Searching for Anammox Bacteria in Aquaria Biofilters at the Marine Resources Center of the MBL

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Abstract. The presence of anammox-like diversity in aquaria biofilters at the Marine Resources Center of the Marine Biological Laboratory was examined in this study. A Nested PCR approach was used to amplify DNA extracted from samples collected from freshwater and saltwater biological filters using primers specific for planctomycetes and anammox bacteria. Phylogenetic analysis of 75 partial 16S rRNA gene sequences with the ARB software package resulted in sequences that fell into three major lineages of the domain Bacteria: Chlamydiae, Verrucomicrobia and Planctomycete. Of the 75 sequenced clones, 62.6% of the clones were affiliated with the division Chlamydiae (all these sequences were retrieved from freshwater samples), 29.3% were affiliated with the division Verrucomicrobia (all these sequences were retrieved from saltwater samples), and only 8% of the sequence clones fell into the division Planctomycete. None of the clones identified were closely related to known anammox bacteria. Fluorescence in situ hybridization (FISH) was also used to examine the presence of anammox using planctomycetes and anammox-specific oligonucleotide probes. Hybridization with planctomycete-specific probe was positive but no signal was detected for anammox-specific probe. These results confirm clone library results that anammox-like bacteria are not present in aquaria biofilters.

Introduction. Ammonium (NH$_4^+$), present in municipal and industrial wastewater is of special interest to the environment because of its toxicity to aquatic species. Consequently, great interest and attention has been paid by the environmental engineering community to design bioreactors that foster specialized consortia of microorganisms capable for the conversion of NH$_4^+$ to nitrogen gas. In these bioreactors two functional groups of microorganisms are involved in the conversion of NH$_4^+$ to nitrogen gas. These include: (1) the autotrophic nitrifies that convert NH$_4^+$ to nitrite (NO$_2^-$) and further to nitrate (NO$_3^-$) using oxygen as the terminal electron acceptor and carbon dioxide as their carbon source; and (2) anoxic heterotrophic denitrifies the convert NO$_3^-$ and NO$_2^-$ to nitrogen gas using various organic compounds as electron donors (e.g. methanol, acetate, ethanol). Normally the two processes (nitrification/denitrification) occur in a combined system of two reactors in series where denitrification occurs in the first reactor followed by nitrification in the second reactor. Despite the wide application of the conventional nitrification/denitrification configuration for the removal of nitrogen, these systems are still quite expensive because of the large amount of external source of aeration for nitrification (4.2 g of oxygen for 1 gram of NH$_4^+$) and organic carbon source for denitrification (e.g. 2.47 g of methanol required per gram of NO$_3^-$) needed to meet the effluent discharge standard of 10 mg N/L (Jetten et al., 2002). Therefore, future challenges for environmental engineers is to design bioreactors that remove nitrogen in a more effective and economical way.

This challenge is not far from reach. It was not until a decade ago when a group of researchers experimentally demonstrated the presence of anaerobic ammonia-oxidizing (Anammox) bacteria in a denitrifying fluidized bed reactor treating wastewater effluent from methanogenic reactor in Delft, The Netherlands (Mulder et al., 1995). These chemolithautotrophic bacteria oxidize ammonia under anoxic conditions in the presence of nitrite as their electron acceptor and CO$_2$ as
the carbon source. The presence of chemolithoautotrophic Anammox bacteria in the environment was already been predicted two decades ago on the basis of thermodynamic calculations (references). Phylogenetic analysis revealed that Anammox bacteria belong to the phylum planctomycetes (Jetten et al., 2001). Since no external oxygen and organic carbon source is required, the Anammox process would lead to a reduction in wastewater treatment operational costs of up to 90% (Strous and Jetten, 2004). After its first discovery in denitrifying fluidized bed reactor, anammox bacteria were discovered in several marine and freshwater habitats and in many wastewater treatment plants all over the world (Strous and Jetten, 2004). Therefore, understanding the global reservoir of diversity of Anammox bacteria and the environmental conditions in which they exist could provide valuable information that can be further invested in the optimization and design of more cost-effective treatment technologies for the removal of high ammonia-rich wastewater. The role of anammox bacteria is not only limited to its application to wastewater engineering. Studies have shown that Anammox bacteria play an important role in the global nitrogen cycle (Schmid et al., 2005) and understanding their diversity is an essential part for maintaining the global nitrogen cycle.

As mentioned above, a prerequisite for the presence of anammox is the simultaneous occurrence of ammonium and nitrite and the absence of oxygen. Examples of environments that satisfy these requirements include wastewater treatment plants, landfill leachate, trickling filters, and rotating biological contactors. In the current study, clone library and FISH were used to test the hypothesis that anammox bacteria are present in aquaria biofilters.

Materials and Methods. Sample Collection and Processing. Five samples were collected from aquaria biofilters from the Marine Resources Center of the Marine Biological Laboratory (MBL), Woods Hole, Ma (Table 1). It should be noted that for sample number one two samples were collected from two different aquariums (one sample per aquarium).

Table 1. The five environmental samples collected.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Description</th>
<th>Observations</th>
<th>Processing</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Siporax ceramic filter media</td>
<td>Freshwater; 28°C; 5 gal aquarium operated without any animals</td>
<td>Smashed with a mallet, sonicated for 1 min in 20 ml 1x PBS (pH 7.2). Supernatant was used for DNA extraction</td>
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<tr>
<td>2</td>
<td>LANPAC 3.5 inch media from trickling filter # 1</td>
<td>Saltwater; 8°C; treating water recirculated throughout the facility</td>
<td>Smashed with a mallet, blended for 1 min in 100 ml 1x PBS (pH 7.2). Allowed to settle, liquid transferred to 50 ml conical tube and centrifuged at 5,000xg for 15 min, pellet was used for DNA extraction</td>
</tr>
<tr>
<td>3</td>
<td>LANPAC 3.5 inch media from trickling filter # 8</td>
<td>Saltwater; 28°C; treating water recirculated throughout the facility</td>
<td>Smashed with a mallet, blended for 1 min in 100 ml 1x PBS (pH 7.2). Allowed to settle, liquid transferred to 50 ml conical tube and centrifuged at 5,000xg for 15 min, pellet was used for DNA extraction</td>
</tr>
<tr>
<td>4</td>
<td>So-Clean, Micron-rated filter bag</td>
<td>Saltwater; 28°C; 50 gal aquarium containing toadfish</td>
<td>Paced directly into 2-ml tube</td>
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**DNA Extraction and PCR Conditions.** DNA from each sample collected was extracted using the Ultraclean soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA.) according to the manufacturer’s instructions. The genomic DNA isolated was used as template material for the polymerase chain reaction (PCR). Nested PCR was used to detect anammox bacteria in the different samples using two sets of primers. Environmental DNA was amplified with the planctomycete-specific forward primer, S-P-Planc-0046-a-A-18 (5’-GGA TTA GGC ATG CAA GTC-3’) with the universal reverse primer 1392r (5’-ACG GGC GGT GTG TAC-3’). PCR product obtained from the first round of PCR was used as a template in the second round of PCR using the planctomycete-specific forward primer, S-P-Planc-0046-a-A-18 (5’-GAC TTG CAT GCC TAA TCC-3’) with reverse primer S-*-Amx-0368-a-A-18 (5’-CTT TTC GGG CAT TGC GAA-3’) (Anammox specific primer) (Schmid et al., 2005). For each set of primers, PCR was performed in 25 µL reaction volume using a reaction mixture of 2X promega master mix, 0.25 µM/L of each primer and 1 µL of genomic DNA or PCR product. Amplification of DNA was performed with the MJ research PTC-200 Peltier Thermal Cycler (BioRad, Hercules, CA) using the following program: an initial denaturing step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 50 s, annealing at 52°C for 1 min, extension at 72°C for 1 min and 30s, and final extension at 72°C for 7 min.

**Cloning, Sequencing and Database Analysis.** The 16S rDNA PCR products from the second round of PCR were cloned into pCR2.1 vector and transformed into *Escherichia coli* using the TOPO TA cloning kit according to the manufacture’s instructions (Invitrogen, California). A total of 96 white colonies were picked with a sterile toothpick and transferred into a 96-well micrititer plate containing 100 µL of LB broth with ampicilin. Sequencing was performed at the DNA sequencing facility in the Marine Biological Laboratory (MBL). Partial 16S rRNA gene sequences were aligned automatically using the ARB software package. Subsequently, sequences were manually aligned by visual inspection when necessary. Evolutionary tree was generated using the maximum parsimony and neighbor joining algorithm in ARB. Bootstrapping was conducted using the PHYLIP DNA-Parsimony tool (100x resampling).

**FISH.** In addition to PCR/clone library, FISH was also used to detect the presence of anammox bacteria in the environmental samples using oligonucleotide probe specific for all anammox bacteria (S-*-Amx-0368-a-A-18). In addition to anammox specific probe, probes that target Planctomycetales S-P-Planc-0046-a-A-18 (5’-GAC TTG CAT GCC TAA TCC-3’) and bacterial lineages (EUB338, S-D-Bact-0338-a-A-18) (Schmid et al., 2005) were used too. Immediately after processing the samples (Table 1), cells were fixed in 4% (w/v) formaldehyde in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 per liter distilled water at a pH of 7.4) and incubated overnight at 4°C. Samples were then washed twice with PBS and resuspended in 1:1 mixture of PBS and ethanol and stored at –20°C. 10 µL of resuspended samples were loaded into each gelatin-coated well and dried for 30 min at 46°C and then dehydrated in solutions of 50%, 80% and 100% ethanol for 3 min each. For hybridization, 9 µL of hybridization buffer (720 µL of 5M NaCl, 80 µL of 1M Tris/HCL, 10% SDS, 15% (w/v) formamide for EUB338 and anammox probes and 25% (w/v) formamide for planctomycetales probe, and filled to 4 mL with MilliQ water) was mixed with 1 µL of carboxycyanin Cy3 fluorescently labeled probe (working solution at 50 ng/µL). The slides were then incubated at 46°C for 90 min in a 50 mL conical tube containing a tissue paper moistened with 4 mL of hybridization buffer. After hybridization the slides were immersed in a washing buffer (500 µL of 0.5M EDTA, 1000 µL of 1M Tris/HCL, 10% SDS, 338 mM NaCl for EUB338 and anammox probes and 159 mM NaCl for planctomycetales probe, and filled to 4 mL with MilliQ water) and incubated for 15 min at 48°C.
Optimal % formamide in the hybridization buffer and mM NaCl in the wash buffer for the different probes used in this study were empirically determined elsewhere (Schmid et al., 2005). After 15 min the samples were air-dried and then counterstained with 4,6-diamidino-2-phenylindole (DAPI) (final concentration of 10 mg/L) mounted in Citifluor. Image acquisition was done with ZEISS AXIO Imager.A1.

**Results and Discussion.** In the current study molecular biology techniques were used to test the hypothesis that anammox bacteria are present in aquaria biofilter at the Marine Resources Center of the MBL. A nested PCR approach was used in order to provide high sensitivity to detect low copy numbers of anammox bacteria. Figure 1 shows the results of agarose gel electrophoresis for nested PCR for the four samples collected from the aquaria biofilters. As can be seen from the figure both planctomycete specific bands (1346 bp) from the first round of PCR and anammox specific bands (322 bp) from the second round of PCR were present in all four samples. These results show that nested PCR worked but it doesn’t tell us if the bands in the gel represent anammox-like bacteria.

![Figure 1](image_url)

*Figure 1. Agarose gel electrophoresis results for the four samples collected from aquaria biofilters using planctomycete and anammox specific primers.*

To determine if anammox-related sequences are present in these samples clone library was constructed from partial 16S rRNA gene sequences (PCR products from second round of PCR). 96 colonies were selected and sequenced. Of the 96 colonies, only 75 sequences of good quality were selected for constructing a phylogenetic tree. Maximum parsimony and neighbor joining methods were used to construct a phylogenetic tree using the ARB software package. Figure 2 presents the phylogenetic tree inferred by maximum parsimony method.
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Figure 2. Phylogenetic tree inferred by maximum parsimony analysis of partial 16S rDNA sequences obtained from 16S rRNA clone libraries for aquaria biofilter samples. Environmental clones labeled with blue color represent freshwater samples (ceramic filters) and environmental clones labeled with green color represent saltwater samples (trickling filter # 1&8, and filter bag sample).

From figure 2 the conclusion is that 75 sequences fell into three major lineages of the domain Bacteria: Chlamydiae, Verrucomicrobia and Planctomycete. Environmental clone clusters in figure 2 represent monophyletic groups of 16S rRNA sequences with at least 90% sequence similarity. Of the 75 sequenced clones, 62.6% of the clones were affiliated with the division Chlamydiae (all these sequences were retrieved from freshwater samples (ceramic filters)), 29.3% were affiliated with the division Verrucomicrobia (all these sequences were retrieved from saltwater samples (trickling filters # 1&8 and filter bag)), and only 8% of the sequence clones fell into the division Planctomycete. The results obtained in Figure 2 were unexpected since the primers used in this study were specific for plantomycete bacteria but the majority of the cloned sequences fell into the division Chlamydiae and Verrucomicrobia. One explanation is that primer specificity was low at the annealing temperature (52°C) employed in this study.

Chouari et al. (2003) used planctomycete specific forward primer Planc46 and universal reverse primer 1390R to amplify extracted DNA from activated sludge. The results of 16S rDNA clone library showed nonplanctomycete 16S rDNA sequences, such as Chlamydiales, Spirochetes, and Verrucomicrobiales. However, the majority of the clone sequences belong to the order Planctomycetales. The annealing temperature employed was 59°C.

None of the clones identified in the present study were closely related to known anammox bacteria. One explanation for this is that anammox bacteria are still underrepresented in the 16S
rRNA gene clone libraries (Schmid et al., 2005) and so the primers that are currently being used to detect anammox bacteria do not necessarily cover all potential anammox-related bacteria present in the environment. Another explanation could be due to low recovery of DNA using the current DNA extraction methods (Schmid et al., 2005). Moreover, in one study using planctomycete-specific forward primer S-P-Planc-0046-a-A-18 (5'-GAC TTG CAT GCC TAA TCC-3') together with the universal 1390R it was shown that in a semitechnical plant where the anammox bacteria represent 99% of the planctomycete population and 40% of the bacterial population only 9 clones out of 25 have 16S rRNA gene sequences related to anammox bacteria (Schmid et al., 2000). This suggests that primer specificity is a critical point in the detection of anammox bacteria.

In addition to phylogenetic analysis, FISH was also used to detect if planctomycete and anammox bacteria are present in the aquaria biofilters. Figure 3 shows FISH results obtained for sample number 1 (ceramic filters) and sample number 2 (trickling filter #1) using the EUB338 general eubacterial probe and planctomycete specific probe (Planc-0046). The results in Figure 3 show that hybridization occurred with EUB338 probe and planctomycete specific probe. Similar results were also obtained for samples 3 and 4 (data not shown). These results imply the existence of planctomycete bacteria and possibly anammox bacteria. However, when anammox specific oligonucleotide probe was used (S-*-Amx-0368-a-A-18) hybridization didn’t occur (data not shown). These results confirm clone library results that anammox-like bacteria are not present in aquaria biofilters.

**Figure 3.** In situ detection of planctomycete in aquaria biofilters. A) Hybridization of ceramic filter sample with Cy3-labeled EUB338 and counterstaining with DAPI. B) Hybridization of trickling filter #1 sample with Cy3-labeled EUB338 and counterstaining with DAPI. C) Hybridization of ceramic filter sample with Cy3-labeled plan46 and counterstaining with DAPI. D) Hybridization of trickling filter #1 sample with Cy3-labeled plan46 and counterstaining with DAPI. Cells labeled with the general plan46 probe or EUB3338 are red; cells labeled with DAPI are blue.
Despite the fact that anammox-related bacteria were not detected in aquaria biofilters, the results from clone library analysis were interesting since novel sequences were discovered for the division Chlamydiae and Verrucomicrobia. Chlamydiae are important obligate intracellular bacteria that cause diseases in vertebrates. They are endosymbionts of amoeba and possibly other ciliates. They are present in several different environments ranging from soil, water conduit systems, sewage sludge, corneal/contact lenses samples, nasal mucosa of healthy individuals (Corsaro, et al., 2002), and in the current study in aquaria biofilters. Their presence in these different environments suggests that there is still a vast diversity of this division yet to be discovered. Similar to Chlamydiae, Verrucomicrobia, which are recently discovered bacterial division, are present in different environments and the number of novel sequences discovered is constantly increasing, which suggest that more Verrucomicrobia-related bacteria are still to be discovered in the future.

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References