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Anaerobic Ammonium-Oxidizing (Anammox) Bacteria and Associated Activity in Fixed-Film Biofilters of a Marine Recirculating Aquaculture System†

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Microbial communities in the biological filter and waste sludge compartments of a marine recirculating aquaculture system were examined to determine the presence and activity of anaerobic ammonium-oxidizing (anammox) bacteria. Community DNA was extracted from aerobic and anaerobic fixed-film biofilters and the anaerobic sludge waste collection tank and was analyzed by amplifying 16S rRNA genes by PCR using anammox-selective and universal GC-clamped primers. Separation of amplified PCR products by denaturing gradient gel electrophoresis and sequencing of the different phylotypes revealed a diverse biofilter microbial community. While *Planctomycetales* were found in all three communities, the anaerobic denitrifying biofilters contained one clone that exhibited high levels of sequence similarity to known anammox bacteria. Fluorescence in situ hybridization studies using an anammox-specific probe confirmed the presence of anammox *Planctomycetales* in the microbial biofilm from the denitrifying biofilters, and anammox activity was observed in these biofilters, as detected by the ability to simultaneously consume ammonia and nitrite. To our knowledge, this is the first identification of anammox-related sequences in a marine recirculating aquaculture filtration system, and our findings provide a foundation for incorporating this important pathway for complete nitrogen removal in such systems.

The continuing decline of marine fisheries and the increased demand for seafood by consumers have created a gap that endangers the world's seafood supply. In order to fill this gap while the pressures on wild fisheries stocks are eased, marine species must be farmed through aquaculture. A major drawback of the traditional open pond and marine net pen aquaculture technology is their negative impact on the marine environment, which includes organic and inorganic pollution of coastal areas, the spread of disease to wild populations, and "genetic pollution" as a result of farmed fish mixing with wild stocks (53). In response to these concerns there is a trend to shift marine fish farming inland using environmentally friendly closed recirculating aquaculture systems. Elimination of waste in these systems is generally managed through mechanical filtration for removal of solids and biofiltration for the conversion of toxic ammonia produced by the fish to nitrate through nitrification. Nitrate is typically maintained at low levels by daily replacement of a portion of the water (48). However, strict environmental regulations for both nitrate levels and salt concentrations in discharge water are motivating the recirculating aquaculture industry to integrate a denitrifying biofiltration stage, which results in the conversion of nitrate to nitrogen gas. Denitrification is performed mainly by facultative anaerobic bacteria that utilize organic (heterotrophic denitrification) or inorganic (autotrophic denitrification) compounds as electron sources to reduce nitrate (40). So far, difficulties associated with the denitrification process, such as the high potential for toxic sulfide production, as well as the requirement for addition of an external electron source, have prevented its use in full-scale commercial marine recirculating systems (51).

One option for closing the nitrogen cycle without exclusive reliance on denitrification is to integrate the anaerobic ammonium oxidation (anammox) process in recirculating systems (39, 51). An important pathway of the nitrogen cycle, anammox allows ammonia to be oxidized by nitrite under anoxic conditions (36) and is performed by autotrophic bacteria that are members of the order *Planctomycetales*. The anammox reaction has been shown to occur in an organelle-like compartment called the anammoxosome, where ammonia is oxidized via hydrazine (N_2H_4) and hydroxylamine (NH_2OH) intermediates (47). Anammox bacteria have been found in the anoxic water column of the Black Sea (22), the Costa Rica shoreline (5), an oceanic oxygen-minimum zone (21), and sediments from the Baltimore inner harbor (38) and several estuaries (24, 43, 44). They have also been shown to play a role in the nitrogen cycle associated with the microbial consortia of activated sludge from wastewater treatment plants (6, 8, 18, 37, 42).

Stimulation of anammox activity for use in wastewater purification has been examined by several research groups (6, 12, 18). Utilization of the process for complete removal of nitrogen from contaminated water is economically viable since ammonia oxidation requires, at a minimum, 50% less oxygen than that required by the conventional nitrification-denitrification process (17). Moreover, compared to heterotrophic denitrification, the anammox process results in complete autotrophic nitrogen removal with no organic electron source requirement.

Previously, we characterized the microbial community and the nitrogen transformation processes in a moving bed biore-

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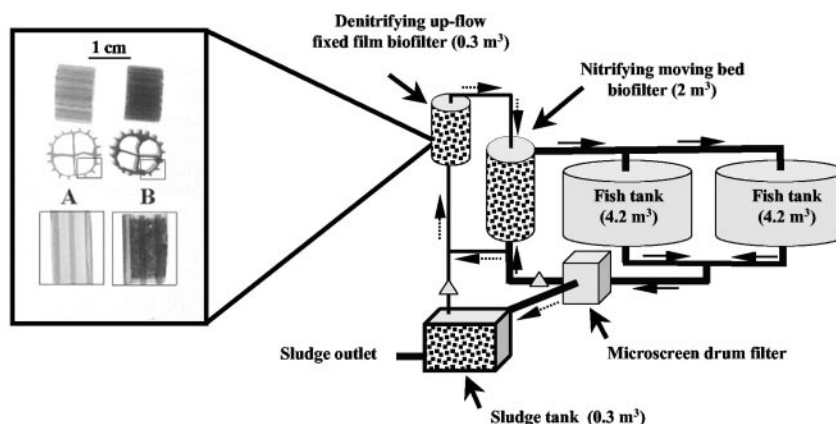

FIG. 1. Schematic diagram of the marine recirculating aquaculture system used in this study. The system included aerobic and anaerobic loops for complete removal of nitrogen by nitrifying and denitrifying processes, respectively. Triangles represent pumps, and arrows indicate the direction of circulating seawater; the high-flow aerobic loop (16 m^3 h^{-1}) is indicated by solid arrows, and the low-flow anaerobic side loop (0.1 m^3 h^{-1}) is indicated by dotted arrows. Stippling indicates compartments containing biofilter beads. The inset is a photograph of top and side views of representative polystyrene beads, as well as a fourfold enlargement of a side area. (A) Sterile bead. (B) Biofilm-containing bead. See Materials and Methods for additional details.

actor connected to a marine recirculating aquaculture system and obtained preliminary evidence for both the presence of *Planctomyces* spp. and the potential for anammox activity (39). In this study, we identified anammox bacteria as part of the microbial consortia of fixed-film biofilters of a closed marine recirculating aquaculture system and measured anammox-related activity in enrichment cultures obtained from these filters. Moreover, we compared anammox and denitrification activities, and we discuss ways to implement the anammox process as an alternative to denitrification in such systems.

MATERIALS AND METHODS

System configuration. A schematic diagram of the marine recirculating system used in this study is shown in Fig. 1. Two 4.2-m^3 tanks containing gilthead seabream (*Sparus aurata*) were operated at a density of 10 to 50 kg m^{-3} and a feeding rate of 1% to 1.6% body weight day^{-1}. The tanks were connected to a 2-m^3 nitrifying moving bed bioreactor filled with 1 m^3 of polyethylene beads (39) that were 1 cm in diameter and had a surface-to-volume ratio of 500 m^2 m^{-3}. A flow rate of 16 m^3 h^{-1} was used to obtain two exchanges of tank water per hour through the nitrifying biofilter. Attached to each tank as a denitrification side loop was a 0.3-m^3 cylindrical up-flow fixed-bed biofilter filled with 0.2 m^3 of polyethylene beads. The flow rate for this anaerobic biofiltration component was 0.1 m^3 h^{-1}, and the water was supplied by two sources, direct water from the fish tank and water recovered from the sludge tank. The average concentrations of ammonia, nitrite, and nitrate for this biofilter component during 350 days of operation were 1.5 ± 0.2, 1.2 ± 0.2, and 121 ± 20 mg of N $liter^{-1}$, respectively, for input water and 0.2 ± 0.04, 0.1 ± 0.02, and 61.6 ± 12 mg of N $liter^{-1}$, respectively, for effluent water (Tal and Schreier, unpublished data). Sludge was collected with a drum screen filter using a backwash system in which tank water was used. Sludge and backwash water were collected in a 0.3-m^3 rectangular tank with 0.1-m^3 beads that provided a means for solids removal, as well as a substrate for bacterial colonization. Water from the sludge tank was pumped back into the system via the anaerobic biofilter, and high-density sludge was collected and removed.

Anammox enrichments. Ninety polyethylene beads from aerobic (nitrifying) and anaerobic (denitrifying) biofilters were incubated in 125-ml glass bottles with 85 ml of autoclaved seawater. The bottles were supplied with 0.7 mmol ammonia and nitrite, flushed with 99.99% nitrogen gas, sealed with gas-tight butyl rubber stoppers, and placed in the dark in a shaking incubator at 37°C for 3 months; once the ammonia and nitrite were consumed, periodic additions were necessary to replenish these substrates. After 3 months of incubation, anammox activity was evaluated by addition of ammonia and nitrite or hydrazine and nitrite in different combinations, and the liquid phase was periodically collected and analyzed to determine the ammonia, nitrite, and hydrazine contents (see below).

Analysis of ammonia, nitrite, and hydrazine. Total ammonia (NH_3 and NH_4^+) contents were determined by the hypochlorite oxidation reaction, as described by Scheiner (31). Nitrite was measured by the sulfanilamide reaction as described by Strickland and Parsons (35). Hydrazine contents were determined spectrophotometrically using the American Society for Testing and Materials standard methods (3) by adding 1 ml of *p*-dimethylaminobenzaldehyde reagent (4 g of *p*-dimethylaminobenzaldehyde dissolved in 200 ml of methyl alcohol and 20 ml of concentrated HCl) and 0.1 ml concentrated HCl to 5 ml of sample and, after 10 min of incubation at room temperature, determining the absorbance at 458 nm. Standard curves for establishing the hydrazine concentration were determined using stock solutions containing between 5 and 200 μg hydrazine $liter^{-1}$. All measurements were carried out in triplicate, and the values were ±10%.

Biofilm DNA extraction. Five polyethylene beads from aerobic or anaerobic biofilters and the sludge tank were sliced into small fragments and were suspended in 2 ml of buffer (0.15 M NaCl, 0.1 M Na_2EDTA, pH 8). One gram of silica beads (diameter, 0.1 mm) was mixed with the polyethylene biofilter bead fragments in 5-ml tubes vigorously for 5 min. After centrifugation at 15,000 × *g* for 5 min, each supernatant fraction was collected, and bacterial DNA was extracted as described previously (39).

PCR amplification. To identify anammox bacteria, a sequential PCR approach was employed, as described by Tal et al. (38). Amplification of *Planctomycetales*-specific 16S rRNA genes was performed using the Pla46 oligonucleotide primer (5′-GAC TTG CAT GCC TAA TCC-3′) (25) with the universal bacterial primer 1392r (5′-ACG GGC GGT GTG TAC-3′) (10). The product of this reaction was then cloned into *Escherichia coli*, and selected transformants were examined further by PCR amplification using primer Pla46 and anammox-specific primer Amx820 (5′-AAA ACC CC TCT ACT TAG TGC CC-3′) (33). Amplification of 16S rRNA gene sequences used for denaturing gradient gel electrophoresis (DGGE) was performed using universal bacterium-specific 16S rRNA gene primer 1055f (5′-TGG CTG TCG TCA GCT-3′), universal bacterial GC clamp primer 1392rGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC ACG GGC GGT GTG TAC-3′) (10), and either biofilter DNA or the amplified anammox-related *Planctomycetales*-related clones as the template. The PCR mixtures (50 μl) contained 5 μl of PCR buffer, 4 μl of deoxynucleoside triphosphates (25 mM each), 3 μl of $MgCl_2$ (25 mM), 0.1 μl of *Taq* polymerase (250 U) (Applied Biosystems, CA), 2 μl of DNA template (10 to 100 ng), and 1 μl of each primer (100 to 200 ng). The reaction cycle parameters included an initial denaturation step consisting of 5 min at 95°C, followed by seven touchdown PCR cycles (7) (30 s of denaturation at 94°C, 30 s of annealing at 62, 60, 59, 58, 57, 56, and 55°C, and 30 s of elongation at 72°C) and then 30 cycles consisting of 30 s of denaturation at 94°C, annealing for 30 s at 54°C, and elongation for 30 s at 72°C and a final extension step consisting of 5 min at 72°C.

The sizes and yields of PCR products were determined by using a 0.8% (wt/vol) agarose gel in TAE buffer (20 mM Tris-HCl, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.0) (29).

DGGE. Analysis of PCR products by DGGE was performed as described by Tal et al. (39), using the D-Code universal mutation detection system (Bio-Rad, Hercules, CA). Products from triplicate PCRs were combined and applied directly to 10% (wt/vol) polyacrylamide gels containing a 35 to 50% gradient of denaturants (100 ml of 100% denaturant contained 42 g of urea, 40 ml of deionized formamide, 25 ml of a 40% acrylamide-bisacrylamide solution [37.5:1], and 1 ml of 50× TAE buffer). Electrophoresis was performed for 5 h at 60°C in TAE buffer at 200 V (constant voltage). After electrophoresis, nucleic acids were stained with SYBR Green II (1:10,000 dilution; Molecular Bio-Probes, Eugene, OR), and the staining pattern was immediately recorded using a fluoroimager (Molecular Dynamics, Sunnyvale, CA). DNA fragments were isolated after gels were stained with ethidium bromide (0.5 μg/ml) and visualized using a UV transilluminator. Gel sections containing fragments were excised, placed in TE buffer (50 mM Tris-HCl [pH 8.0], 1 mM Na_2EDTA), and incubated at 4°C overnight. The eluted DNA was used as a template for the PCR, as described above, and was checked for purity by DGGE.

Cloning, sequencing, and database analysis. PCR-amplified DNA and DNA fragments obtained from DGGE gel slices were introduced into a pCR2A vector and transformed into *E. coli* using a TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen, California). The presence of cloned inserts was verified by PCR amplification, and sequence analysis was carried out using an ABI 3100 automated sequencer (PE Applied Biosystems, Foster City, CA). DNA sequences were examined and edited using DNAman (Lynnon Biosoft, Quebec, Canada) and then checked for chimera formation using the Check Chimera program of the Ribosomal Database Project (23). Sequences were subjected to the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST) (1) and the Ribosomal Database Project (23) to determine the levels of similarity with other 16S rRNA genes. Partial 16S rRNA gene sequences were manually compiled and aligned using the PHYDIT software (4). Evolutionary trees were generated using the neighbor-joining (28), Fitch-Margoliash (11), and maximum-parsimony (20) algorithms in the PHYLIP software (9). Evolutionary distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated as described by Jukes and Cantor (19). The robustness of the inferred tree topologies was evaluated after 1,000 bootstrap resamplings of neighbor-joining data.

FISH analysis. Biofilms extracted from polyethylene beads (five beads from aerobic biofilters and five beads from anaerobic biofilters) were suspended in 1 ml of phosphate-buffered saline (PBS) (pH 8.0) (29) and vortexed gently for 2 min. Material was then fixed with 4% (vol/vol) paraformaldehyde in PBS for 1 h at room temperature, and this was followed by two washes with PBS. After fixing, the samples were suspended in a 50% (vol/vol) PBS–ethanol solution and stored at −20°C. Aliquots (10 μl) were used for fluorescence in situ hybridization (FISH) analysis as described previously (34). Texas Red-labeled anammox-specific probe Amx820 (Sigma Genosys) was used for detection of anammox bacteria, and counterstaining was performed using SYBR Gold (Molecular Probes, Eugene, OR). Texas Red-labeled nonsense probe NONEUB (52) was used to examine background fluorescence. Image acquisition was performed with a Bio-Rad Radiance 2100 laser confocal microscope equipped with the appropriate filter set and a digital camera. Densities were determined by comparing the number of cells stained with the Amx820 probe to the number of cells stained with SYBR Gold, and the average number was obtained by counting 100 to 200 cells in each of 10 random fields (100 μm by 100 μm) on five slides of biofilm samples (before and after anammox enrichment).

Nucleotide sequence accession numbers. Sequences of the partial 16S rRNA genes of the predominant restriction fragment length polymorphism types have been deposited in the GenBank database under accession numbers AY769988, DQ146408, and DQ146409.

RESULTS

Isolation and examination of anammox-specific 16S rRNA genes from biofilter components. Our initial survey of the microbial consortium associated with nitrogen transformation processes in a marine recirculating aquaculture system resulted in identification of two 16S rRNA gene segments that exhibited high levels of sequence similarity to members of the order *Planctomycetales* (39). However, both of these segments were most closely related to *Pirullela* spp., which have not been associated with anammox (39). To enhance our ability to amplify anammox-related 16S rRNA gene sequences, we utilized a sequential PCR approach that we previously used to successfully identify analogous sequences from an environmental sample (38). DNA extracted from aerobic and anaerobic biofilters, as well as the system sludge tank, was subjected to PCR amplification using the *Planctomycetales*-specific primer Pla46 and the general bacterial primer 1392r as described in Materials and Methods. Under stringent conditions, amplification produced libraries of partial (~1,350-bp) *Planctomycetales* 16S rRNA genes, which were then cloned into *E. coli*. From several hundred transformants isolated for each library, we chose between 30 and 50 transformants that contained the cloned Pla46-1392r insert. Screening of these clones using Pla46 and the anammox-specific Amx820 primer resulted in identification of three clones which yielded an approximately 750-bp PCR product, clone 3-8b6 from the denitrifying biofilter, clone 9

A

Amx 820	GGGCACTAAGTAGAGGGGTTTT
B. Anammoxidans	GGGCACTAAGTAGAGGGGTTTT
K. stuttgartiensis	GGGCACTAAGTAGAGGGGTTTT
Clone 3-8b6	GGGCACTAAGTAGAGGGGTTTT
S. brodae	GGGCACTAAGTAGAGGGGATTA
S. sorokinii	GGGCACTAAGTAGAGGGAATTA
S. wagneri	GGGCACTAAGTAGAGGGAATTA
Clone 9	GAGCACTAGATCGAGGGGGCTC
Clone YC	GAGCACTAGTTCGAGGGGACCC
P. marina	GAGCACTGGATCGAGGGGACTC

B

Amx 1240	GGTTGGTACAAAGGGATGCTAAA
B. anammoxidans	GGTTGGTACAAAGGGATGCTAAA
K. stuttgartiensis	GGTCGGTACAAAGGGATGCTAAG
Clone 3-8b6	GGTCGATACAAAGGGATGCTAAA
S. brodae	GGTCGGTACAAACGGAAGCGAAG
S. sorokinii	GGTCGGTACAAACGGAAGCGAAG
S. wagneri	GGTCGGTACAAAAGGAAGCAAGA
Clone 9	GGCGCGTACAAAGGGACGCAAAC
Clone YC	GGCACGTACAAAGCGACGCAAGA
P. marina	GGTGCGAACAAAGGGACGCAAAC

C

Amx 368	TTCGCAATGCCCGAAAGG
B. anammoxidans	TTCGCAATGCCCGAAAGG
K. stuttgartiensis	TTCGCAATGCCCGAAAGG
Clone 3-8b6	TTCGCAATGCCCGAAAGG
S. brodae	TTCGCAATGCCCGAAAGG
S. sorokinii	TTCGCAATGCCCGAAAGG
S. wagneri	TTCGCAATGCCCGAAAGG
Clone 9	TCCGCAATGGGCGAAAGC
Clone YC	TCCGCAATGGGCGCAAGC
P. marina	TTGGCAATGGGCGCAACG

FIG. 2. Comparison of the aquaculture system *Planctomyces* species sequences to anammox and nonanammox *Planctomycetales* sequences in anammox signature regions. Clone sequences in regions that are around 770, 1,200, and 350 bp from the 5′ (Pla46) end are compared to the Amx820 (A), Amx1240 (B), and Amx368 (C) oligonucleotide sequences and corresponding *B. anammoxidans* (accession number AF375994), *K. stuttgartiensis* (accession number AF375995), *S. brodae* (accession number AY254883), *S. sorokinii* (accession number AY257181), *S. wagneri* (accession number AY254882), and *P. marina* (accession number X62912) sequences. Bases that differ from the bases in oligonucleotide probe sequences are underlined. The GenBank accession number for clones 3-8b6, 9, and YC are AY769988, DQ146408, and DQ146409, respectively.

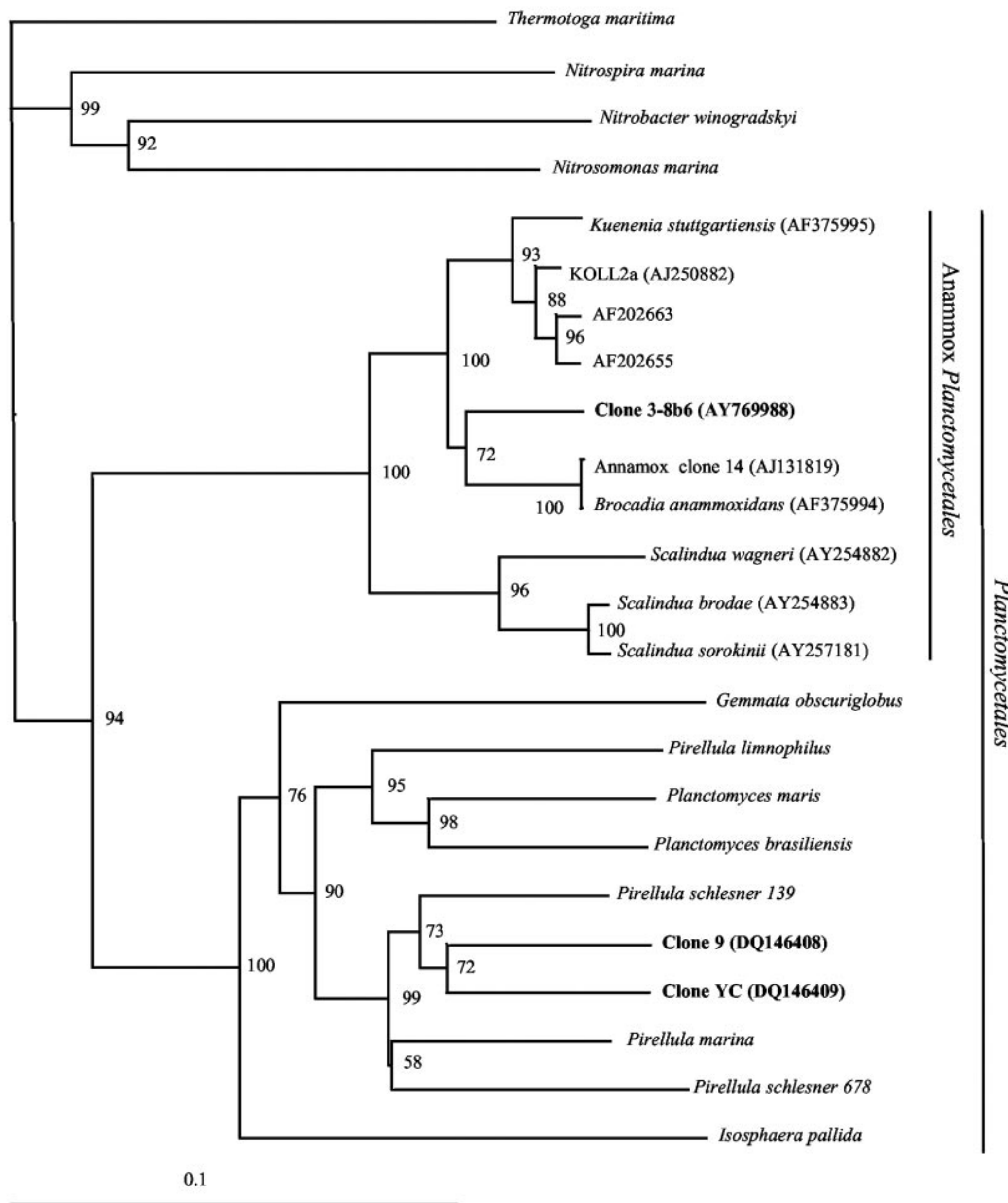

FIG. 3. Consensus phylogenetic tree generated by neighbor-joining (23), Fitch-Margoliash (11), and maximum-parsimony (19) analysis methods for a comparison of 1,010 bp of 16S rRNA genes. The phylogenetic reconstruction shows the relationship of the 16S rRNA gene sequences retrieved from the aquaculture system to *Planctomycetales* sequences. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1,000 resampled data sets (only values greater than 50% are shown). *Thermatoga maritima* was used as an outgroup. Scale bar = 10 substitutions per 100 nucleotide positions. The numbers in parentheses are accession numbers.

from the nitrifying biofilter, and clone YC from the sludge tank.

DNA sequence analysis of the three Pla46-1392r segments (GenBank accession numbers are shown in the legend to Fig. 2 and in Fig. 3) revealed that clones 9 and YC were 89% similar to each other over the entire sequence but were only 78 to 79% similar to clone 3-8b6. A comparison of the anammox signature Amx820, Amx1240, and Amx368 (33) regions indicated that clone 3-8b6 was identical to Amx820 and Amx368 sequences (Fig. 2A and C) and nearly identical (with a two-base difference) in the Amx1240 region (Fig. 2B). In addition to the high levels of sequence identity in these regions, clone 3-8b6 was also found to exhibit between 89 and 93% sequence similarity to several anammox *Planctomycetales* spp., including *Brocadia anammoxidans*, *Kuenenia stuttgartiensis*, *Scalindua brodae*, and *Scalindua wagneri*, as well as the marine bacterium *Scalindua sorokinii*, but only 78% similarity to *Pirullela marina*, a nonanammox *Planctomycetales* sp. On the other hand, clones 9 and YC were found to differ at several positions in the anammox signature regions, and the sequences

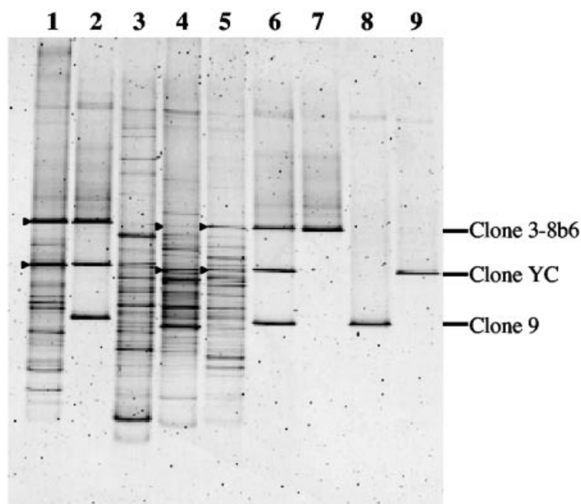

FIG. 4. DGGE analysis of PCR-amplified 16S rRNA gene fragments. DNA was extracted from biofilter and sludge tank samples, and PCR amplification with the universal 1055 and 1392rGC clamp primers followed by electrophoresis was performed as described in Materials and Methods. Lane 1, denitrifying biofilter after enrichment in the presence of ammonia and nitrite; lane 3, nitrifying biofilter; lane 4, sludge tank sample; lane 5, denitrifying biofilter before anammox enrichment. The same primer set was used to amplify plasmid DNA of clones 3-8b6 (lane 7), 9 (lane 8), and YC (lane 9). Lanes 2 and 6 contained all three clones together. The arrowheads indicate fragments that were subjected to DNA sequence analysis.

in these regions were more closely related to *P. marina* sequences (Fig. 2). Furthermore, clones 9 and YC were 88 to 89% similar to *P. marina* sequences but only 77 to 79% similar to the anammox *Planctomycetales* sequences.

A phylogenetic tree of the 16S rRNA gene sequences was assembled, and this tree included representative members of the order *Planctomycetales*, the known anammox bacteria, and the three new clones (Fig. 3). Consistent with our sequence analyses, clone 3-8b6 was found to fall deep within the anammox cluster, branching along with *B. anammoxidans*. Similarly, clones 9 and YC branched with the nonanammox *Pirullela* group. Thus, not only did the microbial consortium in the main aquaculture wastewater treatment compartments include *Planctomycetales* spp., as we have demonstrated previously (39), but the system also included one organism whose 16S rRNA gene sequence was characteristic of the sequences associated with the anammox *Planctomycetales* members.

DGGE analysis. The presence of *Planctomycetales* spp. within the bacterial consortium of the biofiltration system was examined by DGGE analyses (Fig. 4). Electrophoresis was performed using PCR products generated with universal bacterial primers and microbial community DNA from nitrifying and denitrifying biofilter beads, as well as heavily colonized beads obtained from the sludge tank. As shown by the band patterns in Fig. 4, lanes 3 to 5, there were significant differences in microbial diversity for each compartment. In addition, bands that comigrated with two of the *Planctomycetales* clones could be identified in at least one of the compartments. A fragment that migrated with clone 3-8b6 was detected in the denitrifiying biofilter (lane 5), as well as in the sludge tank (lane 4). This fragment was extracted from the gel, cloned, and purified as described in Materials and Methods, and it was found to have a DNA sequence identical to that of the clone 3-8b6 fragment (data not shown). Similarly, the denitrifying biofilter yielded a product that comigrated with the fragment from clone YC, which was initially amplified from the sludge tank (lanes 4 and 5). Bands from either of the biofilters or the sludge tank that comigrated with the amplified clone 9 fragment were not detected. Moreover, clone 3-8b6 was not detected in samples from the nitrifying biofilter (lane 3).

To examine the occurrence of the *Planctomycetales* clones under conditions favorable for anammox activity, we incubated beads from the denitrifying biofilter under anammox-enriching conditions as described in Materials and Methods and examined the microbial population by DGGE analysis. As noted above, the anaerobic denitrifying biofilter had been found to contain sequences that coincided with both clone YC and 3-8b6 sequences (Fig. 4, lane 5). As shown in Fig. 4, lane 1, while there was a significant difference in the DGGE banding patterns before and after enrichment (compare lanes 1 and 5), bands that comigrated with clones 3-8b6 and YC were clearly evident in the enrichment population. These bands were extracted and were found to have DNA sequences identical to the sequences of the fragments in clones 3-8b6 and YC (data not shown).

FISH analysis. The presence of anammox-related *Planctomycetales* in the bacterial consortia of the biofilm from the denitrifying biofilter before and after anammox enrichment was examined by FISH using the anammox-specific Amx820 probe labeled with Texas Red and SYBR Gold as a counterstain. As shown in Fig. 5, the inner biofilm of beads from the denitrifying biofilter contained SYBR Gold-staining bacteria (Fig. 5A, C, and E) that were also labeled with the Amx820 probe (Fig. 5B, 5D, and 5F) and exhibited the characteristic spherical morphology of the *Planctomycetales*. In no case were we able to detect any cells that hybridized with the Texas Red-labeled nonsense NONEUB primer (data not shown). By determining the number of cells that hybridized with the Amx820 probe and comparing the results to the number of cells stained by SYBR Gold, we found that anammox-related bacteria accounted for 1.1% $\pm$ 0.1% of the total bacterial population before enrichment (compare Fig. 5A to Fig. 5B and Fig. 5C to Fig. 5D) and 12.5% $\pm$ 1.6% of the total bacterial population after anammox enrichment (compare Fig. 5E and F).

Assessment of anammox and denitrification activity in biofilters. In the presence of nitrite, anammox bacteria are able to oxidize either hydrazine or ammonia under anaerobic conditions (30, 33). To examine whether the bacterial consortia from nitrifying and denitrifying biofilters were capable of anammox activity, samples from these biofilters were cultured under anammox-enriching conditions, and their abilities to utilize either ammonia and nitrite or hydrazine and nitrite were determined under anaerobic conditions. After 3 months of incubation under anammox-enriching conditions (see Materials and Methods), beads from the denitrifying biofilter were assayed for the ability to oxidize hydrazine as described in Materials and Methods. As shown in Fig. 6A, hydrazine oxidation occurred at a rate of approximately 0.16 μM bead^{-1} h^{-1} over a 50-h period. However, no significant hydrazine removal was observed for beads from the nitrifying biofilter that were treated under the same conditions (Fig. 6B). Although nitrifying bac-

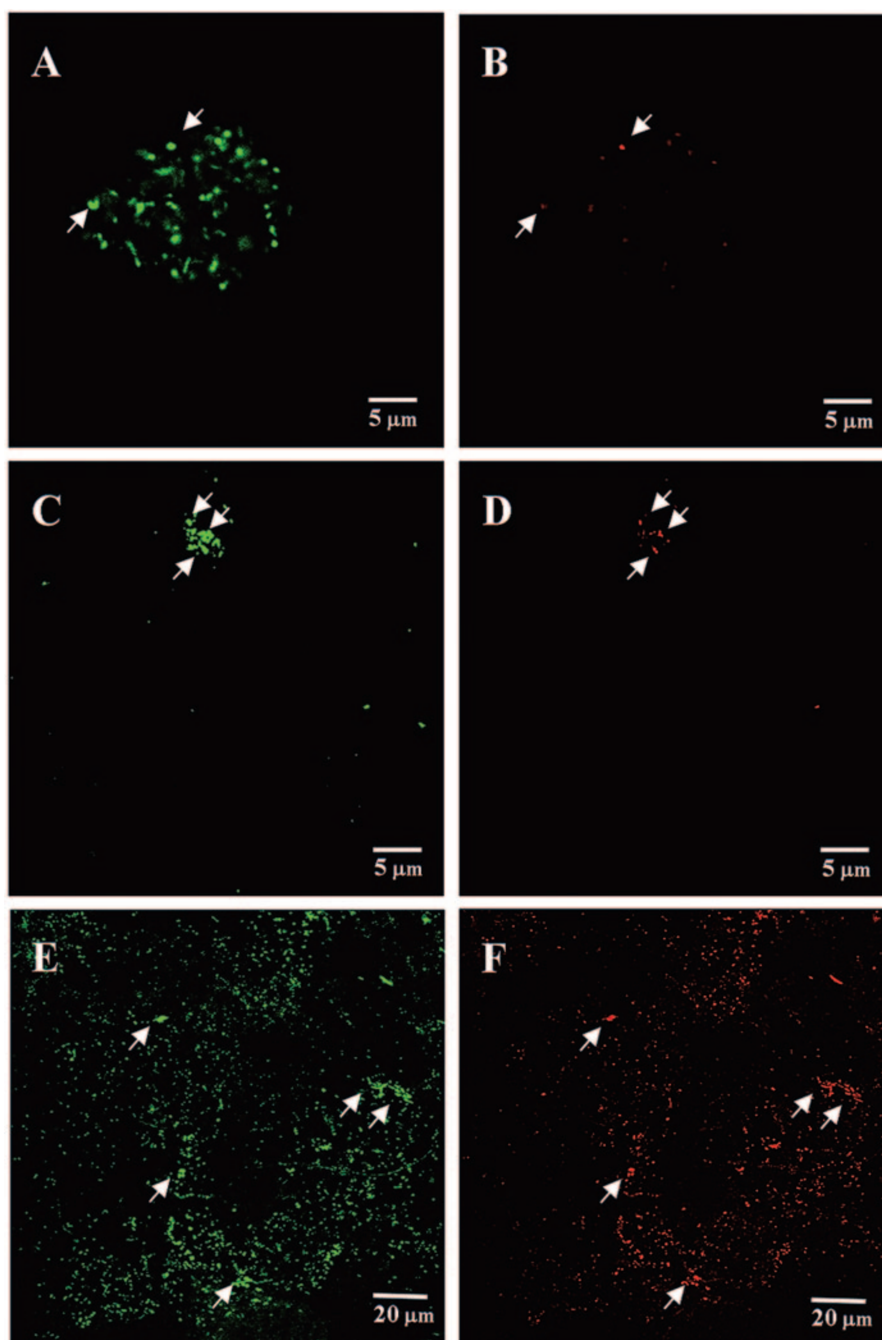

FIG. 5. Identification of anammox bacteria in denitrifying biofilter material by FISH analysis. Aggregates of biofilm before (A, B, C, and D) and after (E and F) enrichment were stained with SYBR Gold (A, C, and E) and visualized with Texas Red filters after hybridization with anammox probe Amx820 (B, D, and F) as described in Materials and Methods. The arrows indicate representative SYBR Gold-stained cells that also reacted with the Amx820 probe.

teria are capable of utilizing hydrazine (2, 27), the hydrazine removal rates for nitrifying sludge have been shown to be as much as 10-fold lower than the hydrazine removal rates for anammox sludge (30), which might explain the absence of detectable activity under our experimental conditions. Hydrazine removal could be attributed to biological activity as no significant uptake was detected using beads taken from either biofilter that was autoclaved prior to incubation (data not shown). Beads from the anammox-enriched denitrifying culture were also able to completely remove 0.66 mM ammonia in the presence of 0.76 mM nitrite (Fig. 6D) over 90 h of incubation under anaerobic conditions, which corresponded to an ammonia removal rate of 0.098 μM NH_3 $bead^{-1}$ h^{-1}, which is approximately 1.6-fold lower than the hydrazine removal rate of the same culture (Fig. 6A). In the absence of nitrite, however, ammonia removal could not be detected (data not shown). These results indicated that the anaerobic denitrifying biofilter was able to carry out anammox activity, which is con-

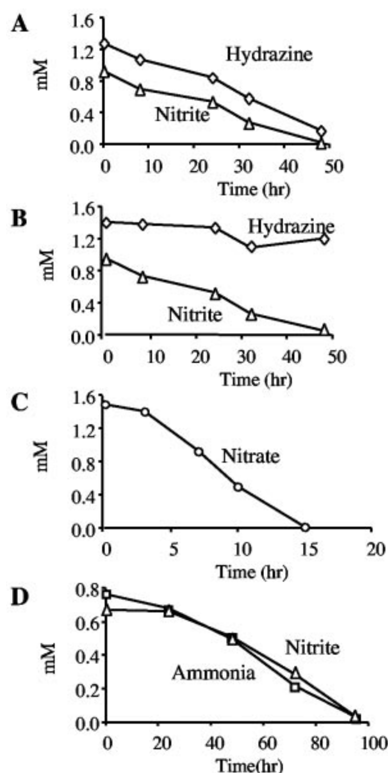

FIG. 6. Anammox and nitrate-reducing activities of denitrifying and nitrifying biofilter beads. Denitrifying (A and D) and nitrifying (B) biofilter beads were incubated under anammox enrichment conditions for 3 months, and the abilities to utilize hydrazine (◇) or ammonia (□) and nitrite (△) were measured as described in Materials and Methods. (C) Nitrate uptake by denitrifying biofilter beads that were not incubated under anammox enrichment conditions.

sistent with the presence of anammox-related *Planctomycetales* in such biofilters. Moreover, the molar ratio of ammonia to nitrite removal for the denitrifying biofilter (Fig. 6D) was between 1.1 and 1.3, which is characteristic of anammox activity (16).

To distinguish between anammox and denitrification activities, nitrate reduction rates were determined using beads from the denitrification biofilter that were incubated under anoxic conditions in the presence of only nitrate (Fig. 6C). Under these conditions, the organic carbon incorporated into the biofilm served as an electron donor to support denitrification (39). The nitrate removal rate was determined to be 1.08 μM NO_3^- $bead^{-1}$ h^{-1} over 15 h of incubation. Thus, the capacity for nitrogen removal via denitrification was as much as 11-fold greater than the ammonia consumption rate.

DISCUSSION

Our ability to measure anammox activity in enrichment cultures established from a denitrifying biofilter of a recirculating aquaculture system and our identification of anammox-related bacteria both microscopically and by isolation of associated 16S rRNA gene sequences constitute the first report of the occurrence of anammox-related bacteria in marine recirculating aquaculture systems. Moreover, this study provides a foundation for use of the anammox process for removing inorganic nitrogen waste from recirculating systems.

Identification of anammox-related *Planctomycetales*. Using an approach for detecting anammox *Planctomycetales* in environmental samples, we isolated three new *Planctomycetales*-related 16S rRNA gene sequences from different components of a marine aquaculture system biofilter. One of the clones, 3-8b6, exhibited high levels of sequence similarity to known anammox bacterial 16S rRNA gene sequences in signature anammox-specific regions (Amx820, Amx1240, and Amx368), as well as across the entire sequence. Interestingly, clone 3-8b6 sequences were found to fall in the *Brocadia* branch of anammox *Planctomycetales* rather than the *Scalindua* branch, which includes the marine *Planctomycetales* (22), and additional studies are necessary to determine the relationship between 3-8b6 and the other anammox *Planctomycetales* members. We hypothesize that clone 3-8b6 represents a new anammox-capable *Planctomycetales* taxon that was part of the bacterial community of the denitrifying biofilter of the marine recirculating system. The ability to detect anammox activity in enriched cultures from the biofilters, the presence of an Amx820-hybridizing population as determined by the FISH analysis, and the identification of clone 3-8b6 sequences in both untreated and anammox-enriched biofilter preparations are consistent with the notion that 3-8b6 is involved in anammox activity. Whether the anammox activity that we measured can be directly attributed to this clone or was due to some other uncharacterized *Planctomycetales* sequence could not be determined from our studies and requires future isolation and characterization of the microorganisms. However, while the Amx820 probe used for library screening is selective for a subgroup of anammox *Planctomycetales* (33), PCR amplification of the *Planctomycetales* clone library created from the denitrifying filter before and after anammox enrichment using the universal anammox Amx368 (33) and 1392r primers resulted in identification of clones having the same sequence as 3-8b6 (Tal and Schreier, unpublished), suggesting that 3-8b6 is the predominant anammox *Planctomycetales* clone in this system.

In addition to the anammox-related clone, we identified two 16S rRNA sequences, clone 9 and clone YC, that were closely related to each other, as well as to sequences that we identified previously (reference 39 and data not shown), and also exhibited high levels of sequence similarity to the nonanammox *Planctomycetales*, which include the *Pirellula* spp. Although clones 9 and YC were obtained by the anammox-specific PCR approach, a phylogenetic and 16S rRNA gene sequence analysis of these clones did not indicate that there is a strong relationship to known anammox bacteria. It is conceivable that these sequences were detected due to the bias of *Planctomycetales* PCR clone libraries toward anammox 16S rRNA (33). Alternatively, the wide genus diversity of the anammox *Planctomycetales* (32) may indicate that these clones may represent new anammox bacteria that are not related to known anammox bacteria. The presence of clone YC sequences in the anammox enrichment culture from the denitrifying biofilter is consistent with the detection of related *Pirellula*-like *Planctomycetales* spp. in the autotrophic nitrogen-removing biofilms in wastewater systems (26), and the nature of the participation of these organisms has not been determined.

Aquaculture biofilters and wastewater treatment environments. With the exception of natural environments and wastewater treatment plants, there have been no reports of anam-

mox-related *Planctomycetales* in water treatment systems that have characteristics of the organisms encountered in recirculating aquaculture systems. The important differences between water quality parameters of aquaculture systems and wastewater plants include the concentrations of nutrients and the organic loads. While the concentration of ammonia in raw sewage may reach 20 to 40 mg liter^{-1} (ammonia nitrogen, NH_3-N) (51), the maximum ammonia concentrations allowed in aquaculture systems are less then 1 mg NH_3-N liter^{-1}, as higher concentrations of ammonia are lethal to fish (41). Moreover, the biological oxygen demand values for organic loads in raw sewage reach between 200 and 500 mg liter^{-1} (13), while they are usually less than 50 mg liter^{-1} in recirculating aquaculture systems (15). Furthermore, the growth structures of the bacterial communities are different. While activated sludge suspensions of wastewater treatment plants promote granule formation, a thin fixed film is the main structure of bacterial growth in moving bed biofilters of recirculating aquaculture systems. The ability of anammox bacteria to populate these distinct synthetic environments is consistent with the belief that the anammox process is amenable to many wastewater treatment applications with minimal apparent restrictions with respect to nutrient and salt concentrations, organic loads, or bacterial growth configurations (37).

Anammox versus denitrification. Anaerobic incubation of beads from the denitrifying biofilter showed that the removal rates were 1.08 μM nitrate nitrogen bead^{-1} h^{-1} under denitrifying conditions and 0.098 μM ammonia nitrogen bead^{-1} h^{-1} and 0.16 μM hydrazine bead^{-1} h^{-1} under anammox conditions. Although the fraction of anammox bacteria in the consortia of the anammox-enriched beads was 12.5% $\pm$ 1.6%, as determined by FISH, the nitrogen removal rates were between 7- and 11-fold lower than the denitrification rates of untreated beads. This discrepancy was likely due to the low activity and growth rate (11 days) of the anammox bacteria and indicates that there is a major limitation in using the anammox process for nitrogen removal in these systems. Ammonia oxidation rates for cultures of anammox bacteria with nitrite as the electron acceptor have been determined to be 55 nmol min^{-1} mg protein^{-1} (16), compared to, for example, 400 nmol min^{-1} mg protein^{-1} for nitrate reduction by the heterotrophic denitrifyer *Pseudomonas stutzeri* (50). This more-than-sevenfold difference suggests that denitrifying bacteria may outcompete anammox bacteria, especially in an organic carbon-rich environment, and could explain the relatively low numbers of anammox bacteria in the denitrifying biofilter, which was populated predominantly by fast-growing heterotrophic denitrifiers (51). Recent studies have shown that anammox bacteria are capable of myxotrophic growth in the presence of fatty acids, such as acetate and propionate (14), which are common organic waste products of aquaculture filtration systems (49), supporting the notion that anammox and denitrifying bacteria can share the same environment. To further assess the specific contributions of anammox and denitrifying bacteria in the nitrogen removal process of the recirculating system will require the use of ^{15}N-labeling experiments in which isotope pairing distinguishes between these two processes (16, 45).

Applying the anammox process. Development of anammox as the major nitrogen removal process in recirculating aquaculture systems is advantageous due to the reduced oxygen demands and the autotrophic nature of the process, which allow complete nitrogen removal without a need for organic carbon. As noted by other workers (26, 46), the low growth rates and activity exhibited by anammox bacteria should be reflected in two major features of anammox-based biofilters, inoculation time and biomass retention efficiency. Long initiation periods under conditions favorable for anammox are required in order to establish a stable anammox population. Shortening this period may be possible by inoculation of lab-enriched anammox cultures, although the success of such an approach will depend largely on the type and origin of the anammox culture used. Inoculation of marine recirculating systems with anammox cultures that are already a component of the natural bacterial consortium of the anaerobic biofilters should improve the likelihood of success. High biomass retention efficiency of an anammox-based biofilter is essential to overcome the low activity of the anammox bacteria. High surface-to-volume ratios of the biofilter substrate should increase the anammox bacterial biomass and improve the overall activity per volume of biofilter.

Successful operation of an anammox biofilter requires a constant supply of ammonia and nitrite along with minimal input of organic carbon. Such an environment is necessary to maintain the anammox consortium, while reducing competition from denitrifying bacteria. One approach for enhancing anammox activity in the recirculating system used in this study would be to partially aerate the sludge tank to stimulate incomplete denitrification in order to promote nitrite accumulation. The nitrite-containing water would then be supplied to the anaerobic biofilter along with ammonia-rich wastewater from the fish tank. We are presently addressing whether this combination promotes growth and activity of the anammox bacteria in an anaerobic biofilter.

Conclusion. Stricter environmental regulations for effluent discharge from aquaculture facilities are currently motivating the aquaculture industry to reduce effluent volumes and nitrogen loads. Closing the nitrogen cycle in the water treatment system by utilizing the anammox process is a desirable alternative to heterotrophic denitrification, especially in marine recirculating systems. Our finding that bacteria capable of anammox and their activity are associated with the consortia of a denitrifying biofilter makes this approach feasible for implementation and will direct future advances in building an environmentally sustainable land-based mariculture industry.

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