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ORIGINAL ARTICLE

Ecological patterns, diversity and core taxa of microbial communities in groundwater-fed rapid gravity filters

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Here, we document microbial communities in rapid gravity filtration units, specifically serial rapid sand filters (RSFs), termed prefilters (PFs) and after-filters (AFs), fed with anoxic groundwaters low in organic carbon to prepare potable waters. A comprehensive 16S rRNA-based amplicon sequencing survey revealed a core RSF microbiome comprising few bacterial taxa (29–30 genera) dominated by *Nitrospira*, *Proteobacteria* and *Acidobacteria*, with a strikingly high abundance ($75\text{--}87 \pm 18\%$) across five examined waterworks in Denmark. Lineages within the *Nitrospira* genus consistently comprised the second most and most abundant fraction in PFs ($27 \pm 23\%$) and AFs ($45.2 \pm 23\%$), respectively, and were far more abundant than typical *proteobacterial* ammonium-oxidizing bacteria, suggesting a physiology beyond nitrite oxidation for *Nitrospira*. Within the core taxa, sequences closely related to types with ability to oxidize ammonium, nitrite, iron, manganese and methane as primary growth substrate were identified and dominated in both PFs ($73.6 \pm 6\%$) and AFs ($61.4 \pm 21\%$), suggesting their functional importance. Surprisingly, operational taxonomic unit richness correlated strongly and positively with sampling location in the drinking water treatment plant (from PFs to AFs), and a weaker negative correlation held for evenness. Significant spatial heterogeneity in microbial community composition was detected in both PFs and AFs, and was higher in the AFs. This is the first comprehensive documentation of microbial community diversity in RSFs treating oligotrophic groundwaters. We have identified patterns of local spatial heterogeneity and dispersal, documented surprising energy–diversity relationships, observed a large and diverse *Nitrospira* fraction and established a core RSF microbiome.

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Introduction

Microbial ecology seeks to find out what forces shape and maintain the composition and structure of microbial communities; elucidate the origin of community and guild diversity and their link to system function, stability and resilience; and identify the interactions that stabilize or constrain microbial communities; among others. Answering those questions requires access to microbial communities that can be observed, sampled and described at relevant spatiotemporal scales, and with sufficient replication, while the system's environmental conditions and performance can be measured. Technical systems—where open microbial communities are subject to some degree of management—might provide useful alternatives to field systems—without suffering

from the simplicity of laboratory-based model systems. Here, we introduce the microbial communities that populate rapid sand filters (RSFs), which are constantly fed with anoxic groundwater (GW) after vigorous aeration, as model technical systems, and present our initial analysis on their composition and ecological patterns.

Rapid gravity filtration is a conventional treatment process whereby GW is intentionally passed through a porous medium bed, often consisting of sand (then called rapid sand filtration), to produce high-quality drinking water. Rapid gravity filters receive continuous inputs from aquifers over multiple years to decades and this input may vary depending on the temporal and spatial dynamics of the aquifer (Griebler and Lueders, 2009). Conditions in source aquifers are similar in that they lack the photosynthetic activity, are at low temperature, carry no dissolved oxygen and typically have low contents of organic carbon, yet they can differ in their content of inorganic (e.g., NH_4^+ , Mn^{2+} , Fe^{2+} , H_2S) as well as some organic electron donors (mainly CH_4) (Danielopol *et al.*, 2000), all potential energy sources for microbial growth.

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Studies on the diversity, abundance and distribution of microorganisms in GW-fed RSF have only recently been reported. Using 16S rRNA gene cloning, van der Wielen and van der Kooij (2009) and White *et al.* (2012) identified *Nitrosomonas oligotropha*, *Nitrosomonas marina* and *Candidatus Nitrosopumilus maritimus* as the dominant ammonia-oxidizing prokaryotes in three GW treatment plants. Pinto *et al.* (2012) and Albers *et al.* (2015), using 16S rRNA pyrosequencing, identified *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Nitrospira*, *Bacterioidetes* and *Planctomycetes* as the major phyla in the filtration units. Using a 16S rRNA-targeted DGGE approach, de Vet *et al.* (2009) identified a shift from iron-oxidizing bacterial dominance in the subsurface-aerated GW to ammonia-oxidizing archaea in above-ground RSF. In a previous study, using 16S rRNA pyrosequencing, we identified *Nitrosomonas europaea*, *Candidatus Nitrosoarchaeum* and *Nitrospira moscoviensis* as dominant nitrifiers in a GW-based full-scale RSF (Gülay *et al.*, 2014).

Reports on RSF microbial communities have typically involved limited sampling sites with insufficient replication within and across RSFs and across drinking water treatment plants (DWTPs) to allow inference of distribution patterns, identify the degree of filter-specific versus shared taxa across DWTPs or assess spatial variation of community structure within DWTPs.

We contend that further knowledge about the microbial community structure of RSFs and its core members is important, and RSF communities can provide suitable model systems to answer central microbial ecological questions. Core taxa have been identified in anaerobic digesters (Rivière *et al.*, 2009), marine environments (Pommier *et al.*, 2007) and activated sludge systems (Zhang *et al.*, 2012; Saunders *et al.*, 2015). Distribution patterns of shared taxa can reveal to what extent environmental conditions vis-à-vis dispersal drive microbial community structure across habitats (Shade *et al.*, 2012) and help identify the microbial taxa that govern central system functions. In addition, the effects of dispersal limitation (Bell, 2010), environmental heterogeneity (Ramette and Tiedje, 2007), energy input (Bienhold *et al.*, 2011) and system design and operation (Sundberg *et al.*, 2013) on microbial community structure are central to many environments, and RSFs might be ideally suited for their investigation.

Here, we extensively characterize the microbial communities at five different DWTPs. Both the feed GWs and the serial filtration units (termed prefilters (PFs) and after-filters (AFs)) were analyzed via 16S rRNA amplicon sequencing. Replicate samples were taken within individual filters and between filters. We first identified and quantified taxa shared among DWTPs. We then evaluated to what extent energy availability could explain bacterial richness and evenness patterns within DWTPs.

We also explored distance–decay relationship between aquifer microbial communities and the extent of dispersal within a DWTP. Finally, we examined the extent of local (within filter) spatial heterogeneity in microbial community structure at all RSFs as initiated before (Gülay and Smets, 2015).

Materials and methods

Study sites

Filter materials and GW samples were obtained from five DWTPs in Denmark (Supplementary Figure S1): first line of Langerød (DWTP1, 55°41'45''N, 11°40'44''E); second line of Langerød (DWTP2); Sjælsø-1 (DWTP3, 55°52'25''N, 12°28'36''E); Sjælsø-2 (DWTP4, 55°52'20''N, 12°28'32''E); Islevbro (DWTP5, 55°42'14''N, 12°27'21''E). Study sites are detailed in Supplementary Information.

Sampling procedures and physicochemical characteristics of GW and discharge water

Typical values of the dominant chemical constituents in the GW and performance at the different DWTPs are in Table 1. Water quality data (between 2009 and 2011) of raw and discharge water were obtained from the JUPITER database (Hansen and Pjetursson, 2011) and from direct contact with the water utilities, respectively. GW samples were collected from the main influent lines under anoxic conditions before the aeration unit. Microbial mass was then harvested from 250 ml GW using a sterile 0.2 µm pore size (Millipore, Billerica, MA, USA) membrane filter and stored at –20 °C. Filter samples were collected by inserting a 60-cm-long core sampler at a single PF and two separate AF units at each DWTP 6 days AF backwash (Supplementary Figure S2). Sampling procedures are detailed in the Supplementary Information.

DNA extractions, PCR amplification and pyrosequencing

DNA was extracted, amplified and subject to 16S rRNA amplicon 454 pyrosequencing, essentially following the protocol by Sundberg *et al.* (2013), as described in the Supplementary Information.

Real-time qPCR

Quantification of total bacteria (Eubacteria), the sum of *Nitrosomonas* plus *Nitrospira*, and *Nitrospira* was performed by 16S rRNA gene targeted quantitative PCR (qPCR) on replicates of DNA extracts and the results are described elsewhere (Tatari *et al.*, in preparation). The qPCR primers, PCR conditions and procedures used are described in the Supplementary Information. Results, as reported by Tatari *et al.* (in preparation), are used here to calculate relative and absolute abundance values.

Table 1 Influent conditions and performance of the studied drinking water treatment plants

Compound	Effluent guidelines ^a	DWTP1-2 ^b	DWTP3 ^c	DWTP4 ^c	DWTP5 ^c
Temperature (°C)	—	9.1 ± 0.4	9.5 ± 0.3	8.8 ± 0.3	9.3 ± 0.4
pH	7.0–8.5	7.4 ± 0.1	N/A	7.5 ± 0.1	7.4 ± 0.3
Dissolved oxygen (mg O ₂ l ⁻¹)	5	0.26 ± 0.11	0.65 ± 0.27	0.87 ± 0.44	1.09 ± 0.59
Ammonium (mg NH ₄ l ⁻¹)	0.05	1.06 ± 0.18	0.51 ± 0.21	1.08 ± 0.44	0.41 ± 0.2
Iron (mg Fe l ⁻¹)	0.1	3.0 ± 0.2	2.9 ± 1.7	1.9 ± 1.1	1.8 ± 1.1
Manganese (mg Mn l ⁻¹)	0.02	0.115 ± 0.010	0.14 ± 0.09	0.07 ± 0.06	0.06 ± 0.02
Hydrogen sulfide (mg H ₂ S l ⁻¹)	0.05	0.02 ± 0.00	0.03 ± 0.02	0.12 ± 0.27	0.02 ± 0.00
Methane (mg CH ₄ l ⁻¹)	0.01	0.06 ± 0.03	0.12 ± 0.17	3.73 ± 2.67	0.02 ± 0.03
Phosphorus (mg total P l ⁻¹)	0.15	0.269 ± 0.051	N/A	N/A	0.025 ± 0.01
Bicarbonate (mg HCO ₃ l ⁻¹)	—	361 ± 6	N/A	N/A	346 ± 48
Chloride (mg Cl l ⁻¹)	250	55 ± 15	51 ± 36	41 ± 15	64 ± 32
Sulfate (mg SO ₄ l ⁻¹)	250	7 ± 3	36 ± 22	12 ± 17	102 ± 28
NVOC (mg C l ⁻¹)	4	2.6 ± 0.3	2.7 ± 0.4	2.3 ± 0.3	2.3 ± 0.3
Treatment plant performance (with regard to guidelines)	—	Problems with incomplete ammonium removal Average effluent NH ₄ DWTP1: 0.061 ± 0.091 mg NH ₄ l ⁻¹ DWTP2: 0.193 ± 0.245 mg NH ₄ l ⁻¹ (values are averages of 27 measurements from 2010 until 2011) ^c None of the other above parameters exceeded the effluent guideline from 2009 until 2011 ^b	None of the above parameters exceeded the effluent guideline from 2009 until 2011 ^b (annotation: data only available for combined effluent of DWTP3 and DWTP4)	None of the above parameters exceeded the effluent guideline from 2009 until 2011 (n: 15—except for HCO ₃ ⁻ , CH ₄ and H ₂ S: six measurements) ^b	

Abbreviations: DWTP, drinking water treatment plant; N/A, no data available; NVOC, nonvolatile organic carbon.

Raw water values for DWTP5 and DWTP1–2 are weighted averages ± s.d. (weighting based on raw water abstraction data). Raw water values for DWTP3 and DWTP4 are average values ± s.d.

^aMiljøministeriet (2011). BEK nr 1024 af 31/10/2011: Bekendtgørelse om vandkvalitet og tilsyn med vandforsyningsanlæg—Danish Ministry of the Environment: Ministerial order on water quality and monitoring of water supply systems. Date of publication: 08/11/2011.

^bSee Jupiter database (Hansen and Pjetursson, 2011).

^cWater quality raw data obtained from the respective water utilities.

Bioinformatic and statistical analysis

Raw sequence data were quality checked (denoised) with Ampliconnoise (Quince *et al.*, 2011) and all analyses were performed using the QIIME 1.5.0 software (Caporaso *et al.*, 2010). Subsequent bioinformatics and statistical analyses are described in the Supplementary Information, and β-diversity significance testing was performed as described earlier (Gülay and Smets, 2015).

Nucleic acid sequences

Raw SFF files were deposited into the Sequence Read Archive at GenBank under the study accession number SRP045492.

Results

Overall measures of diversity

A total of 8 57 835 (74.7% of total) amplicon sequences (average length 421 nucleotides) passed all quality checks, including denoising and chimera removal, and were distributed across 33 019 operational taxonomic units (OTUs), defined at 97% sequence identity, hereafter called OTU_{0.03}. We rarefied to 4000 sequences per sample for further diversity and taxonomic comparisons.

While rarefaction analysis suggests some under-sampling of the metacommunity at all DWTPs based on individual or merged samples (Supplementary

Figures S3 and S4), the large degree of replication provided many subsamples of the metacommunity. Comparison of microbial diversity in RSFs with other environments showed a high degree of richness in RSFs, notwithstanding the much lower input of nutrients (Supplementary Table S2).

The Shannon index of the GW community was twofold higher compared with that of the PF and AF communities; consistently, the Gini coefficient was lowest in the GW communities. Hence, the GW community had the most even distribution of OTU_{0.03}s. The community richness as well as evenness was consistently higher in the AF compared with the PF (Table 2 and Supplementary Figure S5).

Microbial community composition

Of all identified OTU_{0.03}s, 99% (32 627) could be taxonomically assigned using NCBI BLASTN analyses (Altschul *et al.*, 1997) at a minimum identity of 90%. Thousand seventy-five taxa were identified at the genus level from 21 941 OTU_{0.03}s after sample size normalization. The communities were dominated by bacteria comprising 89.9%, 99.5% and 99.7% of all sequences in GW, PF and AF, respectively (Supplementary Table S3). At the phylum level, a large fraction of the sequences in PFs and AFs (89.56 ± 19% and 87.62 ± 15%, respectively) were *Nitrospirae*, *Proteobacteria* and *Acidobacteria* (Figure 1). *Proteobacterial* OTU_{0.03}s in

Table 2 Diversity estimates of the microbial communities at different locations and units at investigated waterworks

Water works	Unit	Zone	Number of sequences ^a	Observed OTUs ^b	Richness estimates			Evenness	Diversity
					Singleton OTUs _{0.035}	Chao1	ACE	Gini _{coeff}	H
DWTP1	GW		6758	372	192	1005	890	0.841	8.5 ± 0.02
	PF	Top	12 050 ± 2396	322 ± 4	175 ± 3	546 ± 81	641 ± 99	0.890 ± 0.01	4.1 ± 0.18
		Depth	12 414 ± 3826	361 ± 10	193 ± 34	604 ± 130	742 ± 159	0.881 ± 0.01	4.2 ± 0.24
	AF	Top	13 908 ± 787	439 ± 66	257 ± 56	779 ± 130	909 ± 161	0.862 ± 0.01	4.8 ± 0.17
Depth		14 361 ± 1988	497 ± 69	296 ± 47	959 ± 155	1100 ± 178	0.855 ± 0.01	4.8 ± 0.40	
DWTP2	GW		6392	1105	772	3046	3352	0.683	6.1 ± 0.03
	PF	Top	7738 ± 553	393 ± 47	284 ± 31	1142 ± 182	1337 ± 182	0.895 ± 0.01	4.3 ± 0.20
		Depth	7113 ± 622	406 ± 30	286 ± 26	1406 ± 59	1410 ± 97	0.888 ± 0.01	4.5 ± 0.05
	AF	Top	9869 ± 3891	512 ± 45	340 ± 49	1361 ± 362	1525 ± 330	0.862 ± 0.01	4.6 ± 0.09
Depth		11 757 ± 4330	480 ± 62	301 ± 71	1275 ± 473	1371 ± 505	0.868 ± 0.01	4.5 ± 0.02	
DWTP3	GW		18746	863	527	1695	1895	0.775	6.8 ± 0.04
	PF	Top	6803 ± 1332	470 ± 69	343 ± 55	1639 ± 302	1719 ± 336	0.929 ± 0.01	3.1 ± 0.60
		Depth	6988 ± 751	564 ± 50	427 ± 58	1893 ± 233	2241 ± 401	0.879 ± 0.04	3.3 ± 0.43
	AF	Top	6406 ± 1228	802 ± 81	589 ± 79	2694 ± 494	3008 ± 520	0.721 ± 0.03	7.6 ± 0.01
Depth		6803 ± 1332	784 ± 105	590 ± 114	2580 ± 591	2973 ± 708	0.716 ± 0.03	7.5 ± 0.41	
DWTP4	GW		16 480	1169	676	2170	2373	0.667	8.6 ± 0.03
	PF	Top	6397 ± 930	239 ± 45	161 ± 39	750 ± 202	770 ± 296	0.879 ± 0.01	4.0 ± 0.37
		Depth	7498 ± 888	357 ± 42	258 ± 34	1222 ± 165	1311 ± 236	0.852 ± 0.01	4.6 ± 0.26
	AF	Top	6880 ± 952	1106 ± 192	850 ± 184	4368 ± 1194	4932 ± 1526	0.784 ± 0.02	6.4 ± 0.08
Depth		6390 ± 782	1104 ± 176	844 ± 164	4599 ± 1715	5013 ± 1689	0.792 ± 0.01	6.3 ± 0.47	
DWTP5	GW		16 648	962	593	1808	2183	0.735	7.5 ± 0.03
	AF	Top	8084 ± 740	343 ± 68	241 ± 45	1131 ± 317	1233 ± 354	0.909 ± 0.01	3.4 ± 0.36
		Depth	7650 ± 1026	562 ± 181	422 ± 142	1913 ± 742	2136 ± 853	0.859 ± 0.04	4.6 ± 1.02

Abbreviations: ACE, abundance-based coverage estimator; AF, after-filter; DWTP, drinking water treatment plant; G_{coeff}, Gini coefficient; GW, groundwater; H, shannon index; OTU, operational taxonomic unit; PF, prefilter.

All sample sizes were equalized at 4000 sequences. Standard deviations (±) were obtained from biological replicates; standard deviations in the top and bottom layers were assessed from triplicate samples from the same filter. GW samples were taken as individual samples.

^aNumbers of sequences in PF and AF rows show the total amount of high-quality sequences that were obtained from 10 ng of DNA extracted from 0.5 g of drained wet sand grains. Mean values were calculated by grouping the samples according to zones, units and waterworks.

^bOTU calculations were carried out at 97% phylogenetic similarity.

the PFs and AFs were distributed across the *gamma* (19.8 ± 15%), *alpha* (13.9 ± 5%), *beta* (7.4 ± 5%) and *delta* (4.7 ± 7%) subphyla.

In PFs and AFs, absolute bacterial abundances, expressed in gene copies per gram of filter material, based on broad and clade-specific 16S rRNA-targeted qPCR ranged from 2 to 12 and 1 to 4 × 10⁹ total bacteria, 6 to 23 and 0.4 to 15 × 10⁷ AOB (*Nitrosomonas* plus *Nitrosospira*), 8 to 370 and 3 to 19 × 10⁴ *Nitrobacter* and 2 to 24 and 1 to 9 × 10⁸ *Nitrospira*, respectively, across the examined DWTPs (Tatari *et al.*, in preparation). Comparing qPCR results on individual samples confirmed the high relative abundance of *Nitrospira* in all samples (Figure 2a). The qPCR estimated abundances are somewhat lower compared with those estimated by amplicon sequencing, but overrepresentation of abundant taxa when using universal primers in PCR-based surveys is well documented (Gonzalez *et al.*, 2012). Nevertheless, even qPCR estimates reveal an extremely high *Nitrospira* (on average 17 ± 11.6%) and much lower AOB abundance (2.26 ± 2.79%) consistent with amplicon sequencing estimates (Figure 2b).

The microbial communities in the PFs were dominated (30.3 ± 24%) by sequences of an uncultured genus of the *Methylococcales* (*γ-proteobacterial* methane oxidizers) and this taxon decreased in abundance in the AF communities (5.7 ± 3%). The most abundant bacterial lineage in the AFs (45.2 ± 23%) and the second most abundant in the PFs (27 ± 23%) was the genus *Nitrospira* (phylum *Nitrospirae*), typically known as nitrite oxidizer (Pester *et al.*, 2014).

Core taxa among water works

We speculated that similarities in influent, design and operational parameters of the DWTPs would lead to dominant DWTP-shared RSF microbial communities. Hence, shared taxa in the microbial communities of the GW, the PFs and the AFs at all DWTPs were determined. Shared taxa were classified at the phylum, class, order and genus level, and taxa (at the genus level) present at all DWTPs at more or >1% mean relative abundance were defined as dominant or rare core taxa (Gobet *et al.*, 2010), respectively (Figure 3).

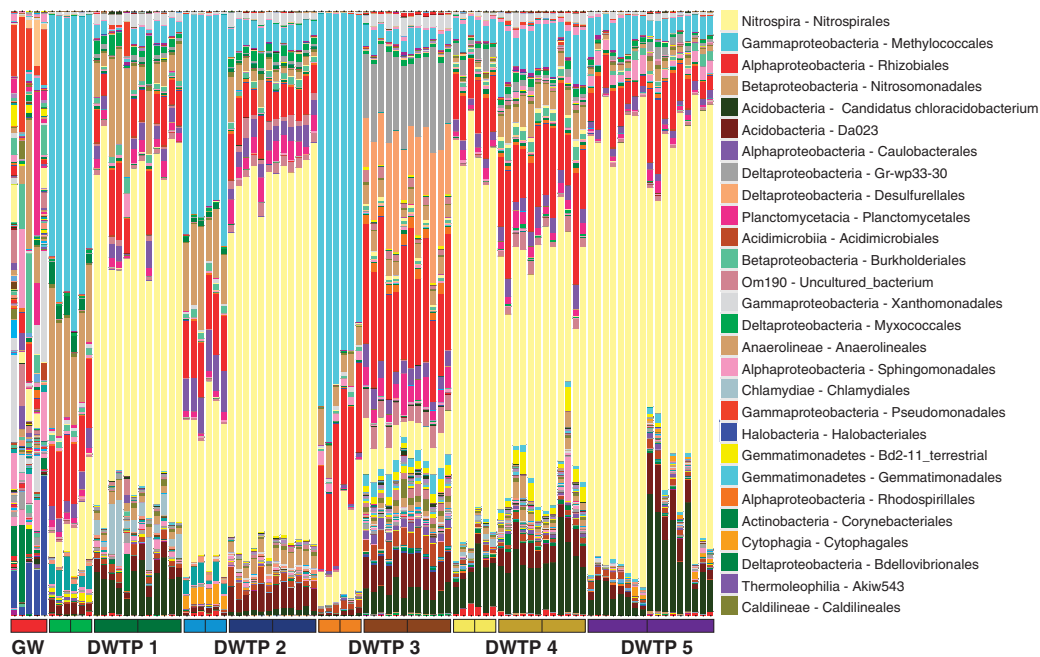


Figure 1 Relative abundance and order-level taxonomic classification of 16S rRNA amplicons across all DWTPs against the Silva108 prokaryotic reference database. For each DWTP, different PFs (bright boxes) and AFs (dark boxes) are plotted along the x axis. The first and second box, in each color, refers to the top sample and composite depth sample of the filter, respectively. GW microbial communities (GW) are shown in first five columns.

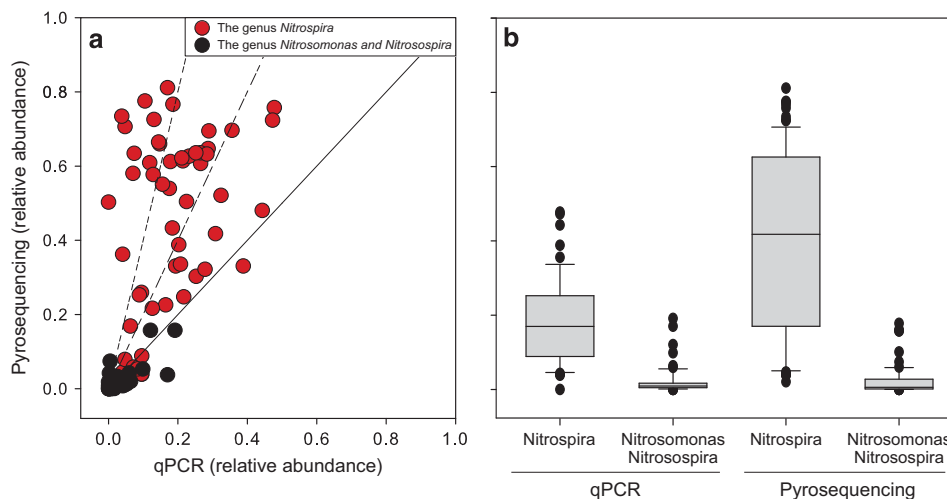


Figure 2 Relative abundance (a) and inferred ratios (b) of *Nitrosomonas* plus *Nitrosospira* and *Nitrospira* clades measured by 16S rRNA gene targeted qPCR and inferred from 16S rRNA amplicon libraries.

At the genus level, the GW comprised 73 core taxa (9.5% of all core genera) in 14 phyla accounting for $68.9 \pm 13\%$ of the total sequence abundance (Figure 3a). In the PF microbial communities, 30 core taxa (4% of all core genera) were identified, comprising $87 \pm 5\%$ of the total sequence abundance (Figure 3a). Most of the PF core taxa (72% of core genera) were also in the GW core (Figure 3b), although the habitats of anoxic GWs and highly oxyc PFs are different. Twenty-nine taxa (3% of all core genera) constituted the core of AF microbial communities, representing $75 \pm 18\%$ of the total

sequence abundance (Figure 3a). Six of the *rare* core taxa in the PF were *dominant* core taxa in the AF, including *Nitrosomonadales*, *OM190*, *Chloracidobacterium*, *Xanthomonadales*, *Anaerolineales* and *Da023*. Both PF and AF community cores were dominated by *Caulobacteriales*, *Nitrospirales*, *Methylococcales*, *Rhizobiales* and *Myxococcales*.

Although few genera constituted the core in both PF and AF communities, the number of OTU_{0.03}s per core genus varied significantly. The number of OTU_{0.03}s per taxon exponentially related to its abundance (i.e., sequence number) in both PF

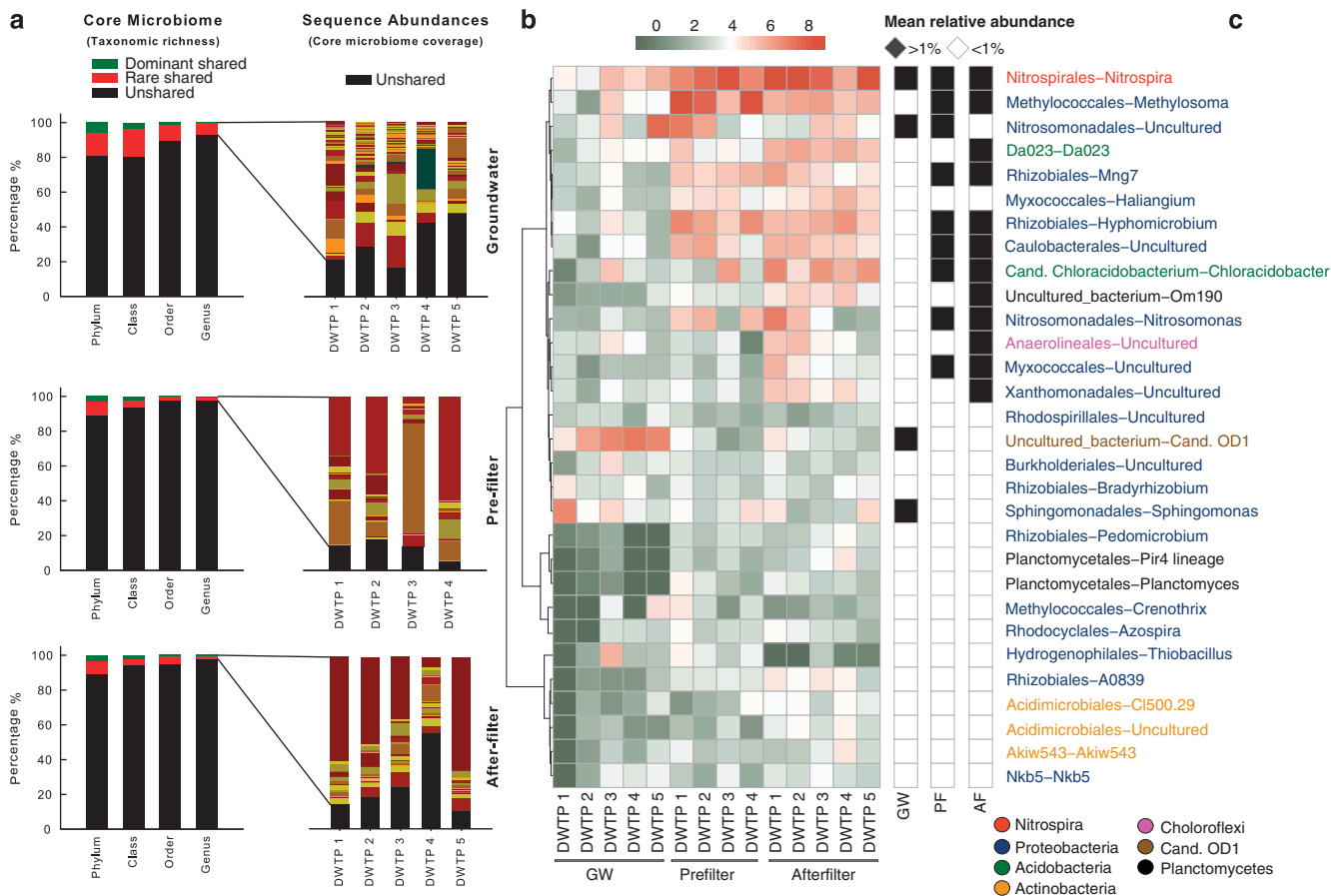


Figure 3 Richness, abundance and identity of taxa shared across all DWTPs. (a) Richness and sequence coverage of shared taxa in GW, PF and AF units. (b) Shared genera and their absolute abundance. Sequence abundances were log transformed and colored from red-to-black to present higher-to-lower abundance. Heatmap boxes without any sequences were assigned 10^{-6} (darkest green). (c) Identification of shared genera as dominant (>1% relative sequence abundance) or rare (<1% relative sequence abundance).

($r^2 = 0.75$; $P = 4.21e-08$) and AF communities ($r^2 = 0.55$; $P = 1.03e-04$ with *Da023*, $r^2 = 0.82$; $P = 3.39e-08$ without *Da023*), except for genus *Da023*. The sequence abundance and intragenus OTU_{0.03} richness of *Nitrospira*, *Methylosoma*, *Hypomicrobium*, *Rhizobiales* (uncultured) and *Caulobacterales* (uncultured) was, thus, highest of all core taxa. *Da023* displays an anomalously high OTU_{0.03} richness for its total sequence abundance of 8674 across all AF communities (on average abundance 131 ± 69) (Supplementary Figure S6).

Physiological typing of the core taxa

Phylogenetic trees were constructed containing sequences from the core taxa and their closest cultured relatives. Assuming shared metabolic pathways among phylogenetically related organisms, this suggests a physiology of the core taxa (Langille et al., 2013). A high number of OTU_{0.03}s within the core taxa of the PFs (eight taxa) and AFs (seven taxa) were closely related ($\geq 95\%$ sequence similarity) to sequences of types with the ability to oxidize ammonium, nitrite, iron, manganese and

methane as primary growth substrate (Figure 4). Furthermore, these OTU_{0.03}s represent up to $79 \pm 2.3\%$ and $66 \pm 0.6\%$ of all sequences in the PF and AF core, respectively (Table 3).

In PFs and AFs, 31 OTU_{0.03}s had *Nitrosomonas oligotropha*, a high-affinity chemolithotrophic ammonia oxidizer (Koops et al., 2006) as closest relative; in addition, *Nitrosoccus mobilis*, another oligotrophic ammonium oxidizer, was a close phylogroup to many sequences in PF (25 OTU_{0.03}s), and *Nitrosomonas* sp. BF16c52, a *Nitrosomonas mobilis* strain isolated a nitrifying freshwater system (Burrell et al., 2001) clustered with 73 OTU_{0.03}s of AF core taxa (Figure 4).

Within the nitrite-oxidizing clades, a majority of sequences phylogenetically affiliated with uncultured representatives of the *Nitrospira* genus (169 OTU_{0.03}s in the PF core, and 243 in the AF core). In addition, less OTU_{0.03}s grouped with Cand. *Nitrospira defluvii* (25) and *Nitrospira moscoviensis* (80) in the PF and *Nitrospira marina* (36), *Nitrospira calida* (105) and Cand. *Nitrospira defluvii* (1) in the AF.

In PFs, 43 OTU_{0.03}s of the core genera had *Crenothrix polyspora*, a methane-oxidizing bacterium

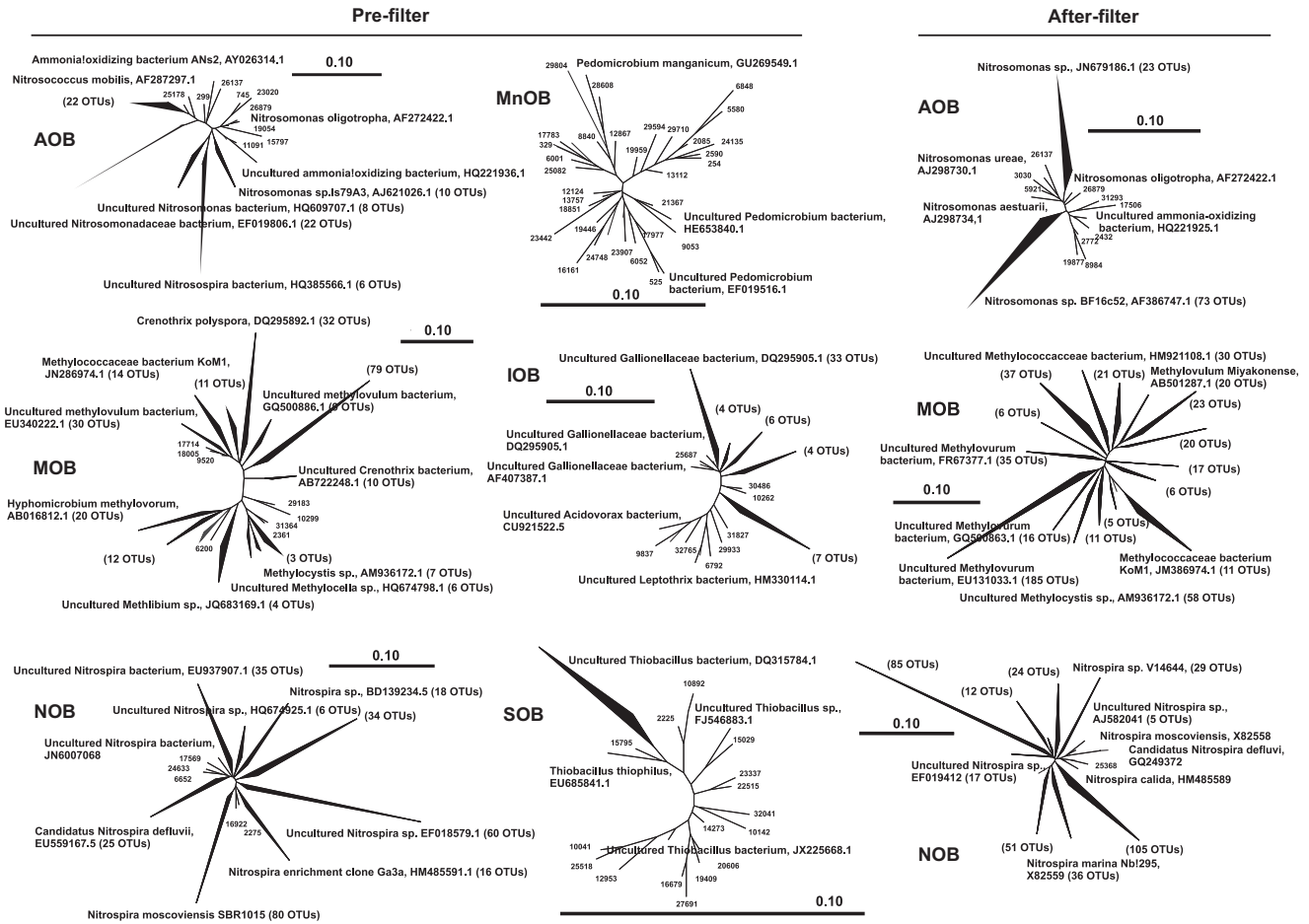


Figure 4 Phylogenetic trees of a subset of OTU_{0.03s} of the shared taxa in PF and AF together with reference isolates with identified physiologies (AOB, ammonium oxidizers, NOB, nitrite oxidizers, MOB, methane oxidizers, IOB, iron oxidizers, MnOB, manganese oxidizers, SOB, sulfide oxidizers). The number of OTU_{0.03s} in each cluster is within parentheses. The name for each clade was selected from a reference isolates in that clade. The scale bar represents 0.10 substitutions per nucleotide position. GenBank accession numbers of reference sequences are indicated.

Table 3 Abundance of taxa (%) with known physiology in the core taxa at each waterworks

	Pre-filter				After-filter				
	DWTP1	DWTP2	DWTP3	DWTP4	DWTP1	DWTP2	DWTP3	DWTP4	DWTP5
AOB	1.55	4.00	3.50	0.09	8.79	2.54	1.00	0.12	0.11
NOB	9.82	29.24	11.89	73.64	60.40	70.49	44.97	19.11	71.45
MOB	54.46	39.70	62.69	4.09	2.37	4.26	9.66	5.76	4.09
IOB	12.77	6.72	0.73	0.23	—	—	—	—	—
MnOB	0.16	0.15	0.24	0.38	—	—	—	—	—
SOB	0.70	0.53	0.60	0.20	—	—	—	—	—
Total	79.46	80.34	79.65	78.62	71.55	77.29	55.63	25.00	75.65

Abbreviations: AOB, ammonium oxidizers; DWTP, drinking water treatment plant; IOB, iron oxidizers; MOB, methane oxidizers; MnOB, manganese oxidizers; NOB, nitrite oxidizers; SOB, sulfide oxidizers.

originally isolated from an RSF (Wolfe, 1960), as a closest relative. The presence of such phylotypes indicates methane residuals in the influent water, although aeration units preceding the PFs have methane removal as central objective. *C. polyspora*-related phylotypes were not detected in the AF core;

here, abundant taxa were identified with another methane oxidizer *Methylovulum miyakonense* (43) and uncultured representatives of the *Methylovulum* genus as closest relatives. OTU_{0.03s} with iron- and manganese-oxidizing strains as closest phylotypes were mostly detected in the PF core. Thirty-two

OTU_{0.03S} had *Pedomicrobium manganicum*, a dimorphic prosthecate and oligotrophic manganese-oxidizing bacterium (Poindexter, 2006), as a closest relative, whereas 15 OTU_{0.03S} clustered with sequences assigned to uncultured *Gallionellaceae* putatively strains with iron-oxidizing capability.

Relationships of diversity with energy availability

We investigated whether richness and evenness patterns in the RSFs related to energy availability within DWTPs. Energy sources for chemolithotrophic growth are gradually consumed between the serial filtration units (Lopato et al., 2013; Gülay et al., 2014), and within individual units removal is spatially stratified (Lopato et al., 2011, 2013; Tatari et al., 2013; Lee et al., 2014). Hence, a gradient in energy supply is present along the four sampling points in the DWTPs: from top to bottom of the PF and from top to bottom of the AF. In DWTP2, 3 and 4 decreases in total bacterial abundance were

estimated from PF to AF, whereas in DWTP5, where typical AF units were absent, total bacterial density was clearly stratified with filter depth (Tatari et al., in preparation). Abundance of the *Nitrosomonas* plus *Nitrospira* clade, followed the pattern of total bacterial abundance: decreasing from PF to AF units at DWTPs 2, 3, 4, whereas total abundance of the *Nitrospira* clade also decreased at DWTPs 3 and 4, but significantly increased from PF to AF at DWTPs 1 and 2 (Tatari et al., in preparation).

At community level, OTU richness correlated strongly and positively with sampling location in the DWTP ($r^2_{\text{adj.}} = 0.69$, $P = 0.0001$; Figure 5a), whereas a weaker and negative correlation was observed for OTU evenness (Gini coefficients, $r^2_{\text{adj.}} = 0.11$, $P = 0.003$; Figure 5b). This pattern was consistent across DWTPs and at each DWTP: OTU richness in all five DWTPs increased from PF to AF ($r^2_{\text{adj.}} = 0.57 \pm 0.2$, $P < 10^{-2}$; Supplementary Figure S7) and Gini coefficient significantly decreased ($r^2_{\text{adj.}} = 0.54 \pm 0.12$, $P < 10^{-2}$; Supplementary Figure S8).

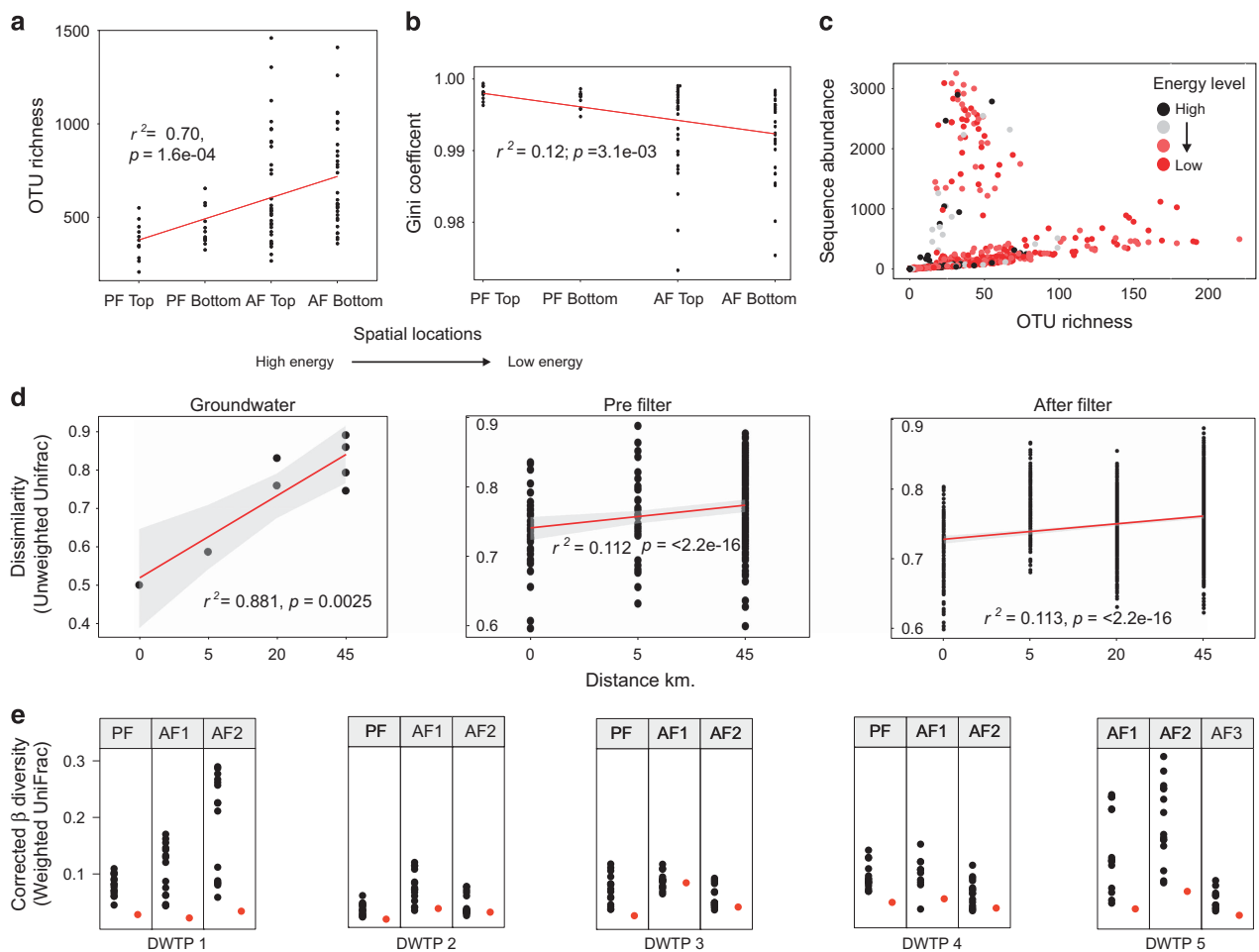


Figure 5 Ecological patterns in RSFs. Changes in OTU richness (a) and evenness (b) along sampling location. (c) Sequence abundance versus OTU richness of phyla at four sampling locations across DWTPs. Each individual dot represents a phylum at a sampling location. (d) Phylogenetic dissimilarity between *Nitrospira* OTUs across aquifers, PFs and AFs of DWTPs, based on unweighted UniFrac diversity estimates. (e) β -diversity analysis applied to six biological replicate samples from a filter unit based on weighted UniFrac diversity estimates. Corrected β -diversity values of replicate samples (black circles) and estimated meta-community subsamples (red circles) are plotted.

For 4 and 21 core genera (of 30 total) in both PFs and AFs, evenness (0.17 ± 0.04) and richness (0.53 ± 0.24) were related to sampling location along the DWTP, respectively (Supplementary Table S4 and Supplementary Figure S9). Richness decreased with location for genera *Akiw543*, *Crenothrix*, *Thiobacillus*, *Pedomicrobium*, *OD1*, *Pir4* and uncultured representatives of *Burkholderiales*, *Nitrosomonadales*, *Rhodospirillales*; richness increased with location for genera *Haliangium*, *Chloracidobacterium*, *Da023*, *Nitrospira*, *Om190* and uncultured representatives of *Xanthomonadales*, *Anaerolineales* and *Acidimicrobiales* (Supplementary Figure S9).

Spatial heterogeneity

The microbial community structures at different sampling locations within an individual RSF were all significantly different (β -value exceeded the meta-community-derived threshold) at all DWTPs (Figure 1e), and the magnitude of dissimilarities were similar across PFs and AFs (Supplementary Figure S11). In PFs, however, communities were on average less spatially heterogeneous than in AFs, both in the horizontal plane and with depth. Community dissimilarity between DWTPs was greater than within DWTPs, and within a DWTP dissimilarities decreased along the treatment train (from GW to AF) (Supplementary Figure S12).

Microbial dispersal within DWTPs and between aquifers

Logically, microbial communities from GWs would seed and influence the taxonomic composition of the PFs communities; similarly, the PFs would seed the AFs. To assess this occurrence, each dominant OTU_{0.03} in a unit was checked for its occurrence in the upflow units. Dominant phylotypes (OTU_{0.03}s) in the downflow units should also occur in the upflow units, if seeding were significant. On average, $75.4 \pm 20\%$ of the dominant OTU_{0.03}s in PF communities were detected in the GWs, be it as rare members. For the dominant OTU_{0.03}s in the AFs, $99 \pm 2\%$ were found as PF OTU_{0.03}s, of which 49% were dominant and 51% rare (Supplementary Table S5). Clearly, the taxonomic inventory of the community in upflow process units, including the rare members, affects the downflow units.

Second, because deep aquifers are isolated ecosystems (Fredrickson and Onstott, 2001), a decay in taxon similarity is expected with distance. We investigated the degree of phylogenetic dissimilarities between *Nitrospira* OTU_{0.03}s across DWTPs and the associated feed GWs. The pairwise *Nitrospira* dissimilarities increased with distance between aquifers ($r^2_{\text{adj.}} = 0.88$, $P = 0.002$; Figure 5d—GW). This distance effect was significantly less when comparing *Nitrospira* OTUs in the PFs ($r^2_{\text{adj.}} = -0.11$, $P < 2.2e - 16$; Figure 5d) or AFs ($r^2_{\text{adj.}} = -0.11$, $P < 2.2e - 16$; Figure 5d) at different DWTPs.

Discussion

Microbial communities in RSFs

Proteobacteria were the dominant phylum in the PFs and remained abundant in the AFs, consistent with other, more limited, surveys of GW-fed RSFs (Gülay et al., 2014; Albers et al., 2015), and suggest their central role in RSF processes. Largest decreases in abundance from PF to AF were noted for phylotypes of the *Nitrosomonadales*, an ammonium-oxidizing *Beta*-proteobacterial taxon, and *Methylococcales*, a *Gamma*-proteobacterial methane consuming taxon in line with the removal of their substrates from PF to AF (Macalady et al., 2013; Tatari et al., 2013; Lee et al., 2014). Even though dissolved oxygen concentrations in AFs are typically near saturation, *Chloroflexi* of the order *Anaerolineales*, assumed to be strict anaerobes (Yamada et al., 2006), were major phylotypes in AFs, suggesting anoxic microniches or another physiology. We report for the first time the presence of *Gemmatimonadetes* phylotypes in RSFs, but the ecology and physiology of this phylum is undescribed (DeBruyn et al., 2011).

Nitrospira was consistently the most abundant phylum in the AFs and the second most abundant phylum in the PF amplicon libraries. This high abundance was confirmed by targeted qPCR (Figure 2), although the high *Nitrospirae* abundance caused a potential overrepresentation in the amplicon libraries (Figure 2) as observed before (Gonzalez et al., 2012). Significantly high *Nitrospira* occurrence, be it at lower abundance, has been seen in full-scale RSFs fed with GW (detectable *Nitrospira* DGGE bands (de Vet et al., 2009) and 6% of 16S rRNA amplicons (Albers et al., 2015)), fed with GW/surface water mixtures (2% of 16S rRNA amplicons (Pinto et al., 2012) and 50.4% of 16S rRNA clone libraries (White et al., 2012)) or fed with surface waters (13% to 21% of 16S rRNA amplicons (LaPara et al., 2015) and 17% of 16S rRNA clone libraries (Feng et al., 2012)). *Nitrospirae* were also dominant in a model drinking water distribution system carrying water from a GW-fed RSF (5–27% in biofilm and 30–40% in bulk water clones; Martiny et al., 2005).

All detected *Nitrospirae* OTU_{0.03}s belonged to the *Nitrospira* genus (275 and 814 for PFs and AFs, respectively). The subgenus level analysis showed a broad diversity (Figure 4), suggesting a number of ecophysiologicaly distinct *Nitrospira* species, as observed elsewhere (Daims et al., 2001; Pester et al., 2014). In PFs, 214 and 39 OTU_{0.03}s belonged to *Nitrospira* lineage I and lineage II, respectively, whereas 39 *Nitrospira* OTU_{0.03}s were of undefined lineage (Supplementary Figure S13—PF). In contrast, in the AFs, the majority of the *Nitrospira* OTU_{0.03}s (328) were of undefined lineage, with few OTU_{0.03}s belonging to lineage I(2), II(5), IV(2) and VI(36), respectively (Supplementary Figure S13—AF).

Of all possible co-occurrence relations among the *Nitrospira* OTU_{0.03}s (45.958 ± 15.228) in each DWTP

community, few were detected as significant and positive (5590 ± 3951 , $13 \pm 8\%$) or significant and negative (123 ± 95 , $0.30 \pm 0.28\%$) (Supplementary Table S6). Hence, the vast majority of relations between *Nitrospira* OTU_{0.03}s appear neutral instead of competitive. Taken together with the high intra-genus diversity, this suggests a high degree of specialization within the *Nitrospira* clade, permitting the exploitation of slightly distinct niches.

The high abundance of these *Nitrospira* sequences in the community (Figure 2a) is puzzling given the low influent nitrite concentrations ($<0.01 \text{ mg l}^{-1}$) and the relatively low abundance of known ammonium-oxidizing prokaryotes (*Nitrospira* over AOB ratios: 45 ± 90 and 210 ± 431 based on qPCR and amplicon libraries, respectively; Figure 2b) in the filters. Unusually high *Nitrospira*/AOB ratios were recently reported by others in other GW-fed filtration systems (*Nitrospira*: $33.4 \pm 8\%$, *Nitrosomonas*: $3.7 \pm 2.5\%$ —16S rRNA amplicons; Nitzsche *et al.*, 2015; *Nitrospira*: $16.9 \pm 12\%$, *Nitrosomonas*: $3.2 \pm 2\%$ —16S rRNA clones; White *et al.*, 2012), and even in surface water-fed filters (*Nitrospira*: 17.3% , *Nitrosomonas*: 0.82% —16S rRNA clones; Feng *et al.*, 2012 and *Nitrospira*: $17 \pm 5\%$, *Nitrosomonas*: 0.07% —16S rRNA amplicons; LaPara *et al.*, 2015). Differences in 16S gene copy number (Nitzsche *et al.*, 2015), differences in specific growth rates of *Nitrosomonas* vs *Nitrospira* (Feng *et al.*, 2012), presence of unidentified AOBs (LaPara *et al.*, 2015), multiple energy metabolisms in *Nitrospira* (LaPara *et al.*, 2015; Nitzsche *et al.*, 2015), primer bias in PCR (Nitzsche *et al.*, 2015) and the presence of dormant *Nitrospira* cells (Martiny *et al.*, 2005) have all been invoked to explain these unexpected ratios. While all these explanation may contribute to high *Nitrospira*/*Nitrosomonas* ratios (Figure 2), the observed ratios in our study remain much higher than previously reported.

Possibly, novel physiological features support the presence of these *Nitrospira* phylotypes. This is in line with the co-occurrence analysis on all core taxa; significant co-occurrence patterns of *Nitrospira* with other taxa were exclusively negative, suggesting competitive interactions (Supplementary Figure S14). It has been documented that *Nitrospira*-like bacteria in activated sludge can assimilate pyruvate (Daims *et al.*, 2001), and simple organic compounds can stimulate growth of *Cand. Nitrospira defluvii* and *Nitrospira marina* (Watson *et al.*, 1986; Spieck *et al.*, 2006). In addition, the genome of *Cand. Nitrospira defluvii* encodes pathways for the degradation and assimilation of acetate, pyruvate and formate (Lucker *et al.*, 2010), and *Nitrospira* can efficiently use formate as carbon and energy source (Gruber-Dorninger *et al.*, 2015). It is possible, then, that part of the *Nitrospira* abundance is supported by their growth on assimilable organic carbon substrates, present in the GW. In addition, the very recent identification of novel *Nitrospira* species carrying and expressing the *amo* operon

(*Cand. Nitrospira inopinata* (Daims *et al.*, 2015), *Cand. Nitrospira nitrosa* and *Cand. Nitrospira nitrificans* (van Kessel *et al.*, 2015) suggest that *Nitrospira* may well be involved in ammonium oxidation in these RSFs. Confirmatory experiments are needed to support the actual ecophysiology of the *Nitrospira* clade in the RSFs.

The candidate division *OD1* phylum, present at $1.8 \pm 1\%$, has not previously been reported as dominant in GW-fed filters, although it has been detected at low densities in raw surface waters (Eichler *et al.*, 2006) and treated surface and GW mixtures (Pinto *et al.*, 2012). Marine members of *OD1* phylum have been implicated in sulfur cycling (Harris *et al.*, 2004); a similar role is possible in RSFs. Little is known about the physiology of *Actinobacteria* phyla beyond the general notion that *Actinobacteria* can use a range of labile organic carbon compounds (Pernthaler, 2013).

Identification of core taxa and their likely physiological role

Filter-specific OTU_{0.03}s accounted, on average, for $2.91 \pm 2.3\%$ and $6.41 \pm 4.8\%$ of the total sequence abundance within PFs and AFs, respectively; most ($96 \pm 2.1\%$) were singletons or doubletons. The relative abundance of the shared genera at each DWTP was strikingly high with $87 \pm 5\%$ and $75 \pm 18\%$ of all sequences in PFs and AFs, respectively. The existence of core genera shared across the DWTPs indicate that environmental conditions in the RSF are sufficiently similar, and DWTP-specific conditions—or source water communities—have a secondary influence on the microbial community composition. Abundance of few shared taxa has been reported before in technical systems: 70 core genera (of 744 total) accounted for 63.7% of all sequences in 15 activated sludge communities, sampled from around the world (Zhang *et al.*, 2012), 86 core genera accounting for 68% of all sequences in activated sludge communities of 13 Danish wastewater treatment plants (Saunders *et al.*, 2015), 23% of all sequences in seven anaerobic digesters across Europe could be assigned to six core OTU_{0.03}s (Rivière *et al.*, 2009). Core taxa were abundant at individual DWTP locations—suggesting a functional role (Fuhrman, 2009)—and would indicate selection as a dominant force for community assembly.

By clustering OTU_{0.03}s in the PF and AF core taxa with the closest cultured strains beyond the genus level ($>95\%$ pairwise similarity), putative physiologies could be assigned. Physiological types included ammonium, nitrite, methane, manganese and iron oxidizers, which are expected in the DWTPs (Figure 4). These phylotypes dominated the core taxa in both PFs ($73.6 \pm 6\%$; Table 3) and AFs ($61.4 \pm 21\%$; Table 3), supporting the notion that core genera are functionally important in RSFs. Nevertheless, for a fraction of OTUs in the core taxa ($21 \pm 0.6\%$ of all OTUs in the PF core and $34 \pm 0.4\%$

of all OTU_{0.03}s in the AF core), no close cultured isolates could be identified. Most of the unassigned OTU_{0.03}s in the core belong to the taxa *Cand. Chloracidobacter*, *Da023* (*Acidobacteria*), *Myxococcales*, *Rhizobiales* (α -*proteobacteria*), *Xanthomonadales* (γ -*proteobacteria*) and *Anaerolineales* (*Chloroflexi*). These taxa are also dominant in other GW-fed RSFs (Albers *et al.*, 2015; Nitzsche *et al.*, 2015). *Acidobacteria* are abundant in a suite of environments (Barns *et al.*, 1999), harbor broad metabolic capabilities and can cope with nutrient limitations (Ward *et al.*, 2009; Hartmann *et al.*, 2015); they may be central to carbon cycling in RSFs. *Myxococcales* are widely distributed in terrestrial and marine environments, are often surface attached (Ganesh *et al.*, 2014) and have a predatory lifestyle or live off polymer hydrolysis (Shimkets *et al.*, 2006; Huntley *et al.*, 2011); they might have a central role in recycling microbial decay products in RSFs. The physiology of *Xanthomonadales* is largely undescribed, but some are, similarly, known to have high extracellular hydrolytic activity (Jacobson Meyers *et al.*, 2014).

It should be pointed out that, while an RSF core is evident at the genus level, the core taxa still retain subgenus variation across DWTPs, potentially due to dispersal limitation between the source aquifers (Gibert *et al.*, 2009), leading to different evolutionary trajectories for any clade. Indeed, the observed distance–decay relationship for *Nitrospira* OTU_{0.03}s in the source aquifers is consistent with previous studies on aquifer communities (Franklin *et al.*, 1999; Hug *et al.*, 2015) and a weakened signal remains in the PF and AF *Nitrospira* clade.

Ecological patterns in RSFs

The consistent decrease in community evenness and richness from GW to filter might be due to the strong environmental changes (shift in DO level from below detection to saturation), resulting in loss of many, potentially oxygen-intolerant, taxa from GWs, and enrichment and selection of few taxa, adapted to the new environmental conditions. While the total OTU richness decreased, the OTU richness of few PF genera strikingly increased.

At the community level, OTU richness and evenness were consistently higher in the AF compared with the PF communities; this is surprising as available energy would decrease (because of microbial removal of energy sources) moving down-gradient in the DWTP (Lopato *et al.*, 2011, 2013; Tatari *et al.*, 2013; Lee *et al.*, 2014) and available energy would promote richness by its positive effect on community size, per the species-energy theory (Wright 1983). Increasing community richness with decreasing energy input contradicts previous observations on natural and technical systems (Bienhold *et al.*, 2011 and Supplementary Table S2).

Direct evidence of total community size decrease from PF to AF was difficult to obtain (small decreases were detected for DWTP2, 3 and 4 with qPCR), and suggests a significant degree of density redistribution by detachment/deposition, among other processes. The pattern of diversity increase is detected at the community, phylum, order and even at the genus level (Supplementary Table S7). OTU richness did, however, remain correlated with relative (Supplementary Table S8) abundance (*Nitrospira* and *Nitrosomonas*) within specific taxa, confirming that increasing taxon abundance increases taxon diversity (Figure 5c). These taxa were, primarily, the dominant members of the AF community.

Negative relationships between diversity and energy input have not been reported at the community level, but they have been observed at the level of specific taxa (Bienhold *et al.*, 2011). Oligotrophy was suggested as the cause for taxa to exhibit negative relationships with energy input (Bienhold *et al.*, 2011), and could be an explanation for the increase of abundance, and hence richness, with decreasing energy levels in RSFs. The abundance of *Nitrospira* as well as genera *Xanthomonadales*, *Nkb5*, *Haliangum*, *A0839* (*Proteobacteria*), *Acidomicrobiales*, *Cl500* (*Actinobacteria*), *Anaerolineales* (*Chloroflexi*), *Cand. Chloroacidobacter*, *Da023* (*Acidobacteria*) and *OM190* (*Planctomycetes*) significantly increased with sampling location. Potentially, *Nitrospira* OTUs become more dominant and diverse in AFs (Figure 3b), as they are favored under low substrate conditions (Schramm *et al.*, 1999; Attard *et al.*, 2010; Lucker *et al.*, 2010) (Supplementary Table S9). On the other hand, the relative abundance of core genera with putatively assigned physiology, such *Nitrosomonadales*, *Crenothrix*, *Thiobacillus*, *Methylosoma*, *Hyphomicrobium* and *Azospira*, decreases with sampling location in a DWTP, consistent with consumption of their specific energy substrates (Lopato *et al.*, 2011, 2013; Tatari *et al.*, 2013; Gülay *et al.*, 2014; Lee *et al.*, 2014).

Here, we extend our previous observation (Gülay and Smets, 2015) that RSF microbial communities display spatial heterogeneity within a single PF or AF at all DWTPs. This heterogeneity is more pronounced in AFs than PFs. In all cases, rare taxa (which are more dominant in AFs than PF) explain the dissimilarities (Table 1 and Gülay and Smets, 2015), as observed in other environments (Youssef *et al.*, 2010). The contribution of observed community dissimilarity to differences in filter activity (Lopato *et al.*, 2011, 2013) is uncertain, especially given the abundance of the core taxa, shared across all samples. Across DWTPs, communities are as a result less dissimilar in the RSFs than in the GWs that feed them (Supplementary Figure S12), confirming similar selective forces driving community structure (Table 1), and an identifiable resultant RSF core microbiome.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

All authors contributed to this study and the final manuscript. The original concept and sampling strategy were developed by AG, SM, HJA and BFS. Sampling was performed by AG and SM. DNA preparation and PCR experiments were performed by AG and SM. WAAS performed the pyrosequencing, supported by SJS. AG performed all the bioinformatic analyses. AG and BFS lead the analysis and interpretation of the results, the drafting, revision and completion of the manuscript. All authors read and approved the manuscript before submission

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