Nitrospira-Like Bacteria Associated with Nitrite Oxidation in Freshwater Aquaria

TIMOTHY A. HOVANEC,1,2*, LANCE T. TAYLOR,1 ANDREW BLAKIS,1 AND EDWARD F. DELONG†

Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, Santa Barbara, California 93106,1 and Aquaria Inc., Moorpark, California 93021†

Received 4 September 1997/Accepted 27 October 1997

Oxidation of nitrite to nitrate in aquaria is typically attributed to bacteria belonging to the genus Nitrobacter which are members of the α subdivision of the class Proteobacteria. In order to identify bacteria responsible for nitrite oxidation in aquaria, clone libraries of rRNA genes were developed from biofilms of several freshwater aquaria. Analysis of the rDNA libraries, along with results from denaturing gradient gel electrophoresis (DGGE) on frequently sampled biofilms, indicated the presence of putative nitrite-oxidizing bacteria closely related to other members of the genus Nitrospira. Nucleic acid hybridization experiments with rRNA from biofilms of freshwater aquaria demonstrated that Nitrospira-like rRNA comprised nearly 5% of the rRNA extracted from the biofilms during the establishment of nitrification. Nitrite-oxidizing bacteria belonging to the α subdivision of the class Proteobacteria (e.g., Nitrobacter spp.) were not detected in these samples. Aquaria which received a commercial preparation containing Nitrobacter species did not show evidence of Nitrobacter growth and development but did develop substantial populations of Nitrospira-like species. Time series analysis of rDNA phytophtypes on aquaria biofilms by DGGE, combined with nitrite and nitrate analysis, showed a correspondence between the appearance of Nitrospira-like bacterial ribosomal DNA and the initiation of nitrite oxidation. In total, the data suggest that Nitrobacter winogradskyi and close relatives were not the dominant nitrite-oxidizing bacteria in freshwater aquaria. Instead, nitrite oxidation in freshwater aquaria appeared to be mediated by bacteria closely related to Nitrospira moscoviensis and Nitrospira marina.

Chemolithoautotrophic NOB are phylogenetically diverse, occurring in several subdivisions of the class Proteobacteria (Fig. 1). The most well-studied members of this group of organisms (i.e., Nitrobacter spp. and close relatives) belong to the α subdivision of the class Proteobacteria (16). Nitrospira gracilis and Nitrooccus mobilis, which were first isolated by Watson and Waterbury (16), were determined to be members of the 8 and γ subdivisions of the class Proteobacteria, respectively (14). Another NOB, Nitrospira marina, is phylogenetically affiliated with non-NOB such as Leptospirillum ferrooxidans (7, 14, 16). Based on phylogenetic analysis of 16S rRNA sequences, Erlich et al. (7) proposed a new phylum within the domain Bacteria for these organisms (Fig. 1). A newly discovered NOB from a freshwater environment (a corroded iron pipe in a heating system), Nitrospira moscoviensis, was recently found to be phylogenetically related to N. marina (7).

Whether in pure culture or on biofilters, NOB are slowly growing organisms with doubling times from 12 to 32 h (3, 5, 7). Therefore, in newly set up aquaria, ammonia and nitrite can reach concentrations toxic to fish before a sufficient biomass of AOB and NOB becomes established. To reduce the length of time for establishment of NOB on biofilters, commercial preparations of these organisms, in various forms of preservation, are available to seed the aquarium environment. These preparations range from essentially pure cultures of Nitrobacter species to mixed cultures of autotrophic AOB and NOB organisms and to products which combine autotrophic nitrifying bacteria with various species of heterotrophic bacteria. Past studies have generally shown these mixes to be ineffectual but have not elucidated specific reasons for their poor performance (4, 15).

In this study, we observed that Nitrospire-like species rather than Nitrobacter species appeared responsible for oxidation of nitrite to nitrate in freshwater aquaria. A combination of

The oxidation of nitrite to nitrate by chemolithoautotrophic nitrite-oxidizing bacteria (NOB) in fish culture systems, ranging from home aquaria to commercial aquaculture systems, is an important process. The accumulation of high concentrations of nitrite, which is toxic to fish and other aquatic organisms, is prevented by active nitrite removal by nitrifying microorganisms. Nitrite is formed in aquarium systems from the oxidation of ammonia, the principal nitrogenous waste of teleosts, by autotrophic ammonia-oxidizing bacteria (AOB). Thus, closed aquatic filtration systems usually provide a solid substratum, which is termed a biological filter or biofilter, to promote the growth of AOB and NOB. A variety of materials can form the substratum of a biofilter, ranging from gravel to specially engineered molded plastics. Biofilters can be submerged in the flow path of the filtration system or can be located such that the water trickles or percolates through a medium situated in the atmosphere outside the aquarium, before flowing back into the tank.

Traditionally, the bacteria responsible for the oxidation of ammonia and nitrite in aquariums were considered to be Nitrosomonas europaea and Nitrobacter winogradskyi or their close relatives, respectively (17, 18). However, there is some indication that both N. europaea and N. winogradskyi may not be predominant components of actively nitrifying freshwater aquaria (9). In seawater aquaria, however, N. europaea and close relatives do appear to comprise a significant proportion of the total eubacterial community, but N. winogradskyi was below detection limits (9).

* Corresponding author. Mailing address: Aquaria Inc., 6100 Condor Dr., Moorpark, CA 93021. Phone (805) 529-1111. Fax (805) 529-3030. E-mail: hovanec@lifesci.ucsb.edu.
† Present address: Monterey Bay Aquarium Research Institute, P.O. Box 628, 7700 Sandholdt Rd., Moss Landing, CA 95039.
methods was used to investigate concurrently the appearance of NOB on biofilters and the oxidation of nitrite to nitrate. Oligonucleotide probes, which target Nitrospira and close relatives, were developed and used to quantify this group at different times during the establishment of nitrification. Detuning gradient gel electrophoresis (DGGE) was used to monitor the appearance of Nitrospira-like bacteria during the onset of nitrite oxidation. The effectiveness of a commercial mix of AOB and NOB was also evaluated.

MATERIALS AND METHODS

Nucleic acid sampling and extraction. For rRNA extractions from aquarium gravel, individual gravel samples (10 g) were placed in a polypropylene tube and covered with 2.5 ml of low-pH buffer (50 mM sodium acetate, 10 mM disodium EDTA) and processed as previously described (9). For DNA extraction, gravel samples were resuspended in cell lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) and processed as described previously (9). Samples were stored at −20°C until extraction.

DNA was quantified by Hoechst type 33258 dye binding and fluorometry (DynaQuant 200; Hoefer Pharmacia Biotech, Inc., San Francisco, Calif.). RNA was quantified by measuring A$_{260}$ (Lambda 3B; Perkin Elmer), assuming that 1 A$_{260}$ U corresponds to 40 μg of RNA per ml.

Clone libraries of PCR-amplified rRNA genes. Clone libraries were derived from nucleic acid extracts of aquarium samples. Bacterial rRNA gene fragments were amplified with the primers S-D-Bact-0011-a-S-17 (8f; GTT TGA TCC TGG CTC AG) and 1492r (eubacterial; GGT TAC CTT GTT ACG ACT T) or S*-Univ-0519-a-A-18 (519r; GWA TTA CCG CGG CKG CTG). PCR conditions, cycle parameters, and reaction components were as previously described (6). PCR products were evaluated by agarose gel electrophoresis. PCR fragments were cloned with a TA cloning kit (Invitrogen, Carlsbad, Calif.), as previously described (6).

DGGE analysis and profiling. For DGGE analysis, ribosomal DNA (rDNA) fragments were amplified with the forward primer 35S (eubacterial; CCT ACG GGA GGC AGC CGG AG) with a 40-bp GC clamp on the 5’ end as described by Murray et al. (11) and the reverse primer S*-Univ-0519-a-A-18 (519r; GWA TTA CCG CGG CKG CTG). PCR was performed on a Stratagene Robocycler Gradient 96 (La Jolla, Calif.) with the manufacturer’s reagents. PCR conditions included a hot start (90°C) and a touchdown procedure (11). Initial denaturation at 94°C for 3 min was followed by a denaturation at 94°C for 1 min, a touchdown annealing from 65 to 55°C for 1 min and 29 s (the annealing time during the touchdown increased by 1.4 s per cycle), and primer extension at 72°C for 56 s (the extension time was increased 1.4 s per cycle). The final temperature series of the above thermal cycle was repeated for 20 total cycles, followed by a final extension at 72°C for 5 min. Amplicons were examined by agarose gel electrophoresis.

DGGE was performed with a Bio-Rad D-GENE System (Bio-Rad Laboratories, Hercules, Calif.). All gels were 8.5% acrylamide–bis with Bio-Rad reagents (D-GENE Electrophoresis Reagent kit). Gel gradients were poured with Bio-Rad reagents (D-GENE Electrophoresis Reagent kit) with a denaturing gradient of 20 to 60% (where 100% denaturant is a mixture of 40% deionized formamide and 7 M urea) and the Bio-Rad gradient delivery system (model 475; Bio-Rad). All gels were run at 200 V for 6 h. The gels were visualized in one of two ways. For visualization and recovery of discrete DNA bands, the gels were first stained for 10 min in 250 ml of 1× Tris-acetate-EDTA (TAE) buffer, in which 100 μl of ethidium bromide (1 mg/ml) was added, and then were washed for 10 min in 1× TAE buffer. For documentation purposes, some gels were stained in Vistra Green (diluted 1:10,000) (Molecular Dynamics, Sunnyvale, Calif.) for 20 min, followed by a 20-min wash in 1× TAE buffer, and then were scanned with a FluorImager SI (Molecular Dynamics).

Individual bands were excised from the DGGE gels with alcohol-sterilized scalpels. Extraction of DNA from the gel followed the methods of Ferris et al.

FIG. 1. Phylogenetic relationships of autotrophic NOB in the α subdivision of the class Proteobacteria and the Nitrospira group. Clone 710-9, an rDNA clone originating from aquaria with active NOB populations, is most similar to NOB of the Nitrospira group. The specificities of two oligonucleotide probes designed for Nitrospira spp. are indicated by the boxed sections.

<table>
<thead>
<tr>
<th>Probe*</th>
<th>Position (nucleotides)*</th>
<th>Base sequence (5’ to 3’)</th>
<th>$T_d$ (°C)</th>
<th>Wash temp (°C)</th>
<th>Targeted group</th>
<th>Nontarget bacteria with exact match to probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-G-Ntspa-0685-a-A-22</td>
<td>664–685</td>
<td>CAC CGG GAA TTC CGC GCT CCT C</td>
<td>63.0</td>
<td>60.0</td>
<td>N. moscoviensis, N. marina, and 710-9 clone</td>
<td>None</td>
</tr>
<tr>
<td>S*-Ntspa-0454-a-A-19</td>
<td>435–454</td>
<td>TCC ATC TTC CCT CCC GAA AA</td>
<td>58.5</td>
<td>56.0</td>
<td>N. moscoviensis 710-9 clone</td>
<td>None</td>
</tr>
</tbody>
</table>

* Probe names designated by the standard proposed by Alm et al. (1).

* $T_d$, temperature at which 50% of the bound probe is released from the homologous hybrid.

TABLE 1. The nucleotide sequences and positions of oligonucleotide probes for NOB
nucleotide probes were designed which specifically hybridize with these tests, 200 ng of template was immobilized on a nylon membrane (Hybond-N; Amersham) and hybridized overnight at 45°C with32P-labelled probe. After hybridization, the membrane was washed at room temperature in 1 mmol of filter-sterilized (0.2-μm-pore-size filter) ammonium chloride was added to each tank, followed by an additional dosing of 5.0 mmol of NH₄Cl on the fourth day. On days 10, 15, 18, 23, and 30, further ammonium additions of 8.9 mmol were made to each aquarium. During the test, a total of 50.4 mmol of ammonium was added to each aquarium. Water samples were collected daily.

Two 10-g samples of gravel were collected from each aquarium daily for 33 days. To one sample, 2 ml of lysis buffer was added and the sample was frozen (–20°C) until rDNA was extracted by previously described methods. rDNA was subjected to DGGE after undergoing PCR with the primers and conditions described above. The other sample was preserved with 2 ml of bead beating buffer.

(iii) Time series. Three aquariums were set up as previously described with 4.53 kg of gravel and were filled with 30 liters of city water which had been passed through activated carbon. The test was run for 138 days, during which the aquarium was individually dosed with 8.9 mmol of filter-sterilized (0.2 μm) ammonium (as ammonium chloride) on the first and second days of the test. From days 12 to 78 of the test, further additions of 8.9 mmol of ammonium were done on average every 3 days. A total of 246 mmol of ammonium was added to each tank during the test. The water was sampled three times a week for chemical analysis. The aquarium was run for 80 days with freshwater, at which time the water was switched to seawater (32 ppt) by draining and refilling with water mixed with artificial sea salts (MarineLab Commercial Aquariums, Moorpark, Calif.). After the switch, the testing continued for an additional 57 days.

Nucleotide sequence accession no. The nucleotide sequence reported in this paper for clone 710-9 has been deposited in the GenBank database under accession no. AF035813.
**RESULTS**

**Isolation of putative NOB.** Two approaches were taken to identify NOB in aquarium samples. The first approach was to develop clone libraries from gravel samples from an aquarium at several times during the establishment of nitrification. Samples were taken 17 and 31 days after the aquarium establishment and ammonia additions started. A third library was constructed from DNA extracted from the material of a commercial biofilter constructed of thermoplastic material (model CBW-1; Aquaria, Inc.). This filter had been set up for 109 days in a system with daily dosing of ammonium chloride.

The second approach used to monitor and identify nitrifying microorganisms was DGGE. The DNA extracted from aquarium gravel samples taken during the establishment of nitrification was subjected to DGGE to produce a pattern of discrete bands. The banding patterns were compared to each other and to band patterns produced by a mix of known nitrifiers. Unique bands were excised from the gels and sequenced.

The sequences from the clone libraries and DGGE were compared to bacterial sequences found in public databases (BLAST [2] and RDP [10]). Some clones, which showed a close similarity to those of known nitrite-oxidizing organisms, were more completely sequenced.

**Identification of putative *Nitrospira*-like NOB.** Five samples were screened for NOB by either clone library development or DGGE. A total of 96 clones or excised bands were partially sequenced. Of these, 11 were highly similar to members of the *Nitrospira* group but none were similar to *Nitrobacter* spp. The partial sequences were most highly similar to those of *N. ma-

\[ \text{TABLE 3. Results of probing rRNA extracted from aquarium biofilms with nucleic acid probes for NOB} \]

<table>
<thead>
<tr>
<th>Sample label</th>
<th>Aquarium environment</th>
<th>Biofilm substrate</th>
<th>Daily ammonia amount</th>
<th>Ammonia source</th>
<th>Ammonia</th>
<th>Signal with the following oligonucleotide probes (^a)</th>
<th>NoB</th>
<th>S(^{-a})-Nbs-1017- (a)-A-20</th>
<th>S-G-Ntpsa-0685- (a)-A-22</th>
<th>S(^{-a})-Ntpsa-0454- (a)-A-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>710r</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>32.1 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>711r</td>
<td>Freshwater</td>
<td>Polyfiber</td>
<td>32.1 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T825</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>0.8 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T825</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>0.8 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WDF1036</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>3.2 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WDF1036</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>3.2 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WDF1026</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>2.0 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WDF1039</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>3.2 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WDF1038</td>
<td>Freshwater</td>
<td>Sponge</td>
<td>2.0 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WDFP1035</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>2.0 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLRT6</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>2.0 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EXP5B</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>1.4 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FWSW4</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FWSW6</td>
<td>Freshwater</td>
<td>Polypp</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BC2-8</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BC2-10</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BC2-12</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BC2-13</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BC2-16</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BC2-4</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>2.0 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BC2-16a</td>
<td>Freshwater</td>
<td>Gravel</td>
<td>2.0 g</td>
<td>Fish</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>714r</td>
<td>Seawater</td>
<td>Polyfiber</td>
<td>714 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>715r</td>
<td>Seawater</td>
<td>Polyfiber</td>
<td>714 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FWSW2</td>
<td>Seawater</td>
<td>Polypp</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FWSW3</td>
<td>Seawater</td>
<td>Polypp</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FWSW8</td>
<td>Seawater</td>
<td>Polypp</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FWSW9</td>
<td>Seawater</td>
<td>Polypp</td>
<td>5 mM</td>
<td>NH(_4)Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) Type of aquarium water.

\(^b\) Media from which the bacterial cells were extracted. Polypp, polypropylene.

\(^c\) Fish, the aquarium had a fish population and ammonia was generated by the fish; NH\(_4\)Cl, the tank had no fish and the ammonia was from daily dosing with ammonium chloride.

\(^d\) Values in grams are the amounts of fish feed put into the aquarium each day; molar or millimolar values are the concentrations of ammonia added to the aquarium or system in which the biofilter was located each day.

\(^e\) +, signal detected by probe; -, no signal detected.


**FIG. 3. Specificities of the oligonucleotide probes targeting NOB of the *Nitrospira* group and the 710-9 clone identified in this study.** Probe order was eubacterial probe S-D-Bact-0338-a-A-18 (A), *Nitrospira*-like NOB probe S-G-Ntpsa-0685-a-A-22 (B), and *Nitrospira*-like NOB probe S\(^{-a}\)-Ntpsa-0454-a-A-19 (C), with rRNA, transcribed RNA (trRNA), or PCR-amplified rDNA, in the following arrangement: slot A-1, Comamonas testosterone; slot A-2, Alcaligenes eutrophus; slot A-3, Alcaligenes faecalis; slot A-4, Comamonas acidovorans; slot A-5, *N. winogradskyi* (rDNA); slot A-6, *Nitrobacter* agilis (rDNA); slot B-1, clone 710-9 (rDNA); slot B-2, clone 710-9 (trRNA); slot B-3, *N. marina* (trDNA); slot B-4, *N. marina* (trRNA); slot B-5, *N. gracilis*; slot B-6, Shewanella putrefaciens. See text for description of methods.
and *N. moscoviensis* (data not shown). The 16S rDNA of a representative clone which contained the *Nitrospira*-like rDNA was fully sequenced, and a phylogenetic tree was inferred. Phylogenetic analysis indicated a high similarity between this cloned rDNA (710-9) and members of the *Nitrospira* group, *N. moscoviensis* and *N. marina* (Fig. 1). The rDNA contained in clone 710-9 was 96.1% similar to that of *N. moscoviensis* and 87.4% similar to that of *N. marina* (Table 2).

**Oligonucleotide probe specificity.** Oligonucleotide probe sequences, positions (*Escherichia coli* numbering), *T*<sub>a</sub>, wash temperatures and target groups for the probes are indicated in Table 1. For probe S*-Ntspa-0454-a-A-19, the *T*<sub>a</sub> was 58.5°C, while the *T*<sub>d</sub> was 63.0°C for the S-G-Ntspa-0685-a-A-22 probe (Fig. 2).

Slot blot experiments confirmed that the probe S-G-Ntspa-0685-a-A-22 was specific to the known NOB of the *Nitrospira* group, as well as to the clone 710-9 (Fig. 3). As predicted, probe S*-Ntspa-0454-a-A-19 hybridized to clone 710-9, but not *N. marina*. Furthermore, experiments demonstrated that neither probe hybridized with NOB which are members of the α or δ subdivisions of the class *Proteobacteria* (Fig. 3).

**Detection of NOB in aquaria.** Table 3 summarizes the results from the probing of several aquarium biofilms with the NOB probes. Probe S-G-Ntspa-0685-a-A-22 yielded a positive signal with all freshwater and saltwater aquaria tested. The probe S*-Ntspa-0454-a-A-19 detected *Nitrospira*-like bacteria in all freshwater aquaria, but not in all the saltwater aquaria (Table 3). There were no cases of positive detection by a probe which targets α proteobacterial *Nitrobacter* species (Table 3).

**Time series.** The ammonia, nitrite, and nitrate values for a representative test aquarium dosed with ammonium chloride for 138 days are shown in Fig. 4. The data show the expected pattern for the establishment of nitrification in aquaria. Initially, the concentration of ammonia increased and then decreased to undetectable levels by day 12 (the saw-toothed pattern of the ammonia values is the result of the increasing frequency of ammonia additions). By day 12, the amount of nitrite increased, reaching its maximum value on day 22. By day 38, the amount of nitrate was essentially 0 and that of nitrate was steadily increasing (Fig. 4). The change from freshwater to seawater at day 80 resulted in an immediate increase in the amounts of ammonia and, subsequently, nitrite. It took nearly 20 days for ammonia oxidation to become reestablished. Reestablishment of nitrite oxidation took approximately 40 days.

A DGGE profile for selected days over the first 101 days for this aquarium shows that the *Nitrospira*-like rDNA sequence appeared faintly on day 15, corresponding to the onset of nitrite oxidation (Fig. 5). By day 22, the band corresponding to the *Nitrospira*-like rDNA sequence increased in relative intensity and remained intense over the next two sampling dates. After the switch to seawater, the relative intensity of the *Nitrospira*-like band diminished. The general band pattern also changed qualitatively between freshwater and seawater sampling dates. The banding pattern for day 87 (7 days after the switch) appeared to more closely resemble the pattern for day 57 (freshwater) than the pattern for day 101 (seawater) (Fig. 5).

**Time of *Nitrospira*-like bacterial appearance.** The daily concentrations of ammonia, nitrite, and nitrate over the first 33 days after setup of a new aquarium are presented in Fig. 6. The trends were as expected, with ammonia peaking about day 12. Nitrite values increased starting at day 12, peaked at day 21, and decreased to below detection limits by day 26. Nitrate values increased from day 15 onwards. DGGE showed that the band corresponding to clone 710-9, the putative NOB, first appeared on day 12, with the relative intensity of the 710-9 band increasing daily based on relative fluorescence units of rDNA amplicons (Fig. 7).

![Figure 4](Image 4.png)

**FIG. 4.** Ammonia (A), nitrite (B), and nitrate (C) chemistry for an aquarium from startup through 138 days. The saw-toothed pattern for ammonia is the result of the increasing frequency of dosing with ammonium chloride as nitrification was being established. The water was switched from freshwater to seawater on day 80.

![Figure 5](Image 5.png)

**FIG. 5.** DGGE time series profile from a biofilm of a freshwater aquarium during the establishment of nitrification. The aquarium was switched to seawater on day 80. Lanes A, G, and J contain two clones, including clone 710-9, a putative NOB showing close similarity to the *Nitrospira* group. The band corresponding to this organism first appears with significant intensity on day 22. Lanes B, C, D, E, and F are sampling dates before the switch to seawater. Lanes H and I are sampling dates after the switch to seawater. The water chemistry for the establishment of nitrification in aquaria. Initial concentrations of ammonia, nitrite, and nitrate over the first 33 days are shown in Fig. 4. The data show the expected pattern for the establishment of nitrification in aquaria. Initially, the concentration of ammonia increased and then decreased to undetectable levels by day 12 (the saw-toothed pattern of the ammonia values is the result of the increasing frequency of ammonia additions). By day 12, the amount of nitrite increased, reaching its maximum value on day 22. By day 38, the amount of nitrate was essentially 0 and that of nitrate was steadily increasing (Fig. 4). The change from freshwater to seawater at day 80 resulted in an immediate increase in the amounts of ammonia and, subsequently, nitrite. It took nearly 20 days for ammonia oxidation to become reestablished. Reestablishment of nitrite oxidation took approximately 40 days.

A DGGE profile for selected days over the first 101 days for this aquarium shows that the *Nitrospira*-like rDNA sequence appeared faintly on day 15, corresponding to the onset of nitrite oxidation (Fig. 5). By day 22, the band corresponding to the *Nitrospira*-like rDNA sequence increased in relative intensity and remained intense over the next two sampling dates. After the switch to seawater, the relative intensity of the *Nitrospira*-like band diminished. The general band pattern also changed qualitatively between freshwater and seawater sampling dates. The banding pattern for day 87 (7 days after the switch) appeared to more closely resemble the pattern for day 57 (freshwater) than the pattern for day 101 (seawater) (Fig. 5).

**Time of *Nitrospira*-like bacterial appearance.** The daily concentrations of ammonia, nitrite, and nitrate over the first 33 days after setup of a new aquarium are presented in Fig. 6. The trends were as expected, with ammonia peaking about day 12. Nitrite values increased starting at day 12, peaked at day 21, and decreased to below detection limits by day 26. Nitrate values increased from day 15 onwards. DGGE showed that the band corresponding to clone 710-9, the putative NOB, first appeared on day 12, with the relative intensity of the 710-9 band increasing daily based on relative fluorescence units of rDNA amplicons (Fig. 7).
Commercial additive. The addition of a commercial bacterial mixture which contained *Nitrobacter* sp., but not *Nitrospira* sp., did not result in the detection of *Nitrobacter* species by oligonucleotide probe hybridization experiments (Fig. 8). However, a band which comigrated with a control derived from pure *Nitrobacter* DNA could be detected in the original commercial mixture by DGGE analysis (data not shown). *Nitrospira*-like rRNA was readily detected in the aquarium. *Nitrospira* group-specific probes indicated that the tank which received the additive had a significantly greater percentage of the *Nitrospira* species rRNA (Fig. 8). By day 16, approximately 5% of the eubacterial rRNA hybridized with the general *Nitrospira* group-specific probe, compared to only 0.33% of the eubacterial rRNA in the tank which did not receive an additive (Fig. 8). By day 50, the values were 3.39 and 1.52% for the additive and nontreated aquaria, respectively (Fig. 8).

Nitrite concentrations in the two aquaria decreased as the relative percentages of *Nitrospira*-like rRNA increased. By day 22, the nitrite value had reached a maximum in the tank which received the additive. Nitrite concentrations reached maxima in the nonadditive aquarium on about day 32. By day 38, the nitrite levels in both aquaria were essentially below our limits of detection, and nitrate levels were equivalent in the treated and nontreated aquaria (Fig. 8).

**DISCUSSION**

Our results from DGGE analysis, rRNA probing, and sequencing generally indicate that *Nitrospira*-like bacteria are the most likely candidates responsible for nitrite oxidation in freshwater aquaria. The combined use of molecular phylogenetic techniques and monitoring of water chemistry suggested a correspondence between changes in the biofilm microbial community which coincided with the onset of ammonia and nitrite oxidation. The commencement of nitrite oxidation coincided with the appearance of the putative nitrite-oxidizing *Nitrospira*-like bacterium. The results lend support to the conclusion of an earlier study, which suggested that a subdivision proteobacterial NOB (*Nitrobacter* types) were not major components of nitrite oxidation bacterial populations in freshwater or marine aquaria (9).

Results regarding the beneficial effects of the addition of a bacterial additive containing *Nitrobacter* species were equivocal. While nitrite levels in treated aquaria decreased earlier than those in nontreated aquaria, there was no evidence that *Nitrobacter* species were actively growing in these aquaria. It is possible that the levels of *Nitrobacter* species were below the limits of detection of our techniques. However, since *Nitrospira*-like bacteria were readily detected and that their establishment coincided with nitrite oxidation we postulate that *Nitrospira*-like organisms, and not *Nitrobacter* species, are the major nitrite oxidizers in the freshwater aquarium environment. It is possible that the addition of bacterial mixtures supplies vitamins and other nutrients which generally stimulate the growth of the nitrifying assemblages, fostering their growth and development and indirectly stimulating nitrite oxidation.

In the present study, we identified *Nitrospira*-like putative NOB by amplification of rDNA with general bacterial PCR primers and DGGE analyses. We chose to use universal and domain primers rather than group-specific primers, since previous analysis suggested that nitrite oxidizers other than...
Nitrate (B) are for two tanks, i.e., tank 4 (freshwater aquarium during the first 57 days after startup. Nitrite (A) and for NOB. Probes S-G-Ntspa-0685-a-A-22 and S-*-Ntspa-0454-a-A-19 target (relative to that of a eubacterial probe, S-D-Bact-0338-a-A-18) to probes specific to Nitrobacter might be involved in nitrification in aquaria (9). Combined monitoring of environmental conditions (water chemistry) with bacterial assemblage analysis (DGGGE) allowed us to detect a correspondence between nitrate oxidation and Nitrospira-like rRNA. By monitoring samples over time, changes in the microbial assemblage were evident. This approach permitted a more focused effort in the search for links between environmental processes and the microbes which mediate them.

When comparing biofilters, researchers in the past have been generally limited to assessing mainly water chemistry changes, such as ammonia disappearance and nitrate appearance. The use of molecular probes for the relevant nitrifying bacteria in different systems should provide a more detailed understanding of the interaction between the biology and chemistry of the systems. This in turn provides information relevant to better filter design and may allow the effects of various conditions to be assessed with respect to their effects on the biology as well as the chemistry of the system.

ACKNOWLEDGMENTS

We thank Ellen Ko, Quynh Lu, and Michelle Waugh for helpful assistance and Alison Murray for assisting with the DGGGE. We also thank Julia Sears-Hartley, Melissa Lokken, and Les Wilson for water chemistry analysis.

This work was supported in part by National Science Foundation grants OCE95-29804 and OPP94-18442 to E.F.D. and by assistance from Aquaria, Inc. to T.A.H.

REFERENCES