assembly and an air diffuser at the bottom. To 20-L seawater (salinity 30), 10 mg L^{-1} NH_4^+–N and 2 mg L^{-1} PO_4^-–P (as NH_4Cl and KH_2PO_4) were added, pH adjusted to 7.5 using Na_2CO_3 and inoculated with AMOPCU-1 to get 1×10^7 cells mL^{-1}. Delignified wood powder (400 g) from *A. altissima* as the substratum

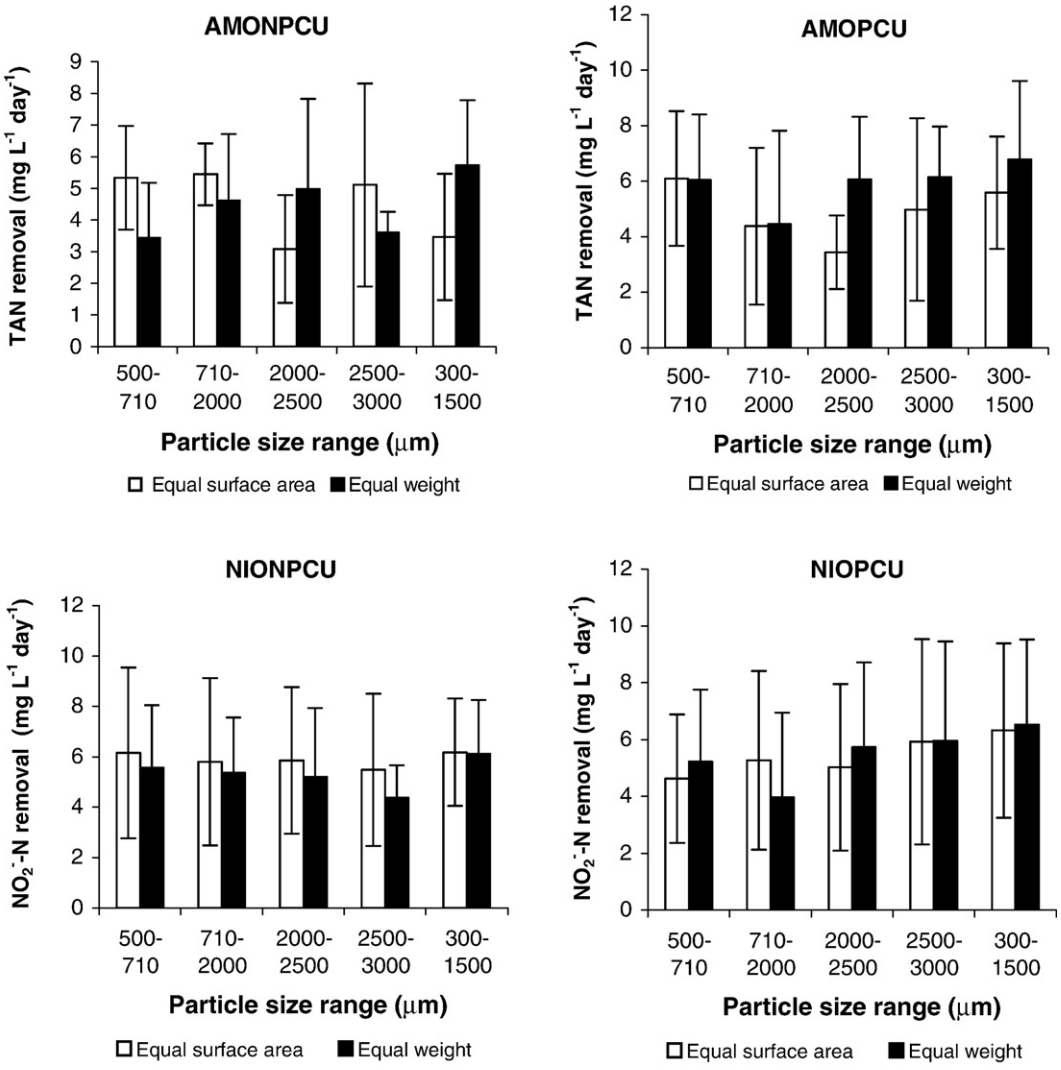

Fig. 3. TAN and NO_2^-–N removal by NBC during immobilization on wood particles derived from *A. altissima* having different particle size ranges. Number of replicates (n) = 5.

was added to the immobilization tank and aeration set at 6 L min^{-1}. Samples were analyzed daily for pH, TAN, NO_2^-–N and NO_3^-–N. On depletion of NH_4^+–N by 90%, additions were made to bring up the concentration to 10 mg L^{-1}. During the process three samples were drawn daily, washed gently in sterile medium (Watson's 1965) and transferred to fresh aliquots for analyzing the nitrifying potency as described above.

2.11. Evaluation of NBC immobilized wood particles in shrimp culture system

The immobilized NBC envisaged as products for bioremediation of ammonia in shrimp grow out systems has been named TANOX (Total Ammonia Nitrogen Oxidizer). Preliminary assessment of TANOX was done in a shrimp pond, where high TAN loading was experienced during routine analysis. Two shrimp ponds of 1 ha in area, 1 m in depth and having stocking density of 8.5 shrimps/m^{-2}, were used for the preliminary trial. The immobilized NBC were applied uniformly only in the test pond at the rate of 400 g per ha after mixing with pond water, and water quality parameters such as TAN, NO_2^-–N, NO_3^-–N, alkalinity and pH were monitored.

2.12. Statistical analysis

All measured variables (TAN, NO_2^-–N, NO_3^- –N, alkalinity, pH, BOD, turbidity, salinity and temperature) were analyzed by one-way ANOVA for all experiments conducted in simulated system and for experiments to select the carrier material. Consistency of nitrification activity, during the primary immobilization of NBC to different carrier materials was assessed by coefficient of variance. The effect of initial dosage on nitrification was assessed by simple correlation. Statistical analysis was performed using Excel package (Microsoft).

3. Results

3.1. Nitrifying bacterial consortia

After 7 days of activation in Watson's medium (1965), average TAN consumption was 4.0 ± 0.53 mg L^{-1} day^{-1} by AMONPCU-1 and 6.2 ± 0.78 mg L^{-1} day^{-1} by AMOPCU-1, and NO^{2-}–N consumption was 5.37 ± 1.2 mg L^{-1} day^{-1} by NIONPCU-1 and 6.89 ± 0.98 mg L^{-1} day^{-1} by NIOPCU-1. In all four NBC, products of nitrification were below detectable levels.

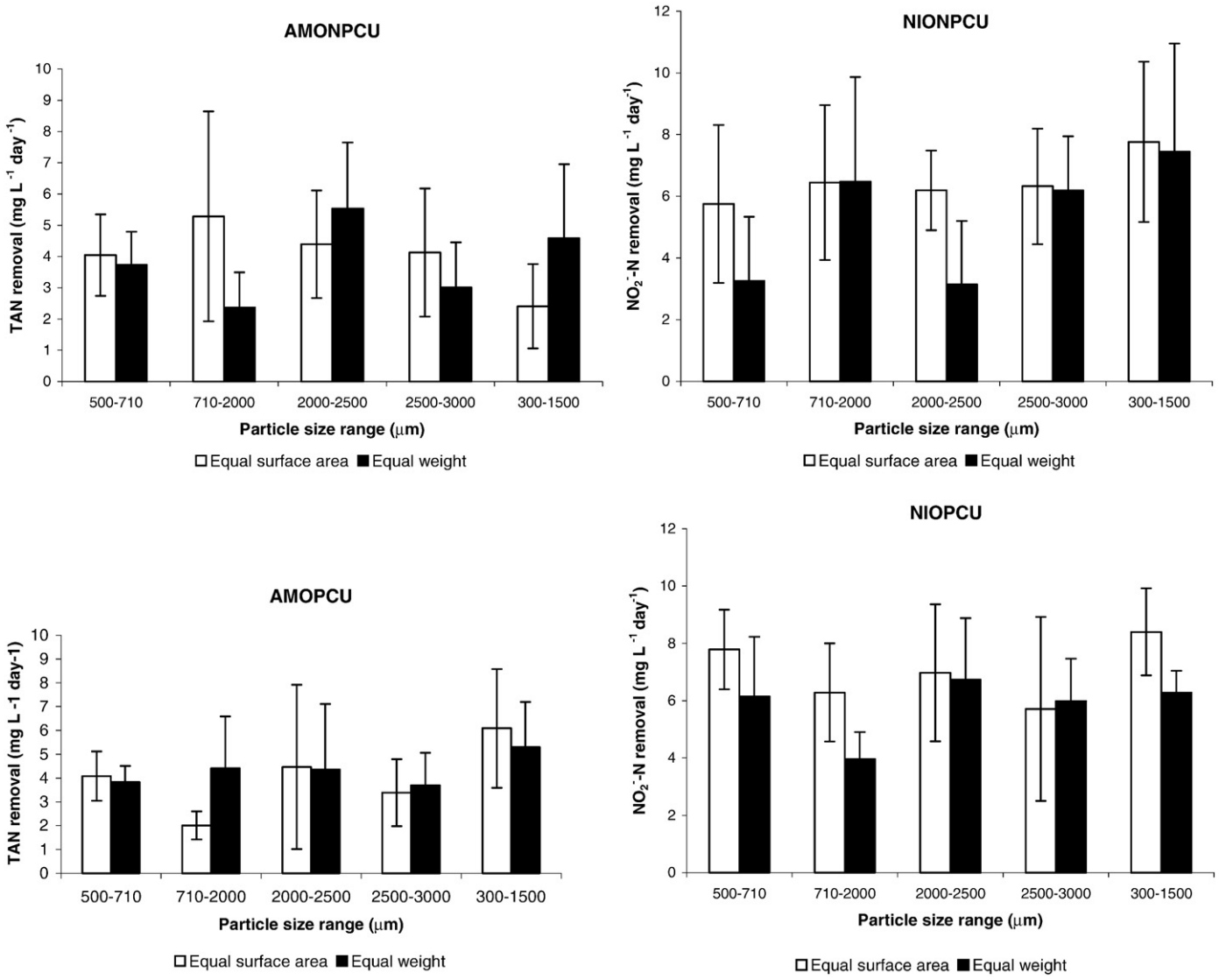

Fig. 4. TAN and NO_2^-– N removal by NBC immobilized on wood particles derived from *A. altissima* having different particle size ranges. Number of replicates (n) = 5.

On enumerating the ultrasonicated samples by epifluorescence microscopy, the highest number of cells obtained was at 125 W exposed for 4 min (Fig. 1).

3.2. Carrier materials

Delignification of wood powder with 1% (v/v) H_2O_2 in tap water for 3 to 5 h at pH 11.5 oxidized lignin to the point of no release subsequent to chlorination.

Surface profiles of wood particles and chitin flakes as carrier materials were documented and evaluated by SEM. Among the samples examined delignified *A. altissima* (300–500 μm and 500–710 μm) particles exhibited the highest level of corrugations (Fig. 2). Among the four species of woody plants examined the particles of *A. altissima* of size ranges 300–500 μm and 500–710 μm were found to have the highest surface area of 0.7 and 0.6 $m^2\ g^{-1}$ respectively. Meanwhile, carrier particles having the same range of particle sizes generated from *M. peltata*, *H. braziliensis* and *M. indica* were having the surface area 0.6 and 0.4 $m^2\ g^{-1}$, 0.44 and 0.3 $m^2\ g^{-1}$ and 0.56 and 0.27 $m^2\ g^{-1}$ respectively. Chitin flakes having sizes ranging from 300–500 μm and 500–710 μm registered surface area 0.36 and 0.3 $m^2\ g^{-1}$ respectively.

3.3. Immobilization of the nitrifying bacterial consortia on support materials

Although all carrier materials showed similar nitrification activities, selection of the most suitable support material was accomplished based on the consistency in nitrification during immobilization with all four NBC, and subsequently transferred to fresh medium expressed in terms of coefficient of variance (Cv). The growth support material derived from *A. altissima* with particle size 300–500 and 500–710 μm was found to have the least Cv (7.03 and 5.8% respectively) (Table 1) during and after immobilization. This approach was necessitated due to the requirement of utilizing one substratum for all the four nitrifying bacterial consortia.

3.4. Effect of particle size of the selected carrier material (A. altissima) on nitrification

The carrier materials derived from *A. altissima* of five different particle size ranges were used for immobilizing all the four NBC and the rates of nitrification in fresh medium assessed. TAN and NO_2^-–N removal by the consortia immobilized on wood particles of different particle size ranges was not significantly different ($p > 0.05$). When transferred to fresh medium after immobilization, the substratum with particle size 300–1500 μm showed better performance in removing TAN and TNN (Figs. 3 and 4). Specific surface area of the wood particles derived from *A. altissima* (300–1500 μm particle size), measured by BET analysis was 1.87 $m^2\ g^{-1}$.

3.5. Nitrifying potency of immobilized consortia and its shelf life

Immobilized nitrifying bacterial consortia, on storage at room temperature for a month, when revived, exhibited lowering of nitrification efficacy from the initial levels (Fig. 5).

3.6. Time course of immobilization of NBC

Scanning electron microscopy revealed the progressive biofilm formation of NBC on the growth support medium (Fig. 6). In general, attachment of cells could be seen at 12nd h exposure to the consortia. At 24th h of incubation, progression in biofilm formation could be observed and almost got completed by 72nd h.

3.7. Optimum cell count of NBC required for immobilization

On applying NBC (ammonia oxidizing) at cell counts of $10^5, 10^6, 10^7$ and 10^8 mL^{-1} for immobilizing on 1 g substratum (area 1.87 $m^2\ g^{-1}$), the highest TAN removal rate, during and after immobilization was observed with an initial cell count of 1×10^7 cells mL^{-1} (Fig. 7). However, the difference between the treatments was not significant ($p > 0.05$) suggesting that 1×10^5 to 1×10^8 cells mL^{-1} could effectively be used without having significant variations in performance between them.

3.8. Quantity of NBC immobilized wood powder for treating unit volume of seawater

Different quantities (0.2, 0.4, 0.6, 0.8 and 1 g) of NBC (AMONPCU-1 and AMOPCU-1) immobilized wood powder were applied in 20 L seawater, and the differences in TAN removal and NO_2^-–N production summarized in Table 2. Significant differences in TAN removal were observed in the case of AMONPCU-1 (p 0.012819) and AMOPCU-1 (p 0.0000205) with positive correlations between NBC administered and TAN removed. In the same way with respect to NO_2^-–N production the treatments with AMONPCU-1 also registered significant differences (p 0.044638), however, it was not significant when AMOPCU-1 was applied despite its positive correlations.

3.9. Evaluation of efficacy of NBC immobilized wood powder in a bioassay system

On administering 3 g (wet weight) immobilized nitrifying bacterial consortia in to shrimp rearing tanks kept under salinity 10, 20 and 30 where the TAN loadings were 10–20 mg L^{-1} nitrification was found to get established with in 24–48 h and progressed rapidly culminating in total TAN removal with in eight days. Simultaneous and corresponding nitrite production also could be noticed. Both these events, even though could be noticed in the control tanks, were of less magnitude (Fig. 8). Drop in alkalinity exhibited stronger correlation in the experimental tanks than in the corresponding controls. Application of the substratum with immobilized NBC did not increase the BOD of the system, which stood

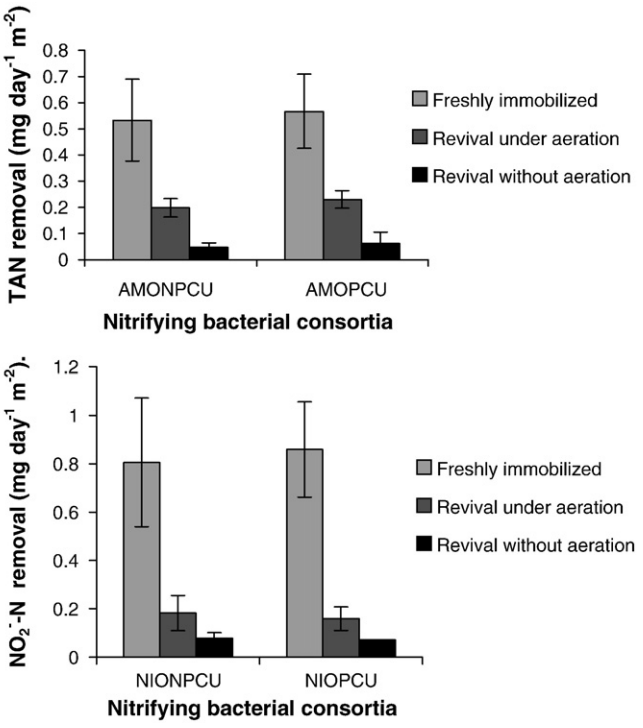

Fig. 5. Nitrifying potency of immobilized NBC and their shelf life. Number of replicates (n) = 3.

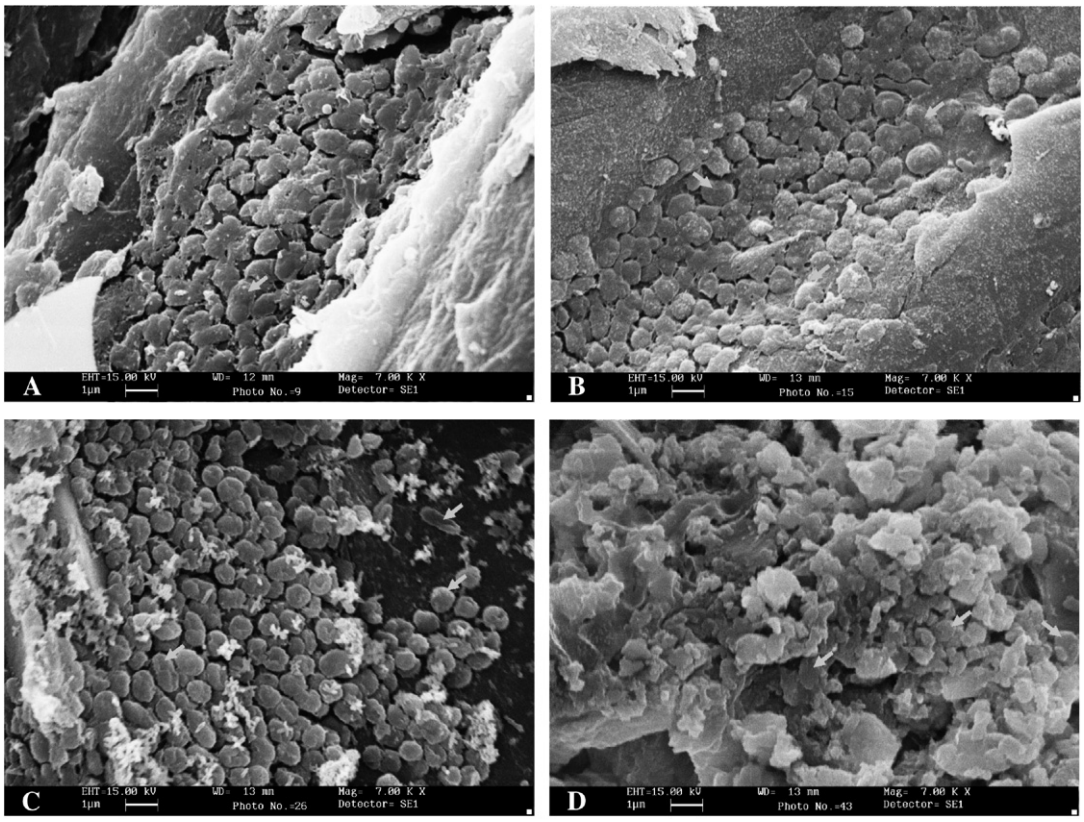

Fig. 6. Time course of immobilization of NBC (NIOPCU-1) on wood particles of *A. altissima* having particle size range 300–1500 μm. (A. 6 h; B. 12 h; C. 24 h and D. 72 h).

well below 50 mg L^{-1} at the end of the experiment (Fig. 9) and there was no increase in turbidity as well. Meanwhile, in the control tanks the substratum without consortia showed black discolouration and contributed to bottom detritus. Strikingly, the wood particles with NBC could retain their integrity and texture even on 60th day of administration.

3.10. Mass immobilization of AMOPCU - 1 on wood powder

The nitrifying bacterial consortium AMOPCU-1 was immobilized on the particles of *A. altissima* in a mass immobilizing device (Fig. 10). When AMOPCU-1 was immobilized on the substratum with particle size 300–1500 μm (20 g L^{-1}), average TAN removal observed during immobilization was 3.5 ± 1.52 mg L^{-1} day^{-1}, while NO_2^-–N and NO_3^-–N concentrations were below detectable limits. Immobilized wood powder sampled on day 4 and 5, exhibited substantial TAN removal potency at the rate 5.67 ± 1.6 mg L^{-1} day^{-1} (0.3 ± 0.085 mg m^{-2} day^{-1}) and 5.23 ± 1.91 mg L^{-1} day^{-1} (0.28 ± 0.1 mg m^{-2} day^{-1}) respectively, in fresh medium. There was no significant increase in TAN removal rate by the NBC after 4 days of immobilization ($p > 0.05$).

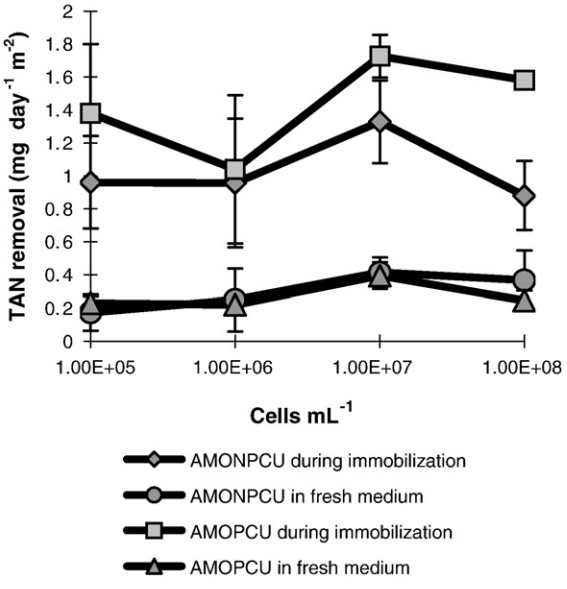

Fig. 7. Optimum cell count of NBC required for immobilization. Number of replicates (n) = 3.

3.11. Evaluation of immobilized nitrifying bacterial consortia in shrimp grow-out system

Immobilized NBC (AMOPCU-1) was administered in a shrimp culture pond (1 ha) where sudden TAN loadings were reported. Consequently, the TAN loading was brought down by a half (from 1 mg L^{-1} to 0.5 mg L^{-1}) within 24 h, while in the control pond the situation remained unaltered.

4. Discussion

Shrimp grow out systems are specialized dynamic aquaculture production facilities which when operated under zero water exchange mode, demand constant remediation of ammonia and nitrite nitrogen. In this context objective of the study was to develop an inexpensive and user-friendly technology employing immobilized nitrifying bacterial consortia (NBC) as bioaugmentors to address the issue of excessive dissolved inorganic nitrogen (DIN). Through this investigation, immobilization of indigenous NBC on wood powder derived from *A. altissima* could be achieved as well as a simple method of mass production. The NBC used in this study were generated through a previous investigation (Achuthan et al., 2006), and the cultures were under storage at 4 °C till activation in a seawater based mineral medium prior to immobilization. Enumeration of the aggregate forming NBC could be accomplished by

Table 2
TAN removal and NO_2^-–N production in bioassay systems treated with different quantities of NBC immobilized wood powder (*A. altissima*).

Quantity of immobilized substrata/20 L seawater	Average TAN removal (mg L^{-1})			Average nitrite production (mg L^{-1})		
	Day 1*	Day 2**	Day 3***	Day 1*	Day 2**	Day 3***
Salinity 15						
AMONPCU 0.2 g	1.29 ± 0.297	0.52 ± 0.170	0.61 ± 0.113	0.044 ± 0.0004	0.006 ± 0.006	0.036 ± 0.013
AMONPCU 0.4 g	1.11 ± 0.127	0.62 ± 0.226	0.76 ± 0.057	0.038 ± 0.003	0.023 ± 0.003	0.019 ± 0.001
AMONPCU 0.6 g	1.15 ± 0.184	0.72 ± 0.141	0.64 ± 0.014	0.061 ± 0.014	0.01 ± 0.006	0.017 ± 0.01
AMONPCU 0.8 g	0.85 ± 0.325	0.91 ± 0.453	0.96 ± 0.085	0.056 ± 0.014	0.017 ± 0.0003	0.018 ± 0.009
AMONPCU 1.0 g	0.62 ± 0.170	0.63 ± 0.156	1.2 ± 0.113	0.057 ± 0.014	0.039 ± 0.014	0.078 ± 0.013
Control	0.35 ± 0.042	0.4 ± 0.141	0.19 ± 0.003	0.028 ± 0.003	0.013 ± 0.003	0.035 ± 0.007
Salinity 30						
AMOPCU 0.2 g	0.65 ± 0.170	0.63 ± 0.297	0.66 ± 0.028	0.093 ± 0.028	0.019 ± 0	0.017 ± 0.004
AMOPCU 0.4 g	0.79 ± 0.141	0.64 ± 0.113	0.95 ± 0.099	0.073 ± 0.007	0.047 ± 0.01	0.048 ± 0.003
AMOPCU 0.6 g	1.04 ± 0.028	0.82 ± 0.141	1.21 ± 0.141	0.091 ± 0.014	0.021 ± 0	0.094 ± 0.0001
AMOPCU 0.8 g	0.93 ± 0.170	0.74 ± 0.198	1.2 ± 0.113	0.088 ± 0.003	0.034 ± 0.006	0.049 ± 0.004
AMOPCU 1.0 g	1.05 ± 0.113	0.87 ± 0.368	1.03 ± 0.004	0.088 ± 0.0006	0.054 ± 0.006	0.12 ± 0.147
Control	0.57 ± 0.113	0.4 ± 0.283	0.19 ± 0.071	0.055 ± 0.014	0.035 ± 0.001	0.02 ± 0.0004

Number of replicates (n) = 2.
r Quantity and TAN removal in salinity 15 *0.01044, **0.74304, ***0.93152.
r Quantity and TAN removal in salinity 30 *0.92427, **0.90799, ***0.83198.
p = 0.012819 (salinity 15), p = 0.0000205 (salinity 30).
r Quantity and nitrite production in salinity 15 *0.84769, **0.67763, ***0.36500.
r Quantity and nitrite production in salinity 30 *0.61063, **0.44022, ***0.82215.
p = 0.044638 (salinity 15)and p = 0.302089 (salinity 30).

ultrasonication at 125 W for 4 min, and this method of disaggregation of bacterial flocs and biofilms had been proven earlier (Salhani and Deffur, 1998).

Use of pre-acclimatized indigenous nitrifying bacteria has been reported by Shan and Obbard (2001) for tackling TAN problem in prawn culture ponds. Such consortia have their own merits with respect to reduced inter-specific competition and shorter acclimation period (Shan and

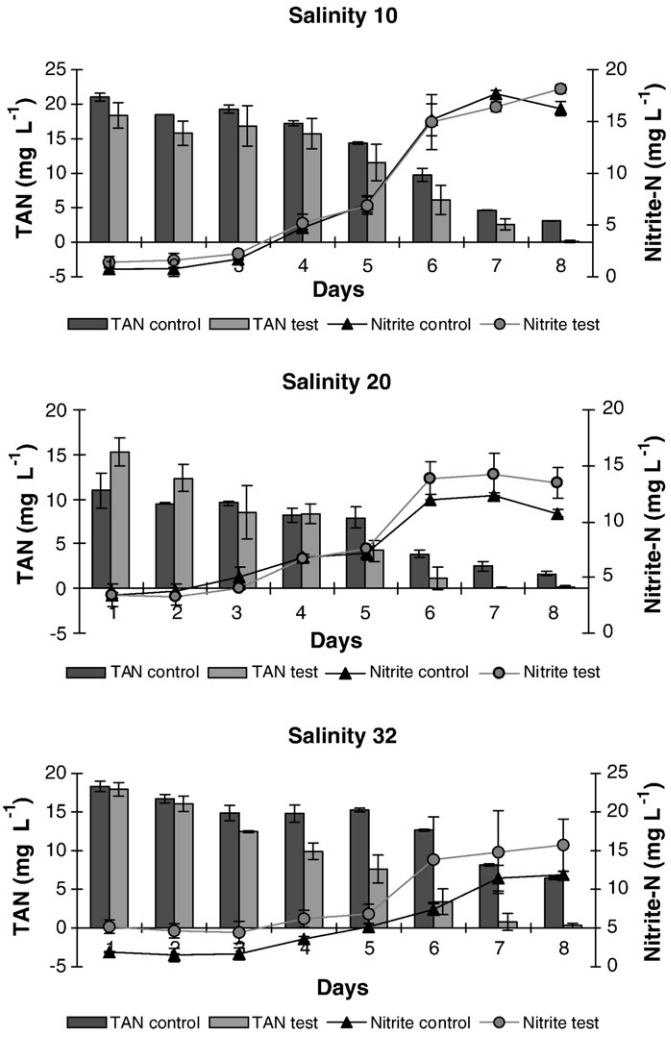

Fig. 8. Efficacy of NBC immobilized wood powder in a bioassay system (Days after application of NBC). Number of replicates (n) = 3.

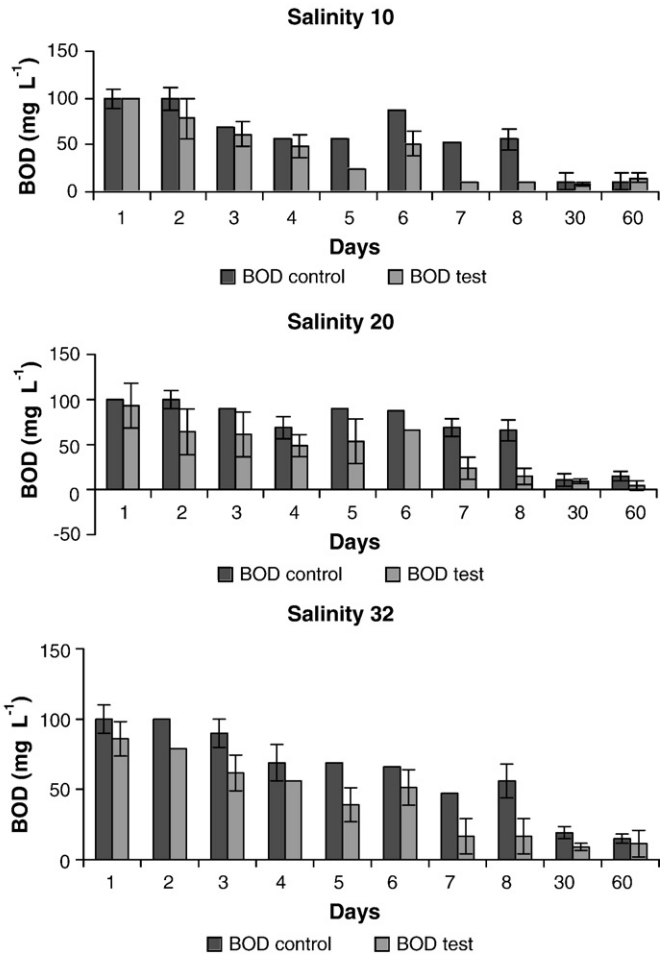

Fig. 9. Biological oxygen demand in the shrimp bioassay system supplemented with immobilized NBC (Days after application of NBC). Number of replicates (n) = 3.

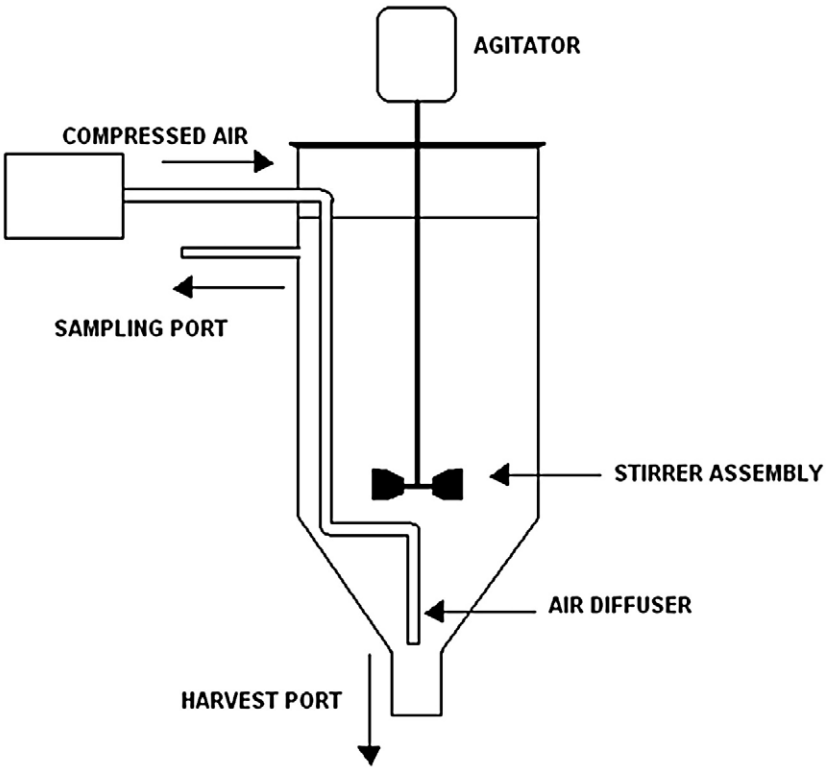

Fig. 10. Layout of NBC immobilization device.

Obbard, 2001). Bioaugmentation using non-indigenous cultures is not recommended as they will be edged out by the competing native microorganisms, besides facing growth inhibition and prolonged acclimation period (Stephenson and Stephenson, 1992). Microorganisms immobilized or attached to a support have several advantages such as longer biomass retention in the system, high microbial cell density, optimized microbial growth and metabolic rates, protection from inhibitory compounds and adverse conditions. Immobilization has been proven to circumvent the inherently slow rate of biological nitrogen removal due to the modest growth of the microorganisms (Khin and Annachhatre, 2004).

Several commercial carrier materials are available for immobilizing bacteria, most of which are made of chemically modified polymers or synthetic compounds like nylon and plastic. In fact, they are inhibitory to growth (Shin et al., 2002) and non-biodegradable and cannot be used in aquaculture. Meanwhile, polymers like cellulose, chitin, and chitosan are commonly used as support media for immobilization of microorganisms through adsorption. Among them cellulose based media, especially specialized porous cellulose derivatives (United States Patent 5935844, Matsumura et al., 1999), and 'biopop' (Kim et al., 1997) are reported for nitrifying bacteria. However, they are prepared under stringent conditions with extensive chemical processing, which render them more resistant to biodegradation and are costly and less suitable for aquaculture systems, though they are more congenial for wastewater treatment facilities. Immobilization techniques like entrapment in hydrogels (Vogelsang et al., 2002) or immobilization on polymeric substrates like carrageenan (Wijffels and Tramper, 1989) lead to a common constraint of oxygen depletion inside the gel matrix owing to increased growth and cell density, overriding the rate of oxygen diffusion across the gel matrix even though such techniques yield higher retention levels of the bioaugmentors (Fouratt et al., 2003). Over a period, this may manifest in oxygen deficiency in the core of the gel matrix (Catalan-Sakairi et al., 1997), resulting in poor nitrification rates. Moreover, they are not cost effective and involve laborious processing. Dried and expanded light weight clay aggregates (product name 'leca') have been used as support medium in nitrifying trickling filters (Lekang and Kleppe, 2000). Shan and Obbard (2001) used sterile buoyant porous clay pellets for immobilizing nitrifying bacteria, and proposed for *in situ* bioremediation of ammonia in aquaculture systems.

For *in situ* applications in aquaculture systems, biodegradable carriers are preferred, and on this ground wood powder was selected as the support medium that too from a softwood tree *A. altissima* having very low lignin content, to facilitate rapid microbial attack and mineralization. *Ailanthus altissima* (Family: Simaroubaceae) commonly known as 'white mutty' is extensively used in match box industry for making high quality splints. The wood particles from *A. altissima* selected as the carrier material in this study have specific surface area of 1.87 $m^2\ g^{-1}$ for attachment, which is comparable to that of the buoyant clay pellets (1.3–3.4 $m^2\ g^{-1}$) used for immobilizing nitrifying bacteria for *in situ* application in prawn culture systems (Shan and Obbard, 2001). SEM studies have revealed the suitability of the wood particles as support medium with sufficiently extensive corrugations and attachment sites. Considering all these, wood particles from *A. altissima* of the size range 300–1500 μm have been proposed as a viable alternative to any other specialized carrier material and it is available as waste byproduct of local wood based industries.

Selection of the support medium was also based on preference of the NBC in terms of adsorption onto the particles, biofilm formation and their subsequent nitrification potential. Wood powder derived from *A. altissima* with particle size in the range of 500–710 μm was found to support uniformly all the four NBC with better consistency in activity, exhibiting least variance. In order to minimize the carrier processing steps, the substratum with particle size in the range of 300–1500 μm was identified and confirmed as the one for immobilization. During immobilization, biofilm development was found progressed substantially within 24 h and completed within 72 h under optimum conditions, as revealed by SEM.

Initial cell density required for affecting immobilization in unit weight/area of the wood particles suggested that 1×10^5 to 1×10^8 cells $mL^{-1}\ g^{-1}$ did not make significant differences in nitrification when NBC immobilized wood powder was tested in fresh medium.

This led to the realization that the lowest initial density of 1×10^5 cells mL^{-1} would be well sufficient to have the cells attached in sufficient quantity onto the wood powder for effective nitrification. This indicated that, even with lower initial cell density, adsorption and colonization progressed rapidly forming biofilm, and nitrification rate observed was similar to that of higher initial cell density.

Minimum dosage of the NBC immobilized wood powder required for treating 20 L sea water was within the range 0.2 to 1.0 g. Progressive increase in nitrification with increase in the dosage was observed on the third day with positive correlation between the activity and the quantity of the immobilized NBC. TAN removal rate of 2.4–2.7 mg L^{-1} achieved within 3 days in all the treatments was comparable to that obtained by Shan and Obbard (2001). Moreover, competent removal rates were apparent in the present experiment with the lowest dosage of 0.2 g wet weight (surface area 1.87 m^2 g^{-1}) wood powder immobilized with NBC in 20 L seawater (salinity 15 and 30). Assuming a dosage rate of 0.2 g per 20 L pond water, treatment of 1 ha aquaculture pond of 1 m depth requires about 100 kg wet weight of the wood powder immobilized with NBC. Whereas, Shan and Obbard (2001) calculated a requirement of 1×10^6 clay pellets weighing 1 g each (1000 kg) for treating a pond of the same dimension. This demonstrated the improvisation attained in the technology. By optimizing the application mode, the requirement of the immobilized NBC can further be reduced.

While considering commercial application of the immobilized NBC, shelf life happens to be a major issue to be sorted out. Though there was a reduction in TAN removal potential during storage, the activity had not been lost totally even after a month-long storage at room temperature. Moreover, the activity could be regained on incubating in fresh medium with aeration and agitation. However, considering the requirement of active immobilized NBC for application it is recommended to accomplish the immobilization of NBC at field level as per demand employing the simple mass immobilization device. This will turn out the shelf life an irrelevant issue.

In the NBC mass immobilization device the process could be accomplished within three days under ambient conditions. Shan and Obbard (2001) reported 30 to 72 h incubation for effective immobilization of nitrifiers on clay pellets. Nevertheless, they (Shan and Obbard, 2003) had experienced a lag of 2 to 4 days for initiation of nitrification on application in fresh water aquaria. Meanwhile, wood powder samples retrieved from the mass immobilization device after the immobilization phase did not exhibit any lag in TAN removal when administered in fresh medium with varying salinities indicating stability and adaptability of the immobilized NBC. It has to be pointed out that the device designed for immobilization is simple, user-friendly and can be manufactured with less investment and operated with least expense. However, more studies are warranted on the kinetics of immobilization.

On evaluating the nitrifying potency of immobilized NBC in a simulated bioassay system, the extent of nitrification was twice that of the control suggesting their probable efficacy under field conditions especially at salinities 10, 20 and 32. The application of wood particles in bioassay systems did not contribute to BOD, and there was no increase in turbidity as well. On preliminary evaluation of AMOPCU-1 immobilized wood powder in a shrimp grow out system, 50% reduction in TAN concentration within a day was observed. However, several field trials are required before arriving at a foolproof evaluation of the product.

In all experiments, products of nitrification were below detectable limit, which could be due to the denitrification potency of the NBC. In a subsequent study, *nirK* and *nirS* genes encoding enzymes involved in denitrification could be confirmed in isolates resolved from the NBC (unpublished). The bioaugmentor generated is named TANOX (Total Ammonia Nitrogen Oxidizer). Accordingly, by employing TANOX ammonia could be eliminated from the system with no accumulation of intermediate nitrite and nitrate, and such a situation was highly desirable as a single bioaugmentation product on application could bring forth nitrification and denitrification simultaneously, which otherwise are difficult to be managed together (Khin and Annachhatre, 2004).

5. Conclusion

Wood particles with high surface area derived from the soft wood tree *A. altissima* were identified as immobilization medium for indigenous nitrifying bacterial consortia, and their *in situ* application demonstrated it as a viable tool for the elimination of TAN from tropical shrimp culture systems. This technology, which has used a biodegradable carrier, is a feasible option in terms of economics and environmental concerns. SEM studies revealed the suitability of the wood particles (300–1500 μm) as a support medium with sufficiently large corrugations and attachment sites for effective immobilization. The NBC immobilized wood powder was stable and adaptable to different salinity regimes. The NBC immobilization device designed, fabricated and demonstrated is user friendly and economically viable. The immobilized product TANOX is able to eliminate ammonia from the system with no accumulation of nitrite and nitrate, a situation highly desirable, as a single bioaugmentation product is sufficient to bring forth nitrification as well as denitrification. Overall, a simple, inexpensive and environment friendly approach using NBC immobilized wood powder for the *in situ* bioremediation of ammonia in shrimp culture systems could be developed and is proposed for adoption in aquaculture.

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