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Comparative Analysis of Nitrifying Bacteria Associated with Freshwater and Marine Aquaria

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Three nucleic acid probes, two for autotrophic ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* and one for α subdivision nitrite-oxidizing bacteria, were developed and used to study nitrifying bacterial phylotypes associated with various freshwater and seawater aquarium biofilters. *Nitrosomonas europaea* and related species were detected in all nitrifying seawater systems and accounted for as much as 20% of the total eubacterial rRNA. In contrast, nitrifying bacteria belonging to the β-proteobacterial subdivision were detected in only two samples from freshwater aquaria showing vigorous nitrification rates. rRNA originating from nitrite-oxidizing α subdivision proteobacteria was not detected in samples from either aquarium environment. The data obtained indicate that chemolithotrophic ammonia oxidation in the freshwater aquaria was not due to β-proteobacterial phylotypes related to members of the genus *Nitrosomonas* and their close relatives, the organisms usually implicated in freshwater nitrification. It is likely that nitrification in natural environments is even more complex than nitrification in these simple systems and is less well characterized with regard to the microorganisms responsible.

The pathways of the nitrogen cycle are highly dependent on microbial activities and transformations. One important pathway in the nitrogen cycle is nitrification, the oxidation of ammonia to nitrite and subsequently to nitrate (17). Traditionally, nitrification has been studied by chemical measurement of ammonia or nitrite disappearance, measurement of the production of nitrite or nitrate, or a combination of these methods (see reference 25 for a review of autotrophic nitrification). Nitrification occurring in a wide range of environments, such as soils (17), ocean water (36), freshwater lakes (11), wastewaters (24), and aquaria (16), is assumed to be due to autotrophic bacteria. While heterotrophic nitrification can occur and may contribute substantially to nitrification in certain environments (17, 29), it is not coupled to energy generation and, therefore, is thought to be a minor component of overall nitrification (4, 25).

A primary concern in fish culture systems ranging from high-density aquaculture operations to the home tropical fish aquarium is the toxic effects of ammonia on fish. To control and maintain safe ammonia levels in fish culture systems, biological filters have been designed to promote the growth of ammonia- and nitrite-oxidizing bacteria. Biological filters use a variety of materials as supports on which the bacteria are cultured. Generally, no special effort is made to distinguish between the types of supports used in different seawater or freshwater culture systems. The general assumption is that species of ammonia- and nitrite-oxidizing bacteria are identical in the two types of environments and that they require only a solid support, good aeration, and an energy source (ammonia or nitrite) to become successfully established.

In freshwater systems, the bacterial genera responsible for the oxidation of ammonia and nitrite are presumed to be predominantly the genera *Nitrosomonas* and *Nitrobacter*, both of which are chemolithoautotrophic members of the class *Proteobacteria* (14, 38). Recent studies in which comparative 16S rRNA analyses of ammonia- and nitrite-oxidizing bacteria were performed have clarified the phylogenetic relationships of these bacteria and have demonstrated that they belong to two separate lineages within the *Proteobacteria* (12, 30). Teske et al. (30) concluded that the nitrifying bacteria may have multiple phylogenetic origins. These authors speculated that nitrifiers have developed independently many times, perhaps from different lineages of photosynthetic bacteria (30). The freshwater autotrophic ammonia-oxidizing bacteria that have been characterized belong exclusively to the β subdivision of the *Proteobacteria* and are typified by *Nitrosomonas europaea* (Fig. 1). These bacteria form a distinct group within the β subdivision and are affiliated with an iron-oxidizing bacterium (*Gallionella ferruginea*) and the photosynthetic bacterium *Rhodocyclus purpureus*, along with methylotrophic bacteria. One ammonia oxidizer, *Nitrosococcus oceanus*, is a marine species that belongs to the γ-proteobacterial lineage.

The most commonly studied autotrophic nitrite-oxidizing bacteria belong to the α subdivision of the *Proteobacteria*, of which *Nitrobacter winogradskyi* is a representative species (Fig. 1). Other chemolithoautotrophic nitrite-oxidizing bacteria that have been characterized are phylogenetically widespread in the class *Proteobacteria*, occurring in the α, δ, and γ subdivisions (Fig. 1). Phylogenetic analysis of the α subdivision of the *Proteobacteria* has shown that *Nitrobacter winogradskyi* is most closely related to *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris* (9, 23, 27, 39).

In this study, we used oligonucleotide probes which target chemolithoautotrophic ammonia-oxidizing and nitrite-oxidizing bacteria to examine nitrifying bacterial populations associated with freshwater and marine aquaria. Various microbial habitats associated with aquarium systems were investigated, including the gravel, water, and biofilter support medium, which is a substratum designed to encourage the growth of nitrifying bacteria. Specific differences between nitrifying bacterial assemblages on freshwater and seawater aquarium biofilters were also investigated.

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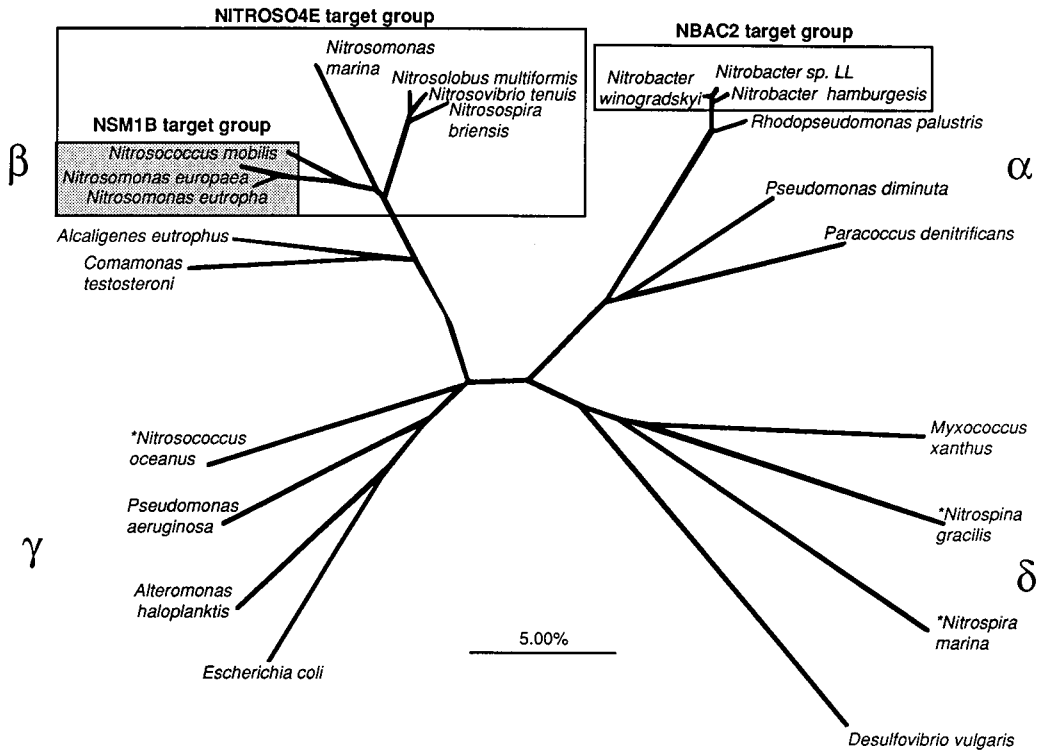

FIG. 1. Phylogenetic relationships of the chemolithoautotrophic ammonia- and nitrite-oxidizing bacteria. Most known ammonia-oxidizing autotrophs belong to the β subdivision of the *Proteobacteria*; the only exception is *Nitrosococcus oceanus*, which is affiliated with the γ subdivision. The nitrite-oxidizing bacteria are more widespread in the *Proteobacteria*, occurring in the α, δ, and γ subdivisions. Nucleic acid probes which correspond to (i) all known β subdivision ammonia oxidizers (probe NITROSO4E), (ii) a clade on a deep branch in the β subdivision (probe NSM1B), and (iii) the nitrite oxidizers belonging to the α subdivision (probe NBAC2) were developed. Nitrifying bacteria which are not targeted by the probes designed in this study are indicated by asterisks. Recent studies indicate that the genus *Nitrospira* may be affiliated with a group outside the δ subdivision of the *Proteobacteria*, in a separate phylogenetic lineage (8).

MATERIALS AND METHODS

Bacterial culture and nucleic acid extraction techniques. Ammonia- and nitrite-oxidizing bacteria were obtained from the American Type Culture Collection or were kindly provided by J. B. Waterbury of Woods Hole Oceanographic Institute, Woods Hole, Mass., and were grown in organic-free media in batch culture by standard methods (Table 1) (2).

Isolation of ribosomal DNA genes of nitrite oxidizers. As expected, the nitrite-oxidizing bacteria grew slowly with low cell yields, and so the PCR was used to generate sufficient ribosomal DNA template to test probe specificities. Prior to the PCR, DNAs from *Nitrobacter winogradskyi* and *Nitrobacter agilis* were extracted. Cells were placed in lysis buffer (40 mM EDTA, 50 mM Tris; pH 8.3) to which lysozyme was added to a final concentration of 1 mg/ml. After incubation at 37°C for 30 min, 50 μl of proteinase K (stock solution concentration, 10 mg/ml) and 50 μl of 20% sodium dodecyl sulfate (SDS) were added to each sample, and then the preparations were incubated at 55°C for 30 min. Cell lysis was monitored by phase-contrast microscopy. In some cases, additional proteinase K and SDS were added and the sample was incubated at 55°C for another 30 min.

After cell lysis, DNA was extracted by sequential extractions with phenol (pH 8.0), phenol-chloroform-isoamyl alcohol (24:24:1), and finally chloroform-isoamyl alcohol (24:1). Each sample nucleic acid was precipitated with 0.3 M sodium acetate and 2 volumes of ethanol and stored at −20°C. The sample was collected by centrifugation, dried, and resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA). The concentration of DNA was determined by Hoechst type 33258 dye binding and fluorometry (model TKO 100 minifluorometer; Hoefer Pharmacia Biotech Inc., San Francisco, Calif.). Ribosomal DNA was amplified by using primers specific for eubacterial rRNA, as previously described (7).

Isolation of rRNA. Cells of the ammonia-oxidizing and heterotrophic bacteria were harvested by centrifugation for 20 min at 8,000 rpm (model RC5C centrifuge; Sorvall Instruments). Total rRNA was extracted from bacterial cells by cell disruption with glass beads, using a Mini Beadbeater (BioSpec Products, Bartlesville, Okla.). After disruption, a three-step purification procedure (with phenol [Tris buffered, pH 5.1], phenol-chloroform-isoamyl alcohol [24:24:1], and chloroform-isoamyl alcohol [24:1]) was performed (28). The resulting crude nucleic acid was precipitated overnight at −20°C after 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol were added. After precipitation, the nucleic acids were collected by centrifugation and resuspended in 100 μl of TE buffer (pH 8.0). RNA was quantified by measuring A_{260} with a Perkin-Elmer Lambda 3B spectrophotometer by assuming that 1 A_{260} unit corresponds to 40 μg of RNA per ml (28).

Oligonucleotide probe design. 16S rRNA sequences of chemolithoautotrophic ammonia-oxidizing bacteria were aligned in a database by using sequence data obtained from the Ribosomal Database Project (20). Two regions were identified as having potential specificity for the target groups. One 20-nucleotide probe (designated NITROSO4E) targeted all known ammonia-oxidizing members of the β subdivision (Fig. 1), and a second probe (NSM1B) targeted three members of the clade containing *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis.*

A third probe (NBAC2) was designed to target the α subdivision nitrite-oxidizing bacteria *Nitrobacter winogradskyi*, *Nitrobacter agilis*, and *Nitrobacter hamburgensis*. The probes were synthesized by Operon Tech, Inc., Alameda, Calif. The nucleotide sequences and positions of the probes are shown in Table 2.

Probe hybridization procedures. To determine the specificity of each probe, probe binding to rRNAs from target and nontarget bacteria was monitored by autoradiography. A temperature series spanning the estimated dissociation temperature of each probe was used to determine the wash temperature empirically.

All probe hybridization experiments were conducted with a slot blot device (Millipore Corp., New Bedford, Mass.). rRNAs from pure stock preparations and samples were denatured with 3 volumes of 2% (vol/vol) glutaraldehyde and then diluted to the final volume (1:100) with dilution water (1 μg of polyriboadenosine per liter, 0.0004% bromophenol blue). The plasmid stock preparations of *Nitrobacter winogradskyi* and *Nitrobacter agilis* were diluted with an equal volume of a mixture containing 1 N NaOH and 3 M NaCl. Samples were applied to nylon filters (Hybond N; Amersham Corp., Arlington Heights, Ill.) fitted into the slot blot device. After air drying, the filters were cross-linked by exposure to 1,200 J of UV irradiation (UV Stratalinker; Stratagene Corp., San Diego, Calif.).

For hybridization experiments, membranes were placed in a heat-sealable bag, 6 or 12 ml (depending on the number of membranes in the bag) of hybridization buffer (0.9 M NaCl, 50 mM $NaPO_4$, 5 mM EDTA, 0.5% SDS, 10× Denhardt's solution, 0.5 mg of polyadenosine per ml) was added, and the bag was sealed and placed in a hybridization oven (model 136500; Boekel Industries, Inc.) for 30 min at 45°C. After 30 min, the bags were removed, and 2×10^7 cpm of ^{32}P-end-

TABLE 1. Sources of the bacteria utilized in the nucleic acid probe validation studies and culture media used to grow them

Species	Proteobacterial subdivision	Strain	Growth medium
Chemolithoautotrophic ammonia-oxidizing bacteria			
Nitrosomonas europaea	Beta	ATCC 19718	ATCC 221
Nitrosococcus mobilis	Beta	NC2 Waterbury[a]	ATCC 928 (25%)[b]
Nitrosolobus multiformis	Beta	ATCC 25196	ATCC 929
Nitrosospira briensis	Beta	C128 Waterbury[a]	ATCC 221
Nitrosovibrio tenuis	Beta	NV12 Waterbury[a]	ATCC 929
Nitrosococcus oceanus	Gamma	ATCC 19707	ATCC 928
Chemolithoautotrophic nitrite-oxidizing bacteria			
Nitrobacter winogradskyi	Alpha	ATCC 25391	ATCC 480
Nitrobacter agilis	Alpha	ATCC 14123	ATCC 96
Nitrococcus mobilis	Gamma	ATCC 25380	ATCC 481
Nitrospira marina	Delta	NB295 Waterbury[a]	ATCC 480
Nitrospina gracilis	Delta	NB211 Waterbury[a]	ATCC 480
Heterotrophic bacteria closely related to ammonia or nitrite oxidizeers			
Alcaligenes eutrophus	Beta	ATCC 17697	Luria-Bertani
Alcaligenes faecalis	Beta	ATCC 15554	Luria-Bertani
Comamonas acidovorans	Beta	ATCC 15668[c]	Luria-Bertani
Comamonas testosteroni	Beta	ATCC 11975[c]	Luria-Bertani
Paracoccus denitrificans	Alpha	ATCC 17741	Luria-Bertani
Rhodopseudomonas palustris	Alpha	ATCC 17001	Luria-Bertani
Pseudomonas diminuta	Alpha	501[c]	Luria-Bertani
Shewanella putrefaciens	Gamma	ATCC 8071	Luria-Bertani
Pseudomonas nautica	Gamma	ATCC 27132	Marine broth
Pseudomonas aeruginosa	Gamma	ATCC 17503	Luria-Bertani

[a] Kindly provided by J. B. Waterbury, Woods Hole Oceanographic Institute.
[b] The medium used was 25% ATCC 928 medium in distilled water.
[c] Received from P. Baumann.

labeled probe was added. Each bag was resealed and returned to the oven. The membranes were incubated overnight in the hybridization oven at 45°C.

After the overnight washing described above, the membranes were removed and washed in a solution containing 1× SET (150 mM NaCl, 1 mM EDTA, 20 mM Tris; pH 7.8) and 1% SDS at room temperature for 30 min on a shaker table. The membranes were then washed in fresh 1× SET–1% SDS at appropriate wash temperatures for 30 min (with shaking every 10 min). After washing, the membranes were allowed to air dry. Autoradiographic signals were quantified by using a gas proportional radioisotope detection system (Ambis, Inc., San Diego, Calif.). Film autoradiographs were also recorded with an intensifier screen for 20 to 24 h at −76°C.

The relative rRNA-specific hybridization signal attributable to each probe was determined by calculating a slope (counts per minute bound per nanogram of RNA) for the serially diluted sample. Values were normalized by using a correction factor determined by dividing the group-specific probe slope derived from known rRNA standards by the slope derived from the eubacterial probe for the same standards (10). Group-specific hybridization signal was calculated by dividing the normalized group-specific probe slope by the eubacterial probe slope of the same sample.

Sampling and extraction of nucleic acids from aquarium samples. A variety of locations in small (water volume, <400 liters) aquaria having two general types of environments (inorganic and organic) were sampled for the presence of chemolithoautotrophic nitrifying bacteria.

The samples consisted of aquarium gravel, aquarium water, and pieces of the aquarium biological filter media. Gravel was collected with a scoop, weighed to the nearest 0.1 g, placed in a polypropylene tube, and immediately covered with low-pH buffer (50 mM sodium acetate, 10 mM disodium EDTA) for rRNA extraction or with cell lysis buffer for DNA extraction. Samples were stored at

TABLE 2. Nucleotide sequences and positions of the three oligonucleotide probes for nitrifying bacteria

Probe	Position (nucleotides)[a]	Base sequence (5′ to 3′)	T_d (°C)[b]/ wash temp (°C)	Targeted group	Nontarget bacteria with exact match to probe sequence
NITROSO4E	639-658	CAC TCT AGC YTT GTA GTT TC	43.2/53.0	β-Proteobacterial ammonia oxidizers	*Nodularia* sp.[c]
NSM1B	479-495	TCT GTC GGT ACC GTC AT	41.2/53.0	*Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosococcus mobilis*	None[d]
NBAC2	1017-1036.1	GCT CCG AAG AGA AGG TCA CA	49.4/53.0	*Nitrobacter winogradskyi*, *Nitrobacter hamburgensis*, *Nitrobacter agilis*	*Afipia clevelandensis*, *Afipia felis*, *Rhodopseudomonas palustris* strain, *Bradyrhizobium japonicum*[e]

[a] *Escherichia coli* numbering.
[b] T_d, dissociation temperature. Wash temp, experimentally determined wash temperature (see Materials and Methods).
[c] The following two nontarget bacteria have a one-base mismatch with the probe sequence: *Oscillatoria* sp. and *Cylindrospermum* sp.
[d] There are 50 nontarget bacteria or strains of bacteria that have a one-base mismatch with the probe sequence. These bacteria include *Ehrlichia*, *Rhodovulum*, *Rhodobacter*, *Rhodoplanes*, and *Fusobacterium* species, as well as *Anaplasma marginale*, *Thiobacillus thioparus*, *Sebaldella termitidis*, and *Streptobacillus moniliformis*.
[e] The following four nontarget bacteria have a one-base mismatch with the probe sequence: *Photorhizobium thompsonianum*, *Photorhizobium* sp. strain IRBG 230, *Bradyrhizobium* sp., and *Photorhizobium* sp. strain MKAa 2.

−20°C until extraction. Aquarium water was collected in prewashed glass jars and filtered through a Sterivex GV filter by using autoclaved pump tubing and a peristaltic pump. Between 1,000 and 4,000 ml of water was filtered depending on the sample. After filtering, 1.8 ml of cell lysis buffer was added to each unit with a sterile syringe, and the filters were stored at −20°C until processing. Various biological filter media were collected by cutting a piece of material from the filter with alcohol-sterilized scissors and forceps. Each medium sample was placed in a polypropylene tube, covered with 2.0 to 2.5 ml of cell lysis buffer or bead beating solution, and then stored at −20°C until extraction.

The gravel samples were extracted by adding 200 μl of 20% SDS and 3 ml of phenol (Tris buffered to pH 5.1) and shaking the preparations by hand for 5 min; this was followed by incubation in a 60°C water bath for 7 min. After shaking for 3 min, the samples were centrifuged at 1,500 rpm for 10 min (model 1550 centrifuge; Hamilton Bell, Montvale, N.J.). The nucleic acids were aliquoted into three tubes, and the contents of each tube were extracted by using the bead beating protocol described above.

The nucleic acids in the aquarium water samples were extracted by adding 40 μl of lysozyme (from a stock solution containing 25 mg of lysozyme in 500 μl of distilled water) to each thawed sample. The filter was placed on an agitator and shaken at 37°C for 30 min. Then 500 μl of proteinase K (stock solution concentration, 10 mg/ml) was added, and the filter was incubated at 55°C for 1 h with shaking. The solution was drawn out of the Sterivex filter with a syringe into a polypropylene tube. Phenol-chloroform-isoamyl alcohol extraction was performed, and this was followed by a series of chloroform-isoamyl alcohol extractions. The solution was concentrated with a Centricon 100 concentrator (Amicon, Beverly, Mass.), and nucleic acids were precipitated.

Freshwater and seawater aquarium biofilter comparison. Six all-glass aquaria (capacity, 34 liters) were used along with a standard home aquarium filtration system (Penguin model 160B; Marineland Aquarium Products, Moorpark, Calif.). There was no substratum or other material in the aquaria. In the model 160B system the main body of the filter unit hangs on the outside upper back edge of the aquarium. On the upper weir of the filter unit is the dedicated biological filter (BioWheel; referred to below as the biofilter), which sits perpendicular to the water flowing back into the aquarium. The water flow causes the biofilter to continuously rotate such that it functions as a rotating biological contactor, and, therefore, the filter surface alternates between a partially submerged phase and an air-exposed phase.

Initially, the tanks were filled with dechlorinated (activated carbon-treated) tap water; 5 mM ammonia (made with ammonium chloride) was added to each aquarium daily for the first 20 days and then every other day or so. Aquarium water was sampled several times a week and was analyzed by performing a flow injection analysis (FIAstar system; Tecator AB, Höganäs, Sweden) for ammonia (gas diffusion membrane method), nitrite (azo dye method), nitrate (cadmium reduction-azo dye method), and acid-neutralizing capacity (methyl orange to an end point of pH 4.5) as recommended in the manufacturer's application notes. The pH was determined with an electrode and a specific ion meter (Orion Instruments).

After all of the aquaria were exhibiting nitrification, as determined by nitrate production, the water in one group of three aquaria was changed from freshwater to seawater (prepared with artificial sea salts [Marineland Commercial Aquariums, Moorpark, Calif.]). Three additional aquaria were also set up with artificial seawater and filter units with BioWheels which had never been run. Water quality data were collected for the nine aquaria as previously described for another 75 days. At 43 and 72 days after the one freshwater group had been switched to seawater, the biofilm on each biofilter was sampled by cutting out a small piece of the filter. rRNA and ribosomal DNA were extracted as described above. rRNA was analyzed by using oligonucleotide probes as described above.

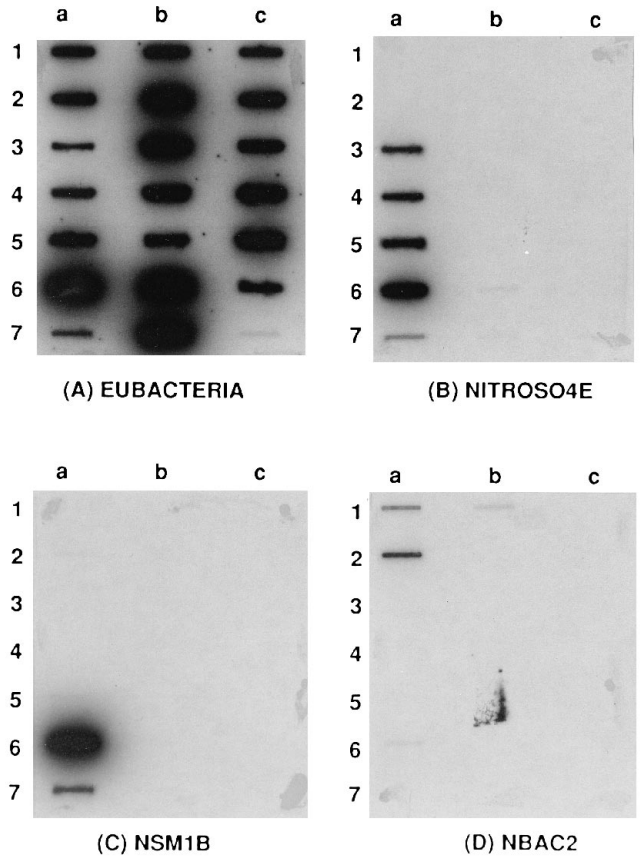

FIG. 2. Autoradiographs demonstrating the specificity of eubacterial probe EUBAC (A), ammonia-oxidizing bacterial probes NITROSO4E (B) and NSM1B (C), and nitrite-oxidizing bacterial probe NBAC2 (D). The rRNA extracts from chemolithoautotrophic nitrifying bacteria and closely related bacteria were blotted in the slots in the following arrangement: slot a-1, *Nitrobacter winogradskyi*; slot a-2, *Nitrobacter agilis*; slot a-3, *Nitrosovibrio tenuis*; slot a-4, *Nitrosospira briensis*; slot a-5, *Nitrosolobus multiformis*; slot a-6, *Nitrosomonas europaea*; slot a-7, *Nitrosococcus mobilis*; slot b-1, *Rhodopseudomonas palustris*; slot b-2, *Pseudomonas diminuta*; slot b-3, *Paracoccus denitrificans*; slot b-4, *Comamonas acidovorans*; slot b-5, *Alcaligenes faecalis*; slot b-6, *Comamonas testosteroni*; slot b-7, *Alcaligenes eutrophus*; slot c-1, *Nitrococcus mobilis*; slot c-2, *Nitrosococcus oceanus*; slot c-3, *Shewanella putrefaciens*; slot c-4, *Pseudomonas nautica*; slot c-5, *Pseudomonas aeruginosa*; slot c-6, *Nitrospina gracilis*; slot c-7, *Nitrospira marina*.

RESULTS

Oligonucleotide probe specificity. The specificities of three of the four ammonia- or nitrite-oxidizing group-specific probes developed in this study are shown in Fig. 2. Database searches and hybridization experiments performed with rRNAs extracted from phylogenetically diverse bacteria indicated that the probes were sufficiently specific to identify various chemolithoautotrophic nitrifying bacteria with the following provisions. There is one nontarget organism for the NITROSO4E probe and there are four nontarget organisms for the NBAC2 probe in which the probe sequence compliments the nontarget sequence exactly (Table 2). In the case of probe NSM1B the sequences of about 50 nontarget organisms out of the database of more than 3,000 sequences have only one mismatch with the target sequence (Table 2).

A range of wash temperatures was tested to determine the optimal conditions for probe specificity. Under appropriate hybridization and wash conditions, the NITROSO4E probe bound the rRNAs of all of the β subdivision ammonia-oxidizing bacteria examined, but not the rRNAs of the closely related heterotrophic bacteria (Fig. 2). The NSM1B probe yielded positive signals with the two targeted β subdivision ammonia-oxidizing bacteria (*Nitrosomonas europaea* and *Nitrosococcus mobilis*) but not with other nitrifying bacteria or closely related heterotrophic bacteria belonging to the same subdivision (Fig. 2). We tested a third probe for the β subdivision oxidizers, NLB1, but this probe cross-reacted with the closely related heterotrophic bacteria at all wash temperatures tested (data not shown). None of the nucleic acid probes designed for the β subdivision ammonia-oxidizing bacteria hybridized to *Nitrosococcus oceanus*, a marine species which is the only known autotrophic ammonia oxidizer not in the β subdivision (Fig. 2).

The results of the specificity test for the nitrite-oxidizing bacterial probe (NBAC2) show that this probe is specific for two known α subdivision nitrite oxidizers (*Nitrobacter winogradskyi* and *Nitrobacter agilis*) and does not cross-hybridize with either the δ or the γ subdivision nitrite-oxidizing bacteria

or closely related α subdivision heterotrophic bacteria, such as *Rhodopseudomonas palustris* (Fig. 2).

The data indicated that three of the four nucleic acid probes tested were sufficiently specific to distinguish autotrophic nitrifying bacteria from closely related heterotrophic bacterial species.

Detection of autotrophic nitrifying bacteria in aquaria. We tested the nitrifying bacterial rRNA probes with nucleic acids extracted from a wide range of samples obtained from actively nitrifying freshwater and seawater aquaria (Table 3). Some of the samples came from biofilters in aquaria which received more than 82 g of fish food or were dosed with 32 mM ammonia each day. Only 2 of the 38 freshwater samples gave a positive result with any of the nitrifier-specific probes (Table 3). These two samples, which exhibited positive signals for the two ammonia oxidizer rRNA probes, were from biofilters which had been dosed with ammonium chloride and were never exposed to the fish waste or organic compounds that are normally associated with a fish tank. The positive signals obtained for these biofilters may have resulted from contamination from seawater biofilters located nearby. These biofilters had been in the culturing system for 76 days before sampling. The NBAC2 probe did not indicate the presence of α subdivision nitrite-oxidizing bacteria in any of the freshwater samples (Table 3). There were large amounts of eubacterial rRNA detected by the eubacterial probe in each sample, and so the lack of signal cannot be attributed to insufficient material on the membranes.

A PCR analysis in which two general eubacterial primers (forward primer 8-27 and reverse primer 1492-1510) (19) were used was performed with some samples to increase sensitivity and to determine whether nitrifying bacterium rRNA genes could be detected in the mixed-community DNA. PCR products were blotted, and hybridization experiments were performed with the nitrifier rRNA probes. No signal was detected in the PCR products, which is consistent with the results of the rRNA hybridization experiments.

Positive results with probes specific for ammonia-oxidizing nitrifiers (NITROSO4E and NSM1B) were obtained for all seawater samples, which were dosed daily with ammonium chloride (Table 3). The lengths of time in the systems for the seawater biofilters tested ranged from 53 to 299 days. As with the freshwater systems, negative results were obtained with the probe for nitrite-oxidizing bacteria (NBAC2). Quantitative oligonucleotide probe hybridization experiments indicated that as much as 20% of the eubacterial rRNA was derived from ammonia-oxidizing bacteria belonging to the β subdivision of the *Proteobacteria* (Table 4). This is consistent with the presumed presence of significant numbers of ammonia-oxidizing bacteria on the biofilters. Furthermore, since the signal of the *Nitrosomonas* species probe (NSM1B) is equivalent to the signal of the more general β-proteobacterial ammonia-oxidizing group-specific probe (NITROSO4E), the nitrifiers on the seawater biofilters appear to be dominated by *Nitrosomonas europaea* and its close relatives rather than *Nitrosospira* types.

Freshwater-seawater biofilter comparison. The mean ammonia, nitrite, and pH data for the three groups of biofilters from aquaria that received different water treatments are presented in Fig. 3. It is clear that established freshwater aquarium biofilters experienced a complete loss of nitrification when the water in the aquaria was switched to seawater. This caused an increase in the ammonia concentrations in the aquaria (Fig. 3). After the switch to seawater, it took the previously freshwater biofilters nearly as long to reestablish ammonia oxidation as it took the newly set up seawater biofilters. However, the maximum ammonia concentration reached during the establishment period was less in the switched biofilters than in the newly set up seawater biofilters (Fig. 3). There was a small, temporary increase in the ammonia concentration in the freshwater aquaria from day 9 to day 17 (after the switch), which coincided with a drop in the pH to less than 7.00. The pH rose (and ammonia disappeared) after the addition of $NaHCO_3$.

Nitrite oxidation was established faster in the newly set up seawater biofilters than in the biofilters switched from freshwater, with complete oxidation occurring by day 50 and by day 60 (after the switch), respectively (Fig. 3). Furthermore, the nitrite concentration reached a much higher value and remained higher for a longer period of time in the switched biofilters than in the newly set up seawater biofilters. The nitrite concentration in the continuously freshwater biofilters was low for the duration of the measuring period (Fig. 3). A partial water change was performed on day 29 (after the switch) in all aquaria, and this change is reflected by the sudden drop in the nitrite concentrations in the seawater and freshwater-to-seawater groups. The nitrite concentration steadily increased again after day 29 in both groups until it finally decreased before the end of the measuring period because of establishment of nitrite oxidation.

The pH trends for the three groups of biofilters were similar except for a period of 8 days early in the test (days 9 to 17) when the pH in the freshwater biofilter group fell to less than 7.00. This pH change was compensated for by the addition of $NaHCO_3$.

Oligonucleotide probe hybridization experiments revealed positive signals with both ammonia-oxidizing bacterial probes for all seawater filters regardless of age (newly set up filters and filters switched from freshwater) (Fig. 4). Freshwater biofilters consistently yielded negative results with all of the nitrifier-specific probes (Fig. 4). The results indicated that *Nitrosomonas europaea* or its close relatives were well represented on the seawater biofilters. The results obtained with the probe for nitrite-oxidizing bacteria were negative for all samples (Fig. 4). Thus, both *Nitrobacter winogradskyi* and *Nitrobacter agilis* were either absent or present only at concentrations below our limits of detection, even though the nitrate concentrations steadily increased during the test.

DISCUSSION

Definitive studies correlating nitrification rates with nitrifying microorganisms in natural samples are difficult. Until recently there were few available methods for identifying and quantifying specific bacteria or groups of bacteria in environmental samples without cultivation, an approach known to sometimes lead to biased representation (1, 32). Cultivation of nitrifying bacteria is especially challenging because of the slow growth rates of these bacteria and the frequent occurrence of culture contamination by heterotrophic bacteria (22, 31). Ward (35) utilized immunofluorescence techniques to enumerate nitrifying bacteria, but this technique also required cultivation of the target group to raise antibodies. More recently, PCR primers have been developed and used to detect *Nitrosomonas* spp., *Nitrosospira* spp., and *Nitrobacter* spp. in diverse environments (6, 13, 21, 22, 31). Wagner et al. (33) developed fluorescent in situ hybridization probes specific for certain β-subdivision proteobacterial ammonia oxidizers. These authors found that up to 20% of the total bacteria in activated sludge samples from an animal waste-processing facility could be ammonia oxidizers.

In this study, oligonucleotide probes were used successfully to detect ammonia-oxidizing chemolithoautotrophic bacteria in environmental samples (i.e., seawater aquarium biofilters). Furthermore, the data obtained indicated that the bacteria responsible for ammonia oxidation in freshwater aquaria are

TABLE 3. Results of probing rRNAs extracted from biofilms attached to various aquarium biofiltration media or aquarium water with domain- and group-specific oligonucleotide probes[a]

Sample	Aquarium environment[b]	Biofilm substrate[c]	Daily amt of ammonia[d]	Ammonia source[e]	Signal detected by the following oligonucleotide probes[f]:			
					Bacterial	NITROSO4E	NSM1B	NBAC2
1301	Freshwater	Bulk water	4 g	Fish	+	−	−	−
1302	Freshwater	Gravel	4 g	Fish	+	−	−	−
1303	Freshwater	Gravel	4 g	Fish	+	−	−	−
1304	Freshwater	Filter fiber	4 g	Fish	+	−	−	−
1306	Freshwater	Bulk water	4 g	Fish	+	−	−	−
1307	Freshwater	Gravel	4 g	Fish	+	−	−	−
1309	Freshwater	Polypropylene	4 g	Fish	+	−	−	−
1312	Freshwater	Bulk water	32.1 mM	NH_4Cl	+	−	−	−
1315	Freshwater	Bulk water	32.1 mM	NH_4Cl	+	−	−	−
1316	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
7501	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
7502	Freshwater	Bulk water	82.84 g	Fish	+	−	−	−
7503	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
7504	Freshwater	Polyfiber	82.84 g	Fish	+	−	−	−
710r	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
711r	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
CAQBW	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	+	+	−
CAQBW	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	+	+	−
E8T32B	Freshwater	Sponge	1.4 g	Fish	+	−	−	−
E8T33B	Freshwater	Polypropylene	1.4 g	Fish	+	−	−	−
E8T34B	Freshwater	Filter fiber	1.4 g	Fish	+	−	−	−
Flwrte5	Freshwater	Gravel	10 mM	NH_4Cl	+	−	−	−
Flwrte8	Freshwater	Gravel	10 mM	NH_4Cl	+	−	−	−
Flwrte9	Freshwater	Gravel	10 mM	NH_4Cl	+	−	−	−
FWSW4	Freshwater	Polypropylene	5 mM	NH_4Cl	+	−	−	−
FWSW6	Freshwater	Polypropylene	5 mM	NH_4Cl	+	−	−	−
MejBW-A	Freshwater	Polypropylene	82.8 g	Fish	+	−	−	−
MejBW-B	Freshwater	Polypropylene	82.8 g	Fish	+	−	−	−
T408	Freshwater	Detritus	3.5 g	Fish	+	−	−	−
T408	Freshwater	Gravel	3.5 g	Fish	+	−	−	−
T825	Freshwater	Gravel	0.8 g	Fish	+	−	−	−
T825	Freshwater	Gravel	0.8 g	Fish	+	−	−	−
WDF1025	Freshwater	Sponge	2.0 g	Fish	+	−	−	−
WDF1026	Freshwater	Polypropylene	2.0 g	Fish	+	−	−	−
WDF1036	Freshwater	Polypropylene	3.2 g	Fish	+	−	−	−
WDF1036	Freshwater	Gravel	3.2 g	Fish	+	−	−	−
WDF1039	Freshwater	Polypropylene	3.2 g	Fish	+	−	−	−
WDF1039	Freshwater	Gravel	3.2 g	Fish	+	−	−	−
714r	Seawater	Polyfiber	714 mM	NH_4Cl	+	+	+	−
715r	Seawater	Polyfiber	714 mM	NH_4Cl	+	+	+	−
FWSW2	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
FWSW3	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
FWSW8	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
FWSW9	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
SW117	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW123	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW129	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW134	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW148	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW152	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW159	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW202	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−

[a] A continued negligible concentration of ammonia in the systems which had daily inputs of fish food or ammonium chloride was considered evidence that nitrification occurred.

[b] The type of aquarium water.

[c] The medium from which the bacterial cells were extracted.

[d] The values in grams are the amounts of fish food put into the aquaria each day; the molar and millimolar values indicate the amounts of ammonia added to the aquaria or systems in which the biofilters were located each day.

[e] Fish means that the aquarium had a fish population and ammonia was generated by the fish; NH_4Cl means that there were no fish in the tank and the ammonia was from ammonium chloride added daily.

[f] +, signal detected; −, no signal detected.

different from the bacteria responsible for ammonia oxidation in seawater aquaria. In seawater aquaria, *Nitrosomonas europaea* and related phylotypes appear to be present at high levels and are presumably the active ammonia-oxidizing bacteria, which is consistent with the results of previous studies. However, previously characterized β subdivision ammonia-oxidizing bacteria were detected in vigorously nitrifying freshwater aquaria in only 2 of 38 samples.

TABLE 4. Levels of hybridization (normalized to the eubacterial probe) of the two probes specific for β-subdivision ammonia-oxidizing bacteria to rRNAs extracted from the biofilms of two seawater aquarium filters[a]

Sample	Age of biofilter (days)	Daily ammonia dose (mol)	% Hybridization to:	
			NITROSO4E probe	NSM1B probe
SW202	53	2.5	20.4	23.8
SW148	98	2.5	18.5	17.4

[a] The biofilters were part of a larger group of 35 filters dosed daily with 2.5 mol of ammonia. The NITROSO4E probe targets all β ammonia oxidizers, while the NSM1B probe targets a subgroup of these bacteria (Fig. 1).

There are three possible explanations for our observations: (i) there were few nitrifiers relative to other bacteria in the samples examined, and so the method used was not sensitive enough; (ii) heterotrophic bacteria were responsible for the oxidation of ammonia and nitrite in the environments studied; or (iii) the responsible species of autotrophic ammonia-oxidizing bacteria belong to another phylogenetic group which the probes did not detect. These possibilities are discussed below.

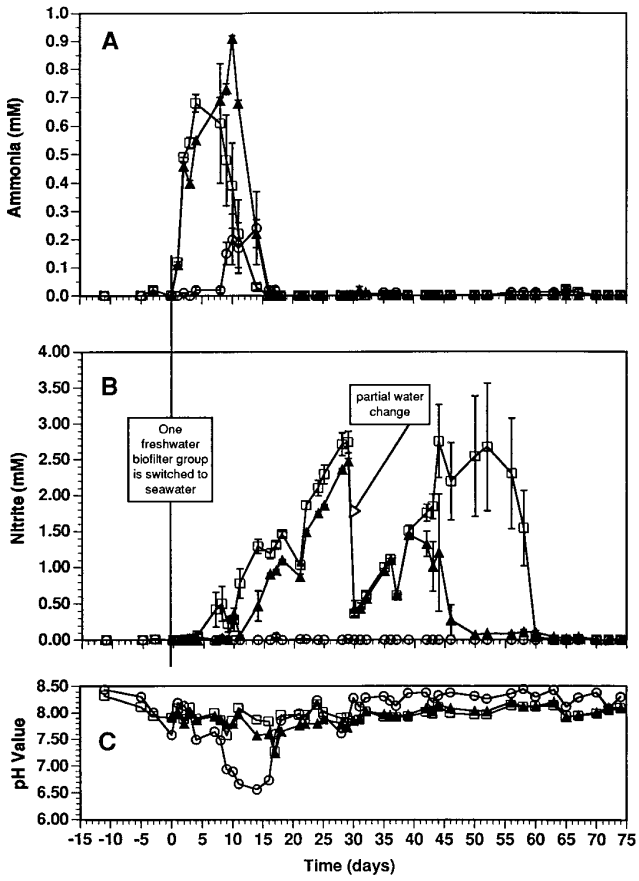

FIG. 3. Mean values ($n = 3$) for ammonia concentration (A), nitrite concentration (B), and pH (C) for the biofilters from the following three aquarium environments: freshwater changed to seawater (□), freshwater (○), and seawater (▲). Bars indicate standard errors. Each aquarium received 5 mM ammonia (as ammonium chloride) each day for the first 20 days and then nearly every other day; none of the aquaria contained fish. Establishment of nitrification is shown by the sudden decrease in the ammonia concentration for the seawater group and the group in which freshwater was changed to seawater near day 15 (after the switch). This was followed by a rapid decrease in the nitrite concentration between days 45 and 60. A partial water change was performed on all aquaria on day 29, and this resulted in the large temporary decrease in nitrite concentration evident at this time.

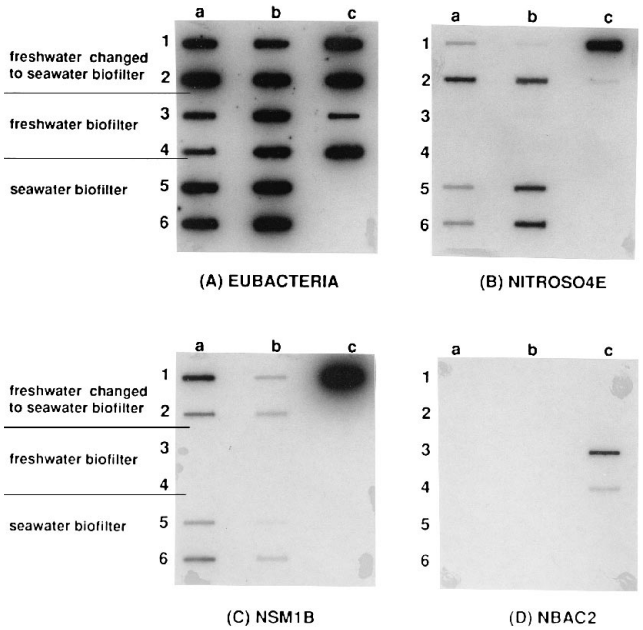

FIG. 4. Slot blot analysis of rRNAs extracted from the biofilters of two freshwater aquaria changed to seawater (rows 1 and 2), two continuously freshwater aquaria (rows 3 and 4), and two seawater aquaria (rows 5 and 6) and hybridized with the eubacterial probe (A), the NITROSO4E probe (B), the NSM1B probe (C), and the NBAC2 probe (D). Water chemistry was tested three times a week for these filter units (see Fig. 3), and the data confirmed that active nitrification occurred. Lanes a, rRNA samples taken 43 days after the switch from fresh water to seawater; lanes b, rRNA samples taken 72 days after the switch; lanes c, rRNAs extracted from control strains (slot c-1, *Nitrosomonas europaea*; slot c-2, *Comamonas testosteroni*; slot c-3, *Nitrobacter winogradskyi*; slot c-4, *Rhodopseudomonas palustris*).

The minimum detection limit for radiolabelled nucleic acid probes is between approximately 0.1 and 1.0% of the total rRNA (1). While absolute bacterial cell numbers cannot be inferred from the results of hybridization experiments, this method does provide a reasonable indication of the relative biomass or metabolic activity of the targeted group. The biofilter experiments demonstrated that our method was sufficiently sensitive to detect nitrifiers in this environment, since all seawater samples produced a strong positive signal. It is reasonable to assume that in the aquaria, whose sole energy input was ammonia, the bacteria responsible for nitrification were active and constituted a large fraction of the total bacterial assemblage. The positive results obtained with the autotrophic ammonia-oxidizing bacterial probes for seawater biofilters exposed to the same environmental conditions as parallel freshwater filters indicate that the extraction and hybridization procedures were sufficiently sensitive to detect ammonia oxidizers belonging to the β-proteobacterial subdivision on filters.

Heterotrophic nitrification has been shown to be potentially greater than autotrophic nitrification in certain environments (26). There are several species of heterotrophic bacteria which use ammonia as a substrate and produce either nitrite, nitrate, or a less common nitrogen cycle intermediate, such as hydroxylamine (18). Tate (29) found insufficient numbers of *Nitrosomonas* and *Nitrobacter* cells to account for the nitrate production in histosols. Instead, using inhibitors, he determined that an *Arthrobacter* population was responsible for a major

portion of the nitrification occurring in these soils. Castignetti and Hollocher (5) identified six heterotrophic bacteria, including *Pseudomonas denitrificans*, *Pseudomonas aeruginosa*, and two strains of *Pseudomonas fluorescens*, that exhibited nitrification activity. While there is no direct evidence that heterotrophic nitrification was the dominant process in the present study, this possibility cannot be totally discounted. However, such heterotrophic nitrification seems unlikely since the aquaria received only inorganic ammonia (ammonium chloride) as an energy source. Carbon dioxide was the sole carbon source available as there were no significant inputs of organic carbon to support heterotrophic bacterial growth beyond trace contamination. It is doubtful that heterotrophic bacterial growth was significant in this lithotrophic environment.

The possibility that the autotrophic nitrifying bacteria in the freshwater aquaria studied belong to subdivisions other than the β subdivision of the *Proteobacteria* seems to be the most likely explanation for our observations. The probes were sensitive enough to detect ammonia-oxidizing bacteria in seawater systems. The freshwater systems had similar rates of nitrification, but ammonia oxidizers were not detected. Furthermore, *Nitrosomonas* spp. were detected on biofilters only after a shift from freshwater to seawater. This suggests that there were changes in the population of nitrifying bacteria, as indicated by the appearance of *Nitrosomonas* spp. and their relatives, as well as the transient decrease in nitrification immediately following the shift from freshwater to seawater. In total, our data suggest that microorganisms other than the usually implicated nitrifiers (members of the β subdivision of the *Proteobacteria*, such as *Nitrosomonas* spp. and their relatives) are the major agents responsible for nitrification in the freshwater aquarium environments examined.

To date, only one ammonia-oxidizing bacterium which does not belong to the β subdivision of the *Proteobacteria* has been cultured. The emphasis on *Nitrosomonas* types, especially *Nitrosomonas europaea*, as the major ammonia oxidizers in environments may be partially a result of culture bias. It is possible that *Nitrosomonas europaea* grows better in enrichment cultures and pure cultures than other, more ecologically significant nitrifiers which flourish and outcompete *Nitrosomonas* spp. in mixed populations. Belser and Schmidt (3) observed selectivity among the different genera of ammonia oxidizers, with *Nitrosomonas* spp. generally dominant over *Nitrosospira* and *Nitrosolobus* spp., possibly because of a faster growth rate. Furthermore, these authors found that while a medium could support the growth of either *Nitrosomonas* species or *Nitrosospira* species, these bacteria generally never grew together in the same enrichment culture. This may explain the data of Hiorns et al. (13), who suggested that *Nitrosomonas* spp. were prevalent only in enrichment cultures that were not obtained from environmental samples.

In the case of nitrite-oxidizing bacteria, the data suggest the possibility that the responsible bacteria were not *Nitrobacter* species, since we were unable to detect *Nitrobacter* cells in any sample examined. Nitrite-oxidizing α subdivision proteobacteria were also not detected by Wagner et al. (34), who examined river water, a nitrifying trickle filter biofilm, and activated sludge samples by using fluorescent probes specific for various *Nitrobacter* species. These authors concluded that the most probable reason for their results was that there were large numbers (or high-level activities) of non-*Nitrobacter* nitrite-oxidizing bacteria present in the systems which they examined. The chance that our results were due to the relatively low level of sensitivity of quantitative rRNA hybridization experiments does exist. However, DNAs from aquarium samples amplified by PCR with general eubacterial primers and subsequent hybridization experiments with the amplified DNAs also yielded negative results with the *Nitrobacter* probe. The fact that there are several nitrite oxidizers in other subdivisions of the *Proteobacteria* could readily explain our results. Three known autotrophic nitrite-oxidizing bacteria are in the δ subdivision, and one such organism is in the γ subdivision, although a recent study has suggested that the phylogenetic placement of the genus *Nitrospira* may need to be reconsidered (8). The δ subdivision nitrite-oxidizing bacteria were isolated from marine environments, but the salinity of the water from which the sample of *Nitrospina gracilis* was isolated was low (12.870 ppt) (37). Ehrich et al. (8) recently isolated a new obligately chemolithoautotrophic nitrite-oxidizing species, *Nitrospira moscoviensis*, which was cultured in freshwater media. Since few strains of the other nitrite-oxidizing bacteria have been cultivated, it may be premature to design probes based on only a few isolates.

There have been no definitive studies of the microbiology of aquarium biofilters. Johnson and Sieburth (15) used scanning electron microscopy to investigate nitrifying bacteria obtained from the biological filters and waters of three aquaculture operations (one freshwater and two seawater). These authors were unable to detect bacteria with *Nitrosomonas*-like cytomorphological features in actively nitrifying freshwater salmon culture systems. In addition, they could not find *Nitrobacter winogradskyi* in any of the natural systems which they sampled, but these bacteria were found in subsequent enrichment cultures. These results are consistent with our results obtained with freshwater aquaria, in which no "classical" *Nitrosomonas* species could be detected.

To a certain extent, models of nitrification are dependent on the known biochemical properties and pathways of the classical nitrifiers. The data from our study indicate that the bacterial species responsible for nitrification in simple freshwater systems remain unknown. It is likely that nitrification and the associated nitrifying bacterium diversity in natural systems are even more complex. Therefore, models which assume, in a general fashion, that *Nitrosomonas* spp. are the major nitrifiers may have to be revised as novel species of nitrifying bacteria are identified, isolated, and characterized and the biochemical properties of these species are determined. Molecular phylogenetic methods, along with classical isolation and culture techniques, all of which are aimed at determining the responsible organisms and their physiological properties, should provide a more complete understanding of biogeochemical processes mediated by nitrifying bacteria.

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REFERENCES

1. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
2. **American Type Culture Collection.** 1992. Catalogue of bacteria and bacteriophages, 18th ed. American Type Culture Collection, Rockville, Md.
3. **Belser, L. W., and E. L. Schmidt.** 1978. Diversity in the ammonia-oxidizing nitrifier population of a soil. Appl. Environ. Microbiol. **36:**584–588.
4. **Bock, E., H.-P. Koops, B. Ahlers, and H. Harms.** 1992. Oxidation of inorganic nitrogen compounds as energy source, p. 414–430. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harper, and K.-H. Schleifer (ed.), The prokaryotes: a handbook on the biology of bacteria. Ecophysiology, isolation, identification, applications, 2nd ed. Springer-Verlag, New York.

5. **Castignetti, D., and T. C. Hollocher.** 1984. Heterotrophic nitrification among denitrifiers. Appl. Environ. Microbiol. **47:**620–623.
6. **DeGrange, V., and R. Bardin.** 1995. Detection and counting of *Nitrobacter* populations in soil by PCR. Appl. Environ. Microbiol. **61:**2093–2098.
7. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
8. **Ehrich, S., D. Behrens, E. Lebedeva, W. Ludwig, and E. Bock.** 1995. A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis sp. nov.* and its phylogenetic relationship. Arch. Microbiol. **164:**16–23.
9. **Gibson, J., E. Stackebrandt, L. B. Zablen, R. Gupta, and C. R. Woese.** 1980. A phylogenetic analysis of the purple photosynthetic bacteria. Curr. Microbiol. **3:**59–64.
10. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
11. **Hall, G.** 1986. Nitrification in lakes, p. 127–156. *In* J. I. Prosser (ed.), Nitrification. IRL Press, Oxford.
12. **Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene-sequences. J. Gen. Microbiol. **139:**1147–1153.
13. **Hiorns, W. D., R. C. Hastings, I. M. Head, A. J. McCarthy, J. R. Saunders, R. W. Pickup, and G. H. Hall.** 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology **141:**2793–2800.
14. **Huguenin, J. E., and J. Colt.** 1989. Design and operating guide for aquaculture seawater systems. Elsevier, Amsterdam.
15. **Johnson, P. W., and J. M. Sieburth.** 1976. In situ morphology of nitrifying-like bacteria in aquaculture systems. Appl. Environ. Microbiol. **31:**423–432.
16. **Kawai, A., M. Sugiyama, R. Shiozaki, and I. Sugahara.** 1971. Microbiological studies on the nitrogen cycle in aquatic environments. Mem. Res. Inst. Food Sci. Kyoto Univ. **32:**7–15.
17. **Killham, K.** 1986. Heterotrophic nitrification, p. 117–126. *In* J. I. Prosser (ed.), Nitrification. IRL Press, Oxford.
18. **Kuenen, J. G., and L. A. Robertson.** 1994. Combined nitrification-denitrification processes. FEMS Microbiol. Rev. **15:**109–117.
19. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley-Interscience, West Sussex, England.
20. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
21. **McCaig, A. E., T. M. Embley, and J. I. Prosser.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol. Lett. **120:**363–368.
22. **Nejidat, A., and A. Abeliovich.** 1994. Detection of *Nitrosomonas* spp. by polymerase chain reaction. FEMS Microbiol. Lett. **120:**191–194.
23. **Orso, S., M. Gouy, E. Navarro, and P. Normand.** 1994. Molecular phylogenetic analysis of *Nitrobacter* spp. Int. J. Syst. Bacteriol. **44:**83–86.
24. **Painter, H. A.** 1986. Nitrification in the treatment of sewage and waste-water, p. 185–211. *In* J. I. Prosser (ed.), Nitrification. IRL Press, Oxford.
25. **Prosser, J. I.** 1989. Autotrophic nitrification in bacteria. Adv. Microb. Physiol. **30:**125–181.
26. **Schimel, J. P., M. K. Firestone, and K. S. Killham.** 1984. Identification of heterotrophic nitrification in a sierran forest soil. Appl. Environ. Microbiol. **48:**802–806.
27. **Seewaldt, E., K.-H. Schleifer, E. Bock, and E. Stackebrandt.** 1982. The close phylogenetic relationship of *Nitrobacter* and *Rhodopseudomonas palustris*. Acta Microbiol. **131:**287–290.
28. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54:**1079–1084.
29. **Tate, R. L., III.** 1977. Nitrification in histosols: a potential role for the heterotrophic nitrifier. Appl. Environ. Microbiol. **33:**911–914.
30. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
31. **Voytek, M. A., and B. B. Ward.** 1995. Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl. Environ. Microbiol. **61:**1444–1450.
32. **Wagner, M., R. Amann, H. Lemmer, and K.-H. Schleifer.** 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59:**1520–1525.
33. **Wagner, M., G. Rath, R. Amann, H. P. Koops, and K.-H. Schleifer.** 1995. *In situ* identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. **18:** 251–264.
34. **Wagner, M., G. Rath, H. P. Koops, J. Flood, and R. Amann.** *In situ* analysis of nitrifying bacteria in sewage treatment plants. Water Sci. Technol., in press.
35. **Ward, B. B.** 1982. Oceanic distribution of ammonium-oxidizing bacteria determined by immunofluorescence. J. Mar. Res. **40:**1155–1172.
36. **Ward, B. B.** 1986. Nitrification in marine environments, p. 157–184. *In* J. I. Prosser (ed.), Nitrification. IRL Press, Oxford.
37. **Watson, S. W., and J. B. Waterbury.** 1971. Characteristics of two marine nitrite oxidizing bacteria, *Nitrospina gracilis nov. gen. nov. sp.* and *Nitrococcus mobilis gen. nov. sp. nov.* Arch. Mikrobiol. **77:**203–230.
38. **Wheaton, F. W., J. N. Hochheimer, G. E. Kaiser, M. J. Kronos, G. S. Libey, and C. C. Easter.** 1994. Nitrification filter principles, p. 101–126. *In* M. B. Timmons and T. M. Losordo (ed.), Aquaculture water reuse systems: engineering design and management. Elsevier, Amsterdam.
39. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.