Molecular characterization of the nitrifying bacterial consortia employed for the activation of bioreactors used in brackish and marine aquaculture systems

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\textbf{ABSTRACT}

The addition of commercial nitrifying bacterial products has resulted in significant improvement of nitrification efficiency in recirculating aquaculture systems (RAS). We developed two nitrifying bacterial consortia (NBC) from marine and brackish water as start up cultures for immobilizing commercialized nitrifying bioreactors for RAS. In the present study, the community compositions of the NBC were analyzed by universal 16S rRNA gene and bacterial amoA gene sequencing and fluorescence in situ hybridization (FISH). This study demonstrated that both the consortia involved autotrophic nitrifiers, denitrifiers as well as heterotrophs. Abundant taxa of the brackish water heterotrophic bacterial isolates were \textit{Pareibacillus} and \textit{Beijerinckia} spp. whereas in the marine consortia they were \textit{Flavobacterium}, \textit{Cytophaga} and \textit{Gramella} species. The bacterial amoA clones were clustered together with high similarity to \textit{Nitrosomonas} sp. and uncultured beta Proteobacteria. FISH analysis detected ammonia oxidizers belonging to \textit{Nitrosomonas} sp. in both the consortia, and \textit{Nitrosooccus mobilis} lineage only in the brackish water consortium and the halophilic \textit{Nitrosomonas} sp. only in the marine consortium. However, nitrite oxidizers, \textit{Nitrobacter} sp. and phylum \textit{Nitrosospira} were detected in both the consortia. The metabolites from nitrifiers might have been used by heterotrophs as carbon and energy sources making the consortia a stable biofilm.

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\textbf{1. Introduction}

The aquaculture industry is driven towards more intensification under recirculation due to the compelling factors such as limited space, water conservation, and restrictions in water discharge and easiness in the management of diseases. RAS reduces the water demand and discharges by recycling water and increases the food conversion efficiency resulting in less waste generation from feed (Losordo et al., 1998). In saltwater systems, RAS plays an important role in the production of healthy and properly sized fingerlings for stocking in net pens or ponds (Fielder and Allan, 1997). The most prominent characteristic of any RAS is the nitrifying biofilter to prevent accumulation of metabolites like ammonia and nitrite; which at high levels undermine the commercial production as their toxic impacts are manifested through impaired growth or chronic diseases (Tomasso, 1994; Cheng et al., 2004; Sovobodova et al., 2005). Fixed film biofilters are commonly used for total ammonia nitrogen (TAN) removal in RAS, where attached growth as biofilm offers several advantages such as handling convenience, increased process stability to shock loading and prevention of the bacterial population from being washed off (Fitch et al., 1998; S\textsc{o} et al., 2001; Shnel et al., 2002).

Nitrification is the biological oxidation of ammonia to nitrate via nitrite by two groups of chemolithotrophic bacteria, ammonia oxidizers and nitrite oxidizers; both having a low specific growth rates (Prosser, 1989; Bock et al., 1991). Recent discoveries of ammonia oxidizers (Strous et al., 1999) and ammonia oxidizing archaea (Koe\textsc{n}eke et al., 2005) have augmented the depth and breadth of ammonia oxidizing microorganisms. Nitrification in biofilters often relies on natural colonization of the nitrifying bacteria in the production systems. However, this natural method can take a relatively long time (4–8 weeks) to establish a healthy and viable population of both ammonia and nitrite-oxidizing bacteria (Manthe and
Malone, 1987; Masser et al., 1999). Moreover, the nitrifying bacterial population is much sensitive to chemical and physical stresses (Malone and Pfeiffer, 2006; Emparanza, 2009). Therefore, a viable start up culture is vital for enhanced performance of RAS, which can overcome the initial lag and can also be used for quick reactivation. Culture is vital for enhanced performance of RAS, which can overcome the initial lag and can also be used for quick reactivation.

The addition of 100 mg l\(^{-1}\) of Na\(_2\)CO\(_3\) to the optimum can also be used for quick reactivation.

2.2. DNA extraction, amplification of the 16S rRNA and amoA genes

DNA from each of the consortia was extracted following Burrell et al. (1998). Aliquots of 2 ml of the active consortia were centrifuged at 12000 g for 5 min and the pellets were resuspended in 500 μl saline – EDTA (150 mM NaCl, 100 mM EDTA, pH 8.0). After the addition of 100 μl of freshly prepared 100 mg ml\(^{-1}\) lysozyme, the mixtures were incubated at 37 °C for 1 h and then subjected to four cycles of freeze-thaw sequentially at −20 and 65 °C. Thereafter, 100 μl 25% (w/v) sodium dodecyl sulfate and 50 μl 2% (w/v) proteinase K each were added to the mixtures and incubated at 60 °C for 1.5 h. This was followed by phenol-chloroform extraction, and the residual RNA was removed by adding 3 μl of 10 mg ml\(^{-1}\) RNase and incubated at 37 °C for 1 h. The DNA at 100 ng concentration was maintained at −20 °C for further use.

Bacterial and archaeal 16S rRNA genes were amplified using primers 1652F (5′-GAGTTTGATCCTGGCTCA-3′)/1652R (5′-ACGTCACGGAGGCAGG-3′) (Lane, 1991) and Arch21F (5′-TCCGGTTGATCCYGGCACCAAG-3′)/Arch958R (5′-YCCGGTTGATCCATATT-3′) (DeLong, 1992) respectively as per the conditions described by the authors.

For the amplification of bacterial amoA gene, primers amoA-A1F (5′-GGGTTTACTCACTGGTGT-3′)/amoA-A1R (5′-CCCCCTCGGAAAAGGTTTTCCTCC-3′) (Rothhauwe et al., 1997) were used. The reactions were performed in a solution containing 1× PCR buffer (10 mM KCl, 10 mM (NH\(_4\))\(_2\)SO\(_4\), 2 mM MgSO\(_4\), 0.1% Triton X-100, 20 mM Tris–HCl, pH 8.8), 20 nmol of each dNTPs, 30 pmol each primer, 1 μl of template DNA (100 ng) and 2.5 μl of Taq DNA polymerase (New England Biolabs). The Taq polymerase was added after the first denaturation step. The reaction cycle followed, 5 min at 94 °C, 30 s at 68 °C and 30 s at 72 °C and a final elongation of 72 °C for 7 min. Aliquots (10 μl) of the PCR products were visualized in 1% agarose gels by standard electrophoresis procedures.

2.3. Cloning and screening

Fresh PCR products of 16S rRNA and amoA genes were cloned into the pGEM-T Easy vector (Promega). The ligation mix (10 μl) consisted of 5 μl ligation buffer (2×), 0.5 μl vector (50 ng ml\(^{-1}\)), 3 μl of PCR product and 1 μl of T4 DNA ligase (3 U μl\(^{-1}\)). This was incubated at 4 °C overnight. The entire ligated mix was used to transform Escherichia coli JM109 competent cells prepared using calcium chloride method. The ligation mix was added to 10 ml glass tube previously placed on ice to which 50 μl of competent cells were added and incubated on ice for 20 min. A heat shock at 42 °C was given for 40 s, immediately the tubes were placed on ice for 2 min and then 600 μl of SOC medium was added and incubated for 2 h at 37 °C with shaking at 250 rpm. The transformation mixture (100–200 μl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 μg ml\(^{-1}\)), IPTG (100 mM) and X-Gal (80 μg ml\(^{-1}\)). After overnight incubation at 37 °C the positive clones were selected using the blue/white screening. The white colonies were selected and streaked to purity on LB-Amp + X-gal + IPTG plates and incubated at 37 °C overnight. To confirm the identity, colony PCR of the white colonies were carried out using the primers T7 (5′-TAAATACGACTCACTATAGGG-3′) and SP6 (5′-GATTAGTGACTACATATAG-3′). White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25 μl) containing 2.5 μl 10× PCR buffer, 2.5 μl 2.5 mM dNTPs, 1 μl 10 pmol μl\(^{-1}\) of T7 and SP6 primers, 0.5 μl Taq polymerase, and the remaining volume was made up with MilliQ water. The thermal cycling conditions were as follows: 95 °C for 5 min; 35 cycles of 94 °C for 15 s, 57 °C for 20 s, 72 °C for 60 s and a final extension of 72 °C for 10 min. Plasmids from the positive clones were extracted using the GenElute HP plasmid miniprep kit (Sigma). Plasmids were further screened by restriction digestion using EcoRI enzyme (New England Biolabs) to release the insert. The reaction mix (20 μl) consisted of 2 μl 10× buffer, 5 μl plasmid DNA, 0.5 μl EcoRI enzyme (20,000 U ml\(^{-1}\)) and the rest was made up using sterile water (MilliQ). The reaction mix was incubated at 37 °C for 1 h and the enzyme was heat inactivated at 65 °C for 20 min.

2.4. Phylogenetic analyses

Screened clones were sequenced using ABI Prism 3700 Big Dye sequencer at Microsynth AG, Switzerland. After removing
the vector regions, the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990). The non-chimeric sequences were aligned with other published sequences using clustalX 1.83 (Thompson et al., 1994). Distance matrices and phylogenetic tree were constructed from alignment data sets of the clone library using ARB software with the neighbor-joining method (with the Jukes-Cantor correction) (Ludwig et al., 2004). Bootstrap values were estimated using 100 replicates. Phylogenetic tree for amoA protein was created using the neighbor-joining algorithm with Dayhoff matrix alignments using Mega 5.0 software (Tamura et al., 2011). The specificity and the hybridization conditions were confirmed with ‘Probebase’ (Lozupone et al., 2006). Actively growing consortia were harvested by centrifugation, and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (prepared at the respective salinity). The samples were stored at −20 °C in a 1:1 mixture of PBS:ethanol until further processing. Hybridizations were performed on 6 well Teflon-coated slides (Electron Microscopy Sciences, USA). Prior to the hybridization, the slides were coated with poly L-Lysin, and 10 μl of the fixed consortia were spread on to the well, dried at 46 °C for 10 min, and dehydrated by successive passage through 50, 80 and 98% ethanol (3 min each). Working solutions of the probes were prepared to obtain a final

2.5. Fluorescence in situ hybridization (FISH)

The FISH analyses of the consortia were carried out using universal bacterial probe (EUB 338) and nitifiers specific probes, NSO 190 (ammonia-oxidizing β subclass proteobacteria), NEU (halophilic and halotolerant members of the genus *Nitrosomonas*), NSV 443 (*Nitrosospira* spp.), NMV (*Nitrosococcus mobilis* lineage), NIT2 (*Nitrobacter* sp.), Ntspa 712 (*Phylum Nitrospira*) and S-Amx-0820-a-A-22 (anaerobic ammonium oxidizing bacteria) (Kumar et al., 2009c). The specificity and the hybridization conditions were confirmed with ‘Probebase’ (Loy et al., 2007). Actively growing consortia were harvested by centrifugation, and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (prepared at the respective salinity). The samples were stored at −20 °C in a 1:1 mixture of PBS:ethanol until further processing. Hybridizations were performed on 6 well Teflon-coated slides (Electron Microscopy Sciences, USA). Prior to the hybridization, the slides were coated with poly L-Lysin, and 10 μl of the fixed consortia were spread on to the well, dried at 46 °C for 10 min, and dehydrated by successive passage through 50, 80 and 98% ethanol (3 min each). Working solutions of the probes were prepared to obtain a final

![VITCOMIC merged mapping results for the nitrifying consortia isolates. Red dots indicate specific taxa of the brackish water consortia isolates; green dots indicates specific taxa of marine consortia isolates and the gray dot indicate common taxa shared by the brackish and marine consortia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
3.1. Molecular analyses

On comparing 16S rRNA gene sequences from the consortia with the GenBank database using the BLAST algorithm, majority of the hits were with the sequences of uncultured bacteria and heterotrophs like *Alcanivorax* sp., *Paenibacillus* sp., *Flavobacterium* sp. and *Gramella* sp. Overall taxonomic compositions of both the consortia could be clearly visualized using VITCOMIC (Fig. 1). The font color of each species name corresponded to its phylum name. Large circle indicated boundaries of BLAST average similarities (inner most circle starting at 80% followed by 85, 90, 95 and 100% similarity of the database sequence). The size of the dots indicated relative abundance of the sequences in the sample. The VITCOMIC diagram showed that most of the communities of both the consortia belonged to the phyla Proteobacteria and Bacteriodetes. The common taxa in both the consortia included *Alcanivorax* sp., *Gramella* sp. and *Pseudomonas* sp. The abundant taxa of the brackish water isolates were *Paenibacillus* and *Beijerinckia* spp., whereas in the marine consortia the genera detected were *Flavobacterium*, *Cytophaga* and *Gramella* spp. Comparative analysis showed a difference between both the communities as indicated by Jacard index, Lenon Index and Yue and Clayton Theta of 0.11, 0.2 and 0.1 respectively.

### Table 1
Fluorescent in situ hybridization results of the nitrifying bacterial consortia.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Reference</th>
<th>Target organisms</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>Amann et al., 1990</td>
<td>Bacteria</td>
<td>+ +</td>
</tr>
<tr>
<td>Nso190</td>
<td>Mоборрy et al., 1996</td>
<td>Ammonia-oxidizing β</td>
<td>+ +</td>
</tr>
<tr>
<td>NEU</td>
<td>Wagner et al., 1995</td>
<td>Halophilic and halotolerant members of the genus Nitrosomonas</td>
<td>− −</td>
</tr>
<tr>
<td>NSV443</td>
<td>Mобильрy et al., 1996</td>
<td>Nitrospira spp.</td>
<td>+ +</td>
</tr>
<tr>
<td>Nm V</td>
<td>Pomerening – Roeser et al., 1996</td>
<td>Nitroccocus mobilis lineage</td>
<td>+ −</td>
</tr>
<tr>
<td>NIT2</td>
<td>Wagner et al., 1996</td>
<td>Nitrobacter species</td>
<td>+ +</td>
</tr>
<tr>
<td>Ntspa712</td>
<td>Daims et al., 2001</td>
<td>Phylum Nitrospira</td>
<td>+ +</td>
</tr>
</tbody>
</table>

+ Present, − Absent.
Moderate difference among the communities were supported by the UniFrac significance and P values (<0.01). During the mass production, brackish and marine nitrifying bacterial consortia exhibited an ammonia removal of 1.24 and 1.95 g l\(^{-1}\) respectively with a maximum specific growth rate of 0.112 and 0.105 h\(^{-1}\) (Kumar et al., 2009a). Tal et al. (2003) characterized a nitrifying microbial consortium from a moving bed bioreactor (MBB) connected to a marine recirculating aquaculture system using DGGE of amplified 16S rRNA gene fragments and found ammonia oxidizer *Nitrosomonas cryotolerans* and nitrite oxidizer *Nitrospira marina* associated with the system as well as a number of heterotrophic bacteria, including *Pseudomonas* sp. and *Sphingomonas* sp. and two *Planctomycetes* sp. The denitrification had not been measured in the studies with the nitrifying bioreactors as the systems were adequately aerated. However, in the present investigation denitrifying *Psuedomonas* spp. were observed in both the consortia while *Paracoccus denitrificans* was limited to marine consortium. Cytryn et al. (2003, 2005) observed *P. denitrificans* in the digestion basin as well as in the fluidized bed reactor of a zero water exchange mariculture system. It was noticed that both the consortia consisted of different hydrocarbon degrading bacterial species like *Alcanivorax*, sp., *Rhodococcus* sp., *Rugeria* sp., *Flavobacterium* sp. and

![FISH images](image-url)

**Fig. 3.** Representative FISH images (600x) and DAPI staining of the nitrifying bacterial consortia. The specific probes hybridized are written below each image.
**Paenibacillus** sp. Many of the recent studies demonstrated the hydrocarbon degradation capability of various ammonia oxidizers by cometabolism (Arp et al., 2001; Wahman et al., 2006; Sayavedra-Soto et al., 2010). However, such phenomena and significance of the existence of these bacteria in the NBC, which are cultured in a minimal mineral based medium, require further analysis.

The amplification of bacterial amoA gene resulted in a PCR product of 491 bp size and this was used to construct clone library. Obtained sequences were aligned with amoA proteins of nitrifiers as present in the GenBank and a phylogenetic tree was constructed (Fig. 2). The evolutionary distances were computed using the Dayhoff matrix based method and are in the units of the number of amino acid substitutions per site. High similarity was observed between the sequences of both the consortia. All the clones were clustered together with high similarity (< 98%) with species of *Nitrosomonas* and uncultured beta Proteobacteria. Ammonia-oxidizing bacteria (AOB) are generally members of the beta-subdivision of the class Proteobacteria (Teske et al., 1994). In a study on marine biofilter, Foesel et al. (2008) noticed *Nitrosomonas* sp. NM143 lineage and *Nitrosomonas marina* species as the dominant AOB in the system. Studies have revealed that *Nitrosomonas* sp. formed an important group involved in ammonia oxidation in marine aquaria and moving bed bioreactors of closed RAS (Hovanec and DeLong, 1996; Tal et al., 2003; Schreier et al., 2010). While studying the nitrifying functional genes in coastal aquaculture systems of India, Krishnani (2010) detected amoA sequences exhibiting 82% identity to *Nitrosomonas europaea* and *Nitrosococcus mobilis* and 81% identity to *Nitrosomonas eutropha*.

Many of the bacterial products available for enhanced nitrification in aquaculture systems usually comprise of ammonia oxidizing and nitrite oxidizing bacteria like *Nitrosomonas eutropha* and *Nitrobacter winogradskyi* (Kuhn et al., 2010; Dhanasiri et al., 2011). Nitrification has been reported to be faster in mixed cultures than in pure cultures (Steinmüller and Bock, 1976). Previous studies on nitrifying biofilms showed the coexistence of a high level of heterotrophs with nitrifiers in autotrophic nitrifying biofilms cultured without an external organic carbon supply (Okabe et al., 1999, 2002). Kindaichi et al. (2004) stated that heterotrophs may be responsible for mineralizing different low and high molecular weight organic compounds produced or released by nitrifiers in an autotrophic nitrifying biofilm, and the heterotrophs may play an important role in the stability of biofilms. This is substantiated by the fact that the biofilms of both the consortia in activated bio-reactors were stable with optimum performance for months under field conditions (Kumar et al., 2011). In addition to bacteria, the members of kingdom Crenarchaeota within the archaeal domain also was found to play an important role in ammonia oxidation (Dhanasiri et al., 2011) in natural and engineered systems (Nicol and Schleper, 2006; Erguder et al., 2009). However, our study could not detect archaeal RNA genes in both the consortia.

### 3.2. Fluorescence in situ hybridization (FISH) of the consortia

Fluorescence in situ hybridization analyses of both the consortia established the presence and diversity of autotrophic nitrifiers (Table 1). Representative images of hybridized probes are shown in Fig. 3. Taking the consortia altogether, except the probe S-Amx-0820-a-A-22 (anaerobic ammonium oxidizing bacteria), all others gave positive signals. The absence of anammox was expected since the consortia were cultured under highly aerobic conditions. Most of the nitrifiers observed in the consortia were in the form of aggregates. FISH with universal bacterial probe in combination with DAPI staining explained the bacteria as the main constituent of the consortia. Ammonia oxidizers belonging to β subclass of proteobacteria and *Nitrosospira* sp. were detected in both the consortia, whereas *Nitrosococcus mobilis* lineage was detected only in the brackish water consortia and the halophilic *Nitrosomonas* sp. was limited to marine consortia. However, nitrite oxidizers, *Nitrobacter* sp. and phylum *Nitrospira* were detected in both the consortia.

The presence of K-specialist bacterial groups with high substrate affinity, *Nitrosospira* and *Nitrospira* in both the consortia, can have an advantage in nitrification at low ammonium and nitrite concentrations (van Kessel et al., 2010). Kindaichi et al. (2004) analyzed eco-physiological interaction between nitrifying and heterotrophic bacteria in an autotrophic nitrifying biofilm by microautoradiography–fluorescence in situ hybridization and found that the biofilm was composed of 50% nitrifiers and 50% heterotrophs. By FISH analyses of nitrifying bacterial enrichment from a shrimp farm, Paungfoo et al. (2007) detected *Cytophaga–Flavobacterium–Bacteroidetes*, and Proteobacteria (beta subdivision) phyla as the dominant groups, while other published FISH probes for *Nitrobacter* and *Nitrospira* were negative. These wide variations in reported molecular analyses of the nitrifying bacterial communities suggest that other unknown communities might also be playing a definite role in nitrification.

### 4. Conclusions

Molecular analyses of the nitrifying consortia based on 16S rRNA and amoA gene sequencing and FISH established that both the consortia consisted of autotrophic nitrifiers, denitrifiers as well as heterotrophs. The PBBRs and SSBRs activated with the consortia showed an instant nitrification under field conditions (Kumar et al., 2009b, c). Current results also emphasize the requirement of a polyphasic approach in nitrifying community analyses, as all the species could not be identified by either 16S rRNA gene, amoA gene sequences or FISH alone. Our earlier studies on FISH of the biofilms from the reactors showed the presence of almost all the nitrifying species present in the original consortia suggesting the capability of the consortia to form a stable biofilm. The heterotrophs in the consortia might be utilizing the metabolites of the nitrifiers and thus forming an integral part of the consortia.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2013.01.002.

### References


