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Ammonia- and Nitrite-Oxidizing Bacterial Communities in a Pilot-Scale Chloraminated Drinking Water Distribution System

John M. Regan, Gregory W. Harrington, and Daniel R. Noguera*

Department of Civil and Environmental Engineering, University of Wisconsin—Madison, Madison, Wisconsin 53706-1691

Nitrification in drinking water distribution systems is a common operational problem for many utilities that use chloramines for secondary disinfection. The diversity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in the distribution systems of a pilot-scale chloraminated drinking water treatment system was characterized using terminal restriction fragment length polymorphism (T-RFLP) analysis and 16S rRNA gene (ribosomal DNA [rDNA]) cloning and sequencing. For ammonia oxidizers, 16S rDNA-targeted T-RFLP indicated the presence of *Nitrosomonas* in each of the distribution systems, with a considerably smaller peak attributable to *Nitrosospira*-like AOB. Sequences of AOB amplification products aligned within the *Nitrosomonas oligotropha* cluster and were closely related to *N. oligotropha* and *Nitrosomonas ureae*. The nitrite-oxidizing communities were comprised primarily of *Nitrospira*, although *Nitrobacter* was detected in some samples. These results suggest a possible selection of AOB related to *N. oligotropha* and *N. ureae* in chloraminated systems and demonstrate the presence of NOB, indicating a biological mechanism for nitrite loss that contributes to a reduction in nitrite-associated chloramine decay.

Many drinking water utilities in the United States have implemented chloramination for secondary disinfection because chloramines are less reactive than free chlorine and have been demonstrated to produce lower concentrations of disinfection by-products, such as trihalomethanes and haloacetic acids (2, 23, 25). However, the addition of chloramines can lead to biological instability in a drinking water distribution system by promoting the growth of nitrifying bacteria, microorganisms that oxidize ammonia to nitrite and nitrate. Chloramination introduces ammonia to the distribution system both as excess ammonia from chloramine formation and released ammonia from chloramine decay. Ammonia-oxidizing bacteria (AOB) are able to grow on the residual and released ammonia in the presence of chloramines and produce nitrite and soluble organic compounds (29, 32). The AOB cell mass and the products of AOB activity deplete the residual disinfectant concentration by exerting a chloramine demand and may sustain the growth of nitrite-oxidizing bacteria (NOB) and heterotrophs. The resulting reduction in chloramine residual and development of a microbial community in the distribution system lead to water quality deterioration and violation of drinking water regulations (49, 50).

Several studies have reported on the disinfection kinetics of AOB with chloramines (4, 18, 50; P. S. Oldenburg, J. M. Regan, G. W. Harrington, and D. R. Noguera, unpublished data). In general, the results of these investigations suggest that AOB should be inactivated effectively at typical chloramine exposure times and doses. Nonetheless, nitrification episodes are common in these systems, even in the presence of

high chloramine residuals (4, 36). A survey of chloraminating utilities in the United States revealed that 63% of the utilities have experienced evidence of nitrification episodes (48), and an analytical survey of five chloraminated distribution systems in South Australia showed 64% of samples testing positive for nitrifying bacteria (4).

One hypothesis for the discrepancy between the laboratory studies and operating results is that there are AOB strains growing in full-scale systems that possess a greater chloramine resistance than those studied in the kinetic experiments. Whether the AOB strains used in earlier kinetic studies are representative of significant strains involved in full-scale nitrification episodes has not been confirmed, since there are no published evaluations of AOB diversity in chloraminated distribution systems.

There also are no reports on the presence of NOB in chloraminated systems and the effectiveness of chloramine inactivation of NOB. The emphasis of past studies on chloraminated systems has been on AOB, as the conceptual model of the escalation of nitrification episodes has involved incomplete nitrification, with nitrite evolving from AOB activity and accumulating in the water. Subsequent nitrite disappearance has been attributed to the chemical reaction between nitrite and chloramines (21, 45) in which the production of nitrate and ammonia occurs, thereby increasing the ammonia available as a growth substrate for AOB. However, it is conceivable that NOB play an important role in the ecology of nitrification and biological regrowth in these systems. Having an active NOB community coupled with AOB within the distribution system may favor the biological oxidation of nitrite to nitrate, thereby reducing the chloramine demand exerted by nitrite. NOB may also contribute to the growth of heterotrophs via the production of soluble organic products (32).

The objective of this study was to characterize AOB and NOB diversity in a pilot-scale chloraminated distribution sys-

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* Corresponding author. Mailing address: Department of Civil and Environmental Engineering, University of Wisconsin—Madison, 3216 Engineering Hall, 1415 Engineering Dr., Madison, WI 53706-1691. Phone: (608) 263-7783. Fax: (608) 262-5199. E-mail: noguera@engr.wisc.edu.
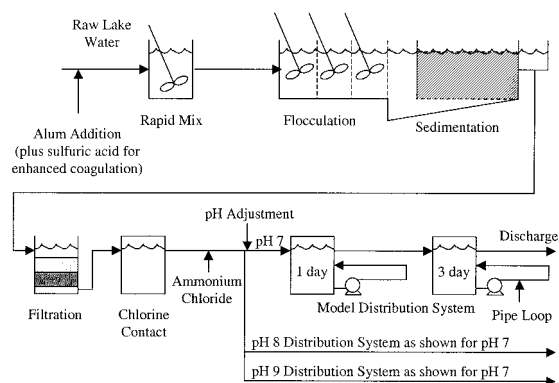
FIG. 1. Pilot plant flow diagram. Two parallel systems were continuously operated, one with conventional alum coagulation and the other with enhanced coagulation. Each treatment train had three distribution systems maintained at pH 7, 8, and 9.

tem. The nitrifier communities were evaluated using a combination of molecular techniques, including a rapid screening approach using terminal restriction fragment length polymorphism (T-RFLP) and a more detailed analysis involving cloning and sequencing of 16S ribosomal DNA (rDNA) fragments. The results provided a characterization of nitrifier diversity in a chloraminated system and the first direct evidence of NOB associated with nitrification episodes in these systems.

MATERIALS AND METHODS

Pilot plant operation and sampling. The pilot-scale drinking water treatment and distribution system used in this study (Fig. 1) was operated as part of a larger investigation of the factors affecting chloramine decay and the onset of nitrification (G. W. Harrington, D. R. Noguera, C. C. Bone, A. I. Kandou, P. S. Oldenburg, J. M. Regan, and D. V. Hoven, unpublished data). The raw water supply for the system was Lake Mendota in Madison, Wisconsin. Parallel treatment trains were operated at an approximate flow rate of 3.0 liters min^{-1} each. Treatment consisted of either conventional or enhanced (i.e., adjustment to pH 6 with sulfuric acid) alum coagulation followed by flocculation, sedimentation using inclined-tube settlers, dual-medium filtration through anthracite and sand, chlorination, and secondary chloramination through the addition of ammonium chloride. Following chloramine formation, the flow from each parallel train was partitioned into three separate model distribution systems with initial pH adjustments to 7, 8, or 9, creating six different treatment and distribution conditions. Each model distribution system consisted of two completely mixed tanks in series to simulate nominal distribution system detention times of 1 and 4 days. Associated with each retention tank was a 4.6-m-long, 1.3-cm (i.e., 0.5-in)-diameter polyvinyl chloride (PVC) pipe loop with a flow velocity maintained at approximately 4.3 cm s^{-1}.

The system was routinely monitored for chemical and biological parameters, including chlorine, chloramines, ammonia, nitrite, nitrate, pH, heterotrophic

plate counts using Standard Method 9215C with R2A agar (5), and most probable number (MPN) for AOB. The MPN enumeration involved a five-tube procedure with three 10-fold dilutions using Soriano and Walker medium (37) containing 0.1 ml of 1% (wt/vol) sodium thiosulfate to neutralize the chloramine residual (4). Tube contents were incubated in the dark for 21 days at 28°C and were tested for nitrite production using dimethyl-naphthylamine and sulfanilic acid (50). For the characterization of nitrifier communities, liquid samples from the six distribution systems were collected at the end of the operation period (day 259) in sterile sample bottles from the 4-day retention tanks, and 500-ml aliquots were filtered through a sterile 0.2-μm-pore-size nylon membrane filter for cell collection. The retained cells were resuspended in 1 ml of Tris-EDTA (TE) buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and were transferred to a sterile 1.5-ml microcentrifuge tube. Biofilm samples were also collected on the same day from each 4-day PVC pipe loop by cutting 15-cm-long sections of PVC, scraping the inside of the pipe section with a sterile spatula, and transferring the detached biofilm to 1 ml of sterile deionized water.

DNA extraction. Biofilm and filtered cells from liquid samples were washed by pelleting for 5 min at $14,800 \times g$ and resuspending in 550 μl of TE. Lysis was performed by the addition of 25 μl of 50 mg of lysozyme ml^{-1} and a 1-h incubation at 37°C, followed by the addition of 3 μl of 20-mg ml^{-1} proteinase K and 30 μl of 20% sodium dodecyl sulfate and a 1-h incubation at 37°C. The suspension was centrifuged at $14,800 \times g$ for 5 min, and 500 μl of supernatant was recovered and transferred to a clean microcentrifuge tube. The DNA was purified by the addition of 500 μl of buffered phenol-chloroform-isoamyl alcohol (25:24:1), gentle mixing, and centrifuging for 5 min at $20,200 \times g$. From the aqueous layer, 300 μl was transferred to a clean microcentrifuge tube, and nucleic acids were precipitated by the addition of 30 μl of sodium acetate (3 M, pH 5.2) and 600 μl of ice-cold 100% ethanol, followed by 20 min of storage at −20°C. The samples were centrifuged for 5 min at $20,200 \times g$, supernatant was drawn off, and the nucleic acid pellet was washed once with 1 ml of ice-cold 70% ethanol. The ethanol was removed, and the tubes were placed in a vacuum desiccator until dry. The nucleic acids were dissolved in 100 μl of TE and stored at −20°C.

T-RFLP analysis. Purified DNA was analyzed by AOB- and NOB-specific 16S rDNA-targeted T-RFLP as well as the *amoA*-targeted T-RFLP protocol described by Horz et al. (10). For the 16S rDNA protocol, amplification of AOB and NOB DNA was performed by nested PCR, using the universal primers 11f and 1492r (Table 1) to achieve an initial increase in template concentration followed by the specific amplification of AOB and NOB 16S rDNA. PCR was performed in 50-μl reaction volumes containing 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 0.1-mg ml^{-1} nonacetylated bovine serum albumin (Sigma), 0.4 μM forward and reverse primer, 2.5 mM $MgCl_2$, and a 0.2 mM concentration of each deoxynucleoside triphosphate. For the universal amplification step, 5 μl of template DNA was used, while 1 μl of universal amplification product was used as the template for the nitrifier-specific reactions. After a 5-min hot start at 80°C, 2.5 U of *Taq* polymerase (Promega) was added to each tube. The universal amplification involved an initial denaturation at 95°C for 5 min, followed by 30 cycles of 1.5-min denaturation at 95°C, 2 min of annealing at 52°C, and 3 min of extension at 72°C, with a final 10-min extension at 72°C. The AOB- and NOB-specific 16S rDNA amplifications involved an initial denaturation at 95°C for 5 min, followed by 30 cycles of 1.5-min denaturation at 95°C, 0.5 min of annealing at 65°C, and a 1 min extension at 72°C, with a final 6-min extension at 72°C. The *amoA*-targeted protocol was performed as previously described (10). Negative controls without template addition were treated identically in the nested 16S rDNA- and *amoA*-targeted reactions to confirm the absence of contaminating DNA. In the AOB- and NOB-specific 16S rDNA and the *amoA* amplifications,

TABLE 1. PCR primers

Primer	Sequence (5′ to 3′)	Specificity	Reference
11f	GTT TGA TCC TGG CTC AG	*Bacteria* 16S rDNA	14
1492r	TAC CTT GTT ACG ACT T	*Bacteria* 16S rDNA	19
EUB338f[a]	ACT CCT ACG GGA GGC AGC	*Bacteria* 16S rDNA	1
Nso1225	CGC CAT TGT ATT ACG TGT GA	β-subclass AOB 16S rDNA	24
NIT3	CCT GTG CTC CAT GCT CCG	*Nitrobacter* 16S rDNA	46
Ntspa0685 M[b]	CGG GAA TTC CGC GCT C	*Nitrospira* 16S rDNA	12
AmoA-1F	GGG GTT TCT ACT GGT GGT	*amoA*	33
AmoA-2R	CCC CTC KGS AAA GCC TTC TTC	*amoA*	33

[a] Reverse complement of EUB338.
[b] Ntspa0685 was modified by eliminating 3 bases from each end to reduce the melting temperature and to eliminate mismatches with several *Nitrospira* sequences.
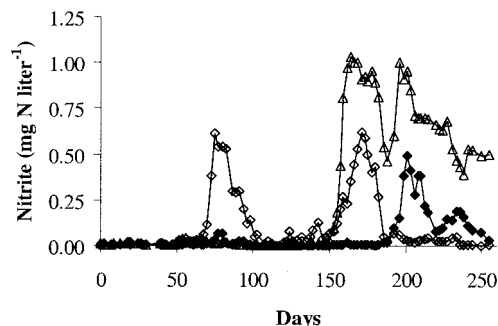
FIG. 2. Nitrite concentration in the 4-day retention tanks of the conventional coagulation treatment train: pH 7 (◆), pH 8 (◇), and pH 9 (△).

the forward primers were labeled with the phosphoramidite dye 6-FAM. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN), and *amoA* PCR products were gel purified using the QIAEX II Gel Extraction Kit (QIAGEN).

Restriction digestion mixtures contained 9 μl of sample, 1 μl of enzyme buffer, and 1 μl of either 10 U of *Msp*I (MBI Fermentas) μl^{-1} for 16S rDNA or 10 U of *Taq*I (MBI Fermentas) μl^{-1} for *amoA*. Samples were analyzed by either capillary electrophoresis with an ABI Prism 310 Genetic Analyzer (Applied Biosystems) or gel electrophoresis with an ABI Prism 377XL Sequencer (Applied Biosystems). For capillary electrophoresis, samples were prepared by denaturing 2-μl aliquots and 1 μl of TAMRA-labeled GeneScan-500 (Applied Biosystems) as an internal size standard in 20-μl deionized formamide at 95°C for 5 min. Analysis involved performance-optimized polymer (POP-4) (Applied Biosystems), a 10-s injection at 15 kV, and an electrophoresis voltage of 15 kV at a column temperature of 60°C. Electropherograms were analyzed using the application GeneScan (Applied Biosystems).

AOB and NOB terminal fragments. To identify primer sequences that selectively target AOB and NOB and restriction sites that allow their differentiation, nearly complete 16S rDNA sequences for 46 AOB and 41 NOB were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). The PCR strategy involved coupling a specific reverse primer with EUB338 as a forward primer. While EUB338 targets the *Bacteria* domain, the use of AOB- and NOB-specific reverse primers would result in only linear PCR amplification of nonnitrifier sequences.

Of the AOB 16S rDNA-targeted primers in the literature (31, 44), Nso1225 was selected as the broadest AOB primer that retains specificity for AOB in the β subclass of the *Proteobacteria* (24). AOB belonging to the γ subclass of the *Proteobacteria* (e.g., *Nitrosococcus oceanus*) were not targeted in this protocol. Of the available β-subclass *Proteobacteria* AOB sequences, only *Nitrosococcus mobilis* does not have a perfect complement to Nso1225, with a single base mismatch near the 5′ primer end.

The NOB constitute a more phylogenetically diverse group than the AOB (42). No single primer was identified that would target all of the characterized NOB diversity. Therefore, two primers were selected to separately target *Nitrobacter* and *Nitrospira* strains. *Nitrococcus* and *Nitrospina* were not targeted in this protocol. NIT3 was chosen to target the *Nitrobacter* sequences (46). This primer is an exact match with all of the deposited *Nitrobacter* sequences. The closest nontarget sequences that were identified by database searches have one mismatch and are from *Bradyrhizobium japonicum*, *Afipia clevelandensis*, *Afipia felis*, and *Rhodopseudomonas palustris*, which are all α-subclass *Proteobacteria* closely related to *Nitrobacter*. For the *Nitrospira* representatives, Ntspa0685 was modified by removing 3 nucleotides from each end to produce a primer (Ntspa0685 M) with a melting temperature compatible with EUB338 that exactly matches all but one of the available *Nitrospira*-type sequences, the exception having one mismatch (12). This primer is an exact match with one non-NOB 16S rDNA, a *Cytophaga* sp. recovered from deep-sea sediment. The next nearest sequences have one mismatch with Ntspa0685 M and are from the thermophilic sulfate reducer *Thermodesulfovibrio*, *Ammoniphilus oxalaticus*, and the thermophilic *Bacillus schlegelii*. Details of the primers are presented in Table 1.

Twenty-six restriction enzymes with different recognition sequences were evaluated by determining both the 5′ and 3′ terminal fragment lengths that would be generated from the PCR product of each AOB and NOB sequence. Based on this analysis, the 4-base cutter *Msp*I was selected due to its generation of two distinct 5′-terminal fragments corresponding to the *Nitrosomonas* and *Ni-*

trosospira AOB genera and distinct 5′-terminal fragments for *Nitrobacter* and *Nitrospira* sequences. The terminal fragment lengths for these genera are 160 to 166 bases for *Nitrosomonas*, 105 to 107 bases for *Nitrosospira*, 141 bases for *Nitrobacter*, and 265 to 267 and 277 bases for *Nitrospira* (except for several *Nitrospira* clone sequences, which give terminal lengths of 134, 321, and 335 bases). Distinction among 16S rDNA-inferred subclusters within the *Nitrosomonas* and *Nitrosospira* genera was not possible with this T-RFLP analysis.

Cloning and sequencing. Unlabeled PCR products were cloned following the instructions in the pGEM-T Vector System I kit (Promega). Ligations with pGEM-T were performed overnight at 4°C. Transformations were performed using competent cells of *Escherichia coli* JM109 prepared by a rubidium chloride technique (Protocol and Applications Guide, 3rd ed., Promega Corp., Madison, Wis.). Transformants were selected by ampicillin resistance, and blue-white screening was performed to identify clones with inserts. Following PCR confirmation of insert size, plasmids were extracted from clones using the Wizard *Plus* SV Minipreps DNA Purification System (Promega). Inserts were sequenced from both directions using the SP6 and T7 RNA polymerase promoter sequences on pGEM-T and cycle sequencing with ABI Prism BigDye dideoxy dye terminators. Samples were purified with AutoSeq G-50 purification columns (Amersham Pharmacia Biotech) and sequenced at the University of Wisconsin Biotechnology Center. ClustalX (43) was used for sequence alignment, tree generation using the neighbor-joining method, and bootstrap analysis. Clone sequences were analyzed using the Basic Logic Alignment Search Tool to evaluate similarities with sequences deposited in GenBank. Sequences were also analyzed for chimeric artifacts using the Chimera Check online analysis function from the Ribosomal Database Project II (20) and by comparison of neighbor-joining trees generated from the first and second halves of the aligned sequence fragments. Any chimeric sequences identified in this manner were removed from further analysis.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession numbers AY048920 to AY048968.

RESULTS

Pilot plant operating data. Chemical and biological monitoring of the pilot plant revealed evidence of nitrification in each of the six distribution systems. Nitrite production was observed in the 4-day retention time tanks for the conventional and enhanced coagulation systems (Fig. 2 and 3, respectively) after an operating period of 3 to 9 months. Nitrate production, which can be due to NOB growth or oxidation of nitrite by chloramine, was also detected in each of the distribution systems. Microbial enumeration by MPN and heterotrophic plate counts showed the proliferation of AOB and heterotrophs (Fig. 4). This microbial growth occurred in the presence of average chloramine residuals of 0.2 mg of Cl$_2$ liter^{-1} to 1.1 mg of Cl$_2$ liter^{-1} and an average free ammonia concentration of 0.3 to 0.6 mg N liter^{-1} over the duration of the study (Harrington et al., unpublished).
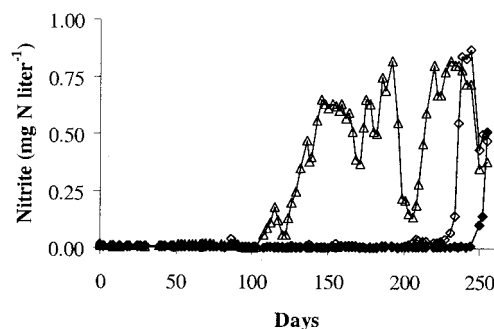

FIG. 3. Nitrite concentration in the 4-day retention tanks of the enhanced coagulation treatment train: pH 7 (◆), pH 8 (◇), and pH 9 (△).
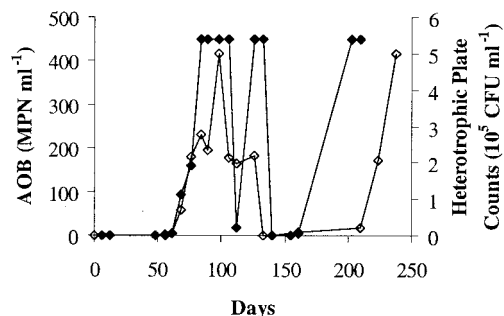
FIG. 4. Ammonia-oxidizing bacteria (◆) and heterotrophic bacteria (◇) enumeration by MPN and heterotrophic plate counts, respectively, for the 4-day retention tank of the pH 8 conventional coagulation system. The dilution series used in the MPN test imposed a maximum AOB quantitation level of 448 MPN ml^{-1}.

AOB community. Distribution system samples were analyzed for AOB via *amoA*- and 16S rDNA-targeted T-RFLP. PCR amplification yielded very little product from both the *amoA* and 16S rDNA gene targets due to low template concentration in the DNA preparations. The low yield resulted in poor T-RFLP electropherograms in which the peak heights of AOB-specific fragments were not distinguishable from background interferences. Therefore, nested PCR was incorporated in the 16S rDNA protocol using universal primers. This provided a significant enhancement to the amplicon yield and the quality of the nitrifier-specific 16S rDNA electropherograms. Sufficient sequencing information was not available to develop a nested protocol for *amoA*-based T-RFLP.

Typical AOB-specific 16S rDNA-targeted T-RFLP electropherograms for the 4-day retention tank samples and a biofilm sample from the pH 8 conventional coagulation distribution system are presented in Fig. 5. Biofilm samples from the other distribution systems did not yield sufficient PCR products, consistent with the visual observation during sampling of minimal attached growth on the pipes of these systems. All six of the distribution systems and the biofilm sample showed a large peak associated with *Nitrosomonas*-type AOB (166 bp), while the peak height for *Nitrosospira*-type template DNA (106 bp) was considerably smaller, representing only 8 to 32% of the sum of the AOB-associated peak heights. This pattern was consistent among the six distribution system conditions. The electropherograms also show the presence of unexpected labeled fragment lengths for the AOB-specific protocol. The peak near 270 bp detected in each of the liquid samples may be due to uncharacterized AOB or incomplete digestion of the labeled products, as the PCR fragments from all *Nitrosomonas* templates have an additional *Msp*I restriction site 270 to 276 bp from the labeled 5′ end. The other extraneous peaks are likely caused by nonspecific amplification of non-AOB sequences, as discussed below.

Cloning and sequencing of AOB 16S rDNA PCR products were performed to increase the resolution of AOB characterization beyond the *Nitrosomonas-Nitrosospira* differentiation achieved by the 16S rDNA T-RFLP protocol. The approximately 900-bp insert sequences were aligned with 16S rDNA sequences from AOB species and other *Proteobacteria* representatives. Figure 6 depicts a neighbor-joining tree generated from the sequence alignment. The results show the presence of

AOB with sequences within the *Nitrosomonas oligotropha* cluster (cluster 6a according to the nomenclature established by Stephen et al. [40]), which is represented by the species *N. oligotropha* and *Nitrosomonas ureae* (31), while no *Nitrosospira*-like sequences were retrieved.

The cloning results also revealed the amplification of nontarget sequences, including false positives (i.e., non-AOB sequences that generate the same labeled fragment length as AOB). The false positives (e.g., clone NsoB8-1-10 in Fig. 6) were confined to other members of the β subclass of the *Proteobacteria*. Some were similar to *Gallionella ferruginea*, which has no mismatches with Nso1225 and generates a *Nitrosospira*-like fragment length, and others resembled *Rhodocyclus* sp. and *Propionivibrio dicarboxylicus*, which have three mismatches with Nso1225 and produce a *Nitrosomonas*-like peak. In addition, several *Sphingomonas*-related clones were detected, which generated a non-AOB, 141-bp terminal fragment, visible in Fig. 5.

NOB community. NOB were analyzed by the nested-PCR strategy using the universal primers, followed by PCR separately targeting *Nitrobacter* and *Nitrospira*. For simplicity in the analysis, the *Nitrobacter* and *Nitrospira* T-RFLP results are superimposed in Fig. 7, which summarizes typical results for the liquid samples and the biofilm sample from the pH 8 conventional coagulation system. *Nitrobacter* (141 bp) was detected in the pH 7 conventional coagulation system, albeit at a low intensity. In the enhanced coagulation systems, the *Nitrobacter*-related peaks were more evident and exhibited a decreasing peak height from pH 7 to pH 9. *Nitrobacter* was also detected in the biofilm sample from the pH 8 conventional
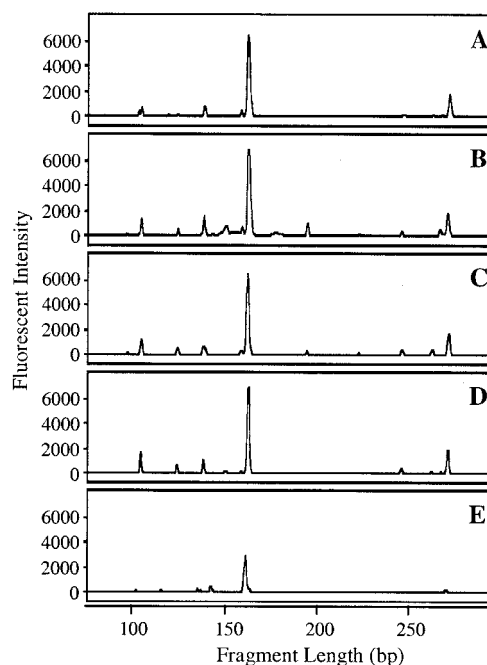

FIG. 5. AOB-targeted T-RFLP electropherograms for liquid samples collected from the 4-day retention tanks (A to D) and a biofilm sample from the pH 8 conventional coagulation system (E). Panel A is for the pH 8 conventional coagulation system, and panels B through D are for the pH 7, 8, and 9 enhanced coagulation systems, respectively.
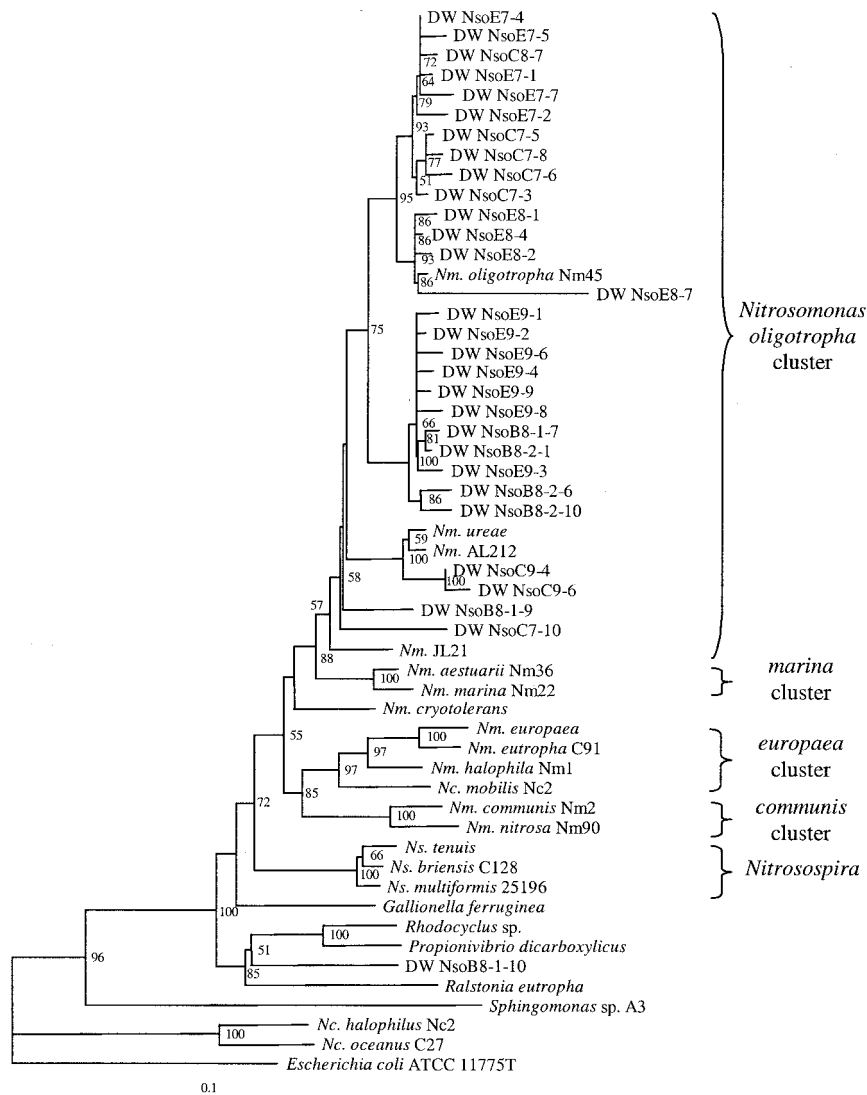
FIG. 6. Neighbor-joining tree generated from an alignment of 16S rDNA sequences from AOB and other *Proteobacteria*. AOB genus abbreviations are Nm for *Nitrosomonas*, Nc for *Nitrosococcus*, and Ns for *Nitrosospira*. Clones recovered from the drinking water distribution systems are designated DW Nso, followed by B for biofilm or C and E for conventional and enhanced coagulation, respectively; the nominal pH of the distribution system; and the clone number. Bootstrap values above 50 are shown at each node. Scale bar represents 10% sequence difference. Redundant clone sequences are not shown.

coagulation system, despite its absence or low numbers in the liquid sample from this distribution system. A significant *Nitrospira* peak (265 and 277 bp) was detected in all of the samples.

Cloning and sequencing were performed with the pH 7 conventional coagulation system for *Nitrobacter* PCR products and with both pH 7 systems and the pH 8 biofilm sample for *Nitrospira* fragments to confirm the positive identity of the NOB peaks. The phylogenetic relationships of the clone sequences relative to NOB sequences and non-NOB representatives are depicted in Fig. 8. The cloned fragments from *Nitrobacter*-targeted PCR did not show a strong association with any one of the *Nitrobacter* species but clearly aligned with these sequences. Most of the *Nitrospira* sequences were related to *Nitrospira moscoviensis* and a *Nitrospira* clone sequence (*Nsr.* sp. AF035813 in Fig. 8) from a freshwater aquarium (12). In

addition, one *Nitrospira* clone did not cluster with any other known species or environmental clones.

DISCUSSION

Figures 2 to 4 represent the typical problem of water quality degradation in chloraminated distribution systems. The appearance of nitrifiers is accompanied by an increase in nitrite concentration and the colonization of the system by heterotrophic bacteria (49). Full-scale systems undergo routine distribution system flushing or breakpoint chlorination to prevent nitrifier growth. When a nitrifier community is established, however, efforts to eradicate these organisms from the distribution system require more drastic and costly approaches as the communities appear to acquire a higher resistance to chloramines (26). The pilot plant used in this study was operated as
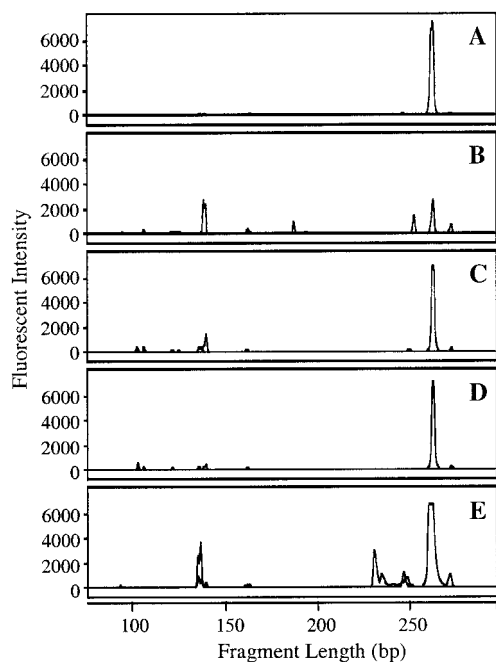
FIG. 7. NOB-targeted T-RFLP electropherograms for liquid samples collected from the 4-day retention tanks (A to D) and a biofilm sample from the pH 8 conventional coagulation system (E). Panel A is for the pH 8 conventional coagulation system, and panels B through D are for the pH 7, 8, and 9 enhanced coagulation systems, respectively. Data from *Nitrobacter*- and *Nitrospira*-specific T-RFLP are superimposed.

part of a larger effort to evaluate operational parameters (i.e., the efficiency of natural organics removal and the pH of the finished water) that may delay the onset of nitrification in chloraminated systems (Harrington et al., unpublished). As such, the system was operated consistently even after nitrification became evident, thus providing an experimental system to evaluate nitrifier communities in chloraminated drinking water systems. This study provides the first glance at the diversity of AOB and NOB present in these communities, which is an initial step towards understanding the persistence of nitrifying microorganisms in chloraminated water.

The 16S rDNA-based T-RFLP analysis using nested PCR was sensitive and effective at discriminating between *Nitrosomonas* and *Nitrosospira* AOB and at detecting *Nitrobacter* and *Nitrospira* NOB. Although the *amoA*-targeted T-RFLP protocol allows a greater discrimination of AOB clusters (10), the amplification was not sensitive enough for the clear detection of target peaks above background noise. While other researchers have used nested PCR for increased sensitivity of *amoA* detection from environmental samples, the primers from these studies were specific for the species *Nitrosomonas europaea* (8) or designed based on *amoA* sequences from *N. europaea* and *Nitrosococcus mobilis* isolates (13). The ability of the primer pairs used by Juretschko et al. (13) to amplify *amoA* from diverse AOB templates is unknown, as the target sequences of these primers are outside the *amoA* fragments that have been sequenced from a wide range of AOB isolates and environmental samples.

The molecular analyses of the AOB community in each

pilot-scale distribution system retrieved a predominance of 16S rDNA PCR products from *Nitrosomonas* template, with considerably less *Nitrosospira*-related product. This is evident from the T-RFLP electropherograms (Fig. 5) and is consistent with the cloning and sequencing, which did not recover a single *Nitrosospira*-like sequence. The majority of cloned sequences were affiliated with the *N. oligotropha* cluster, with clones resembling *N. oligotropha* and *N. ureae*. These analyses are subject to potential biases associated with PCR amplification and cloning. Attempts to evaluate cell numbers of different AOB groups using fluorescent in situ hybridization failed due to nonspecific binding of labeled probe to noncellular material present in the sample matrix, as determined by 4′,6′-diamidino-2-phenylindole staining of cells.

There are only a few phenotypic and ecological characterizations of *N. oligotropha* cluster representatives reported in the literature. *N. oligotropha* is the *Nitrosomonas* species reported to experience substrate inhibition at the lowest ammonia concentrations (i.e., 20 to 70 mM NH_3 or approximately 200 to 1,000 mg of N liter^{-1}), while *N. ureae* can tolerate up to 200 mM (16, 39, 41). Although these studies reveal an ammonia sensitivity by this group of AOB, the inhibitory concentrations are much higher than the concentrations observed in chloraminated drinking water systems (typically <0.2 mM), and thus, it is unlikely that this factor influences their presence in the distribution systems.

N. oligotropha cluster representatives have been isolated from soil, freshwater, estuary sediments, industrial sewage, and laboratory-activated sludge systems (16, 39, 41). They have also been detected by molecular methods in freshwater and sediments (38), shallow soil samples over a pH range of 3.9 to 6.6 (40), full-scale activated sludge treatment plants (30), and a phosphate-removing lab-scale biofilm reactor (7). While Kowalchuk et al. (17) reported the detection of cluster 6 AOB (nomenclature by Stephen et al. [40]) from pig and chicken manure compost and a calf-slurry-activated sludge system, the presence of *N. oligotropha* representatives could not be determined unequivocally in their study because the initial probe designed for this cluster (40) also targets *Nitrosococcus mobilis*, which belongs to the *N. europaea* cluster (cluster 7). The understanding of the ecology of *N. oligotropha* cluster AOB has likely been affected by PCR and probing biases resulting from primers and probes that do not target this group. Several studies of AOB communities in oligotrophic environments have used primer pairs with two to more than five mismatches with *N. oligotropha* and reported the failure to detect AOB in nitrifying systems (11) or the prevalence of *Nitrosospira* and the sporadic detection of *Nitrosomonas* (8, 9, 47). It is possible that these reports provide a biased picture of AOB diversity, particularly in oligotrophic environments in which *N. oligotropha* representatives may outcompete other *Nitrosomonas* organisms and be competitive with *Nitrosospira*.

There may have been a selective advantage for *N. oligotropha* and *N. ureae* in the low ammonia concentrations present in the finished water of the pilot plant, which averaged 0.3 to 0.6 mg of NH_3-N liter^{-1} (i.e., 0.02 to 0.04 mM NH_3) and never exceeded 2.4 mg of NH_3-N liter^{-1} (i.e., 0.17 mM NH_3). This presents an environment with an ammonia concentration near the measured K_m of available *N. oligotropha* strains and more than an order of magnitude below that of several other *Nitro-*
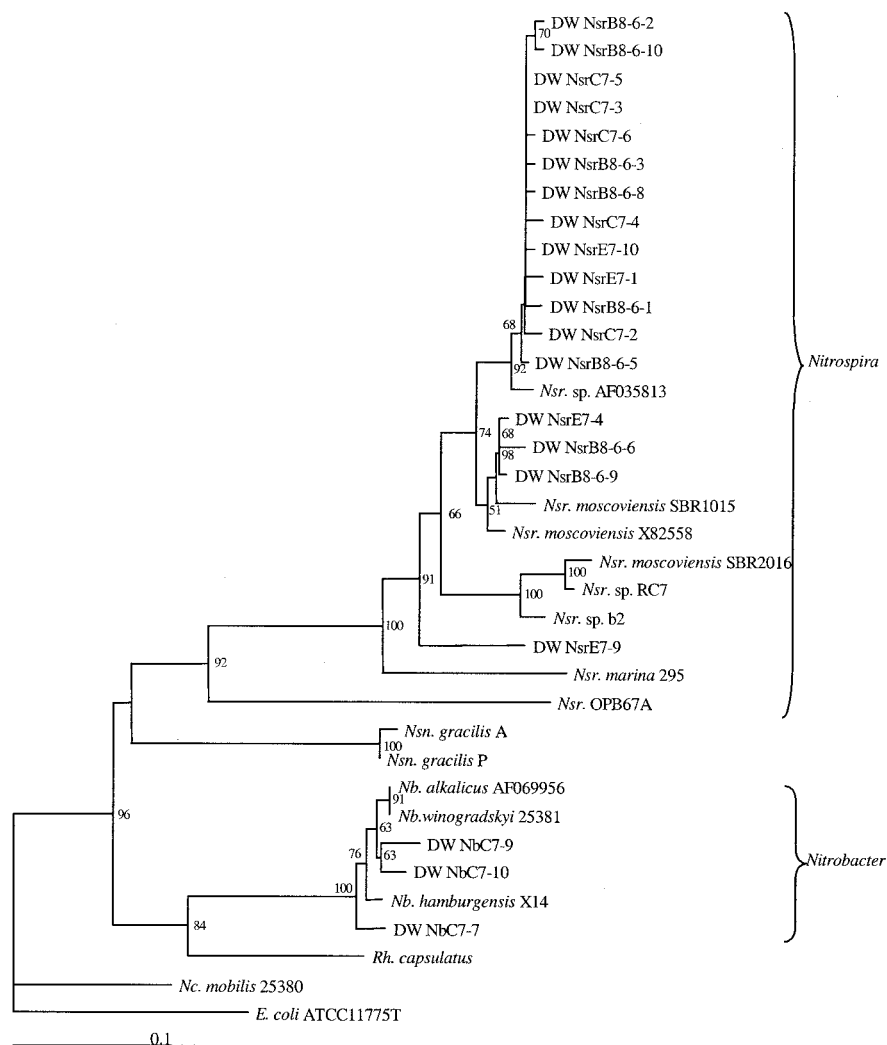
FIG. 8. Neighbor-joining tree generated from an alignment of 16S rDNA sequences from NOB and several *Proteobacteria* representatives. NOB genus abbreviations are Nb for *Nitrobacter*, Nc for *Nitrococcus*, Nsr for *Nitrospira*, and Nsn for *Nitrospina*. Clone designations are the same as in Fig. 6 except for a prefix of Nb or Nsr for *Nitrobacter*- or *Nitrospira*-specific PCR, respectively. Bootstrap values above 50 are shown at each node. Scale bar represents 10% sequence difference. Redundant clone sequences are not shown.

somonas strains (39). Consistent with this hypothesis, Gieseke et al. (7) studied a phosphorus-removing biofilm and observed a deeper penetration of *N. oligotropha*-like AOB than *N. europaea*-like AOB into the biofilm, which corresponded to an environment with lower ammonia and dissolved-oxygen concentrations. Their apparent affinity for ammonia may have allowed them to outcompete the more copiotrophic AOB strains. Although the ammonia concentrations in the distribution system were also of the same order of magnitude as the reported K_m for *Nitrosospira* sp. (34), none of the recovered clones belonged to this cluster. Another possible selection factor may be related to chloramine resistance. Previous studies of AOB inactivation with chloramines have used *N. europaea* (Oldenburg et al., unpublished) and an isolate morphologically identified as a *Nitrosomonas* with no species determination (18, 50), but representatives from the *N. oligotropha* or *Nitrosospira* clusters have not yet been evaluated.

The potential for the growth of NOB in chloraminated sys-

tems has been mentioned (48, 49) but not investigated, presumably because the primary fate of nitrite that concerns drinking water utilities is the reduction of the chloramine residual. The results of this study indicate that *Nitrospira*-like NOB were ubiquitous and that *Nitrobacter* was present in some of the systems, suggesting that complete nitrification may present a significant alternate sink for nitrite produced by AOB growth. This hypothesis is also supported by the chemical monitoring data from the pilot plant, which showed the formation of nitrate during periods of near-zero chloramine residuals. The presence of *Nitrospira* and sporadic detection of *Nitrobacter* in this freshwater environment are consistent with the findings of many recent studies (3, 7, 13, 27, 34, 35), even in a system that was seeded with a *Nitrobacter* inoculum (12). As with the hypothesis presented above for the possible dominance of *N. oligotropha*, this low-nitrite environment may have selected for a *Nitrospira*-dominated NOB community. Schramm et al. (34) estimated a K_m for *Nitrospira* of 0.01 mM

nitrite (i.e., 0.14 mg of NO_2^--N liter^{-1}), and Ehrich et al. (6) reported optimum growth of *Nitrospira moscoviensis* at 0.35 mM nitrite, with inhibition above 15 mM. This K_m estimate is 1 or 2 orders of magnitude lower than reported values for *Nitrobacter* strains (15, 28, 51).

Ideally drinking water utilities would have a means of early detection of a pending nitrification episode to allow the implementation of a control strategy before observation of a measurable deterioration of water quality. Traditional AOB enumeration by MPN requires at least 3 weeks of incubation, thereby yielding operationally irrelevant data. To address this, a number of studies have searched for a correlation between nitrification and a surrogate early warning indicator. Wolfe et al. (50) reported a correlation with heterotrophic plate counts using R2A agar but no consistent correlation with nitrite concentration. The results of this pilot study generally showed a concurrent onset of nitrification and increases in heterotrophic plate counts (Fig. 4). Lieu et al. (18) reported that an increase in nitrite concentration lagged behind AOB growth by 1 to 3 weeks at 15°C in batch disinfection experiments. In a study of chloraminated systems in Australia, Cunliffe (4) reported that an increase in oxidized nitrogen species and a decrease in total chlorine residual served as early indicators of nitrification. Wilczak et al. (48) conducted an analytical survey of 10 chloraminating utilities and reported a lack of correlation between nitrification (operationally defined as >50 μg of nitrite and nitrate N liter^{-1} in their survey) and plant effluent ammonia or pH. Typically, increases in oxidized nitrogen were associated with increases in heterotrophic plate counts and sometimes with a decrease in dissolved oxygen.

Given the inconsistency in these findings, Wilczak et al. (48) concluded that the best indicators of nitrification were a nitrogen balance and the detection of AOB and NOB. The nested-PCR T-RFLP protocol presented here provided a rapid and sensitive screening approach for detecting the presence of nitrifying bacteria that could be useful to drinking water utilities for monitoring AOB and NOB communities. T-RFLP allowed the detection of AOB and NOB within a few days and was not subject to culturability biases, representing a marked improvement over the minimum 3-week incubation period for MPN enumeration (22). While the method is not quantitative in the presented form, it could be applied as a presence/absence tool to detect the presence of nitrifying bacteria in the distribution system, which may indicate the potential onset of a nitrification episode before other water quality parameters are affected.

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