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Genetic diversity of nitrate reducing bacteria in marine and brackish water nitrifying bacterial consortia generated for activating nitrifying bioreactors in recirculating aquaculture systems

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Abstract

Nitrate reducing potency of 88 bacterial isolates segregated from marine and brackish water nitrifying bacterial consortia (NBC), generated for activation of nitrifying bioreactors, was confirmed by determining the nitrate reducing capability under aerobic conditions as maintained in nitrifying bioreactors. All the isolates had the potential to be used as bio-augmentors for activating nitrate dissimilation in recirculating aquaculture system. The existence of nitrate reducers with nitrifiers in NBC and in the reactor configuration negates the requirement of integrating anoxic denitrifying system for effective removal of NO_3^--N. Phylogenetic analyses of representative isolates from each cluster of the dendrograms generated based on phenotypic characterization and amplified ribosomal DNA restriction analysis revealed profound diversity of nitrate reducing bacterial flora in the NBC. They were composed of *Streptomyces enissocaesilis*, *Marinobacter* sp., *Pseudomonas* sp., *Microbacterium oxydans*, *Pelagibacterium halotolerans* and *Alcanivorax dieselolei* from marine NBC and *Streptomyces tendae*, *Nesterenkonia* sp., *Bacillus cereus*, *Microbacterium oxydans* and *Brevibacterium* sp. from brackish water NBC. The diversity indices of the consortia were calculated using Mega 5.0, primer 7 and VITCOMIC softwares. Marine NBC exhibited higher Shannon wiener diversity and mean population diversity than brackish water NBC. The study delineated higher species richness and diversity in marine NBC than in its brackish water counterpart, a possible reflection of the higher biodiversity of marine systems, and hence, the former is more promising to be used as start-up cultures for the activation of nitrifying bioreactors after appropriate acclimatization to the desired salinity.

KEYWORDS
amplified ribosomal DNA restriction analysis, diversity index, nitrate reducer, nitrifying bacterial consortia, nitrifying bioreactor

1 | INTRODUCTION

Accumulation of ammonium to critical levels in recirculating aquaculture systems (RAS) is the major threat faced in aquaculture industry. This affects successful operation of prawn/shrimp hatcheries as it turns out to be toxic to larvae. Integration of bacteriological filters, rotating biological contactors, trickling nitrification filters and submerged flow biofilters to RAS solves the crisis to a limited extend. In this context, the technology was improvised by developing Packed Bed Bioreactor (Kumar, Achuthan, Manju, Philip & Singh, 2009a) and Stringed Bed Suspended Bioreactor (Kumar, Achuthan, Manju, Philip & Singh, 2009b; http://www.nitrifying-bioreactor.com, Patent no. 241648), which could be integrated to existing infrastructure of hatcheries without any modification, transforming them to closed recirculating systems. For the activation of these bioreactors, four nitrifying bacterial consortia (NBC; ammonia and nitrite oxidizing) were developed by enrichment technique from marine (30 ppt salinity) and brackish (15 ppt salinity) water aquaculture systems (Achuthan, Kumar, Manju, Philip & Singh, 2006) and used as start-up cultures for their activation. They have been successfully demonstrated in shrimp larval rearing systems (Kumar et al., 2009a,b), in the maintenance of shrimp juveniles and adults (Kumar, Joseph, Philip & Singh, 2010) and in maturation systems (Kumar, Joseph, Vijai, Philip & Singh, 2011).

A unique characteristic of the nitrifying bioreactors is their nitrate reducing potency. Uniformly in all the systems maintained with the reactors NO_3^--N never used to rise above 8 ppm suggesting efficient nitrate reduction under aerated conditions (Kumar et al., 2011). Community analysis of the NBC by way of random cloning and sequencing and FISH revealed the presence of autotrophic nitrifiers, denitrifiers and heterotrophs in the consortia (Kumar et al., 2013). In a study of ecophysiological interaction between nitrifying and heterotrophic bacteria in autotrophic nitrifying biofilms, the presence of 50% nitrifying and 50% heterotrophic bacteria has been determined using micro-autoradiography and fluorescent in situ hybridization techniques (MAR-FISH) (Kindaichi, Ito & Okabe, 2004). This suggested heterotrophs as the inevitable entity in the system to convey appropriate signals with autotrophs for their cooperative metabolism. However, the information generated through these investigations on denitrifiers is scanty to unravel the interaction of autotrophs and heterotrophs in the nitrifying bacterial biofilms. To encourage the biotechnological processes like bioaugmentation, the interaction of microbial modules within the biofilm has to be fully understood.

Generally, integration of denitrification in to a bioreactor can be accomplished either by creating denitrifying environment inside the bioreactor by intermittent stoppage of aeration or integrating an anoxic compartment by coupling or extending the reactor (van Benthum, 1998) and by incorporating additional electron and carbon donors (e.g., carbohydrates, organic alcohols) or endogenous organic donors originated from the waste (van Rijn, Tal & Schreier, 2006). But with NBC, simultaneous ammonia removal and nitrate removal could be accomplished, which eliminated the requirement of anoxic environment/anoxic compartment in the reactor for the removal of total nitrogen from the system. In this study, marine (comprises ammonia NBC, AMOPCU-1 and nitrite NBC, NIOPCU-1 maintained at 30 ppt salinity) and brackish water (comprises ammonia NBC, AMONPCU-1 and nitrite NBC, NIONPCU-1 maintained at 15 ppt salinity) NBC were resolved on phytagel plates, pure cultures isolated, nitrification—nitrate reduction potency assessed at phenotypic and genotypic level, and the diversity of marine and brackish water isolates was compared using different bioinformatic tools.

2 | MATERIALS AND METHODS

2.1 | Amplification and storage of nitrifying bacterial consortia

Ammonia and nitrite oxidizing bacterial consortia (AMOPCU-1 and NIOPCU-1[marine] generated for aquaculture systems at 30 ppt, and AMONPCU-1 and NIONPCU -1[brackish] for those at 15 ppt) were available (Achuthan et al., 2006). Mineral base media having seawater as the base (15 ppt and 30 ppt) supplemented with 10 mg/L substrates such as $(NH_4)_2$ SO_4 or KNO_2 for ammonia and nitrite oxidizing consortia, respectively, and 2 mg/L KH_2PO_4 were employed at an optimum temperature of 28°C and pH 8.0 in 2 L fermentor (Bioflo 2000, New Brunswick Scientific, USA). On attaining stationary phase, the harvested cultures were maintained at 4°C with intermittent addition of substrate and adjustment of pH.

2.2 | Resolution of the nitrifying bacterial consortia (NBC)

Resolution of the NBC was attempted following conventional spread plate method. The plates were prepared in Watson's medium (1965) solidified with phytagel (1%) (Sigma Chemical Co., USA) prepared in aged seawater of respective salinity (15ppt for brackish and 30 ppt for marine). An aliquot of 20-ml Watson's medium containing 1% phytagel (w/v) was autoclaved at 15 lbs/15 min and poured into Petri plates at a temperature around 60°C. Phytagel plates were preferred for purification as they were devoid of assimilable organic nutrients, and their transparency helped even microcolonies to be distinguished. Plates were inoculated (0.2 ml) by spread plate method and incubated in dark at 28°C in a humidity-controlled environmental test chamber (Remi Instruments, Bombay, India). Colonies developed were isolated and maintained in phytagel slants after recording colony morphology.

2.3 | Purification and stocking

All isolates were purified by repeated streaking on phytagel plates. Purity of the cultures was confirmed based on uniformity of the colony and cellular morphology. Pure cultures were maintained at −80°C as 20% glycerol stocks in Watson's medium (1965) following Brown (2000). Parallel sets of cultures were stocked in screw capped tubes at room temperature (25 ± 1°C) having Watson's medium

(1965) slants solidified with 1% phytagel in triplicate (one sealed with parafilm, the other with paraffin wax and the third overlaid with liquid paraffin).

2.4 | Determination of nitrifying potency of pure cultures

The 88 pure cultures isolated through phytagel plates were examined for their potency to oxidize NH_4^+-N and NO_2^--N, respectively, based on the consortium from which they were isolated. They were inoculated into 100-ml aliquots of the medium according to Watson (1965) in 250-ml conical flasks and incubated under obscurity for 1 week on rotary shaker at 1 *g*. Triplicates and control (without inoculum) were also maintained for the experiment. Substrate uptake (NH_4^+-N/NO_2^--N) (Solorzano, 1969) and product formation (NO_2^--N/NO_3^--N) (Bendschneider & Robinson, 1952; Strickland & Parsons, 1972) were monitored regularly by spectrophotometry.

2.5 | Determination of nitrate dissimilation potency of the pure cultures

Nitrate reducing potency of the 88 isolated cultures was measured both in Watson's medium (1965) and nitrate reduction broth containing 0.25 g peptone, 0.3 g beef extract and 0.25 g yeast extract in 100 ml distilled water (15 ppt/30 ppt salinity) supplemented with 10 ppm NO_3^--N (KNO_3) in test tubes with and without agitation and aeration at an optimum temperature of 28°C and pH 7.5–8.5 as the conditions in bioreactor (Kumar et al., 2009a,b). Triplicates were maintained for all the isolates and un-inoculated tubes served as control. Reduction in NO_3^--N to NO_2^--N (Bendschneider & Robinson, 1952; Strickland and Parsons 1972) and NH_4^+-N (Solorzano, 1969) and the production of gas (N_2) using Durham's tube were assessed qualitatively during 6 days of incubation.

2.6 | Phenotypic characterization of the isolates

For identifying the isolates, they were subjected to phenotypic characterization such as Gram's staining, motility, production of catalase, oxidase, gelatinase, indole, methyl red test, Voges Proskauer reaction, citrate utilization, arginine dihydrolase, β-galactosidase (ONPG) and reduction in nitrate to nitrite (Holding & Collee, 1971; Stanier, Palleroni & Doudoroff, 1966; Stolp & Gadkari, 1981), aesculin hydrolysis (Gemmell & Hodgkiss, 1964), phenyl alanine deaminase (Cowan & Steel, 1974), H_2S production (Cowan & Steel, 1965), lipase production (Tween-20, Tween-40, Tween-60, Tween-80) (Sierra, 1957), 3-ketogluconate (Haynes, 1951) and utilization of thirty carbon sources (sugars and sugar alcohols) such as glucose, sucrose, maltose, mannose, fructose, lactose, galactose, raffinose, rhamnose, ribose, arabinose, xylose, melibiose, cellobiose, trehalose, starch, glycogen, adonitol, erythritol, arabitol, mannitol, dulcitol, inositol, salicin, butanol, glycerol, ethanol, sorbitol, dextrin and inulin at 0.1% concentration in Hugh and Leifson(1953) for the production of acid and gas.

2.7 | DNA extraction and amplification of 16S rRNA gene and *nir*S gene

As part of the molecular characterization of the resolved pure cultures, DNA of all the isolates was extracted following Burrell, Keller and Blackall (1998). Aliquots of 5-ml overnight grown cultures in ZoBell's broth were centrifuged (Eppendorf, Germany) at 11,200 *g* for 5 min (4°C),) and the pellets were resuspended in 500-μl saline EDTA (150 mM NaCl, 100 mM EDTA, pH 8.0). The mixtures were incubated at 37°C for 1.5 hr after adding 100 μl freshly prepared 100 mg/ml lysozyme and four cycles of freeze–thaw were given sequentially at 20 and 65°C. After the addition of 100 μl 25% (w/v) sodium dodecyl sulphate and 50 μl 2% (w/v) proteinase K, the mixtures were incubated at 60°C for 1.5 hr. Phenol–chloroform–isoamyl alcohol mixture (25:24:1) was used to extract DNA, and the aqueous phase was collected after centrifugation at 25,200 *g* for 15 min (4°C) and subjected for further extraction with chloroform isoamyl alcohol mixture (24:1). Final aqueous layer obtained after centrifugation (25,200 *g* for 15 min) was kept overnight (−20°C) after adding 1,000 μl ice-cold ethanol for precipitating DNA. Thereafter, DNA was collected by centrifugation at 20,000 g (4°C) for 15 min and washed with 75% ethanol and subsequently dissolved in MilliQ water. DNA concentration was quantified spectrophotometrically (Hitachi U-2800, Hitachi Corp., Japan) at Abs_{260}.

For the amplification of 16S rRNA gene, 16S-1F (5′-GAGTTTGA TCCTGGCTCA-3′) and 16S-1R (5′-ACGGCTACCTTGTTACGACTT-3′) primer systems were used (Lane, 1991). The reactions were performed as initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 30 s and extension at 68°C for 2 min followed by final extension at 68°C for 10 min.

For the amplification of *nir*S gene, *nir*S1F (CCTA(C/T) T GGCCG CC (A/G) CA (A/G) T) and *nir*S6R (CGTTGAACTT(A/G)CCGGT) primer systems were used (Braker, Fesefeldt & Witzel, 1998). PCR amplifications from pure cultures were performed in a total volume of 25 μl containing 1× PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 20 mM Tris-HCl, pH 8.8), 500 μM each deoxyribonucleoside triphosphate, 1 μl of both forward and reverse primer (7.5 pmol/μl), and 1 μl of template DNA (100 ng/μl). The reactions were performed as follows. After the initial denaturation at 94°C for 5 min, 1 U taq polymerase (New England Biolabs) was added at a holding temperature of 80°C and touch-down PCR was performed (Thermal cycler: Eppendorf Mastercycler Personal). Initial 10 cycles consisted a denaturation step at 94°C for 30 s, primer-annealing step at 45–40°C (0.5°C decreased by every cycle) for 40 s and elongation was performed at 72°C for 1 min. Additional 30 cycles were performed with annealing step performed at 43°C for 40 s and final incubation at 72°C for 10 min.

2.8 | Amplified ribosomal DNA restriction analysis (ARDRA)

The 16S rRNA gene amplified of all the isolates were subjected to ARDRA. Three tetra cutter restriction enzymes namely Alu I, Hpa II

and Hae III (New England Biolabs, Ipswich, MA, USA) with maximum loci in the amplified 16S rRNA gene were randomly selected. The digestion mix was consisted of 15 μl amplified product, 0.5 μl enzyme and 2 μl buffer (supplied with each enzyme). The digestion mixture was incubated at 37°C for 3 hr and the enzyme inactivated at 65°C for 20 min. The digests were separated over 3% agarose gel in TAE buffer. The banding pattern was scored for the absence/presence (0/1) of individual loci.

2.9 | Clustering based on un-weighted average linkage and identification of clusters

Based on the phenotypic characterization and restriction analysis of 16S rRNA gene of pure cultures, data matrix was generated by coding the results obtained from the tests as "1" for positive and "O" for negative. The data matrix prepared in "Excel" spread sheets (Microsoft Office package) was converted to proprietary matrix files by the programme NTedit, version I.1b (Exeter Software, Setauket, NY, USA), and rectangular data matrix generated was analysed by the programme NTSYSpc, version 2.02i (Exeter Software, Setauket, NY, USA). Similarities were calculated by simple matching coefficient using statistical module and sequential agglomerative hierarchical nested cluster method (SAHN), and clustering was achieved by unweighted pair-group method arithmetic average (UPGMA).

2.10 | Phylogenetic analyses of the representative isolates

After constructing the dendrograms based on both phenotypic characterization and ARDRA, the PCR amplified products of 16S rRNA genes of three representative isolates from each cluster were gel eluted and purified using Gen elute Gel extraction kit (Sigma-Aldrich, St. Louis, USA) and subjected for sequencing. The nucleotide sequences obtained were assembled using Gene Tool software, and the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul, Gish, Miller, Myers & Lipman, 1990) at National Centre for Biotechnology Information (NCBI), USA www.ncbi.nlm.nih.gov). Phylogenetic tree was constructed from aligned data sets of sequences by UPGMA statistical method and Kimura 2-parameter substitution model with 1,000 bootstrap replications using Mega 5.0 software (Tamura et al., 2011). The evolutionary history was inferred using the UPGMA method (Sneath & Sokal, 1973), and the evolutionary distances were computed using the Kimura two-parameter method (Kimura, 1980). The mean diversity in entire population of marine and brackish water consortia and the group mean distance between the two were also calculated using Mega 5.0 software (Tamura et al., 2011). The bacterial community structure of the marine and brackish water nitrifying bacterial consortia were analysed using rapid visualization tool VITCOMIC (Mori, Maruyama & Kurokawa, 2010). Similarity indices like Jaccard index, Lenon index and Yue and Theta index (Chao, Chazdon, Colwell & Shen, 2006) were calculated using clustering results of VITCOMIC for the statistical comparison of taxonomic composition between the marine and brackish water NBC. The diversity index included Shannon diversity index, which described species richness, evenness and dominance of both the consortia calculated using primer 7 software (Clarke & Gorley, 2015).

2.11 | Accession numbers

The 16S rRNA gene sequences obtained during the study have been deposited to GenBank under accession numbers KF454838–KF454868.

3 | RESULTS AND DISCUSSION

Altogether, 63 cultures from brackish and 25 from marine NBC were isolated and purified. All the isolated cultures were found to grow on ZoBell's marine agar plates, indicating their heterotrophic nature. On analysing the nitrifying and denitrifying potency, none of the cultures were found utilizing NH_4^+-N and NO_2^--N as the energy sources, which implied that nitrifiers could not be segregated by this method. Although phytagel was free of assimilable organic matter, all colonies grew on it were heterotrophs, and chemolithotrophic nitrifiers could not be obtained. All the isolates were found to reduce NO_3^--N to NO_2^--N and NH_4-N sequentially culminating in the accumulation of gas in Durhams tube. The study revealed that all the cultures were nitrate reducers, which used NO_3^--N as the electron acceptor. As opposed to classical denitrification, low amounts of ammonia were also detected, which did not persist longer as might have got consumed by the producers themselves. Amplification of nitrite reductase (*nirS*) gene from the pure cultures gave the evidence of production of nitric oxide from nitrite in Durham's tube and demonstrated as gas collected within. The gel picture is illustrated in Figure 1.

One of the major factors which regulate the abundance and distribution of denitrifiers is oxygen. Although aerobic denitrification has been reported earlier (Patureau, Bernet, Delgenés & Moletta, 2000), most of the denitrifiers are facultative anaerobes and reduce NO_3^--N only in the absence of oxygen. But, in the present study even under highly aerated conditions, as in the bioreactors and in

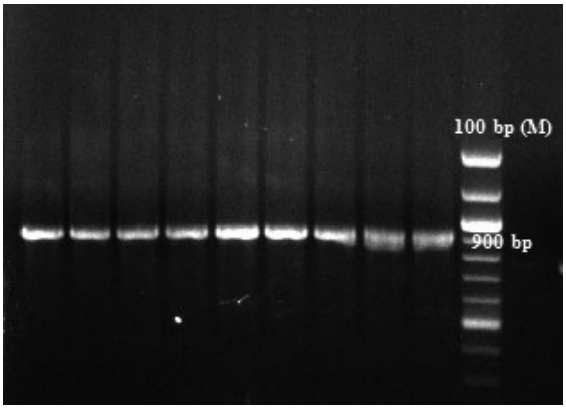

FIGURE 1 *NirS* gene PCR products of nitrate reducing isolates

the laboratory experimental system, active nitrate reduction has been taking place with all the cultures tested. Earlier report of aerobic denitrification by heterotrophic nitrifiers (Robertson, Cornelisse, De Vos, Hadioetomo & Kuenen, 1989) has strengthened this possibility. Moreover, most of the genera identified in this study were the ones previously reported as aerobic denitrifiers (Shoun, Kano, Baba, Takaya & Matsuo, 1998; Takaya et al., 2003; Zhang et al., 2012).

Usually denitrification is incorporated in bioreactor by integrating additional anoxic compartment and providing extra carbon sources. In various studies, simultaneous nitrification and denitrification (SND) were found to be possible by regulating dissolved oxygen concentration through changing aerobic–anaerobic mode (Baek & Pagilla, 2008; Zeng, Lemaire, Yuan & Keller, 2003). Thus, SND has been applied to a wide range of biological wastewater treatment processes, such as aerobic biofilm systems (Helmer & Kunst, 1998); fluidized bed reactor systems (Sen & Dentel, 1998), sequencing batch reactor (SBR) systems (Zeng et al., 2003); and membrane bioreactors (MBRs), such as intermittently aerated MBRs (Yeom, Nah & Ahn, 1999) and sequencing batch reactor MBRs (Buisson, Cote, Praderie & Paillard, 1998). The intermittent aeration was provided in the treatment of high strength wastewater containing high concentrations of ammonium in a staged anaerobic and aerobic membrane bioreactor which resulted in simultaneous nitrification and denitrification in the aerobic zone of the reactor (Zhang & Verstraete, 2002). Tam, Wong and Leung (1992) and Gerber, Mostert, Winter and de Villiers (1986) found that the addition of acetate, methanol and glucose as extra carbon source led to higher denitrification. In the present study, even though we have provided the system with no additional compartmentalization, carbon supply and oxygen demand, TAN and NO_2^--N concentrations were always near to zero, while the NO_3^--N could be observed to a maximum of only 7 mg/L and never exceeded 8 mg/L (Kumar et al., 2011). Thus, the nitrate reducing potency of the resolved isolates under aerobic conditions proved the aerobic denitrifying potency of the consortia, which had got expressed in the nitrifying bioreactor as well.

Isolation of autotrophic pure cultures is more difficult, due to faster growth of heterotrophs on organic carbon contaminants or by-products of nitrifiers. This typically takes several months, and cultures frequently do not survive the continued sub culturing. Laboratory selection also reduces the likelihood of obtaining dominant or representative environmental isolates. Hence, an appropriate protocol has to be adopted following former researchers for successful isolation of nitrifiers. However, it has also been noticed that various autotrophic ammonia oxidizers such as *Nitrosomonas and Nitrosospira*

TABLE 1 Restriction pattern of the nitrate reducing bacterial isolates from marine and brackish water NBC

In vitro restriction fragments			Size of 16S amplicons (bp)	Marine[a] (AMOPCU-1 and NIOPCU-1)	Brackish[a] (AMONPCU-1 and NIONPCU-1)
Alu I(bp)	Hpa II(bp)	Hae III(bp)			
310 + 285 + 205 + 200 + 195 + 120 + 60 + 35	285 + 175 + 165 + 160 + 95 + 90 + 85 + 80 + 75 + 65 + 60 + 55 + 50 + 52 + 40 + 30	305 + 230 + 205 + 155 + 145 + 114 + 85 + 80 + 58 + 55 + 35 + 25 + 20	1,500	43	9
400 + 310 + 285 + 200 + 96 + 46	540 + 255 + 180 + 120 + 95 + 80 + 60 + 56 + 53 + 50 + 30 + 18	300 + 230 + 210 + 200 + 150 + 120 + 100 + 90 + 55 + 25 + 20	1,500	5	1
390 + 200 + 190 + 180 + 165 + 155 + 95 + 52 + 35 + 30	550 + 470 + 150 + 130 + 110 + 80 + 10	480 + 365 + 220 + 170 + 145 + 60 + 35 + 18 + 10	1,500	0	1
570 + 265 + 210 + 205 + 165 + 45 + 40	550 + 340 + 160 + 130 + 115 + 110 + 81 + 12 + 10	565 + 295 + 170 + 155 + 150 + 55 + 35 + 10	1,500	0	10
600 + 225 + 190 + 180 + 175 + 60 + 58 + 20	600 + 390 + 200 + 130 + 100 + 40 + 20	570 + 460 + 295 + 130 + 30 + 25	1,500	0	5
300 + 285 + 210 + 205 + 190 + 105	180 + 120 + 115 + 90 + 85 + 80 + 65 + 60 + 55 + 53 + 50 + 20 + 10	460 + 315 + 180 + 145 + 135 + 85 + 55 + 50 + 40 + 35 + 20	1,500	0	9
615 + 235 + 210 + 205 + 85 + 80 + 70	480 + 300 + 180 + 170 + 130 + 80 + 20	285 + 180 + 175 + 165 + 100 + 95 + 85 + 80 + 75 + 60 + 55 + 52 + 50 + 25 + 20	1,500	0	2
475 + 410 + 285 + 207 + 88 + 30	290 + 220 + 175 + 165 + 155 + 132 + 115 + 102 + 52 + 50 + 18 + 20	490 + 460 + 190 + 100 + 85 + 80 + 55 + 25 + 20	1,500	0	1
300 + 210 + 200 + 190 + 160 + 150 + 145 + 65 + 45 + 40	380 + 250 + 210 + 180 + 150 + 130 + 100 + 55 + 40 + 15	485 + 280 + 230 + 125 + 85 + 65 + 55 + 15	1,500	0	2

[a]Number of positive isolates.

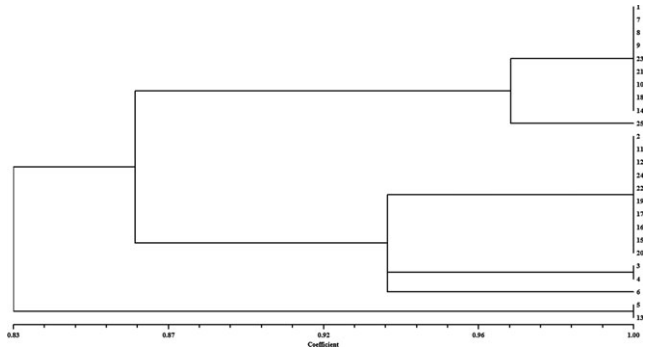

FIGURE 2 Dendrogram of pure cultures resolved from marine NBC (AMOPCU-1 and NIOPCU-1) on the basis of phenotypic characterization and ARDRA

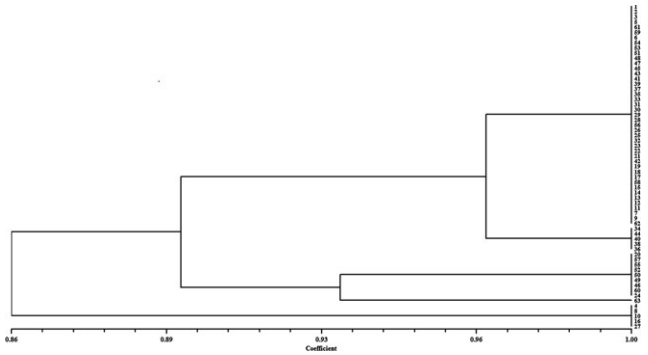

FIGURE 3 Dendrogram of pure cultures resolved from brackish NBC (AMONPCU-1 and NIONPCU-1) on the basis of phenotypic characterization and ARDRA

have been successfully isolated recently from various sources (Bollmann, French & Laanbroek, 2011; Reddy, Subrahmanyam, Naveenkumar, Karunasagar & Karunasagar, 2015) besides heterotrophic nitrifiers such as *Bacillus* sp. from membrane bioreactors (Lin et al., 2007; Zeng, Si & Li, 2010) and *Alcaligenes faecalis* from coke plant waste water (Liu, Li & Lv, 2012) where in 80% TN removal has been observed.

Even though the original objective of the study was to resolve the nitrifying bacterial consortia and to unravel the diversity by way of culture-dependent methods, all colonies developed on phytagel plates were nitrate reducers diminishing the prospects of isolating nitrifiers through this method. Under that circumstance, diversity of nitrate reducers was analysed. However, chemolithotrophic nitrifiers along with heterotrophic aerobic denitrifiers, and heterotrophs as scavengers of extrametabolites could be demonstrated through culture-independent approaches (Kumar et al., 2013).

Based on molecular (ARDRA, Table 1) and biochemical (phenotypic) characteristics, dendrograms of nitrate reducers have been constructed. Development of dendrograms resulted in discrete clusters which revealed the representative isolates and avoided the necessity of sequencing all the resolved ones. Five clusters were obtained from brackish water (Figure 2) and six from marine NBC (Figure 3), and the representatives alone needed to be sequenced. To satisfy the requirement of eliminating un-ambiguity in identifying the clusters, three isolates from each cluster were sequenced. The dendrogram of marine NBC showed distance coefficient of 0.83–1.00, while in brackish water NBC, it was 0.86–1.00. In marine NBC, Cluster-1 joined with Cluster-2 at 0.97r, with clusters 3, 4 and 5 at 0.86r and Cluster-6 at 0.83r. Clusters 3, 4 and 5 showed coefficient of similarity of about 0.94r. Cluster-6 in marine NBC remained singly. In case of brackish water NBC, Cluster-1 joined with Cluster-2 at 0.96r, with 3 and 4 at 0.898 and with Cluster-5 at 0.96r. Clusters 3 and 4 showed coefficient of similarity of about 0.93r. Cluster-5 in marine NBC branched off separately. Based on 16S rRNA gene sequence analysis of representative isolates from each consortium, the nitrate reducers resolved were *Streptomyces enissocaesilis*, *Marinobacter* sp., *Pseudomonas* sp, *Microbacterium oxydans*, *Pelagibacterium halotolerans* and *Alcanivorax dieselolei*, from marine NBC and *Streptomyces tendae*, *Nesterenkonia* sp, *Bacillus cereus*, *Microbacterium oxydans* and *Brevibacterium* sp. from brackish water NBC (Table 2).

As per the phylogenetic trees constructed, the evolutionary history of the nitrate reducers in the consortium was derived matching with the genbank data base (Figures 4 and 5). The phylogenetic tree of nitrate reducers in the marine NBC consisted three clusters (Figure 6), while the same in brackish water NBC two (Figure 7). In marine NBC, Cluster-1 joined with Cluster-2 at 0.11r and with Cluster-3 at 0.14 r. Cluster-1 consisted of *Marinobacter* sp., *Alcanivorax dieselolei* and *Pseudomonas* sp. with coefficient of similarity of 0.06r, and Cluster-3 consisted of *Microbacterium oxydans* and *Streptomyces enissocaesilis* with coefficient of similarity 0.06r. The Cluster-2 with *Pelagibacterium halotolerans* branched off independently. In brackish water NBC, Cluster-1 joined with Cluster-2 containing *Bacillus cereus* at 0.15r. Cluster-1 had *Nesterenkonia* sp and *Brevibacterium* sp having the coefficient of similarity of 0.05r, which joined at 0.07r with *Streptomyces tendae* and *Microbacterium oxydans* having the coefficient of similarity 0.07r. The mean diversity in the entire population of marine NBC was found to be 0.227 $\pm$ 0.013 and with brackish water NBC 0.199 $\pm$ 0.014. The mean distance between the two consortia was 0.251 $\pm$ 0.016 and the mean interpopulation diversity between them 0.025 $\pm$ 0.004.

An overall taxonomic composition of the isolates from marine and brackish water NBC as visualized using VITCOMIC (Figure 8) indicated that most of the taxonomic communities of both the consortia belonged to Actinobacteria. The map showed relative abundance of >10% of Actinobacteria followed by Proteobacteria in marine NBC. In brackish water NBC, a relative abundance of >10% of *Actinobacteria* was observed followed by Firmicutes. Comparative analysis showed a difference between both the consortia as indicated by Jaccard index, Lenon index and Yue and Clayton Theta of 0.0909, 0.1666 and 0.0588 respectively.

Primer 7 software was selected for determining the Shannon wiener diversity index of marine and brackish water NBC. The diversity is depended mainly on two elements: richness and evenness, as the highest diversity occurred in communities with many diverse species present (richness) in relatively equal abundance (evenness) (Torsvik, Sorheim & Goksoyr, 1996), both of which reflected

TABLE 2 Chart showing the outline of segregated nitrate reducers from marine and brackish water NBC with accession numbers

Consortium type	No. of clusters	Name of the representative isolates	Accession numbers
Marine (AMOPCU-1 and NIOPCU-1)	CLUSTER-1	*Streptomyces enissocaesilis*	KF454847
	CLUSTER-2	*Marinobacter* sp.	KF454849
	CLUSTER-3	*Pseudomonas* sp.	KF454850
	CLUSTER-4	Pelagibacterium halotolerans	KF454865
	CLUSTER-5	Alcanivorax sp.	KF454866
	CLUSTER-6	*Microbacterium oxydans*	KF454839
Brackish (AMONPCU-1 and NIONPCU-1)	CLUSTER-1	*Streptomyces tendae*	KF454840
	CLUSTER-2	*Microbacterium oxydans*	KF454838
	CLUSTER-3	*Bacillus cereus*	KF454859
	CLUSTER-4	*Nesterenkonia* sp.	KF454860
	CLUSTER-5	*Brevibacterium* sp.	KF454863

selective pressures that shaped diversity within communities. Here, species richness (d = 2.086) and evenness (J' = 0.9358) looked to be high in marine NBC compared to brackish water NBC (d = 1.476, J' = 0.889), and the Shannon wiener diversity index of marine NBC [H'(log2) = 2.419] was greater than brackish water NBC [H'(log2) = 2.064] even though bacterial dominance appeared to be higher in marine NBC (1 − Lambda' = 0.8724) than brackish water NBC (1 − Lambda' = 0.7725). Accordingly, it has been concluded that marine NBC has more diverse flora within than brackish water NBC.

Streptomyces spp. were found dominant in both the consortia, indicating their importance among diverse groups of organisms which coexist. Kumon et al. (2002) demonstrated for the first time denitrifying Actinomycetes (*S. antibioticus* B-546) producing N_2 as the denitrification product, with a different denitrification pathway designated as co-denitrification. The large-scale production of bio-surfactants by marine *Streptomyces* spp. has also been evaluated by Khopade et al. (2012).

A major group of organism isolated from marine NBC was *Marinobacter* spp. They have been reported to be opportunitrophic, ubiquitous in the global oceans and assumed to give significant impact on various biogeochemical cycles (Singer et al., 2011). It contributes to nitrogen cycle, adapted to survive under different ecological

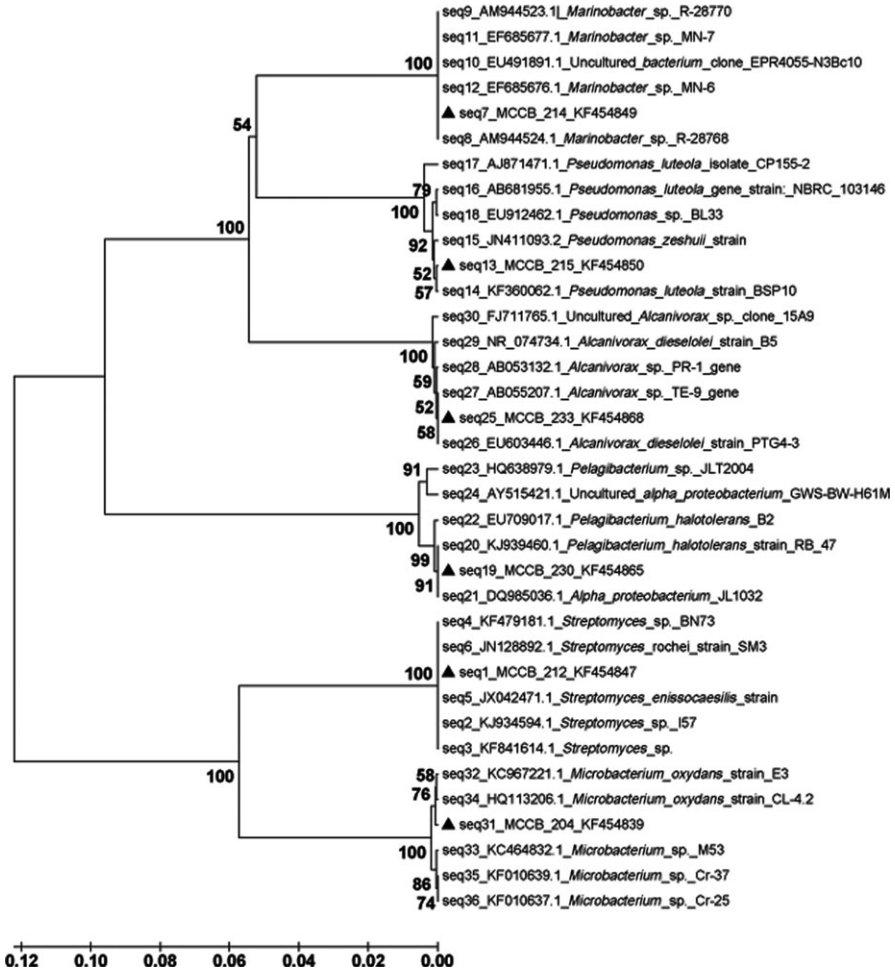

FIGURE 4 Phylogenetic analysis of the 16S rRNA gene sequences from marine NBC (AMOPCU-1 and NIOPCU-1) (indicated using Δ) with the matched sequences from Genbank database using Mega 5.0 software. The UPGMA tree constructed was computed using Kimura-2 parameter substitution model with 1,000 bootstrap replications

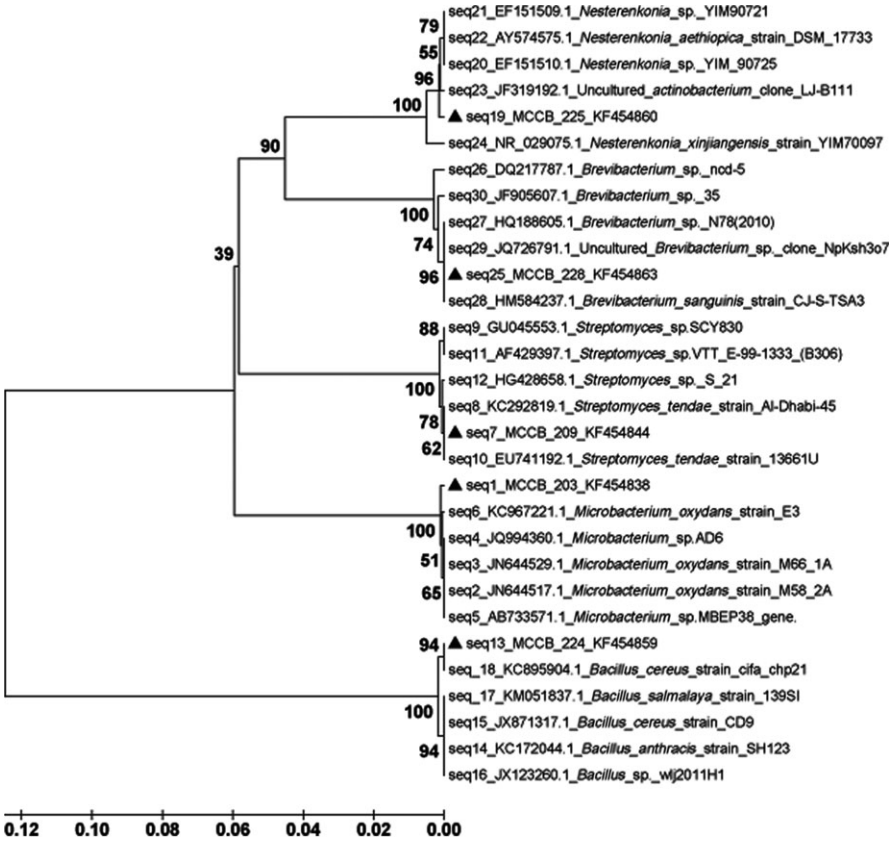

FIGURE 5 Phylogenetic analysis of the 16S rRNA gene sequences from brackish NBC (AMONPCU-1 and NIONPCU-1) (indicated using Δ) with the matched sequences from Genbank database using Mega 5.0 software. The UPGMA tree constructed was computed using Kimura-2 parameter substitution model with 1,000 bootstrap replications

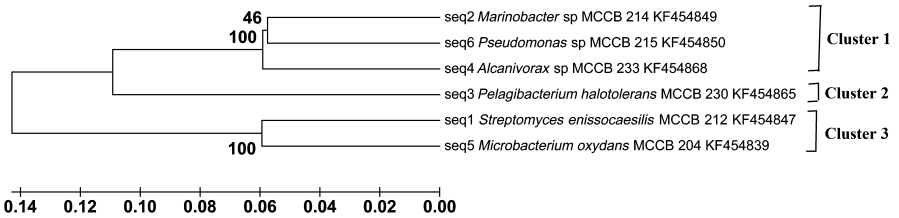

FIGURE 6 Phylogenetic analysis of the 16S rRNA gene sequences from marine NBC (AMOPCU-1 and NIOPCU-1) using Mega 5.0 software. The UPGMA tree constructed was computed using Kimura-2 parameter substitution model with 1,000 bootstrap replications

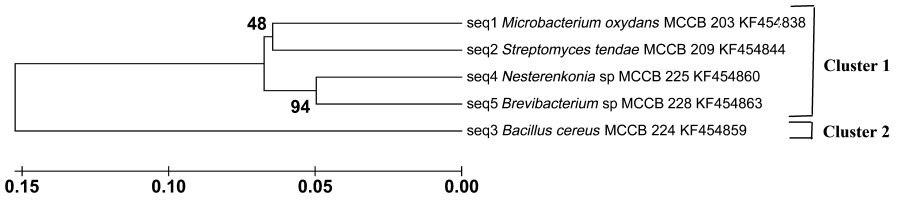

FIGURE 7 Phylogenetic analysis of the 16S rRNA gene sequences from brackish NBC (AMONPCU-1 and NIONPCU-1) using Mega 5.0 software. The UPGMA tree constructed was computed using Kimura-2 parameter substitution model with 1,000 bootstrap replications

conditions, and it has the metabolic capability to use oxygen as well as nitrate as the terminal electron acceptors (Singer et al., 2011). The capability of *Marinobacter hydrocarbonoclasticus* to grow in high saline conditions performing denitrification (Li et al., 2013) has been confirmed recently.

Besides, *Alcanivorax*, *Pseudomonas*, *Pelagibacterium halotolerans* and *Microbacterium oxydans* were segregated from marine NBC. *Alcanivorax* sp. and *Pseudomonas* sp. reported earlier by Kumar et al. (2013) by random cloning and sequencing of 16S rRNA gene from the same NBC could be isolated through this work. *Alcanivorax dieselolei* was reported from ammonia oxidizing consortia, and the denitrifying property was observed when succinate was used as the sole C source (Nakano, Okunishi, Tanaka & Maeda, 2009). However, the *Alcanivorax* sp. isolated in the present study could denitrify even

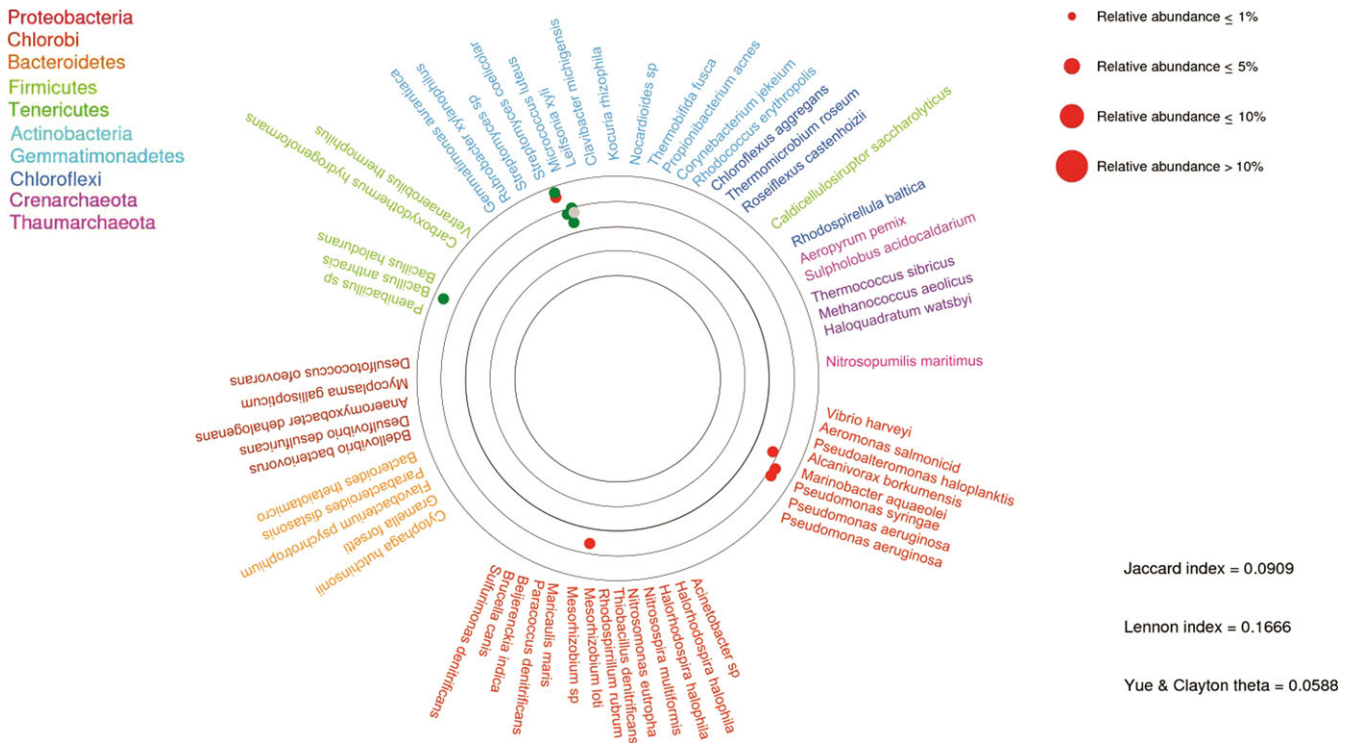

FIGURE 8 VITCOMIC merged map results for marine (AMOPCU-1 and NIOPCU-1) and brackish water (AMONPCU-1 and NIONPCU-1) isolates. Red dots indicate specific taxa of the marine isolates, green dots indicate specific taxa of brackish water isolates, and the grey dots indicate common taxa shared by the marine and brackish water NBC

without supplementing any additional carbon source. *Pseudomonas aeruginosa* was recently observed among ammonia oxidizing consortia for the removal of ammonia in fish hatchery system (Erna, Banerjee, Shariff & Yusoff, 2013). Strains of *Pseudomonas* sp. were observed to utilize many compounds as substrates that might otherwise be toxic to most other prokaryotes and to higher organisms; these properties point to the importance of *Pseudomonas* in bioremediation (Palleroni, Pieper & Moore, 2010), and in the present context as part of the NBC. According to Kostka et al. (2011) coexistence of *Alcanivorax*, *Pseudomonas* and *Marinobacter* within a bacterial community plays a key role in the degradation of dissolved and particulate organic matter besides effective denitrification. *Pelagibacterium* spp. have been reported to be highly halotolerant (Jiang et al., 2012; Xu et al., 2011), which reason out their presence in marine NBC.

Bacillus spp. and *Nesterenkonia* spp. turn out to be the major groups in the isolated denitrifiers from brackish water NBC. Complete removal of ammonia to nitrogenous gas under aerobic growth conditions by different species of *Bacillus* such as *Bacillus cereus*, *B subtilis* and *B licheniformis* indicated their contribution to global nitrogen cycle (Kim et al., 2005). The potential of *Bacillus* as an efficient denitrifier has been confirmed by Verbaendert, Boon, De Vos and Heylen (2011). *Nesterenkonia* spp., a halophilic genus, observed in the microbial communities in bioreactor for treating hydrocarbon-sulphide-containing (HSC) wastewater (Liao, Ji & Cheng, 2008). The data showed that they have profound role in nitrifying bioreactors by performing denitrification.

A minor group observed in brackish NBC was *Brevibacterium* spp. The major features of *Brevibacterium* are glycolipid biosurfactant production, which could be used as a lead compound for the development of novel anti-biofilm agents (Kiran, Sabarathnam & Selvin, 2010). From the above, it could be suggested that the anti-biofilm property of *Brevibacterium* spp. against the biofilm formed of *Vibrio* (an important group of bacterial pathogen in marine environment) has significance in the bioreactors installed in finfish and shellfish hatchery systems as they could be maintained free of the pathogen. It has also to be pointed out that vibrios have never been encountered with the NBC described here.

Microbacterium spp. has been resolved from both marine and nitrite oxidizing consortia, which have been observed as part of microbial community of biofilters meant for the removal of nitrogenous compounds, and they have been demonstrated of their hydrocarbonoclastic capability as well (Kristiansen et al., 2011).

Kesarcodi-Watson, Kaspar, Lategan and Gibson (2008) have proved *Bacillus* and *Pseudomonas* as having probiotic potential and have been identified in bacterial consortia, which may prevent pathogen colonization by producing growth inhibitors or transducing signals (Defoirdt, Boon, Sorgeloos, Verstraete & Bossier, 2007, 2008). The presence of *Pseudomonas*, *Marinobacter*, *Streptomyces* and *Bacillus* in various RAS has been reported, thereby confirming their significance in biological filtration (Schreier, Mirzoyan & Saito, 2010). Sayavedra-Soto, Gvakharia, Bottomley, Arp and Dolan (2010) demonstrated hydrocarbon degradation capability of various ammonia oxidizers by cometabolism. This supports the fact that nitrifiers together with nitrate reducers in the consortia bring out effective bio-augmentation.

The coexistence of heterotrophic denitrifiers along with autotrophic nitrifiers in nitrifying biofilms without any external organic carbon supplement has been reported by Okabe, Naitoh, Satoh and Watanabe (2002). The present study proved that nitrate reduction could be possible within the nitrifying biofilms even under oxic conditions without the addition of any organic carbon source. In addition to nitrate removal, significance of nitrate reducers has been proven with other processes such as increasing alkalinity to replenish inorganic carbon loss, reduction in the organic carbon discharge, reduction in orthophosphate and prevention of accumulation of toxic sulphide, which are all relevant to water quality management in aquaculture systems (van Rijn et al., 2006).

4 | CONCLUSION

Development of nitrifying bacterial consortia for the activation of nitrifying bioreactors in RAS makes a new era in aquaculture industry. Segregation of nitrate reducers from NBC implied the efficacy of the consortia to perform nitrate reduction even under the aerobic conditions persisting in nitrifying bioreactors. This excludes the requirement of incorporation of additional anoxic compartments in bioreactors for the removal of NO_3^--N. By combining results of the previous analysis of NBC with that of the present, it could be established that both two-step nitrification and nitrate reduction could be accomplished using the same consortia within a single bioreactor. The autotrophic nitrifiers along with the heterotrophic denitrifiers could form stable biofilm. The resolution of hydrocarbon degrading and bio-surfactant forming cultures from the consortia highlighted the possibility of their application in bioremediation as well. Occurrence of diverse bacterial flora in marine and brackish NBC could be compared by various molecular tools. Comparative analysis showed a difference between both the consortia as indicated by Jaccard index of 0.0909. The group mean distance was found to be 0.251 ± 0.016. Mean population diversity and Shannon Wiener diversity index were found to be higher in marine NBC. As marine nitrifying bacterial consortia were found to have more diverse bacterial flora than brackish water counter parts, the former is more promising to be used as start-up cultures for the activation of nitrifying bioreactors after appropriate acclimatization to the desired salinity.

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