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Microbial Survey of a Full-Scale, Biologically Active Filter for Treatment of Drinking Water

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The microbial community of a full-scale, biologically active drinking water filter was surveyed using molecular techniques. *Nitrosomonas*, *Nitrospira*, *Sphingomonadales*, and *Rhizobiales* dominated the clone libraries. The results elucidate the microbial ecology of biological filters and demonstrate that biological treatment of drinking water should be considered a viable alternative to physicochemical methods.

Biologically active filtration is commonly used in Europe and Asia for drinking water treatment (5). Biological processes have the potential to cut operation costs by decreasing the amount of chemicals required for treatment and increasing effectiveness in terms of decreased biological regrowth (e.g., corrosion, nitrification, taste, and odor) in the distribution system (DS) and decreased chlorine demand (6, 17, 30). However, biological processes have not been widely accepted in drinking water in the United States, mainly due to issues arising from the negative perception of microorganisms as well as questionable reliability and effectiveness (2, 8, 17). With the lack of published long-term operational data using biological processes and the complexity of microbial systems, there exists a need to both document biologically active systems and design experimental systems to elucidate the microbial consortia and the effects of operational parameters.

One use of biologically active filters for drinking water treatment involves the regulation of nitrate/nitrite nitrogen levels. Excessive ingestion of nitrite and nitrate can be hazardous (H. I. Shuval and N. Gruener, presented at the Panel of Experts on Effects of Agricultural Production on Nitrates in Food and Water with Particular Reference to Isotope Studies, Vienna, Austria, 1974), so the United States Environmental Protection Agency (U.S. EPA) has set source water maximum contaminant levels (MCLs) for nitrite and nitrate at 1 mg/liter N and 10 mg/liter N, respectively. Yet no MCL exists for ammonia. As utilities are required to monitor for only nitrite and nitrate in the source water, concentrations may build in the DS via uncontrolled partial (nitrite accumulation) or full nitrification. When excessive levels of free ammonia are present in the source water or ammonia is added to form chloramines, nitrification may occur with sufficient dissolved oxygen (DO) (34). Nitrification in the DS, and the pH drop associated with nitrification, can impact the corrosion rates of the DS and premise materials (41). In addition, the increased chlorine demand and growth of heterotrophic biofilms produce undesirable taste and odor issues (30). Excess ammonia itself may interfere (by way of chloramine formation) with the maintenance of a free chlorine residual in the distribution system and the chemical oxidation of arsenic(III) in treatment plants utilizing the iron removal process for arsenic removal (18, 19). In the case of arsenic oxidation, excess ammonia will bind with free chlorine used to oxidize arsenic(III) to arsenic(V), thus decreasing arsenic sorption to iron(III) and, ultimately, arsenic removal via iron(III) filtration.

Lytle et al. (18, 19) reported on the use of biologically active filters to oxidize ammonia and arsenic in a full-scale water treatment plant. Briefly, the treatment train aerates groundwater, which is then filtered (loading rate, 85 liters/min/m^2) through dual-medium anthracite over sand filters. The water is finally chlorinated, fluorinated, and distributed. The filter is backwashed every 3 days. They demonstrated that the filters completely and consistently oxidized 1.13 mg/liter of ammonia nitrogen to nitrate nitrogen and 38 μg/liter of arsenic(III) to arsenic(V) without the addition of a chemical oxidant. Preliminary filter analysis and follow-up pilot studies identified bacteria as the probable source of ammonia oxidation and arsenic oxidation (C. N. Green, presented at Rice University, Houston, TX, 19 October 2007). The findings of Green and Lytle were based primarily on culture-dependent methods, with little use of culture-independent (molecular) methods by Green (C. N. Green, presented at Rice University, Houston, TX, 19 October 2007) (18, 19). Molecular microbiological techniques used to characterize microbial communities in wastewater processes are a well-studied field; however, characterization of full-scale, biologically active water treatment systems for drinking water is limited (16, 25, 26, 36, 38). There is a clear need to better identify the diversity of bacterial communities, including the presence of human pathogens, in biologically active drinking water filters to improve the understanding of such a complex system. Identification of the microbial consortia will provide a greater insight into the dynamics of biologically active filters, establish their susceptibility to pathogen growth, and determine their applicability as a viable treatment technology.

The goal of this study was to identify members of the microbial community in a full-scale drinking water filter in Southwest Ohio and identify the specific microorganisms responsible for ammonia oxidation. Specifically, we revisit the microbial oxidation system previously characterized by Lytle et al. (18, 19).

DNA isolations from filter anthracite/sand medium were collected aseptically from a single fluidized filter during a 5-min

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backwash. Approximately 400 g of medium was collected across a 5.9-m^2 filter bed. Samples were taken to the U.S. EPA laboratories in Cincinnati, homogenized via mixing, and processed immediately. A total of 1.2 g of wet medium was placed in 400 μl of lysis buffer (EpiCentre Biosciences, Madison, WI) containing SDS and sonicated three times for 30 s each, vortexing between steps. The tubes were then centrifuged, and the supernatant was removed and placed into a tube containing glass beads. The supernatant was beaten with the beads for 1 min. Two microliters of 50-μg/ml proteinase K (EpiCentre Biosciences, Madison, WI) was added to each tube and incubated at 65°C for 10 min. The supernatant was extracted, and nucleic acids were precipitated in ice-cold isopropanol at 4°C for 30 min. The nucleic acids were pelleted and washed with 70% ice-cold ethanol and desiccated. Samples were rehydrated in 50 μl of sterile water (Chemicon, Temecula, CA) and stored at −20°C.

A PCR was optimized and conducted in a 25-μl volume containing 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.3 μM each primer (16S rRNA gene-Forward, 5′-GTTTGATCCTGGCTCAG-3′, and 16S rRNA gene-Reverse, 5′-ACGGYTACCTTGTTACGACTT-3′), 0.4 U *Taq* polymerase (TaKaRa, Otsu, Japan), 0.6× *Taq* buffer, and 1 μl of template DNA. The *amoA* PCR mixture contained 0.1 μg/μl of nonacetylated bovine serum albumin (BSA). A touchdown cycle was used, starting at 60°C and decreasing 0.5°C each cycle for 10 cycles, followed by 15 cycles at 55°C. PCRs with *amoA* and archaeal *amoA* followed the published protocols of Francis et al. and Rotthauwe et al., respectively (10, 31). PCR products were electrophoresed on a 1.8% agarose gel in 0.5× Tris-acetate-EDTA (TAE) buffer. PCR bands of the16S rRNA gene and *amoA* gene that were used for cloning were excised from the gel using a sterile scalpel and processed using a gel extraction kit (Qiagen, Chatsworth, CA). Products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA).

Sequencing reactions were performed on an ABI 3730 using the BigDye Terminator system with T3/T7 primers. Sequences were edited and aligned in MEGA4 and compared to sequences in the NCBI database using the BLAST function. Highest-similarity sequences were downloaded for each sequence. Duplicate and chimeric sequences were discarded. Chimeric sequences were identified using Mallard (3). Type-cultured and previously identified sequences of bacterial species with close BLAST hits were retrieved from GenBank, and operational taxonomic units (OTUs) were identified using 97% sequence identity as a threshold value.

Phylogenetic trees were constructed using MEGA version 4 (35). Phylogeny was inferred using the neighbor-joining algorithm with 2,000 bootstrap replicates assuming pairwise deletion using the maximum composite likelihood distance correction.

A small amount of filter medium was fixed in a pH 7.2 cacodylate-buffered 1% paraformaldehyde-2.5% glutaraldehyde mixture. The medium was postfixed in 1% OsO_4 and dehydrated in an ethanol series, dehydrated in hexamethyldisilazane (HMDS) for 1 h, and then air dried in a desiccator. The medium was coated with gold/palladium prior to scanning electron microscopy (SEM). Samples were viewed using a JEOL 6490LV SEM at 30 kV under high vacuum. Energy dispersive X-ray analysis (EDX) was performed for 60 live seconds using a process time of 4 and a working distance of 10 mm.

A detailed water quality analysis can be found in previous studies by this lab (18, 19). Lytle et al. reported that raw and influent ammonia values averaged 1.13 mg/liter N, with nitrate and nitrite

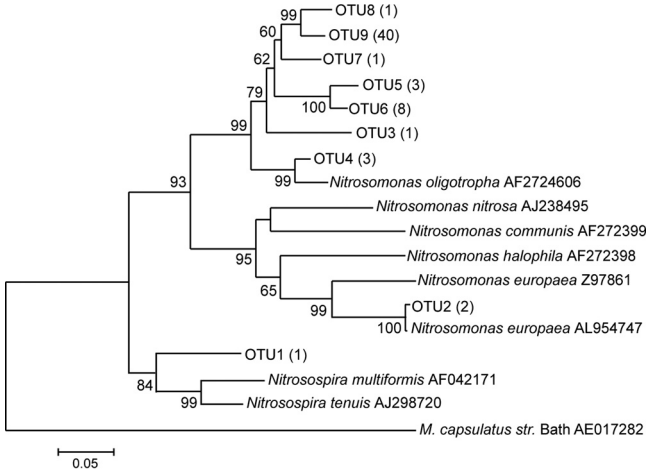

FIG 1 Unrooted neighbor-joining tree of the *amoA* gene sequences. The numbers in parentheses indicate the numbers of sequences of that OTU. *M. capsulatus str.* Bath, *Methylococcus capsulatus* strain Bath.

below the detection limits of 0.01 and 0.02 mg/liter N, respectively (19). After filtration, prior to the addition of chlorine, ammonia was oxidized to below 0.1 mg/liter N, with nitrite and nitrate measuring 0.02 mg/liter N and 1.11 mg/liter N, respectively (19). Ammonia-oxidizing bacteria (AOB) counts were highest on the filter medium and lowest in the plant effluent. Heterotrophic plate counts (HPC) were highest in raw influent and lowest in the plant effluent (19).

DNA isolations from the filter medium provided a sufficient template for PCR. PCR products of 16S rRNA genes and *amoA* genes were the correct length (~1,300 bp and ~450 bp, respectively) and of suitable yield for cloning. A total of 431 16S rRNA genes and 61 *amoA* clones were selected and sequenced. After removing duplicate and chimeric sequences, 297 unique 16S rRNA gene sequences and 31 unique *amoA* sequences were grouped into OTUs and analyzed for phylogeny. Representatives from each OTU were used as query sequences to NCBI BLAST to identify close relatives. These sequences were then downloaded and included in their respective phylogeny.

Unique *amoA* sequences were grouped into 9 OTUs, 4 of which were singletons, close to the Chao-1 estimate of 17 ± 8. Eight OTUs were clustered within the genus *Nitrosomonas*, with the remaining single OTU closely resembling *Nitrosospira* (Fig. 1). Within *Nitrosomonas*, 7 OTUs, comprising 28 total sequences, fell within the *Nitrosomonas oligotropha* lineage. The remaining OTU, comprising 2 sequences, fell within the *Nitrosomonas europaea* lineage. Primers directed to the archaeal *amoA* gene were used in an attempt to create a clone library for subsequent identification of ammonia-oxidizing *Archaea* (AOA), but PCR did not produce any amplification after exhaustive optimization efforts.

Unique 16S rRNA gene sequences were grouped into 65 OTUs, 36 of which were singletons. The Chao-1 estimate (157 ± 31) of the 16S rRNA gene library revealed that rare members of the community were undersampled. With nearly 55% of OTUs singletons, diversity was driven by species captured only once, suggesting a highly diverse system. The 65 OTUs were classified into 9 discrete groups (Table 1). Twenty-seven OTUs (together accounting for 21% of all sequences) were closely related to *Alphaproteobacteria*. Within the *Alphaproteobacteria*, the orders *Rickettsia*, *Rhodobac-*

TABLE 1 GenBank relatives to 16S clone library sequences

Relative in GenBank (accession no.)	Identity (%)[b]	% of clones in clone library that are related[c]	No. of OTUs
Nitrospira sp. (AF035813)	96–98	29.6	2
Nitrospira sp. clone g6 (*Nitrospira* sp. (AJ224039)	95–99	20.8	2
"*Candidatus* Nitrospira defluvii" (EU559167)	99	0.5	1
Rhodospirillaceae bacterium LM22 (FJ455532)	94–98	7.2	5
Rhodospirillaceae bacterium L34 (FJ459988)	92	0.8	1
Sphingomonas sp. UF010 (AB426571)	99	2.1	1
Sphingomonas sp. EZ41 (EU591707)	95–97	2.7	4
Sphingomonas sp. MTR-71 (DQ898300)	95	1.1	1
Sphingomonadaceae bacterium HINF002 (AB426560)	95	0.5	1
Sphingomonas sp. HTCC503 (AY584572)	97	0.3	1
Sphingomonas sp. BAC151 (EU131005)	97	0.8	2
Hyphomicrobium vulgare (Y14302)	97	2.1	1
Hyphomicrobium sp. Ellin112 (AF408954)	95	0.5	2
Hyphomicrobium sp. KC-IT-W2 (FJ711209)	95	0.3	1
Nordella sp. P-63 (AM411927)	92	0.5	1
Bradyrhizobium sp. KC-EP-S3 (FJ711219)	99	0.3	1
Other *Alphaproteobacteria* (FJ203515, AY945895, AF236002, AJ630204, NR 026337, and AF498710)	87–99	5.1	7
Nitrosomonas sp. Nm86 (AY123798)	97–98	5.1	2
Nitrosomonas sp. Nm84 (AY123797)	96	4.3	1
Nitrosomonas sp. Is32 AJ621027)	97	0.3	1
"*Candidatus* Nitrotoga artica" HAM-1 (FJ263061)	99	4.8	1
Methylophilus sp. ECd5 (AY436794)	96–97	0.5	2
Bacterium TG141 (AB308367)	93–96	1.3	3
Other *Betaproteobacteria* (AJ252690, AB271046, AM412133, and DQ386262)	97–98	1.6	4
Gammaproteobacteria (AJ233898)	93	0.5	1
Deltaproteobacteria (DQ295890, DQ145534, AB246770, CP001359, AB245340, NR 025348, and NR 024781)	82–96	2.1	7
Flavobacterium ferrugineum (AM230484)	94	1.9	1
Other *Bacteriodetes* (EF612324, AF070444, and GQ144415)	86–90	0.8	3
Pirellula sp. (X81942)	92	0.8	1
Other *Planctomycetes* (AY162118 and CU925984)	87–98	0.5	2
Uncultured *Chloroflexi* bacterium (FM253645)	98	0.3	1
Opitutus[a] sp. VeSm13 (X99392)	97	0.3	1

[a] Classified as *Verrucomicrobia*.
[b] Percent similarity between each relative in the NCBI database and its closest cloned 16S gene.
[c] Total of 375 clones in the clone library.

terales, *Rhizobiales*, and *Sphingomonadales* were represented. *Betaproteobacteria* accounted for 15% of sequences, with 10 OTUs representing *Nitrosomonadales*, *Methylophilales*, and *Burkholderiales*. *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria* were grouped together and accounted for approximately 3% of the sequences within 8 OTUs. An analysis of 19 sequences representing 6 OTUs identified only unknown bacteria as close relatives. These sequences were placed into the unknown group. *Planctomycetes*, *Bacteroidetes*, *Verrucomicrobia*, and *Chloroflexi* accounted for approximately 4% of sequences within 9 OTUs.

The phylum *Nitrospirae*, known to be a nitrite oxidizer (33, 34), dominated the clone library with over 51% of sequences grouped into 5 OTUs. One OTU (16 sequences) was identified as "*Candidatus* Nitrotoga arctica," a cold-adapted nitrite-oxidizing bacterium previously isolated from activated sludge (1).

SEM-EDX analysis of the filter medium showed particles coated with an inorganic layer with C, O, S, Cl, Fe, Ca, As, Mn, and P (Fig. 2A, B, and D, bottom). No biofilm was observed on the surface of the medium examined. Differential backscatter imaging identified medium particles lacking the outer coating, presumably

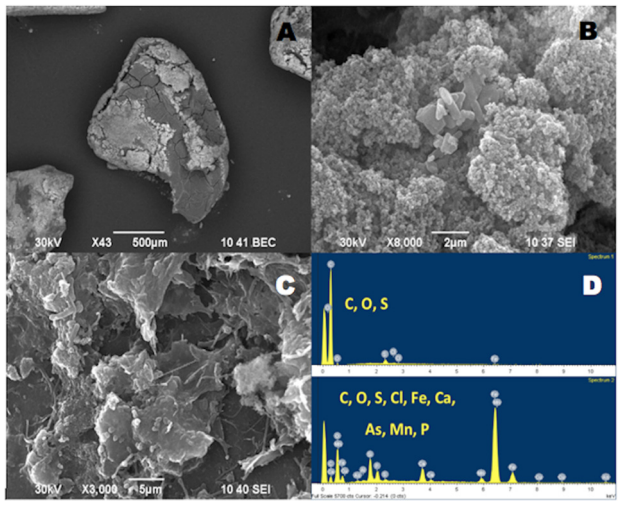

FIG 2 Scanning electron micrograph and EDX spectra of the filter medium. (A) Backscatter image of a representative anthracite grain. (B) Secondary electron image of the outer inorganic layer on the surface of anthracite. (C) Secondary electron image of the biofilm layer beneath the inorganic layer. (D) EDX spectra of the biofilm layer (top) and outer inorganic layer (bottom).

from scouring during backwash. Particles devoid of the outer inorganic layer resolved a complex biofilm containing a mix of spirochetes, bacilli, and cocci within an extracellular matrix (Fig. 2C). SEM-EDX analysis of the biofilm revealed the presence of C, O, and S (Fig. 2D, top).

Lytle et al. (19) observed that raw ground water contained an average of 1.13 mg/liter of ammonia nitrogen prior to filtration and less than 0.1 mg/liter N after filtration (19). Nitrate and nitrite nitrogen levels prior to filtration were below the limit of detection (19). After filtration, nitrate nitrogen was present stoichiometrically with prefiltration ammonia nitrogen. This stoichiometric relationship and presence of nitrate nitrogen after filtration suggested that ammonia was oxidized in the filters (19). As no chemical means of oxidation was included in the treatment train, biological nitrification was determined to be the causative agent. Therefore, the filters were investigated for their microbial diversity.

General congruence was observed between 16S rRNA and *amoA* gene clone libraries. The dominant nitrifying organisms in the 16S rRNA gene clone library were members of the phyla *Proteobacteria* and *Nitrospirae*. Of the sequences in the *amoA* library, species from the genus *Nitrosomonas* were found to be the major ammonia oxidizers, accounting for more than 98% of total sequences. Over 95% of the total sequences clustered in the *N. oligotropha* lineage, and 3% clustered in the *N. europaea* lineage. One sequence was related to the genus *Nitrosospira*. These findings are consistent with other research suggesting that *Nitrosomonas* species, specifically the species *N. oligotropha*, are better adapted to low ammonia concentrations than other AOB and dominate the drinking water distribution system (28, 29).

The filters operate under relatively limited ammonia concentrations and, thus, limited nitrite concentrations, so *Nitrospira* species are expected to be the dominant nitrite oxidizers due to their lower half-saturation coefficient for oxygen and nitrite compared to that of *Nitrobacter* (20, 33). This is supported by the fact that *Nitrospirae* accounted for approximately 51% of the clone library in this study.

Though many clones were related to the order *Rhizobiales*, no sequences were related to the genus *Nitrobacter*. Though *Nitrobacter* has a higher growth rate, its ability to compete for oxygen and substrate is lower than that of *Nitrospira* (13, 21).

Moreover, the detection of "*Candidatus* Nitrotoga artica" may give beneficial operational flexibility to the filter. These organisms have been shown to oxidize nitrite at temperatures near 4°C, possibly adding to the operational temperature range (1). *Rhizobiales*, though not typically related to aquatic environments, are presumably introduced by aquifer transport and may play a novel role in the nutrient-limiting environment (32).

Recent attention has been focused on the dominance of AOA in the environment (10, 36). Specifically, many studies have shown that *Archaea* are the dominant ammonia oxidizers in both soil and marine environments. To this end, archaeal-*amoA* primers were used to determine if AOA were present and to serve as a cloning insert for gene library construction and sequence analysis. No AOA were detected based on the absence of an archaeal-*amoA* amplicon. The lack of detection of AOA indicates that AOB dominate this system. This may be due to the fact that the raw water chemistry may inhibit the growth or physiology of these organisms or that the ammonia levels in the raw water saturate the AOA *amoA* enzyme and prevent the ability to oxidize the ammonia, thus selecting for AOB (12, 40).

Sphingomonas and *Rhizobiales* dominated the 16S rRNA gene clone library. *Sphingomonas* has been shown to degrade complex organic molecules, such as xenobiotics, chloro/nitro phenolics, and large polymers. They accomplish this via numerous dependent and independent metabolisms that may add to the operational flexibility of biologically active filters (4, 11, 15, 22, 37).

Members of *Rhizobiales* have demonstrated the ability to utilize a broad range of carbon sources under aerobic conditions (24). Studies on pure cultures of *Rhizobiales* have shown that they may also be capable of degrading methyl parathion, metolachlor, polyacrylamides, and quaternary ammonium alcohols, all potential source water contaminants (14, 23, 27, 39).

An interesting finding of this study was the fact that no known pathogenic bacteria were identified as the majority of the 16S rRNA gene clone libraries. A primary concern of biologically active filtration is whether or not the filter is hospitable for pathogenic organisms, so this finding is encouraging. Though such organisms may be sensitive to chlorination, the possibility exists for distribution system contamination via slough-off if there is a malfunction in chlorination or the organism is capable of forming endospores. Therefore, there exists a need to further study this question in greater detail.

SEM observations made on the medium indicate that the biofilm formed prior to the deposition of solids during the filtration process. During treatment, the observed outer inorganic layer is constantly forming due to iron(III) filtration and is removed via backwashing every 3 days. This sets up a dynamic environment of constant formation and removal. With the biofilm being the most probable source of nitrification, this layer must allow diffusion to the microorganisms. Based on these observations, it is also worth asking if the outer layer impacts biological activity and if the inorganic layer protects the biofilm from disruption from shear force during backwash. This possible protective function may account for the rapid recovery of biological activity after backwash (7). Previously published culture-dependent studies of this system may support this notion, though a complete analysis is required to draw strong conclusions (9, 19).

The authors acknowledge that phylogenetic identification does not imply physiology, and caution is suggested in interpretation of culture-independent studies of microbial ecology. To this end, the metabolic diversity known to exist in organisms identified in biologically active filters may provide the only means to remove complex contaminants from source waters. With that said, such filters may serve as a unique source for isolation of novel organisms that may be beneficial for bioremediation.

Nucleotide sequence accession numbers. Sequences of one member from each OTU generated in this study were submitted to GenBank. *amoA* sequences fall within accession numbers GU596402 to GU596410. 16S rRNA gene sequences fall within accession numbers HM921089 to HM921151 and JX101440 and JX101441.

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