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Groundwater Chemistry Determines the Prokaryotic Community Structure of Waterworks Sand Filters

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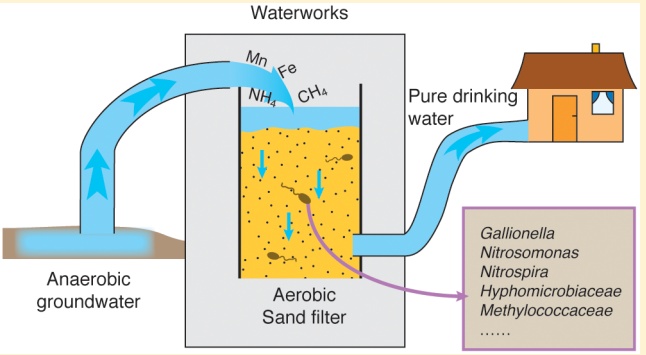
Supporting Information

ABSTRACT: Rapid sand filtration is essential at most waterworks that treat anaerobic groundwater. Often the filtration depends on microbiological processes, but the microbial communities of the filters are largely unknown. We determined the prokaryotic community structures of 11 waterworks receiving groundwater from different geological settings by 16S rRNA gene-based 454 pyrosequencing and explored their relationships to filtration technology and raw water chemistry. Most of the variation in microbial diversity observed between different waterworks sand filters could be explained by the geochemistry of the inlet water. In addition, our findings suggested four features of particular interest: (1) *Nitrospira* dominated over *Nitrobacter* at all waterworks, suggesting that *Nitrospira* is a key nitrifying bacterium in groundwater-treating sand filters. (2) *Hyphomicrobiaceae* species were abundant at all waterworks, where they may be involved in manganese oxidation. (3) Six of 11 waterworks had significant concentrations of methane in their raw water and very high abundance of the methanotrophic *Methylococcaceae*. (4) The iron-oxidizing bacteria *Gallionella* was present at all waterworks suggesting that biological iron oxidation is occurring in addition to abiotic iron oxidation. Elucidation of key members of the microbial community in groundwater-treating sand filters has practical potential, for example, when methods are needed to improve filter function.



INTRODUCTION

Many groundwater aquifers are anaerobic, and to make the water potable, it is often aerated and treated in a biologically active filter, where important processes include removal of dissolved iron (Fe), manganese (Mn), ammonia, and easily biodegradable organic carbon (including methane). This may simply be achieved by aeration followed by rapid filtration through a biologically active sand filter.

In general, the drivers of the prokaryotic community structure in rapid sand filters are poorly understood, and it is unknown to what extent the community structure in different groundwater-treating sand filters vary. However, suggestions have been made that certain microorganisms are generally responsible for the different processes following aeration of the groundwater. For example, Tekerlekopoulou et al.[1] recently proposed *Nitrosomonas* spp. and *Nitrobacter* spp. to be the most important ammonium and nitrite oxidizers in waterworks sand filters, although other studies suggest that ammonium-oxidizing archaea and other ammonium- and nitrite-oxidizing bacteria may be involved as well.[2−4]

The biological oxidation of Fe(II) in sand filters is mainly attributed to *Gallionella* and *Leptothrix* spp.[1,5] Also, the oxidation of Mn(II) is often attributed to *Leptothrix* spp.;[5,6] although, the empirical data supporting this are scarce and many bacteria can oxidize Mn.[7] Knowledge on sand filter organisms responsible for other biological processes related to the shift from anaerobic to aerobic conditions, including methane oxidation and mineralization of organic carbon, is very limited.

Waterworks that treat groundwater differ in filter material, physical structure, operational practice, and geochemistry of the source water. As studies on bacterial community structures in groundwater-treating rapid sand filters are extremely scarce and until now have focused on single waterworks.[2,4,8] We compared the prokaryotic communities in the sand filters of 11 groundwater-treating waterworks. We hypothesized that the prokaryotic communities would differ among treatment filters from different waterworks and that the microbial communities would be shaped by their environment as for example filter design and groundwater chemistry. We selected the 11 waterworks on an east−west line with variation in geological deposits and aquifer characteristics as well as in treatment practice. The aims were to (1) investigate variability in the

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prokaryotic communities of different sand filters by 454 pyrosequencing of 16S rRNA genes, (2) examine links between these communities and their physical−chemical environment, and thereby (3) shed light on the bacterial groups responsible for the most important water-purifying processes occurring in the filters.

MATERIALS AND METHODS

Sample Collection. We collected samples from rapid sand filters of 11 waterworks in Denmark in June 2011 (Figure S1 and Table S1, Supporting Information). All waterworks abstract 8−10 °C anaerobic water for treatment by aeration followed by rapid sand filtration with water retention times of 6−26 min except Lindved waterworks having a retention time of 52 min (Table S1, Supporting Information). No disinfectants or chemical additives are used at any of the waterworks. All filters were backwashed regularly at 2−5 day intervals depending on pressure build-up due to clogging, except in Islevbro and Søndersø waterworks where intervals between backwashing were longer (10−17 days). At waterworks with only a single filter, samples were collected from the top (0−10 cm) and at a depth of 50 cm, being the approximate center of the total filter height. At facilities with two filters in series with no intermediate treatment (Astrup, Aike, and Svenstrup waterworks), these were considered as one large filter to simplify interpretation. At these waterworks, material was collected from the top of both the first and second filter; the latter was considered to be a "depth sample" taken from the approximate center of the total filter height.

We sampled by pushing a PVC tube (15 cm inner diameter) 60−70 cm into the filter. The top 10 cm of the filter material was then taken from the tube by a clean sewer grab and mixed. For 50 cm samples, the grab was used to remove filter material from the PVC tube to a depth of 45 cm before taking the 50 cm sample. Filter material for inorganic analyses was stored in glass jars at 5 °C. Samples for DNA extraction were placed in sterile 50 mL polyethylene vials, immediately frozen in liquid nitrogen and kept frozen until extraction. The grab and PVC tube were cleaned with 70% ethanol before sampling.

Raw water was sampled from the inlet of each waterworks and kept in glass bottles for subsequent analysis of Fe and Mn.

Physical and Chemical Parameters. Fe and Mn in the filter material coatings were determined by extracting 1 g of filter material overnight with 100 mL 0.2 M oxalic acid and 0.2 M ammonium oxalate (pH 3) for atomic absorption spectroscopy (PerkinElmer AANALYST 400, Waltham, MA). Specific surface area was determined for 1 g of material using the Brunauer−Emmett−Teller (BET) method with adsorption of nitrogen gas in a Coulter SA 3100 BET analyzer (Coulter Corp., Miami, FL). Total amounts of Fe and Mn in raw water were determined directly by atomic absorption spectroscopy after addition of 1% (v/v) concentrated HCl. Standards were treated similarly with HCl. The limit of detection was 0.1 mg/L.

Data on pH, nonvolatile organic carbon (NVOC), oxygen, and inorganic ions in water leaving the waterworks were obtained from the Danish National Well Database.[9] The abstracted groundwater was anaerobic in all cases with substantial concentrations of dissolved Fe, Mn, and ammonium and in some cases methane. Therefore, nitrate in the water leaving the waterworks must derive solely from ammonium in the raw water, except in Hvidovre waterworks, where two of seven abstraction wells contained nitrate. The methane and ammonium concentrations in raw water were estimated as the average concentration of methane and ammonium in the wells used to abstract groundwater.

DNA Extraction. DNA was extracted using Fast DNA spin kits for soil (MP Biomedicals, Solon) according to the manufacturer's instructions. Four DNA extractions were performed using four individual subsamples from each filter sample. Prior to extractions, 2 mL of filter material were sonicated for 5−6 min to loosen bacterial biofilms from the sand grains. Samples were vortexed, and 500 mg of the material was recovered into Lysing Matrix E tubes provided with the extraction kit. The four DNA extractions from each sample were pooled and stored frozen until further analysis.

PCR Amplification and Pyrosequencing. Samples were prepared for 454 pyrosequencing using two-step PCR.[10] Initial PCR Mastermix was 1× Phusion HF buffer (with $MgCl_2$; Finnzymes Oy, Espoo, Finland), 0.2 mM of dNTP mixture, 0.5 U Phusion Hot Start DNA polymerase (Finnzymes), 0.5 μM of each primer, 1 μL of template, and sterile Milli-Q water to a final volume of 25 μL. Primers were MPRK341F (5′CCTAYGGGRBGCASCAG-3′) and MPRK806R (5′GGACTACNNGGGTATCTAAT-3′), slightly modified from Yu et al.[11] The primer set targets the 16S rRNA genes flanking the V3 and V4 regions with an overall coverage of 85% and 80% for bacteria and archaea, respectively (Table S3, Supporting Information). PCR conditions were an initial denaturation step of 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 5 s, annealing at 56 °C for 20 s, elongation at 72 °C for 20 s, and a final extension step of 72 °C for 5 min. Immediately before running on 1% agarose gels with ethidium bromide for UV visualization, PCR products were incubated at 70 °C for 3 min and then transferred to ice. The bands of PCR products were cut from the gels and purified using Montage DNA Gel Extraction kits (Millipore, Bedford, MA).

To add adaptor and tags to the PCR products, we performed a second round of PCR using DNA fragments from the purified bands as templates. The second PCR amplification was performed as described above, except that we used the primers MPRK341F and MPRK806R with adaptors and 22 barcodes of 10 nucleotides length (on the forward primer). Further, the number of cycles for denaturation, annealing, and elongation was reduced to 20. The PCR products were processed, run on agarose gels, and purified as described above.

Amplified fragments with adapters and tags were quantified using a Qubit fluorometer (Invitrogen, Life technologies, Carlsbad, CA) and mixed in equal concentrations (10^8 copies μL^{-1}) to ensure equal representation of each sample. A 454 sequencing run was performed on a 70_75 GS PicoTiterPlate using a GS FLX pyrosequencing system according to the manufacturer's instructions (Roche, Mannheim, Germany). Sorting and trimming of sequences >150 bp was done by the Pipeline Initial Process (http://rdp.cme.msu.edu) as previously described.[12]

Bioinformatic Analyses. Sequence processing was performed in Mothur.[13] Primer and sample tag sequences were removed, but the sample association was stored as metadata associated with each sequence. Sequences shorter than 375 bases and longer than 550 bases were removed. High quality sequences were selected by removing singletons and sequences with average read quality <25 (base error approximately 1 in 300) or with bases with a quality score <10. Multiple sequence alignments were performed using the program MAFFT, version

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Table 1. Main Physical and Chemical Characteristics of Filter Material and Water at Each Waterworks[a]

	filter material						raw water				treated water	
	Fe (mg/g)		Mn (mg/g)		BET (m^2/g)		Fe	Mn	CH_4	NH_4^+	NO_3^-	Ca^{2+}
	top	depth[b]	top	depth[b]	top	depth[b]	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Astrup[c]	216/296	2	11/12	18	181/174	5.3	11	0.51	<0.01	0.06	0.25	38
Aike	62	1.1	58	2.2	31	1.3	2.6	0.56	0.2	0.1	0.25	60
Vejle	107	69	45	16	43	42	2.0	0.35	0.05	0.3	1	81
Svenstrup	17	1.8	0.4	14	1.4	0.6	1.7	0.51	0.6	0.4	1.3	150
Odense	74	75	16	16	55	60	5.2	0.26	<0.01	0.3	1.6	100
Lindved	65	66	15	16	39	37	0.9	<0.1	0.03	0.4	1.4	100
Lunde	7.5	N.S	9.6	N.S	6.3	N.S	7.8	0.34	0.5	0.4	1.4	100
Landet	128	107	88	70	72	80	1.2	0.55	N.A.	0.1	0.6	160
Islevbro	118	9.1	80	17	139	5.7	1.7	<0.1	<0.01	0.5	2.0	130
Søndersø	97	11	63	17	92	4.1	2.2	0.12	0.05	0.4	2.2	110
Hvidovre	84	43	19	12	40	26	1.9	<0.1	<0.01	0.5	3.9	180

[a]In all cases, treated water contained less than 0.1 mg/L Fe and less than 0.02 mg/L of both Mn and ammonium. Data on filter materials and raw water are from this study. Data on treated water and methane in raw water from the year of sampling (2011) were derived from the Danish National Well Database[9] BET is the specific surface area. Data on additional chemical and physical parameters included in the statistical analyses may be found in Tables S1 and S2 of the Supporting Information. N.A.: not analyzed. N.S.: not sampled. [b]Depth samples are from 50 cm depth, except in the case of Astrup, Aike, and Svenstrup, where it is the top sample from the second filter in series (see Materials and Methods). [c]Number before slash sign is top sample; number after slash sign is the "intermediate sample" at 40 cm depth in the first filter.

6.925,[14] with the E-INS-i strategy assuming multiple conserved regions and long gaps. After subsequent preclustering to an alignment of known 16S bacterial sequences, chimera check with UCHIME,[15] and removal of singletons, 251,472 sequences passed the initial quality assessment. We then aligned the sequences and clustered them into OTUs using the furthest neighbor algorithm with 97% similarity threshold. We obtained a total of 2877 prokaryote OTUs based on sequence data clustering. These OTUs were taxonomically identified using the Bayesian RDP classifier[16] on the combined SILVA Bacteria and Archaea database, applying a posterior probability threshold of 50. Sequences were unevenly distributed between samples, with n ranging from 558 to 68,918. A standardization to 558 sequences per sample resulted in 946 OTUs; however, a visual inspection of the rarefaction curves (using the rarefaction.single command in Mothur), clearly indicated that subsamples were unsaturated. Excluding four of 22 filter samples (Astrup-T, Aike-T, Odense-D, and Lindved-D) enabled a standardization to 2147 sequences per sample with totally 1617 OTUs giving rarefraction curves much closer to saturation (Figure S3, Supporting Information).

The same taxonomic identification was done for the data set with 22 samples and 558 sequences per filter for comparison. We used the better justifiable standardization to 2147 sequences per sample for the detailed analysis and comparison with the chemical and physical parameters of the water and filter materials.

Sequences have been deposited and are publicly available from the GenBank database (Study Accession No. SRP047414).

Statistical Analyses. Nonmetric multidimensional scaling (NMDS) (PCord, Version 5.0, MjM Software, Gleneden Beach, OR) was used to analyze the similarity of prokaryotic communities. The main matrix consisted of 1617 OTUs from 18 filter samples, while the secondary matrix consisted of the physical–chemical parameters of the raw water (Fe, Mn, methane, and ammonia), treated water (pH, dH-hardness, conductivity, NVOC, Pt-color, O_2, NO_3^-, Cl^-, SO_4^{2-}, P-total, Ca^{2+}, Mg^{2+}, K^+, and Na^+), and filter material (Fe, Mn, BET, age, and backwashing interval). Analysis of the main matrix was done by the Bray–Curtis distance measure using 250 runs followed by 500 iterations to evaluate stability.

RESULTS

Physical and Chemical Characterization of Water and Filters. The studied waterworks are located on an east–west line with clear changes in geological top deposits with fluvial sand in the west (Astrup and Aike waterworks) followed by clay-rich moraine deposits with underlying sandy aquifers used for water abstraction (Vejle S, Svenstrup, Odense, Lindved, Lunde, and Landet) and in the east clay-rich moraine with underlying chalk from which the water is abstracted (Islevbro, Søndersø, and Hvidovre) (Table S1, Supporting Information). Geological settings were generally reflected in the water. For example, water from the western waterworks was softer, with less calcium (Ca) and magnesium (Mg) (Table 1). The raw water from these waterworks also contained less ammonium and hence less nitrate in the treated water. The raw water was anaerobic and in all cases but one without nitrate, which means that nitrate in the treated water reflects the ammonium concentration in the raw water. Hvidovre waterworks was an exception, as two of seven abstraction wells contained nitrate. Nitrite was not detected in any of the treated waters; thus, the nitrification was complete and well-functioning in all filters. The concentration of reduced Mn and especially Fe varied considerably in the raw water, from 10.7 mg/L Fe in Astrup water to less than 1 mg/L Fe in Lindved water. Astrup waterworks received the softest water, low in Ca and Mg, and with the lowest concentration of ammonia and NVOC and highest concentration of Fe.

Fe and Mn in the raw water resulted in extensive coating of the filter material by oxide precipitates (Table 1; Figure S2, Supporting Information). These coatings indicated variation in the function of different parts of the filter material regarding precipitation of metal oxides. For example, at the Astrup and Svenstrup waterworks, samples from the first filters showed reddish Fe oxide coatings, while the second filters were coated mainly by dark Mn oxides. Generally, also in samples from the single filters, more Fe was in the top, compared to the depth samples (Table 1). A strong linear correlation was found

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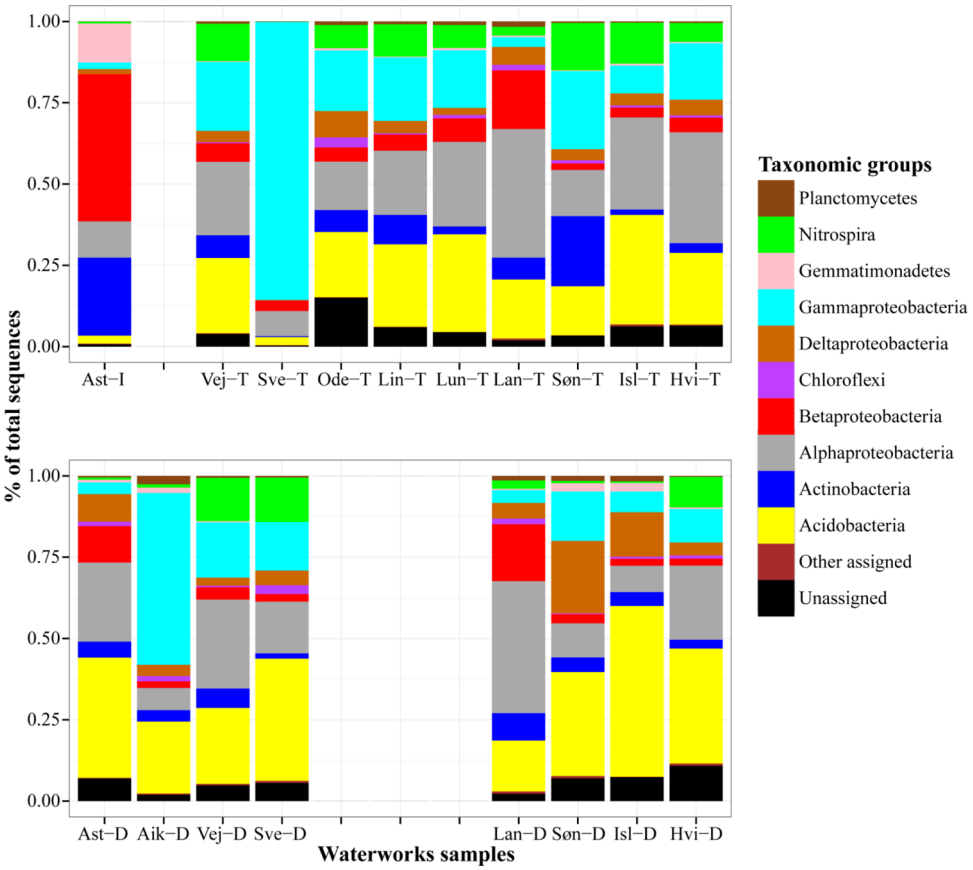

Figure 1. Prokaryotic groups in sand filters from 11 groundwater-treating waterworks (18 filter samples). Bacterial phyla (*Proteobacteria* separated into classes) are shown as percentage of 2147 sequences from each filter sample. For clarity, taxonomic groups not represented by at least 1% in at least one sample are not included. (Upper panel): Top samples (0−10 cm) of first filter and intermediate sample for the Astrup filter. (Lower panel): Samples taken at approximately 50% total filter depth. Only the initial three letters of each waterworks name are shown. Ast-T, Aik-T, Ode-D, and Lin-D were excluded from the analysis due to low number of sequences, but a similar figure with all samples but lower number of sequences is shown in Figure S4 of the Supporting Information.

between the amounts of Fe oxides precipitated and the surface area of the filter material (R^2 = 0.87).

Distribution and Abundance of Bacterial Groups in Sand Filters. A total of 2877 prokaryotic OTUs were found by sequence data clustering of the 251,472 sequences obtained from all 22 filter samples. The overall distribution of the main prokaryotic groups (phyla or classes) showed a dominance of sequences within *Acidobacteria* (27%), *Gammaproteobacteria* (21%), *Alphaproteobacteria* (20%), *Nitrospira* (8%), and *Betaprotobacteria* (6%), which account for 81% of all sequences (Figure S5, Supporting Information). These groups also constituted most (63%) of the OTUs; although, for example, *Nitrospira* constituted only 3% of the OTUs compared to 8% of the sequences, which reflects few but very common species within the *Nitrospira*.

Having standardized the results to a subset of 2147 sequences from 18 filter samples, the average percentage distribution of prokaryotic groups was *Proteobacteria* (54%), *Acidobacteria* (25%), *Actinobacteria* (7%), *Nitrospira* (6%), *Gemmatimonadetes* (1%), *Chloroflexi* (1%), *Planctomycetes* (0.9%), *Cyanobacteria* (0.2%), and *Bacteroidetes* (0.1%). We further found some rare sequences within other phyla and a few archaeal sequences. For 4% of the sequences, a prokaryotic group could not be assigned. Figure 1 shows the distribution of the different phyla for each filter. A further split into the dominant taxonomic orders and families is available in Table S4 of the Supporting Information.

We found only a minor difference on the distribution of main bacterial groups between the analysis on 2147 sequences from each of 18 filter samples compared to 558 sequences from all 22 samples (Figure 1 vs Figure S4, Supporting Information). The distribution of the major bacterial groups of all 22 samples is therefore included in the Supporting Information to get an impression on which groups are abundant in the Astrup-T, Aike-T, Odense-D, and Lindved-D samples as well (Figure S4, Supporting Information). If not stated otherwise, the following Results and Discussion sections are based on the data set with 18 filter samples (Figure 1 and Table S4, Supporting Information).

Alphaproteobacteria dominated in all filter samples, comprising on average 21% of all sequences. Half of these belonged to *Hyphomicrobiaceae* of which 60% were *Hyphomicrobium* and 30% were *Pedomicrobium*. The abundance varied somewhat between filters and depths, but all waterworks had at least one sample with more than 7% *Hyphomicrobiaceae*. This suggests *Hyphomicrobiaceae* to be widespread in groundwater-treating waterworks. No other *Alphaproteobacteria* group was particularly abundant.

Gammaproteobacteria were the second most abundant proteobacterial class and comprised on average 19% of all sequences. Of these, 75% were assigned to *Methylococcaceae*,

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which are Type I methanotrophs that obtain their carbon and energy from oxidation of methane. *Methylococcaceae* were especially abundant in Svenstrup waterworks, which also contains the highest methane concentration (~0.6 mg/L) in its raw water. *Methylococcaceae* constituted an impressive 85% of all sequences in the Svenstrup-T-sample. *Methylococcaceae* were also very abundant in the Aike, Vejle, Lindved, Lunde, and Søndersø samples. These are the five additional waterworks with significant methane concentrations in their raw water (0.05–0.5 mg/L, Table 1).

Betaproteobacteria was the third most abundant proteobacterial class, comprising on average 8% of all sequences. As much as 50% belonged to Fe(II)-oxidizing *Gallionellaceae* and 25% to ammonium-oxidizing *Nitrosomonadaceae* (Table S4, Supporting Information). Both families were present in all waterworks. In the samples from the first Astrup waterworks filter, *Gallionellaceae* (genus: *Gallionella*) was extremely dominating and comprised 41% of the sequences in the Astrup-I sample and even more in the Astrup-T sample (using the 22-sample data set for Astrup-T). *Gallionella* was also abundant in the Landet waterworks and in the Aike-T sample (using the 22-sample data set for Aike-T).

Apart from *Proteobacteria*, *Acidobacteria* was the most dominant phylum, most belonging to *Acidobacteriaceae*. Furthermore, as the only bacterial group, *Acidobacteria* were clearly more abundant in the depth samples (32% on average) than in the corresponding top samples (19%).

The two major remaining bacterial groups, the Gram-positive *Actinobacteria* and nitrite-oxidizing *Nitrospirae*, each constituted 6–7% of all sequences. *Actinobacteria*, primarily *Acidimicrobineae*, were particularly abundant in the samples from the first filters of Astrup and Søndersø. *Nitrospirae* (genus: *Nitrospira*) were abundant in all waterworks and found both in top and depth samples (on average 7.2 and 5.2% of all sequences, respectively).

Analysis of Similarity and Relationship with Physical–Chemical Parameters. To determine the relatedness of the prokaryotic communities, we performed a nonmetric multidimensional scaling (NMDS) analysis on the distribution and abundance of the 1617 OTUs from the 18 filter samples (Figure 2). The *Gallionella*-dominated sample from the first filter of the Astrup waterworks was very distinctive on the second axis. Likewise, the Svenstrup-T sample differed from the other samples but on axis 1 only. This distinctiveness is probably caused by the 85% *Methylococcaceae* sequences in Svenstrup-T. This is supported by the finding that the same *Methylococcaceae* (same OTUs) were abundant also in Søndersø-T, Vejle-T, Vejle-D, Lin-T, and Lun-T, the samples being closest to Svenstrup-T in the NMDS plot. *Methylococcaceae* were also abundant in Aike-D but with dominance of a different *Methylococcaceae* OTU resulting in a different position in the NMDS plot.

At Landet, Vejle, and Hvidovre waterworks in particular, there were only minor differences in the microbial community structure of the top and depth of the filters (Figure 2). The materials from the top and depth of these filters also appeared very similar (Figure S2, Supporting Information) and had similar values for Fe, Mn, and BET surface area (Table 1). In contrast, at Astrup, Svenstrup, and Søndersø waterworks, there were clear differences in the microbial community structures between top and depth samples. Accordingly, there were also clear differences in the coating of the top and depth material by metal oxides (Table 1 and Figure S2, Supporting Information).

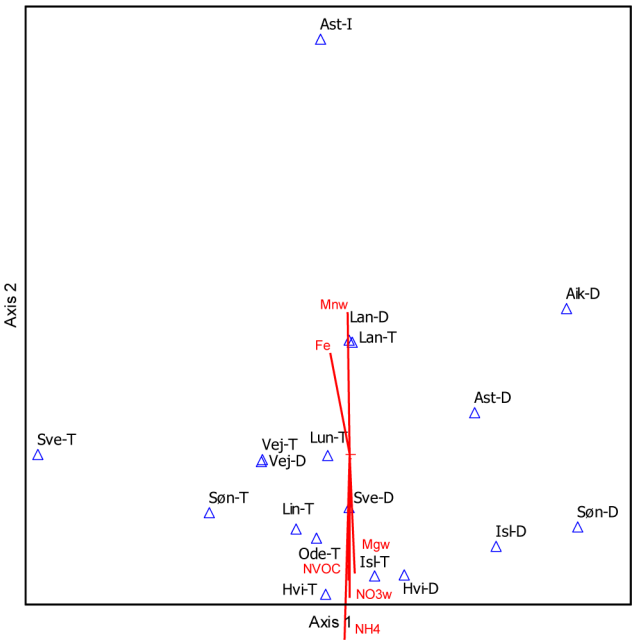

Figure 2. Two-dimensional NMDS plot with Bray–Curtis distances for 18 filter samples based on pyrosequencing data. -T is top-samples. -D is depth samples. -I is intermediate sample from the first filter. Only the initial three letters of each waterworks name are shown. The NMDS plot is superimposed with regressions between 21 chemical and physical parameters and the NMDS plot axes. Lengths of the superimposed lines denote regression coefficients (correlations strengths). Only parameters with regression coefficient (R^2) > 0.35 are shown. Fe is iron precipitates on filter material. Mn_w is dissolved manganese in raw water. Mg_w is Mg^{2+} in treated water. $NO3_w$ is nitrate in treated water. NVOC is nonvolatile organic carbon in treated water. NH4 is ammonium in raw water.

Correlation coefficients between individual physical and chemical parameters and the distribution axes in the NMDS analysis were in general low indicating that several parameters contribute to the development of the bacterial community structure distribution of the filters. Mn and ammonium in inlet water were the parameters with the strongest correlations with the prokaryotic community structure distribution of the filters.

DISCUSSION

On the basis of our results from 11 waterworks, it is clear that rapid sand filters that treat anaerobic groundwater are bacterially diverse. The difference between waterworks is however surprisingly small with the exception of one waterworks dominated by iron oxidizers (Astrup) and another dominated by methane oxidizers (Svenstrup). This dominance may be explained by the high concentrations of iron and methane, respectively, in the raw water of these waterworks.

Most of the bacteria found may be connected to the main functions of the filters (oxidation of Fe, Mn, ammonium, and methane). The bacterial community structure therefore reflects the shift from anaerobic to aerobic conditions that occurs immediately before water enters the filters and the subsequent microbially mediated redox processes.

Nitrosomonas are generally believed to be the major organism responsible for ammonium oxidation in waterworks.[1] For example, *Nitrosomonas* were abundant in groundwater-treating rapid sand filters located in Ohio, U.S.A., and Henan Province, China, respectively.[4,8] We found *Nitrosomonadaceae* including

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Nitrosomonas and/or the related ammonium oxidizer *Nitrosospira* in all waterworks. Generally, they made up 1−2% of all sequences, although up to 9% at one waterworks. We also found the archaeal ammonium oxidizer *Crenarchaeota*[17] in our analysis but in low numbers. It should be noted that the coverage of the primers we used is high for *Nitrosomonas* (94%), *Nitrosospira* (91%), and *Crenarchaeota* (82%). De Vet et al.[2] found that both archaea and bacteria oxidized ammonia in a Dutch sand filter used for groundwater treatment. Also, in other cases, ammonium-oxidizing archaea have been found to be important during water purification, especially at low ammonium concentration.[3] We suggest that future studies should specifically target both ammonium-oxidizing archaea and bacteria to shed further light on their relative importance.

In some waterworks, the nitrite-oxidizing *Nitrospira* was particularly abundant in the top samples, suggesting that nitrification occurs in the top of these waterworks. However, in others, *Nitrospira* were more dominant in the depth samples, suggesting that most of the nitrite oxidation may occur at lower depths in some waterworks. Previous studies have shown that nitrification can occur solely in the top of rapid sand filters[18] or at several depths and even with higher activity at greater depth.[19] The reason for this difference between filters remains unclear. The abundance of *Nitrospira* showed positive relationships with nitrate in treated water and with ammonia in raw water, which further supports *Nitrospira* as responsible for the nitrite oxidation (Figure 3). We did not detect *Nitrobacter* in any of the filters regardless of the range of ammonium concentrations in raw water (from ∼0.06 to ∼0.5 mg/L). This is in agreement with previous reports of *Nitrospira* being the prime nitrite oxidizer in sand filters used to treat aerated groundwater[2,4] and in filters used to treat mixed groundwater and surface water.[20] Our findings contrast the hypothesis that *Nitrobacter* spp. are the dominating nitrite oxidizers at waterworks, including during groundwater treatment.[1]

We recorded the Fe-oxidizing betaproteobacterium *Gallionella* sp. in top samples from all waterworks. Because *Gallionella* can gain energy from oxidation of Fe only,[21] this implies a contribution from biotic Fe oxidation in groundwater-treating waterworks in addition to abiotic Fe oxidation. At the waterworks with very high inlet concentrations of dissolved Fe accompanied by thick Fe oxide coatings on the sand grains, *Gallionella* was particularly dominant. Hence, *Gallionella* is probably the most important iron oxidizer at this waterworks (Astrup), which is also designed specifically to biological Fe oxidation with low pH and low oxygen levels (2−3 mg/L) in the first filter. Our results are consistent with previous findings, which suggested the importance of *Gallionella* in Fe oxidation of water-treatment systems.[2,21,22]

Some filters furthermore contained a high number of the actinobacterial *Acidimicrobineae* (6 and 14% of total sequences at Astrup-I and Søndersø-T, respectively). This family includes Fe(II) oxidizers, for example, *Acidimicrobium ferrooxidans*, suggesting that other Fe oxidizers than *Gallionella* occur in the sand filters.

Oxidation of reduced Mn is an important process in sand filters that treat anaerobic groundwater containing Mn. Hope and Bott[6] considered *Leptothrix* as the dominant Mn oxidizer in sand filters. Similarly, de Vet et al.[2] found some *Leptothrix*-related species in a trickling filter used to treat groundwater and suggested *Leptothrix* to be involved in the oxidation of Mn. *Leptothrix* belongs to the betaproteobacterial *Comamonadaceae*, which is sparsely represented in our samples (Table S4,

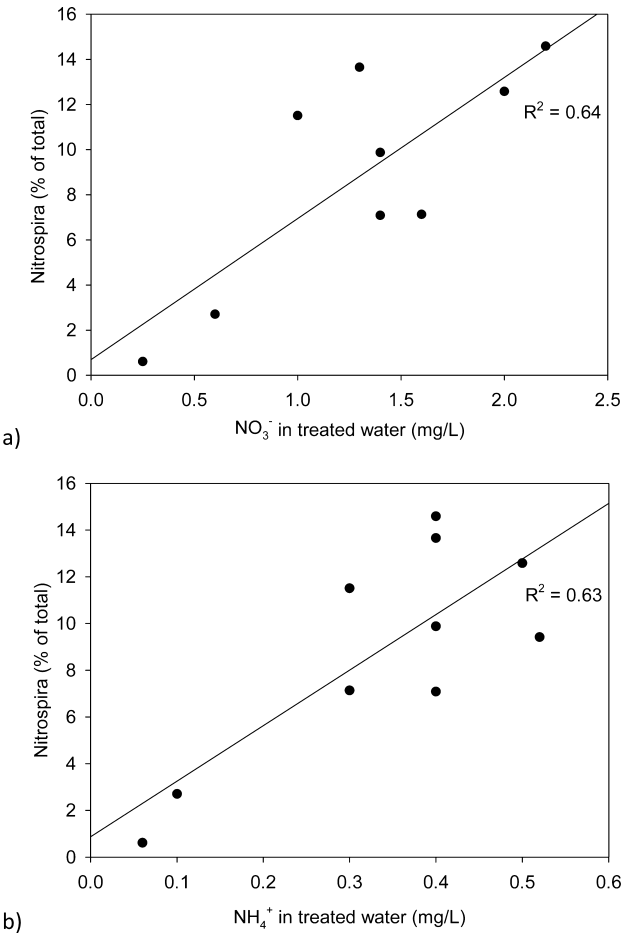

Figure 3. Relationship between *Nitrospira* and nitrification in groundwater-treating waterworks. *Nitrospira* in % of all sequences in top samples vs (a) nitrate in the treated water or (b) ammonia in raw water. Because the nitrite oxidation apparently occurred at lower depths in the Svenstrup and Hvidovre waterworks, *Nitrospira* abundance in depth samples were used for these two waterworks, and furthermore, Hvidovre was excluded from (a) because the raw water contained some nitrate.

Supporting Information). By contrast, *Hyphomicrobiaceae* was dominant in all samples, represented by 60% *Hyphomicrobium* and 30% from the closely related *Pedomicrobium*. Whereas *Pedomicrobium* is a well-known Mn oxidizer found in water distribution systems,[27] *Hyphomicrobium* have not previously been associated with Mn oxidation at waterworks but are normally found in Mn-rich deposits where it probably oxidizes Mn.[7,23−26] We propose that *Hyphomicrobium* and/or *Pedomicrobium* spp. are the key Mn oxidizers at all 11 waterworks, so *Leptothrix* might not be a suitable model organism for Mn oxidation in waterworks sand filters, as previously suggested.[6] In accordance with our results, White et al.[4] found no *Leptothrix* but several *Hyphomicrobium* species in waterworks sand filters, and Pinto et al.[20] found no further identified *Rhizobiales* species. These authors, however, made no connection between their findings and Mn oxidation.

We notice that some members of *Hyphomicrobiaceae* degrade C1 compounds such as methanol and formaldehyde that could originate from methane oxidation by methanotrophs.[28] Only the aquifers that provide water to six of the 11 waterworks contain significant amounts of methane (0.03−0.6 mg/L, Table 1), however. In these six waterworks, members of *Methyl-*

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ococcaceae were dominant and the likely methane oxidizers and could in principle supply C1 compounds to *Hyphomicrobiaceae* members. This would however not explain the dominance of *Hyphomicrobiaceae* in also the remaining five waterworks. In future research, the proposed relationship between *Hyphomicrobiaceae* species and Mn oxidation in groundwater-treating rapid sand filters should be specifically targeted.

Acidobacteria occurred in very high numbers in most of the waterworks, which may be attributed to the generally low content of assimilable organic carbon in groundwater because *Acidobacteria* have been shown to dominate in environments with a low content of assimilable organic carbon.[29−31] More research is needed to determine the role of *Acidobacteria* in waterworks sand filters including their importance for the removal of residual levels of assimilable organic carbon, thereby minimizing the risk of bacterial regrowth in the clean water distribution system.

All in all, most of the variation in microbial diversity observed between different waterworks sand filters could be explained by the geochemistry of the inlet water. This finding is unique as it for the first time demonstrates a strong link between groundwater geochemistry and sand filter microbial diversity. Because all filters have been in operation for more than three years and receive groundwater with no or very limited seasonal variation in geochemistry and temperature, no seasonal variation in the prokaryotic community structure or functionality is expected, and the observed relationship between geochemistry and microbial community reflects some kind of equilibrium state. However, a proportion of the bacteria detected did not correlate with any known filter function or physical or chemical filter parameter. Future studies should investigate the role of these groups as the elucidation of the microbial community in groundwater-treating sand filters is of great practical potential, for example, in situations where methods are needed to improve filter function.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1: Location of the sampled waterworks in Denmark. Figure S2: Images of filter materials. Figure S3: Rarefaction curves. Figure S4: Distribution of bacterial phyla in the analysis with 558 sequences from each of 22 waterworks samples. Figure S5: Prokaryotic groups in the total data set. Table S1: Construction of waterworks and filters. Table S2: Physical and chemical characteristics of filter material and water. Table S3: Primer set coverage of eukaryotic phyla. Table S4: Relative abundance of the bacterial phyla divided into classes, orders, and families. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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