



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

V.J. Rejish Kumar^a, Vrinda Sukumaran^a, Cini Achuthan^a, Valsamma Joseph^a, Rosamma Philip^b, I.S. Bright Singh^{a,*}

^aNational Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin 682016, India

^bDepartment of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Cochin 682016, India

ARTICLE INFO

Article history:

Received 11 October 2012

Received in revised form

24 December 2012

Accepted 2 January 2013

Available online 10 February 2013

Keywords:

Nitrifying bacterial consortium

Molecular characterization

16S rRNA gene analysis

Ammonia monooxygenase

Fluorescence in situ hybridization

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 *Paenibacillus* and *Beijerinckia* spp. whereas in the marine consortia they were *Flavobacterium*, *Cytophaga* and *Gramella* species. The bacterial amoA clones were clustered together with high similarity to *Nitrosomonas* sp. and uncultured beta Proteobacteria. FISH analysis detected ammonia oxidizers belonging to β subclass of proteobacteria and *Nitrospira* sp. in both the consortia, and *Nitrosococcus mobilis* lineage only in the brackish water consortium and the halophilic *Nitrosomonas* sp. only in the marine consortium. However, nitrite oxidizers, *Nitrobacter* sp. and phylum *Nitrospira* 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.

© 2013 Elsevier Ltd. All rights reserved.

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 a 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; Seo 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 anammox (Strous et al., 1999) and ammonia oxidizing archaea (Koenneke 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

* Corresponding author. Tel./fax: +91 484 2381120.

E-mail addresses: bsingh@md3.vsnl.net.in, isbsingh@gmail.com (I.S. Bright Singh).

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 of the system whenever some inhibition occurs. Kuhn et al. (2010) observed a significant improvement in the nitrification efficiency of recirculating systems by the addition of commercial nitrifying bacterial products. Accordingly, for the application in tropical recirculating systems, we developed nitrifying bacterial consortia (NBC) from the marine and brackish water by enrichment method (Achuthan et al., 2006) and mass cultured them in indigenously designed fermentors (Kumar et al., 2009a). Since then, these consortia are being applied as start up cultures for the activation of commercial packed bed bioreactor (PBBR) (Kumar et al., 2009b) and stringed bed suspended bioreactor (SBSBR) (Kumar et al., 2009c) (<http://www.nitrifying-bioreactor.com>, Patent no. 241648). The NBC were successfully demonstrated for activation and performance of the bioreactors in larval rearing systems of *Penaeus monodon* and *Macrobrachium rosenbergii* (Kumar et al., 2009b, c), in the rearing of adult *P. monodon* (Kumar et al., 2010) and also in *P. monodon* maturation systems (Kumar et al., 2011). These consortia were also successfully employed for bioaugmenting nitrification in *P. monodon* grow out systems by immobilizing on wood particles (Manju et al., 2009).

Enumeration, characterization and identification of ammonia- and nitrite-oxidizing bacteria in environmental samples by traditional microscopical and microbiological methods are difficult, because of the limited species specific morphological variety, slow growth rate (Watson et al., 1989), and their low growth yields (Gay and Corman, 1984; Wood, 1986). The use of molecular techniques now enables us to circumvent these limitations. The availability of molecular tools such as 16S ribosomal RNA (rRNA) sequence analyses have made it possible to explore slow growing or uncultivated bacterial species in different environments (Heal et al., 1998; O'Donnell and Gorres, 1999). In the present study, community composition of the NBC enriched from both the marine and brackish water systems have been analyzed using universal 16S rRNA gene and bacterial *amoA* gene sequencing and by FISH analyses.

2. Materials and methods

2.1. Nitrifying bacterial consortia: culturing and storage

NBC were cultured in 2 l fermentor (Bioflo 2000, NewBrunswick Scientific, USA) using simple seawater based medium (salinity adjusted to 15 g l⁻¹ for brackish and 30 g l⁻¹ for marine consortia) supplemented with 10 mg l⁻¹ substrate (NH₄)₂ SO₄ and 2 mg l⁻¹ KH₂PO₄ at an optimum temperature of 28 °C and pH of 8.0. After attaining log phase, the cultures were harvested and maintained at 4 °C with periodic addition of the substrate and adjustment of pH (using 1% Na₂CO₃) to the optimum.

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 consortium each 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 1.5 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 l⁻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 16S1 (5'-GAGTTTGATCCTGGCTCA-3')/16S2 (5'-ACGGC-TACCTTGTACGACTT-3') (Lane, 1991) and Arch21F (5'-TCCGGTTGATCCYGCCGGA-3')/Arch958R (5'-YCCGGCGTTGAMTCCAAT T-3') (DeLong, 1992) respectively as per the conditions described by the authors.

For the amplification of bacterial *amoA* gene, primers *amoA*-1F (5'-GGGGTTTCTACTGGTGGT- 3')/*amoA* 1R (5'-CCCTCKGSAAAGCCTTCTTC-3') (Rotthauwe et al., 1997) were used. The reactions were performed in a solution containing 1× PCR buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton ×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 U 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; pause at 80 °C to add Taq polymerase; then 42 cycles consisting of 90 s at 56.8 °C, 90 s at 72 °C, and 60 s at 94 °C and a final elongation of 72 °C at 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 µl⁻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 90 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 + Xgal + IPTG plates and incubated at 37 °C overnight. To confirm the insert, colony PCR of the white colonies were carried out using the vector primers T7 (5'-TAATACGACTCACTATAGGG3') and SP6 (5'-GATTAGGTGACACTATAG3'). 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 U 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 *EcoR*I 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 *EcoR*I 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). For visualizing the community structure of the consortia, 16S rRNA genes sequences were analyzed using a rapid visualization tool VITCOMIC (Mori et al., 2010). For statistical comparison of taxonomic compositions between consortia at 15 g l⁻¹ and 30 g l⁻¹ salinities, similarity indices like Jaccard index, Lenon index and Yue and theta index (Chao et al., 2006) were calculated using the clustering results of VITCOMIC. Phylogenetic (P)-test and UniFrac significance test were also performed using UniFrac (Lozupone et al., 2006).

2.5. Fluorescence in situ hybridization (FISH)

The FISH analyses of the consortia were carried out using universal bacterial probe (EUB 338) and nitrifiers specific probes, NSO 190 (ammonia-oxidizing β subclass proteobacteria), NEU (halophilic and halotolerant members of the genus *Nitrosomonas*), NSV 443 (*Nitrospira* 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 'Probase' (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 μ l 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

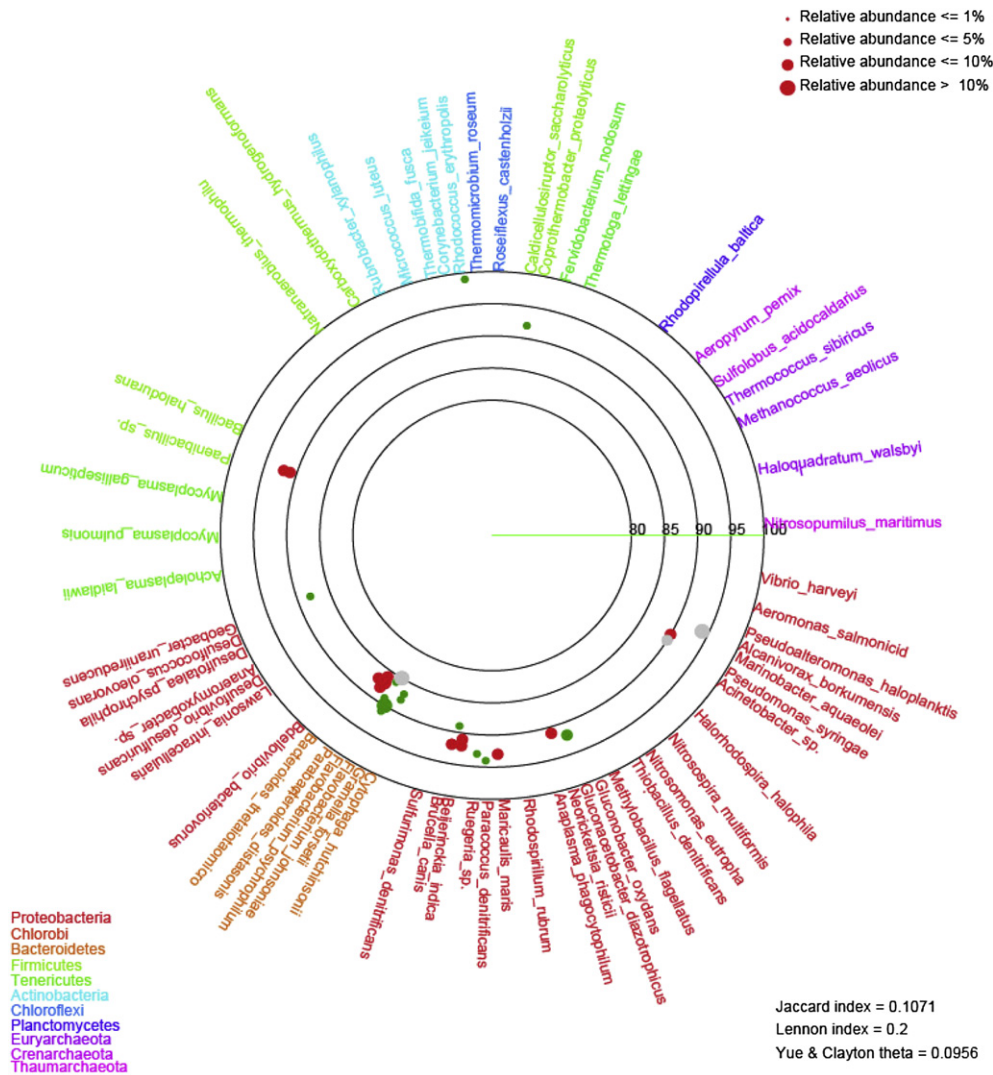


Fig. 1. VITCOMIC merged mapping results for the nitrifying consortia isolates. Red dots indicate specific taxa of the brackish water consortia isolates; green dots indicates specific taxa of marine consortia isolates and the gray dot indicate common taxa shared by the brackish and marine consortia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

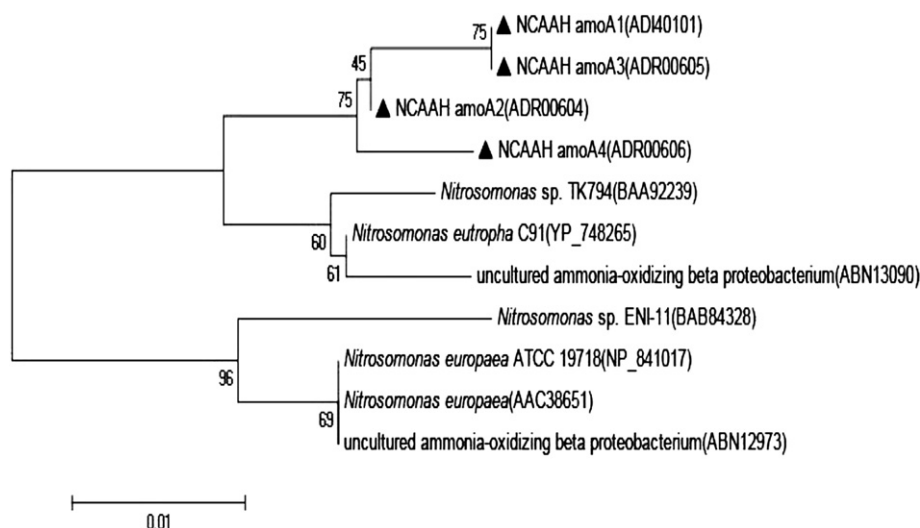


Fig. 2. Phylogenetic analysis of a representative set of amoA proteins with the partial amoA sequences obtained from the nitrifying bacterial consortia (indicated using ▲). The Neighbor-joining tree is drawn to scale and the evolutionary distances were computed using Dayhoff matrix based method and are in the units of the number of amino acid substitutions per site. Bootstrap values (100 replicates) are shown at the branches. Corresponding protein ID of the sequences are written in bracket. The bar represents 1% sequence divergence.

concentration of $5 \text{ pmol } \mu\text{l}^{-1}$ for CY3/5 and $8.3 \text{ pmol } \mu\text{l}^{-1}$ for fluorescein labeled probes. Hybridization buffer (2 ml) containing 360 μl 5 M NaCl, 40 μl 1 M Tris-HCl (pH 8.0), 4 μl 10% SDS, and formamide according to the probe used in MilliQ water was prepared. For hybridization, 10 μl hybridization buffer was dispensed into the wells, and then 1 μl probe stock solution was added. A hybridization tube was prepared by folding a tissue paper onto a 50 ml Falcon tube into which the remainder of the hybridization buffer was dispensed. After addition of probes the slides were immediately transferred into the hybridization tube and incubated for 1.5 h at 46°C in a hybridization oven (Thermo Electron Corp. USA). Washing buffer containing 1 M Tris/HCl, 0.5 M EDTA at pH 8, and NaCl as per the probe used was prepared in separate 50 ml Falcon tubes for each probe and made up to 50 ml by adding MilliQ water. Finally 50 μl of 10% (w/v) SDS was added and the washing buffer was preheated at 48°C in a water bath. On elapse of the incubation period, the hybridization slides were taken out, rinsed and transferred to the washing buffer, where the slides were incubated for 10–20 min at 48°C . After the incubation the slides were rinsed with MilliQ water and dried. The cells were counter-stained with DAPI having the final concentration of $0.2 \text{ } \mu\text{g ml}^{-1}$ for 1 min, washed, dried and added an anti-fading mounting fluid (Vectashield, Vector Laboratories Inc., Burlingame, CA). The slides were observed under Olympus BX 51 epifluorescent microscope (Olympus, USA) equipped with a monochromatic camera (Evolution VF, Media Cybernetics Inc, MD, USA). Images were processed using the “Image pro-express” software (Media Cybernetics Inc, MD, USA).

2.6. Accession numbers

The 16S rRNA gene sequences obtained during this study were deposited to GenBank under accession numbers FJ652050, FJ652-051, FJ665506–FJ665508, FJ711762–FJ711766, GQ221067–GQ221084, GQ243709–GQ243718 and the amoA genes as HM14933, HM590612–HM590614.

3. Results and discussion

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 Bacteroidetes. 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.

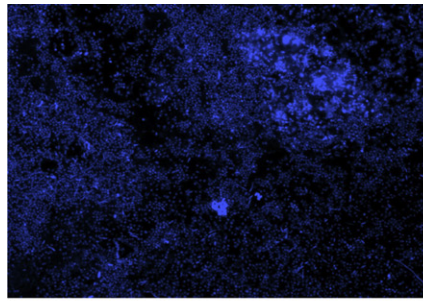
Table 1
Fluorescent in situ hybridization results of the nitrifying bacterial consortia.

Probe	Reference	Target organisms	Results	
			Brackish	Marine
EUB338	Amann et al., 1990	Bacteria	+	+
NSO190	Mobarry et al., 1996	Ammonia-oxidizing β Subclass proteobacteria	+	+
NEU	Wagner et al., 1995	Halophilic and halotolerant members of the genus <i>Nitrosomonas</i>	–	+
NSV443	Mobarry et al., 1996	<i>Nitrosospora</i> spp.	+	+
Nm V	Pommerening–Roeser et al., 1996	<i>Nitrosococcus mobilis</i> lineage	+	–
NIT2	Wagner et al., 1996	<i>Nitrobacter</i> species	+	+
Ntspa712	Daims et al., 2001	Phylum Nitrospira	+	+
S–Amx-0820–a-A-22	Schmid et al., 2000	Anaerobic ammonia-oxidizing bacteria	–	–

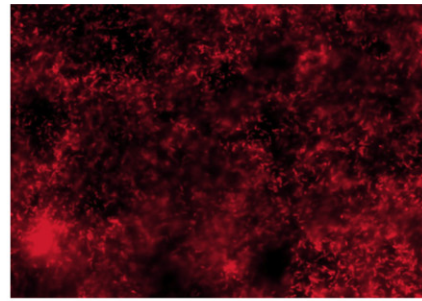
+ Present, – Absent.

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

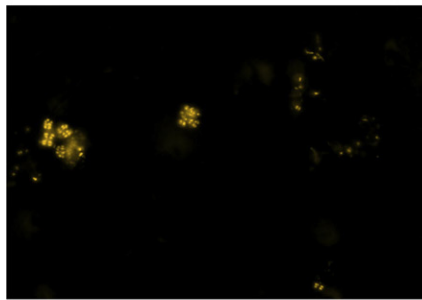
bacteria, including *Pseudomonas* sp. and *Sphingomonas* sp. and two *Planctomycetes* sp. The denitrification had not been measured in the studies with the nitrifying bioreactors as the systems were adequately aerated. However, in the present investigation denitrifying *Pseudomonas* spp. were observed 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



DAPI



EUB338 (Bacteria)



NSO 190 (β ammonia oxidizers)

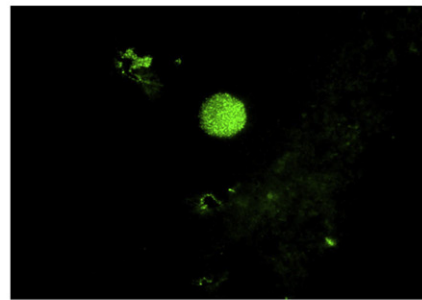
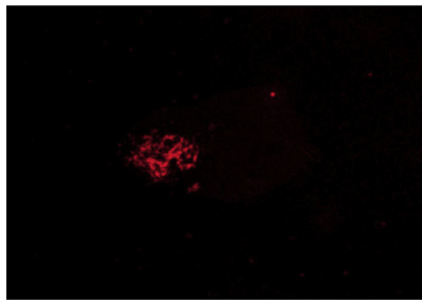
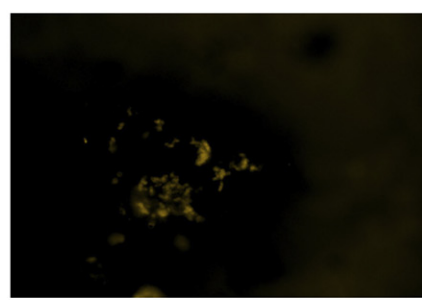
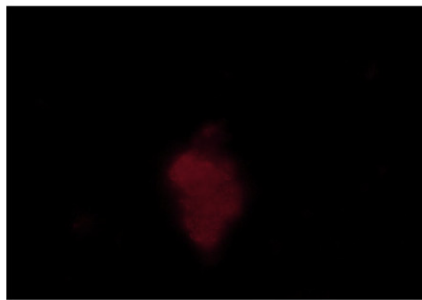
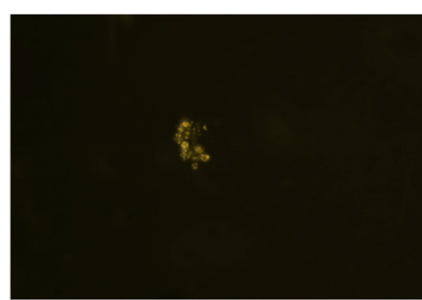
NmV (*Nitrosococcus mobilis* lineage)NIT2 (*Nitrobacter* sp.)NsV 443 (*Nitrospira* spp.)NEU (Halophilic *Nitrosomonas* spp.)Ntspa 712 (Phylum *Nitrospira*)

Fig. 3. Representative FISH images (600×) 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, Foessel 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 bioreactors were stable with optimum performance for months under field conditions (Kumar et al., 2011). In addition to bacteria, the members of kingdom Crenarhaeota 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 rRNA 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 *Nitrosospora* 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, *Nitrosospora* and *Nitrosospora* 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 ecophysiological 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 *Nitrosospora* 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 SBSBRs 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.

Acknowledgments

This work was carried out with the financial assistance from Department of Biotechnology (DBT) and Department of Science and Technology (DST), Government of India (Project nos. BT/PR4012/AAQ/03/204/03 and SR/SO/AS-15/03). The first author acknowledges DST for fellowship.

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

- Achuthan, C., Kumar, V.J.R., Manju, N.J., Philip, R., Singh, I.S.B., 2006. Development of nitrifying bacterial consortia for immobilizing in nitrifying bioreactors designed for penaeid and non-penaeid larval rearing systems in the tropics. *Indian Journal of Marine Sciences* 35, 240–248.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410.
- Amann, R.L., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* 56, 1919–1925.
- Arp, D.J., Yeager, C.M., Hyman, M.R., 2001. Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene. *Biodegradation* 12, 81–103.
- Bock, E., Koops, H.-P., Harms, H., Ahlers, B., 1991. The biochemistry of nitrifying organisms. In: Shively, J.M. (Ed.), *Variations of Autotrophic Life*. Academic Press, London, pp. 171–200.
- Burrell, P.C., Keller, J., Blackall, L.L., 1998. Microbiology of a nitrite-oxidizing bioreactor. *Applied and Environmental Microbiology* 64, 1878–1883.

- Chao, A., Chazdon, R.L., Colwell, R.K., Shen, T.J., 2006. Abundance based similarity indices and their estimation when there are unseen species in samples. *Biometrics* 62, 361–371.
- Cheng, W., Hsiao, I.S., Chen, J.C., 2004. Effect of nitrite on immune response of Taiwan abalone *Haliotis diversicolor* supertexta and its susceptibility to *Vibrio parahaemolyticus*. *Diseases of Aquatic Organisms* 60, 157–164.
- Cytryn, E., Barak, Y., van Rijn, J., Minz, D., 2003. Diversity of microbial communities correlated to physiochemical parameters in a digestion basin of a zero-discharge mariculture system. *Environmental Microbiology* 5, 55–63.
- Cytryn, E., Minz, D., Gelfand, I., Neori, A., Gieseke, A., de Beer, D., van Rijn, J., 2005. Sulfide-oxidizing activity and bacterial community structure in a fluidized bed reactor from a zero-discharge mariculture system. *Environmental Science and Technology* 39, 1802–1810.
- Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.H., Wagner, M., 2001. In situ characterization of *Nitrospira* like nitrite-oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology* 67, 5273–5284.
- DeLong, E.F., 1992. Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences USA* 89, 5685–5689.
- Dhanasiri, A.K.S., Kiron, V., Fernandes, J.M.O., Bergh, O., Powell, M.D., 2011. Novel application of nitrifying bacterial consortia to ease ammonia toxicity in ornamental fish transport units: trials with zebrafish. *Journal of Applied Microbiology* 111, 278–292.
- Emparanza, E.J.M., 2009. Problems affecting nitrification in commercial RAS with fixed-bed biofilters for salmonids in Chile. *Aquaculture Engineering* 41, 91–96.
- Erguder, T.H., Boon, B.N., Wittebolle, L., Marzorati, M., Verstraete, W., 2009. Environmental factors shaping the ecological niches of ammonia oxidizing archaea. *FEMS Microbiology Reviews* 33, 855–869.
- Fielder, S., Allan, G.L., 1997. Inland production of marine fish. In: Hyde, K. (Ed.), *The New Rural Industries: a Handbook for Farmers and Investors*. Rural Industries Research and Development Corporation. Australian Government, pp. 108–113.
- Fitch, M.W., Pearson, N., Richards, G., Burken, J.G., 1998. Biological fixed-film systems. *Water Environmental Research* 70, 495–518.
- Foesel, B.U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., de la Torre, J.R., van Rijn, J., Minz, D., Drake, H.L., Schramm, A., 2008. Nitrosomonas Nm143-like ammonia oxidizers and *Nitrospira marina*-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. *FEMS Microbiology Ecology* 63, 192–204.
- Gay, G., Corman, A., 1984. Comparative study of two strains of *Nitrobacter* in batch and continuous culture. *Microbial Ecology* 10, 99–105.
- Heal, I.M., Saunders, J.R., Pickup, R.W., 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology* 35, 1–21.
- Hovanec, T.A., DeLong, E.F., 1996. Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria. *Applied and Environmental Microbiology* 62, 2888–2896.
- Kindaichi, T., Ito, T., Okabe, S., 2004. Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. *Applied and Environmental Microbiology* 70, 1641–1650.
- Koenneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437, 543–546.
- Krishnani, K.K., 2010. Detection and diversity of nitrifying and denitrifying functional genes in coastal aquaculture. *Aquaculture* 302, 57–70.
- Kuhn, D.D., Drahos, D.D., Marsh, L., Flich Jr., G.J., 2010. Evaluation of nitrifying bacteria product to improve nitrification efficiency in recirculating aquaculture systems. *Aquaculture Engineering* 43, 78–82.
- Kumar, V.J.R., Achuthan, C., Manju, N.J., Philip, R., Singh, I.S.B., 2009a. Mass production of nitrifying bacterial consortia for establishing instantaneous nitrification in saline recirculating aquaculture systems. *World Journal of Microbiology and Biotechnology* 25, 407–414.
- Kumar, V.J.R., Achuthan, C., Manju, N.J., Philip, R., Singh, I.S.B., 2009b. A stringed bed suspended bioreactor (SBSBR) for in situ nitrification in penaeid and non-penaeid hatchery systems. *Aquaculture International* 17, 479–489.
- Kumar, V.J.R., Achuthan, C., Manju, N.J., Philip, R., Singh, I.S.B., 2009c. Activated packed bed bioreactors (PBBR) for the rapid nitrification in brackish water hatchery systems. *Journal of Industrial Microbiology and Biotechnology* 36, 355–365.
- Kumar, V.J.R., Joseph, V., Philip, R., Singh, I.S.B., 2010. Nitrification in brackish water recirculating aquaculture system integrated with activated packed bed bioreactor. *Water Science and Technology* 61, 797–805.
- Kumar, V.J.R., Joseph, V., Vijai, R., Philip, R., Singh, I.S.B., 2011. Nitrification in a packed bed bioreactor integrated into a marine recirculating maturation system under different substrate concentrations and flow rates. *Journal of Chemical Technology and Biotechnology* 86, 790–797.
- Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), *Nucleic Acid Techniques in Bacterial Systematics*. Wiley, New York, pp. 115–147.
- Losordo, T.M., Masser, M.P., Rakocy, J., 1998. *Recirculating Aquaculture Tank Production Systems: an Overview of Critical Considerations*. Southern Regional Aquaculture Center, Publication No. 451 USDA, p. 6.
- Loy, A., Maixner, F., Wagner, M., Horn, M., 2007. probeBase – an online resource for rRNA-targeted oligonucleotide probes: new features. *Nucleic Acids Research* 35, 800–804.
- Lozupone, C., Hamady, M., Knight, R., 2006. UniFrac – an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* 7, 371.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, J., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.-H., 2004. ARB: a software environment for sequence data. *Nucleic Acids Research* 32, 1363–1371.
- Malone, R.F., Pfeiffer, T.J., 2006. Rating fixed film nitrifying biofilters used in recirculating aquaculture systems. *Aquaculture Engineering* 34, 389–402.
- Manju, N.J., Deepesh, V., Achuthan, C., Philip, R., Singh, I.S.B., 2009. Immobilization of nitrifying bacterial consortia on wood particles for bioaugmenting nitrification in shrimp culture systems. *Aquaculture* 294, 65–75.
- Manthe, D.P., Malone, R.F., 1987. Chemical addition for accelerated biological filter acclimation in closed blue crab shedding systems. *Aquaculture Engineering* 6, 227–236.
- Masser, M.P., Rackocy, J., Losordo, T.M., 1999. *Recirculating Aquaculture Tank Production Systems: Management of Recirculating Systems*. Southern Regional Aquaculture Center, Publication no. 452, p. 12.
- Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., Stahl, D.A., 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology* 62, 2156–2162.
- Mori, H., Maruyama, F., Kurokawa, K., 2010. VITCOMIC: visualization tool for taxonomic compositions of microbial communities based on 16S rRNA gene sequences. *BMC Bioinformatics* 11, 332.
- Nicol, G.W., Schleper, C., 2006. Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *Trends in Microbiology* 14, 207–212.
- O'Donnell, A.G., Gorres, H.E., 1999. 16S rDNA methods in soil microbiology. *Current Opinions in Biotechnology* 10, 225–229.
- Okabe, S., Satoh, H., Watanabe, Y., 1999. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Applied and Environmental Microbiology* 65, 3182–3191.
- Okabe, S., Naitoh, H., Satoh, H., Watanabe, Y., 2002. Structure and function of nitrifying biofilms as determined by molecular techniques and the use of microelectrodes. *Water Science and Technology* 46, 233–241.
- Paungfoo, C., Prasertsan, P., Burrell, P.C., Intrasingkha, N., Blackall, L.L., 2007. Nitrifying bacterial communities in an aquaculture wastewater treatment system using fluorescence in situ hybridization (FISH), 16S rRNA gene cloning, and phylogenetic analysis. *Biotechnology and Bioengineering* 97, 985–990.
- Pommerening-Röser, A., Rath, G., Koops, H.-P., 1996. Phylogenetic diversity within the genus *Nitrosomonas*. *Systematic and Applied Microbiology* 19, 344–351.
- Prosser, J.I., 1989. Autotrophic nitrification in bacteria. *Advances in Microbial Physiology* 30, 125–181.
- Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology* 63, 4704–4712.
- Sayavedra-Soto, L.A., Gvakharia, B., Bottomley, P.J., Arp, D.J., Dolan, M.E., 2010. Nitrification and degradation of halogenated hydrocarbons – a tenous balance for ammonia oxidizing bacteria. *Applied Microbiology and Biotechnology* 86, 435–444.
- Schimid, M., Twachtman, U., Klein, M., Strous, M., Juretschko, S., Jetten, M.S.M., Metzger, J.W., Schleifer, K.H., Wagner, M., 2000. Molecular evidence for genus level diversity of bacteria capable of 546 catalysing anaerobic ammonium oxidation. *Systematic and Applied Microbiology* 23, 93–106.
- Schreier, H.J., Mirzoyan, N., Saito, K., 2010. Microbial diversity of biological filters in recirculating aquaculture systems. *Current Opinions in Biotechnology* 21, 318–325.
- Seo, J.K., Jung, I.H., Kim, M.R., Kim, B.J., Nam, S.W., Kim, S.K., 2001. Nitrification performance of nitrifiers immobilized in PVA (polyvinyl alcohol) for a marine recirculating aquarium system. *Aquaculture Engineering* 24, 181–194.
- Shnel, N., Barak, Y., Ezer, T., Dafni, Z., van Rijn, J., 2002. Design and performance of a zero-discharge tilapia recirculating system. *Aquaculture Engineering* 26, 191–203.
- Steinmüller, W., Bock, E., 1976. Growth of *Nitrobacter* in the presence of organic matter. I. Mixotrophic growth. *Archives of Microbiology* 108, 299–304.
- Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., Jetten, M.S.M., 1999. Missing lithotroph identified as new planctomycete. *Nature* 400, 446–449.
- Svobodova, Z., Machova, J., Poleszczu, G., Hoda, J., Hamaakova, J., Kroupova, H., 2005. Nitrite poisoning of fish in aquaculture facilities with water-recirculating systems. *Acta Veterinaria Brno* 74, 129–137.
- Tal, Y., Watts, J.E., Schreier, S.B., Sowers, K.R., Schreier, H.J., 2003. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in closed recirculated mariculture systems. *Aquaculture* 215, 187–202.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731–2739.
- Teske, A., Alm, E., Regan, J., Toje, S., Rittmann, B., Stahl, D., 1994. Evolutionary relationships among ammonia- and nitrite oxidizing bacteria. *Journal of Bacteriology* 176, 6623–6630.

- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.
- Tomasso, J.R., 1994. Toxicity of nitrogenous wastes to aquaculture animals. *Reviews in Fishery Science* 2, 291–314.
- van Kessel, M.A.H.J., Harhangi, H.R., van de Pas-Schoonen, K., van de Vossenbergh, J., Flik, G., Jetten, M.S.M., Klaren, P.H.M., Op den Camp, H.J.M., 2010. Biodiversity of N-cycle bacteria in nitrogen removing moving bed biofilters for freshwater recirculating aquaculture systems. *Aquaculture* 306, 177–184.
- Wagner, M., Rath, G., Amann, R., Koops, H.-P., Schleifer, K.-H., 1995. In situ identification of ammonia-oxidizing bacteria. *Systematic and Applied Microbiology* 18, 251–264.
- Wagner, M., Rath, G., Koops, H.-P., Flood, J., Amann, R., 1996. In situ analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology* 34, 237–244.
- Wahman, D.G., Henry, A.E., Katz, L.E., Speitel Jr., G.E., 2006. Cometabolism of trihalomethanes by mixed culture nitrifiers. *Water Research* 40, 3349–3358.
- Watson, S.W., Bock, E., Harms, H., Koops, H.-P., Hooper, A.B., 1989. Nitrifying bacteria. In: Murray, R.G.E., Brenner, D.J., Bryant, M.P., Holt, J.G., Krieg, N.R., Moulder, J.W., Pfennig, N., Sneath, P.H.A., Staley, J.T., Williams, S. (Eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 3. Williams and Wilkins Baltimore, MD, pp. 1808–1834.
- Wood, P.M., 1986. Nitrification as a bacterial energy source. In: Prosser, J.I. (Ed.), *Nitrification*. IRL Press, Oxford, UK, pp. 63–78.