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Spatial and temporal analogies in microbial communities in natural drinking water biofilms

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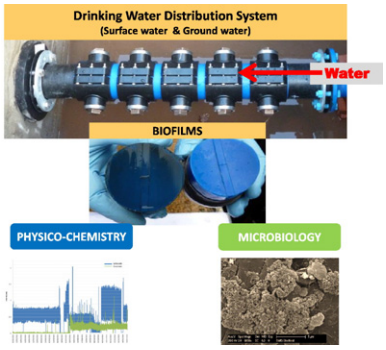
HIGHLIGHTS

- Main abiotic factors affecting microbial distribution were flow, temperature and pH.
- Internal factors were central in shaping biofilm formation and composition.
- Ubiquitous core bacterial community observed in all biofilm samples.
- The less abundant bacteria were responsible for most of the variability.
- This information is essential for the management of drinking water systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Biofilms are ubiquitous throughout drinking water distribution systems (DWDS), playing central roles in system performance and delivery of safe clean drinking water. However, little is known about how the interaction of abiotic and biotic factors influence the microbial communities of these biofilms in real systems. Results are presented here from a one-year study using *in situ* sampling devices installed in two operational systems supplied with different source waters. Independently of the characteristics of the incoming water and marked differences in hydraulic conditions between sites and over time, a core bacterial community was observed in all samples suggesting that internal factors (autogenic) are central in shaping biofilm formation and composition. From this it is apparent that future research and management strategies need to consider the specific microorganisms found to be able to colonise pipe surfaces and form biofilms, such that it might be possible to exclude these and hence protect the supply of safe clean drinking water.

1. Introduction

Drinking Water Distribution Systems (DWDS) are complex ecosystems where biotic and abiotic factors interact along an amalgamate of pipes, storage tanks and other infrastructure extended through vast areas in a buried environment. Many of the interactions are microbially mediated and microorganisms play a central role in determining the quality of the drinking water arriving at customers' taps. Most of the microorganisms living in DWDS are attached to pipe surfaces forming mixed-species biofilms. Biofilms can be considered as microbial factories in constant operation where specific processes can take place such

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as pipe corrosion, residual disinfectant decay or trapping/accumulation of inorganics. Determining the potential for biofilm growth and their composition and structure in DWDS is essential, since biofilm affect the performance of these systems and ultimately the delivery of safe clean drinking water. Understanding the effect of environmental change on biofilm composition and structure in DWDS is challenging mainly due to the difficulty of accessing these buried ecosystems. Commonly observed effects of external factors on biofilms in DWDS are changes in microbial composition and structure (Douterelo et al., 2014), in the components of the extracellular polymeric matrix (EPS) (Fish et al., 2015), changes in density and in chemical and electrical properties (Mukherjee et al., 2012; Janjaroen et al., 2013) and in cell-cell interaction (quorum sensing) (Lee et al., 2014). However, to what extent the combination of biotic and abiotic factors, under realistic conditions, affects the development and composition of natural biofilms in DWDS remains unknown.

We have only a limited vision of the microbial ecology of DWDS since most studies have generally focused on free-living organisms from tap samples (*e.g.* Holinger et al., 2014; Donohue et al., 2015) or water treatment plants (Kasuga et al., 2010; Liu et al., 2013). Studies that have explored biofilms have tended to be in artificial systems in isolation without establishing associations with realistic environmental parameters or between free-living organisms and the attached communities co-habiting the same system (Giao et al., 2008; Moritz et al., 2010). Studies in different environments have shown that natural biofilms respond to their environment and adapt to changes by means of a diverse range of mechanisms (Stewart and Franklin, 2008). Limited information exists regarding whether there are common microbial patterns in biofilms dynamics over time and across different locations. To have a better insight into microbial assembly of natural biofilms and ecological factors influencing their development in DWDS we have used a short-term approach (re-growth every three months) and a long term approach (succession over a one-year period). This will provide a comprehensive vision of how biofilm develop in real systems, allowing for exploration of patterns of behaviour such as seasonal shifts in the structure and composition of biofilms in DWDS.

Previous research suggests that the microbial ecology of DWDS will be affected by source water characteristics (Gomez-Alvarez et al., 2015) the type of treatment (Hwang et al., 2012; Pinto et al., 2012) and hydraulic conditions in the system (Douterelo et al., 2013). However, what remains unknown is to which extend external variation will affect attachment to the pipes of certain microorganisms and biofilm formation, composition and dynamics over time. The objectives of the research reported here are to establish the effect of external factors, including different source waters, on the microbial ecology within operational DWDS and to examine patterns of biofilm formation and growth that can inform efficient management of these systems.

2. Materials and methods

2.1. Biofilm sampling devices and sampling sites

In situ biofilm sampling devices (Fig. 1) made of High Density Polyethylene (HDPE) were used to study two DWDS with different source waters (physico-chemical characteristics shown in Table 1) and hydraulic regimes (Fig. 2). Each sampling device was fitted into a real system in an available space of 150 mm at both sites and contained 10 modified (increased diameter to improve representative sampling) Pennine Water Group (PWG) coupons (Deines et al., 2010) that enable the study of naturally occurring biofilms *in situ* without the need for cutting, scraping or flushing the pipes. In addition, the use of PWG coupons allows for studying biofilms on pipe surfaces without distorting boundary layer hydraulic conditions including shear stress and turbulence driven processes such as nutrient exchange. Using these coupons two different processes were studied: 1) quarterly biofilm re-growth and 2) biofilm succession over a one-year period. The sampling devices were first installed in February 2013, with first assessment of 3 month-old biofilm development used to test a range of different techniques to evaluate best biofilm monitoring practices (Douterelo et al., 2016). From May 2013 and every three months thereafter the same three coupons were replaced with sterile coupons in order to study biofilm re-growth dynamics at different seasons starting from a completely

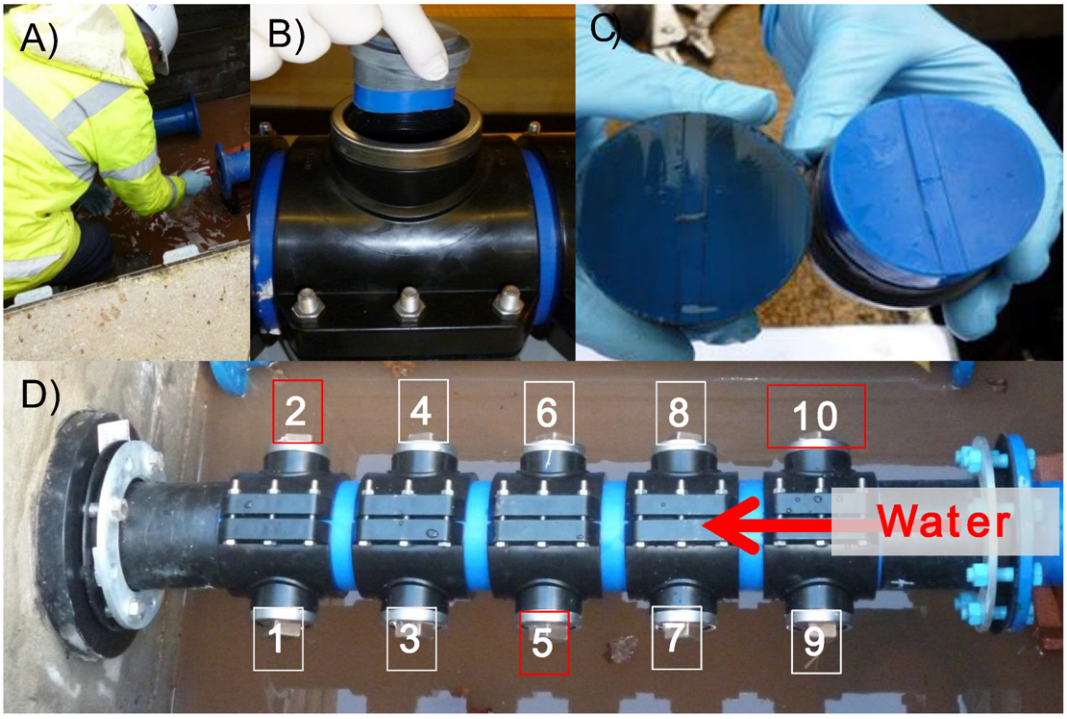


Fig. 1. A) Replacement of a section of pipe for installation of coupon devices. B) Insertion of a coupon in one of the holders of the biofilm sampling device. C) Coupons after sampling showing areas used for biofilm removal. D) Picture showing the distribution of the coupons in the device, coupons in red (2, 5 and 10) were used for re-growth studies, the other coupons were used for long term studies to study succession.

Table 1
Physico-chemical and microbiological parameters measured during every sampling event in August, November and February.

	Surface water			Ground water		
	Aug-13	Nov-13	Feb-14	Aug-13	Nov-13	Feb-14
HPC 37 °C (cfu/ml)	0	0	0	0	0	0
HPC 22 °C (cfu/ml)	0	0	0	0	1.5 ± 0.3	0
E. coli (cfu/100 ml)	0	0	0	0	0	0
Temperature (°C)	21.8 ± 0	7.3 ± 0.1	5.3 ± 0	14.1 ± 0	7.45 ± 0.3	8.05 ± 0
Turbidity (NTU)	0.12 ± 0.01	0.05 ± 0.1	0.2 ± 0.03	0.12 ± 0	0.1 ± 0	0.1 ± 0
Conductivity 20 °C (uS/cm)	311 ± 0	274.5 ± 0.7	244.5 ± 1.5	470 ± 0	461.5 ± 0.3	451.5 ± 11.5
Conductivity 25 °C (uS/cm)	344 ± 0	303.5 ± 0.7	270.5 ± 1.50	520 ± 0	510.5 ± 0.3	499.5 ± 12.5
pH	8 ± 0	7.4 ± 0	7.95 ± 0.05	7.7 ± 0	7.5 ± 0.3	7.6 ± 0
Alkal MO (mg $CaCO_3$/L)	64.5 ± 4.9	50 ± 2.8	52.5 ± 0.5	196.5 ± 2.12	183.5 ± 0.3	[a]
Ammonia as N (mg N/L)	0.02 ± 0	0.07 ± 0.1	<0.02	0.02 ± 0	<0.02	<0.02
Tot oxid N (mg N/L)	0.7 ± 0	1.2 ± 0	1.4 ± 0	8.55 ± 0	8.3 ± 0.3	9 ± 0
Nitrite as N (mg N/L)	0.003 ± 0	0.003 ± 0	0.003 ± 0	0.003 ± 0	0.003 ± 0	<0.003
Nitrate as N (mg N/L)	0.67 ± 0	1.175 ± 0	1.41 ± 0.01	8.54 ± 0.1	8.28 ± 0	9.02
Ammonia (mg NH_3/L)	0.01 ± 0	0.07 ± 0.1	<0.01	<0.01	<0.01	<0.01
Nitrite (mg NO_2/L)	0.01 ± 0	0.003 ± 0	0.01	<0.01	<0.01	<0.01
Nitrate (mg NO_3/L)	2.99 ± 0.01	1.75 ± 0	6.24	37.8 ± 0.42	36.65 ± 0.03	40 ± 0
Orthophosphate (mg P/L)	<0.003	<0.003	<0.03	0.03 ± 0	<0.03	<0.03
Sulphate (mg SO_4/L)	76 ± 0	61 ± 0	47.5 ± 0.5	24.5 ± 0	24 ± 0	16 ± 0
Chloride (mg Cl/L)	21 ± 0	20 ± 0	19 ± 0	18 ± 0	19 ± 0	17 ± 0
Free Cl_2 (mg Cl/L)	0.35 ± 0	0.21 ± 0	0.33 ± 0	0.32 ± 0	0.36 ± 0	0.22 ± 0
Total Organic Carbon (mg C/L)	0.95 ± 0.07	0.01 ± 0	0.95 ± 0.05	0.95 ± 0	0.6 ± 0	0.65 ± 0.05
Al mg/L	0.01 ± 0	0.02 ± 0	0.01 ± 0	<0.01	<0.01	<0.01
Mn mg/L	0.002 ± 0	0.002 ± 0	0.0015 ± 0	<0.001	<0.001	<0.001
Fe mg/L	0.01 ± 0	0.02 ± 00	0.02 ± 0	<0.01	<0.01	<0.01
Cu mg/L	0.01 ± 0	<0.01	<0.01	<0.01	<0.01	<0.01
Zn mg/L	0.01 ± 0	<0.01	<0.01	<0.01	<0.01	<0.01

[a] No measured.

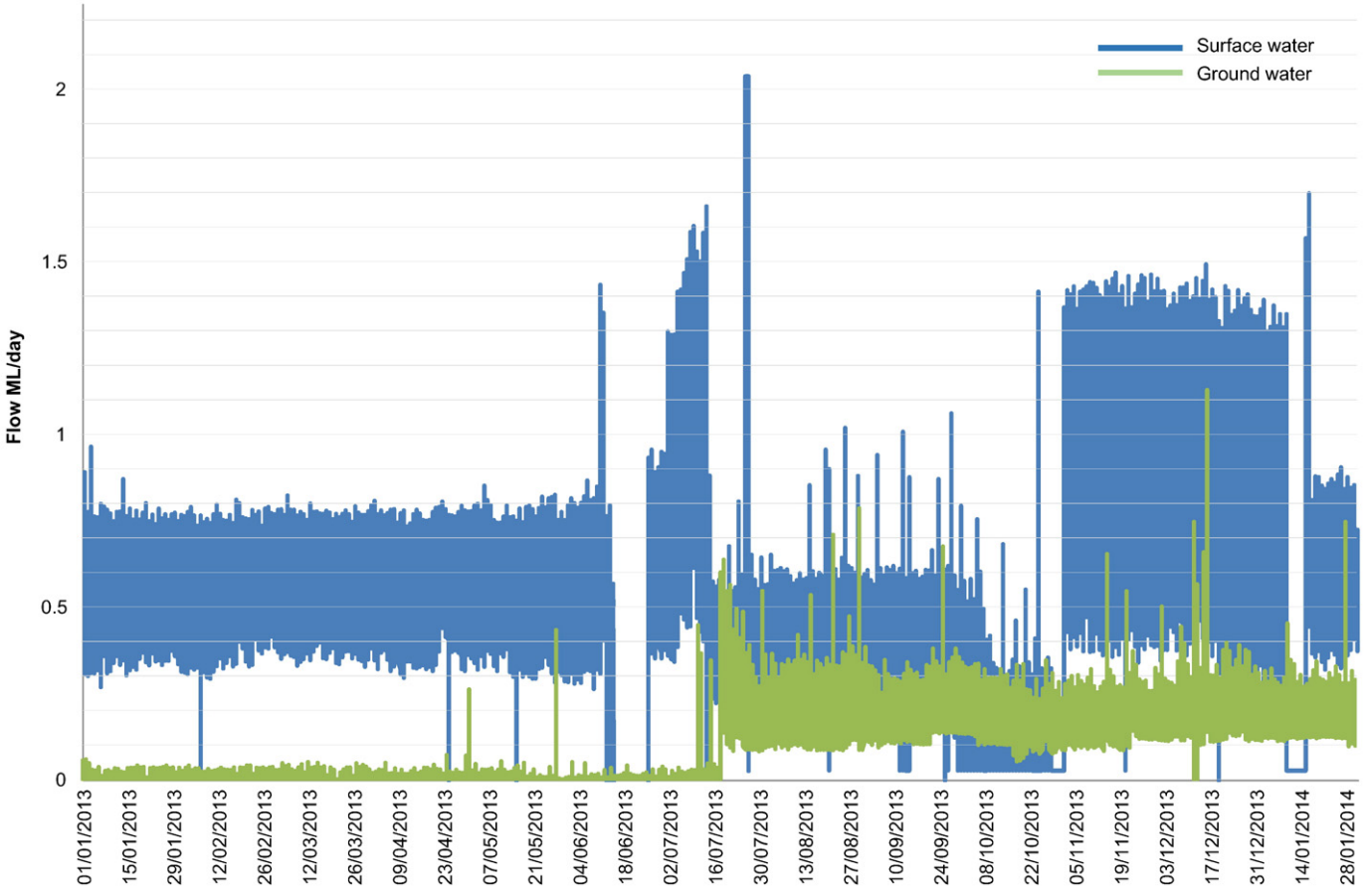


Fig. 2. Flow regimes for the ground water and surface water sampling site.

clean coupon surface (Fig. 1, coupons 2, 5 and 10). Succession coupons (also in triplicate) were sampled at both sites after 3 months (November to February), 6 months (August to February) and 12 months (February to February). Bulk water samples were collected whenever site was visited for the above coupon collection. It should be noted that throughout the period there was no other disturbance of the pipe line other than due to hydraulic changes as captured in Fig. 2, such that the biofilm on the surrounding pipe surfaces was not disturbed.

One of the sites was supplied with surface water from local springs and river abstraction. The water is treated by coagulation with aluminium sulphate, flocculation and removal of flocs by dissolved air flotation. The water is finally filtered using sand filtration and subsequently granular activated carbon is used to absorb and remove organics. A chlorine disinfectant residual is used in the system. The other site is supplied with groundwater from a standalone borehole site, supplied with a mixture of water from 10 boreholes. The water treatment on site includes marginal chlorination using sodium hypochlorite solution for disinfection residual. Both sites are comprised of similar diameter plastic (Medium and HDPE) pipes in and around the sampling locations.

2.2. Water quality analysis

On the dates of coupon collection, samples from the water that supplied the systems were collected for physico-chemical and microbiological analysis *via* sampling taps located immediately upstream of the biofilm sampling devices. Temperature and pH were measured *in situ* using a Hanna portable meter and probe HI 991003 96,711(Hanna Instruments, Leighton Buzzard, UK). All the other parameters (see Table 1) were measured by later analysis of discrete water samples by an UK-accredited drinking water laboratory. Flow was measured at 15 min resolution by magnetic flow meters upstream of the coupon devices. Heterotrophic plate counts were performed after incubation at 37 °C for 48 h (2-day colony) and 22 °C for 72 h (3-day colony) following UK Standard Methods. For *E. coli* counts a volume of 100 ml of sample was filtered through a 0.45 μm membrane and placed onto the surface of a plate containing membrane lactose glucuronide agar. The plate was then placed in an incubator set to provide pre-incubation for 4 ± 0.25 h at 30 °C followed by an incubation period at 37 °C for a minimum of 14 h. After incubation colonies were counted and the number reported as cfu per 100 ml.

2.3. DNA extraction

DNA was extracted from biofilm (n = 29) and water samples (n = 17). For the bulk water samples, three replicates of 2 L per site and sampling event were filtered through 0.22-μm nitrocellulose membrane filters (Millipore, Corp.) for subsequent DNA analysis. To extract biofilm from the coupons surface, first the two symmetric outer areas of each coupon were brushed to remove biofilm following the procedure used by Deines et al. (2010). After brushing biofilm suspensions were concentrated in membrane filters as previously explained (Douterelo et al., 2016). DNA was extracted using a method based on proteinase K digestion using a cetyltrimethylammonium bromide (CTAB) protocol followed by further DNA purification using phenol/isoamyl alcohol protocol (Neufeld et al., 2007).

2.4. Sequencing analysis

Sequencing was performed using Illumina Miseq technology with the pair-end protocol by Research and Testing Laboratories (Lubbock, TX, US) using primers 28F GAGTTTGATCNTGGCTCAG and 519 RGTNTTACNGCGGCKGCTG. Paired end reads were merged and denoised *via* Research and Testing Laboratory Pipeline to remove short sequences, singletons and noisy reads. Chimeras were detected using UCHIME (Edgar et al., 2011) and removed from further analysis. Sequences were clustered in Operational Taxonomic Units (OTUs) and selected using UPARSE (Edgar, 2013). Taxonomic assignments were made with USEARCH global alignment program (Edgar, 2013).

An OTUs table at 97% sequence similarity cut off was imported into the software Explicit 2.140.5 (Robertson et al., 2013) and a heatmap was created representing the most abundant taxonomic groups with a relative abundance >0.5%. All the taxonomic groups with <0.5% of relative abundance are represented as "Other" in the heatmap. The number of shared OTUs between samples at 97% sequence similarity cut off and the Venn diagrams were calculated using the web tool provided by the Bioinformatics & Evolutionary Genomics group at the University of Gent (http://bioinformatics.psb.ugent.be/webtools/Venn/). The software PAST v3.12 (Hammer et al., 2001) was used to estimate Alpha-diversity at 97% sequence similarity and the Shannon diversity index, Chao-I and Dominance-H were calculated. Briefly, the Shannon index (H) measures diversity taking into account the number of OTUs as well as number of taxa, this index varies from 0 for communities with only a single taxon to higher values (max <5 in this study). Chao 1, is an estimate of total OTUs richness using abundance and occurrence. The Dominance index (1-Simpson index) ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely) (Harper, 1999). Approximate confidence intervals for these indexes were computed with a bootstrap procedure (default 9999) and a 95% confidence interval was then calculated. Analysis of similarities (ANOSIM) description was performed based on Bray–Curtis dissimilarity distance matrices to test the differences in community composition among groups of samples using PAST v3.12. The Bray Curtis dissimilarity matrixes were visualised using non-metric multi-dimensional scaling (MDS) diagrams.

Sequencing data were deposited in the National Centre for Biotechnology Information (NCBI), Sequence Read Archive SRA SRP095264.

2.5. Statistics

Correlations between physico-chemical and biological parameters were explored by Spearman's rank non parametric correlations using SPSS 22. Only those parameters showing enough variability between samples were used to establish correlations. Alpha-diversity metrics and the relative abundance of the most abundant OTUs were used as biotic parameters in the establishment of correlations.

3. Results

3.1. Characteristics of the water supplied to the systems

Fig. 2 shows the flows in both systems over the sampled period. Negative or no data on the graph indicate no data collected at that specific time. The surface water (SW) site showed periods of different flow over the studied period; 0.3–0.9 ML/day during January–June 2013 to a maximum increase in July of 1.7 ML/day and minimum values between the end of July and the end of October 0.1–0.6 ML/day and higher flows November to January 0.3 to 1.4 ML/day. The ground water (GW) flow had less change in flow over the monitored period; up to July the monitoring devices showed flows of <0.1 ML/day, after this the average flow increased and was stable ranging typically between 0.1 and 0.35 ML/day. The shifts in flow patterns at both sites were the result of operation changes in the surrounding networks, outside the influence of this study.

No colonies counts (with the exception of November 2013) or *E coli* were detected in any of the discrete samples analysed at the time of coupon collection. Temperature ranged from 5.3 °C for SW and 8.5 °C for GW in February 2014 to maximum values of 21.8 °C for SW and 14.1 °C GW in August 2013. Turbidity was stable for GW 0.1 to 0.12 NTU and fluctuated slightly for SW 0.05 in November 2013 to 0.12 in August 2013. pH values were stable for GW 7.5 to 7.7 and slightly higher for SW 7.4 to 8. Other parameters such as conductivity, alkalinity, nitrate and sulphate were higher for the GW samples. Both sites have a similar

free chlorine residue, 0.2 to 0.35 mg/l, and the levels of chlorine and Total Organic Carbon (TOC) were similar for both sites.

3.2. Seasonality and microbial quarterly dynamics (plankton and biofilms)

3.2.1. Taxonomy variability: heatmap

Changes in the relative abundance of different bacterial OTUs were observed between different habitats and over time (seasonality) (Fig. 3). Please note that from two of the samples no sequencing data was obtained, these are one planktonic GW sample from November 2013 and one biofilm GW sample from November 2013. The Heatmap represents the relative OTU abundance of all replicates but in this section to summarise the information the average of replicates was calculated according to habitat and season. The bacterial community of both biofilm and planktonic samples was dominated by *Proteobacteria* with average relative abundance of up to 79%, (Fig. 3). This phylum was followed by *Actinobacteria* (8%) and *Firmicutes* (5%) that was commonly found in SW planktonic communities. Within the *Alphaproteobacteria* the order *Sphingomonadales* accounted for 17% of the sequences recovered from all samples and the genus *Sphingomonas* (4%) was present in all biofilm samples and in SW planktonic samples. The genus *Hypomicrobium* (5%) was particularly abundant in SW and GW samples in August and February and SW biofilms in August.

SW planktonic communities showed clear seasonal changes, August samples were dominated by *Alphaproteobacteria* and the genus *Hypomicrobium* (>24%) while in November *Gammaproteobacteria* (>30%) was the main represented phylum in the samples with >14% of those affiliated to the *Pseudomonadales* order. *Actinobacteria* were also abundant (>23%) in November. In February there was an increase again in *Alphaproteobacteria* (39%) in the total community mainly represented by *Brucellaceae* (16%) and with 21% of *Actinobacteria* with *Rhodococcus* as the main genus (9%).

GW planktonic communities showed less variability over time than SW but the relative abundance of different taxonomic groups also changed. *Alphaproteobacteria* was the main represented phylum in all the seasons ranging from 38% in August to 25% in February. Within the *Alphaproteobacteria* phylum the order *Sphingomonales* dominated in August (30%) and November (45%). In August *Firmicutes* was highly represented by *Clostridia* (18%), however in November and February this group has a minor representation in the total bacteria community. *Gammaproteobacteria* was presented in all months but was particularly abundant in February (25%) being the main order *Pseudomonales* (16%). During this month *Actinobacteria* (23%) was an important component of the microbial community with the genus *Mycobacterium* (6%) as the main representative.

Biofilm samples maintained several dominant OTUs over time mainly *Pseudomonas* and a high quantity of low abundance (<0.5% relative abundance) or rare OTUs. In SW biofilm samples *Alphaproteobacteria* was highly represented (>73% in August 2013), mainly by genera such as *Brucella* (3%), *Hypomicrobium* (6%), *Sphingomonas* (13%) and *Sphyngopsis* (34%). *Gammaproteobacteria* was commonly found in all seasons but particularly in November (67%) and February (57%) with *Pseudomonas* as the main genus. For GW biofilms *Pseudomonas* was

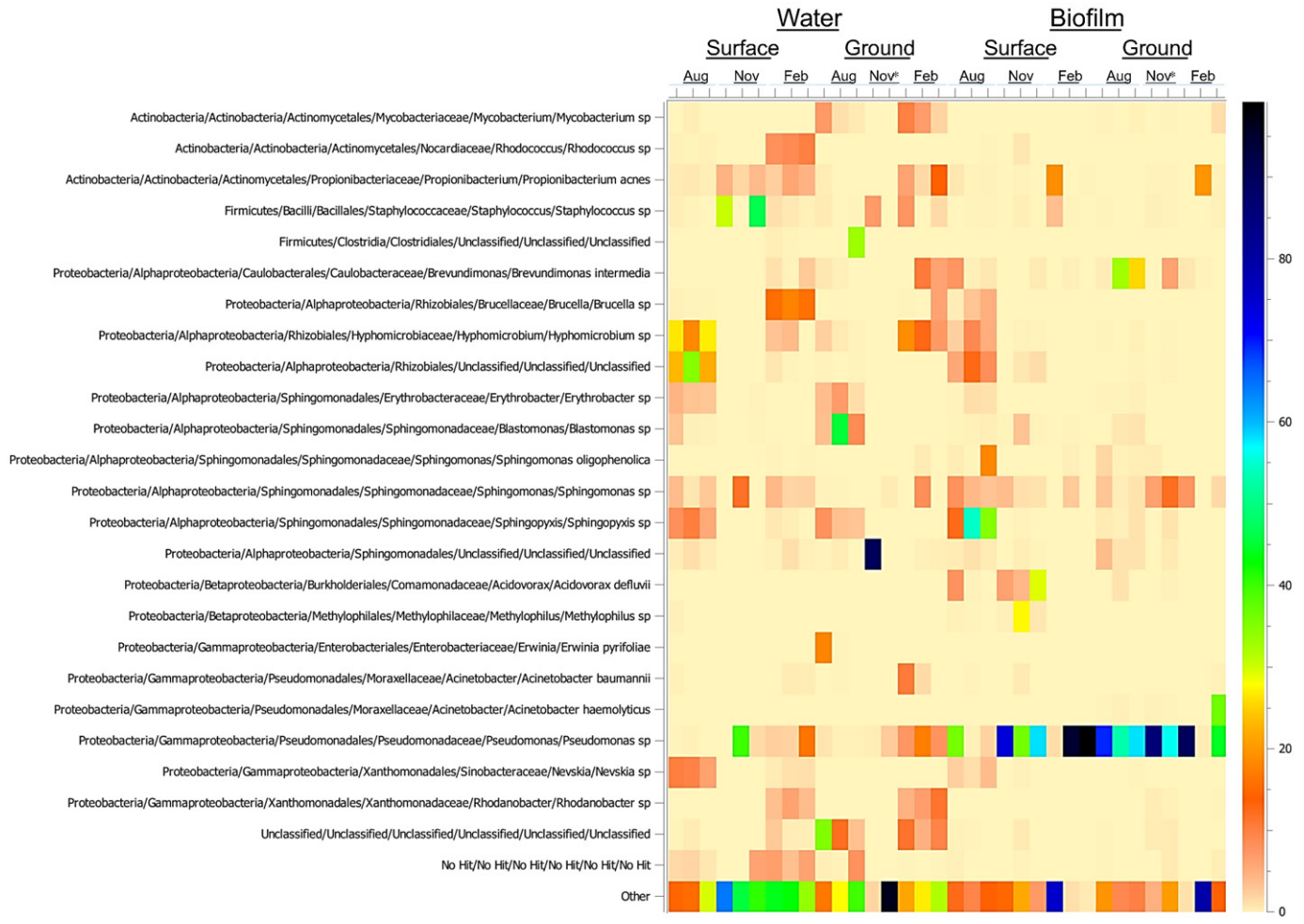


Fig. 3. Heatmap showing the taxonomic distribution of quarterly water and biofilm samples with relative abundance >0.5%. * indicates that only information from two samples was obtained. "Other" indicate the abundance of bacterial groups with <0.5% of relative abundance.

markedly abundant in all the seasons with an average representation always higher than 45%. *Alphaproteobacteria* abundance changed over time from 34% in August 2013 to 13% in February 2014. In August 2013 *Brevundimonas* was representing 20% of the total community but its abundance decreased to only 3% in February. *Sphingomonas* was highly abundant in all seasons but mainly in February (7%). In all the biofilm samples the main differences in microbial community structure over time are determined by several OTUs with <0.5% relative abundance, marked in the heatmap as "Other".

3.2.2. Shared OTUs and MDS

Fig. 4A shows Venn diagrams for each sampling season with the number of unique and shared OTUs between bacterial communities. In August, all habitats shared a large number of OTUs 87 between biofilm samples and 71 between planktonic communities. Conversely, a very low number of OTUs were shared in autumn, where SW and GW biofilm samples only shared 11 OTUs. The MDS analysis (Fig. 4B) using Bray-Curtis dissimilarity matrix (at 97% sequence similarity cut off) showed high variability between planktonic samples and a more stable community over time for biofilm samples from both SW and GW. Bacterial community structure was highly variable for planktonic SW samples. Less variability between biofilm samples was observed indicating that bacterial assemblages tend to re-growth in the same way independently of the water source supplied and the operational parameters. Differences in the community composition between samples were tested using ANOSIM (Fig. 4C). However, no statistical significant differences were observed over time (seasonality) between samples. Statistical differences were observed between habitats (locations), ANOSIM showed significant differences between planktonic samples ($p < 0.05$) from SW and GW. Biofilm and planktonic samples also showed significant difference for each sampling site SW ($p < 0.01$) and GW ($p < 0.01$). No significant differences were obtained for biofilm samples at both sites (SW *vs.* GW).

3.2.3. Alpha-diversity: diversity, richness and dominance

The alpha-diversity metrics of bacterial communities (Fig. 5) indicate clear changes between habitats and seasons. In general, Shannon diversity index (Fig. 5A) exhibited higher values for planktonic communities than for the biofilm ones. SW planktonic samples showed higher diversity when compared with GW. For biofilm samples, diversity was higher for SW biofilms in August 2013 but less in November 2013 and similar levels were showed for February 2014 where all biofilm samples showed an increase in diversity. The high diversity in February 2014 in the planktonic samples does not correspond with the low diversity in SW biofilms for that time.

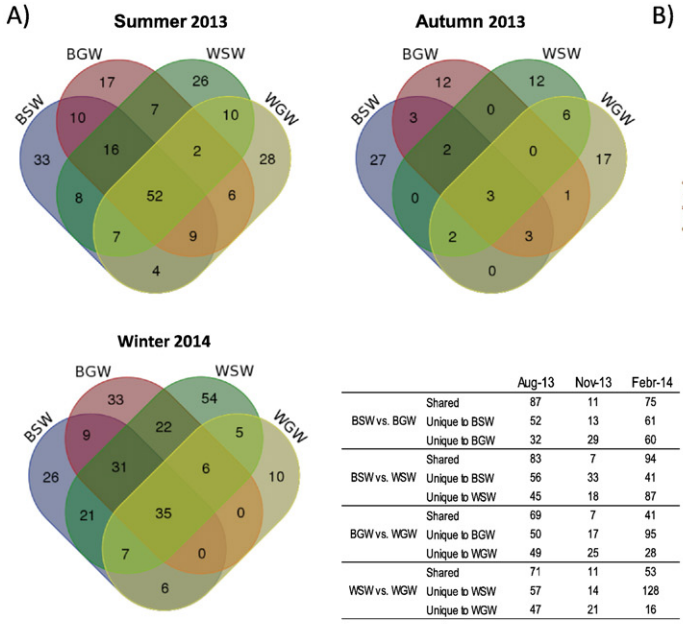
Chao richness (Fig. 5B) for planktonic communities was higher for SW than GW samples, with the exception of November 2013 samples, and similar for biofilm samples from both sites. Dominance (Fig. 5C) was generally low for all water samples but high for biofilms with most of the samples presenting values above 0.3 indicating the dominance of fewer OTUs in these communities.

3.3. Biofilm community composition and structure succession analysis (long term one year experiment)

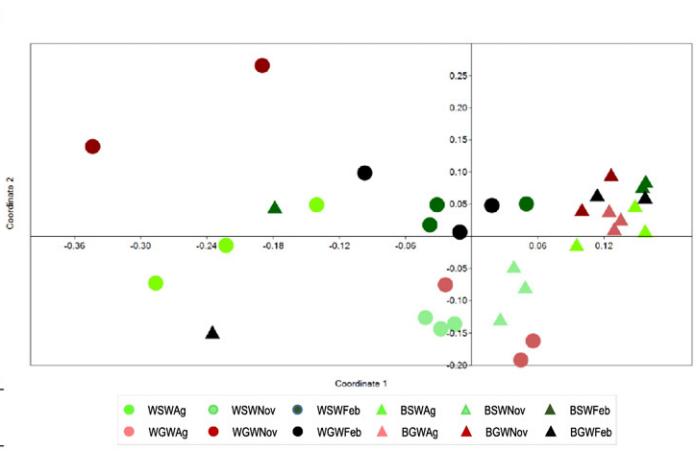
3.3.1. Taxonomic variability

All the samples (Fig. 6) showed high presence of *Gammaproteobacteria*, mainly the genus *Pseudomonas*, particularly the samples experiencing only 3 months of biofilm development. *Sphingomonadales* and *Cyanobacteria* were consistently abundant in all the samples. *Pseudomonas* dominated in SW biofilm samples during the 6 months of biofilm development. Other initially abundant OTUs decreased with time, such as *Rhizobiales* (*Alphaproteobacteria*) from 8% to 2.5% and *Acidovorax* (*Betaproteobacteria*) from 13% in 3 month-old biofilms to 0.7%. However, several OTUs increased over time and in 12 month-old SW biofilm samples a more diverse community was present with high abundance of *Alphaproteobacteria*. *Alphaproteobacteria* increased over time from 14 to 28% (*e.g. Sphingomonas*). *Firmicutes* increased from 0.5% to 15% and *Actinobacteria* from 0.9% to 13% with the main representative genera being *Mycobacterium* and *Propionibacterium*.

The GW community consistently showed predominance of *Pseudomonas* (>35%) and to a certain extent of *Sphingomonas* (1.5% to 14%). Biofilm samples from 6 and 12-month exposure displayed high relative abundance of *Mycobacterium* (0.5–55%) and *Brevundimonas* (3–39%).



		Aug-13	Nov-13	Febr-14
BSW vs. BGW	Shared	87	11	75
	Unique to BSW	52	13	61
	Unique to BGW	32	29	60
BSW vs. WSW	Shared	83	7	94
	Unique to BSW	56	33	41
	Unique to WSW	45	18	87
BGW vs. WGW	Shared	69	7	41
	Unique to BGW	50	17	95
	Unique to WGW	49	25	28
WSW vs. WGW	Shared	71	11	53
	Unique to WSW	57	14	128
	Unique to WGW	47	21	16



C)

R values

	WGW	BSW	BGW
WSW	0.1361*	0.2147*	0.4536**
WGW		0.2765**	0.3775**
BSW	0.2765**		0.04036
BGW	0.3775**	0.04036	

* p <0.05 ; **P<0.01

Fig. 4. A) Venn diagram of the planktonic and biofilm community showing the shared and unique OTUS for samples collected quarterly. B) Non-parametric multidimensional analysis (MDS) of biofilm and water samples. C) Table showing the results from the ANOSIM analysis. Labels: BSW: biofilm surface water, BGW: biofilm groundwater, WSW: water surface water, WGW: water ground water, Ag: August, Nov: November, Feb: February.

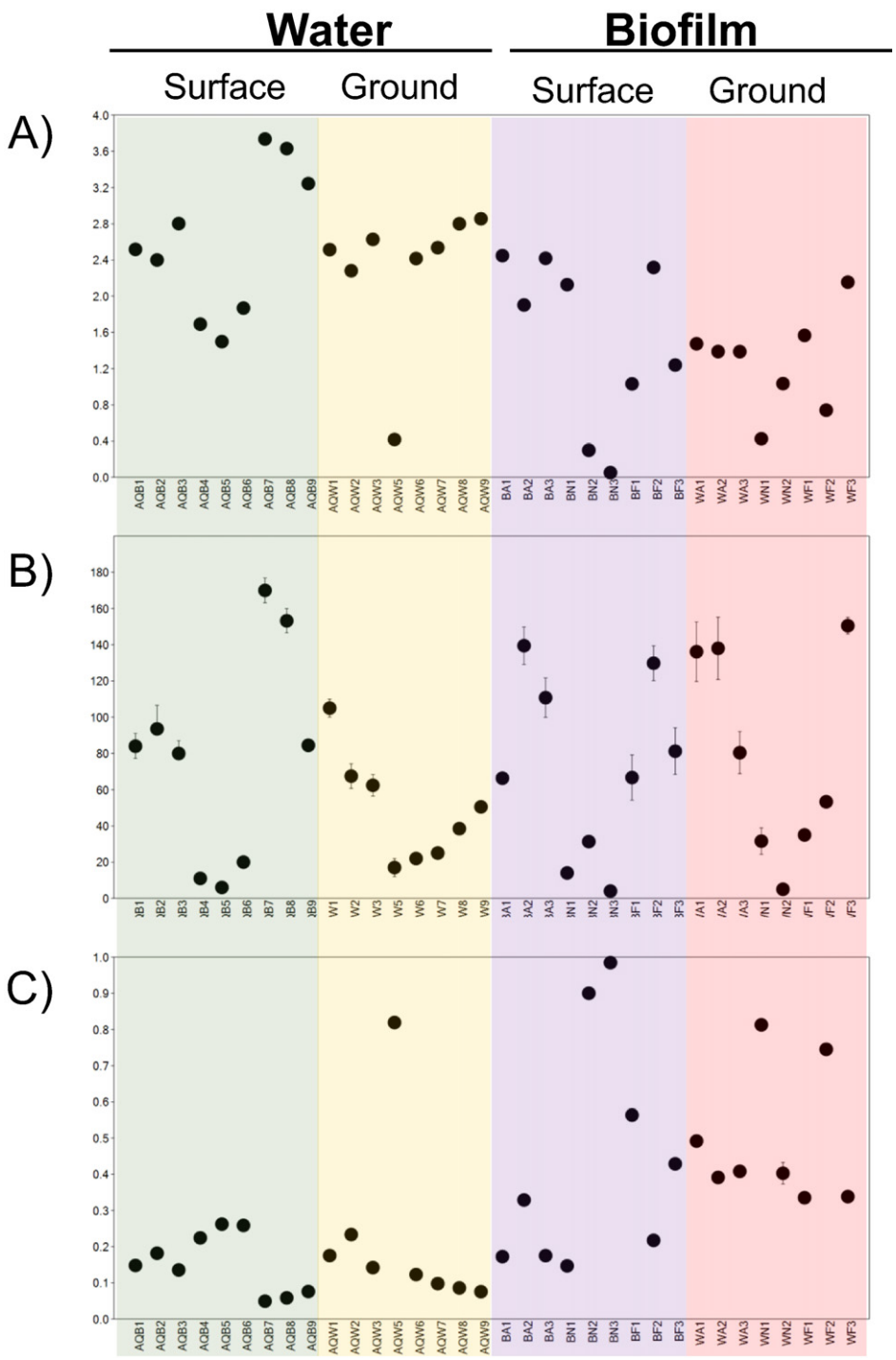


Fig. 5. Alpha diversity metrics for biofilm and water samples used to study biofilm re-growth. A) Shannon diversity index. B) Chao richness indicator. C) Dominance-H. Labels: AQ: water, B: biofilm, A: August, N: November, F: February. 1, 2, 3 are the numbers assigned to the replicates.

3.3.2. Shared OTUs and MDS

In SW biofilm samples, the proportion of unique *vs.* shared OTUs was similar over time (Fig. 7A). GW biofilm communities showed less temporal changes and the number of OTUs shared decreased between 6 and 12 month-old samples. For GW the number of shared OTUs decreased and between 3 and 12 month-old samples (Fig. 7B). The number of OTUs unique to GW biofilm samples on 12 month-old samples when compared to 3 month-old samples was 74. SW samples displayed a slightly increase in the number of OTUs in more mature biofilms but the number of unique OTUs for GW 3 month-old samples decreased. Despite SW and GW samples being more different to start with (3 month-old biofilms), regardless of sharing a relatively high percentage of OTUs, more mature biofilms were less distinctive and GW samples had only 37 unique OTUs when compared with SW samples. The number of OTUs unique to GW 12 month-old samples when compared to SW 12 month-old samples was only 37.

The compositional comparison of samples in a non-metric multidimensional scaling (MDS) plot (Fig. 7C) showed difference over time among sites and high variability for SW replicates. Temporal changes were more marked for SW samples, those showed high variability

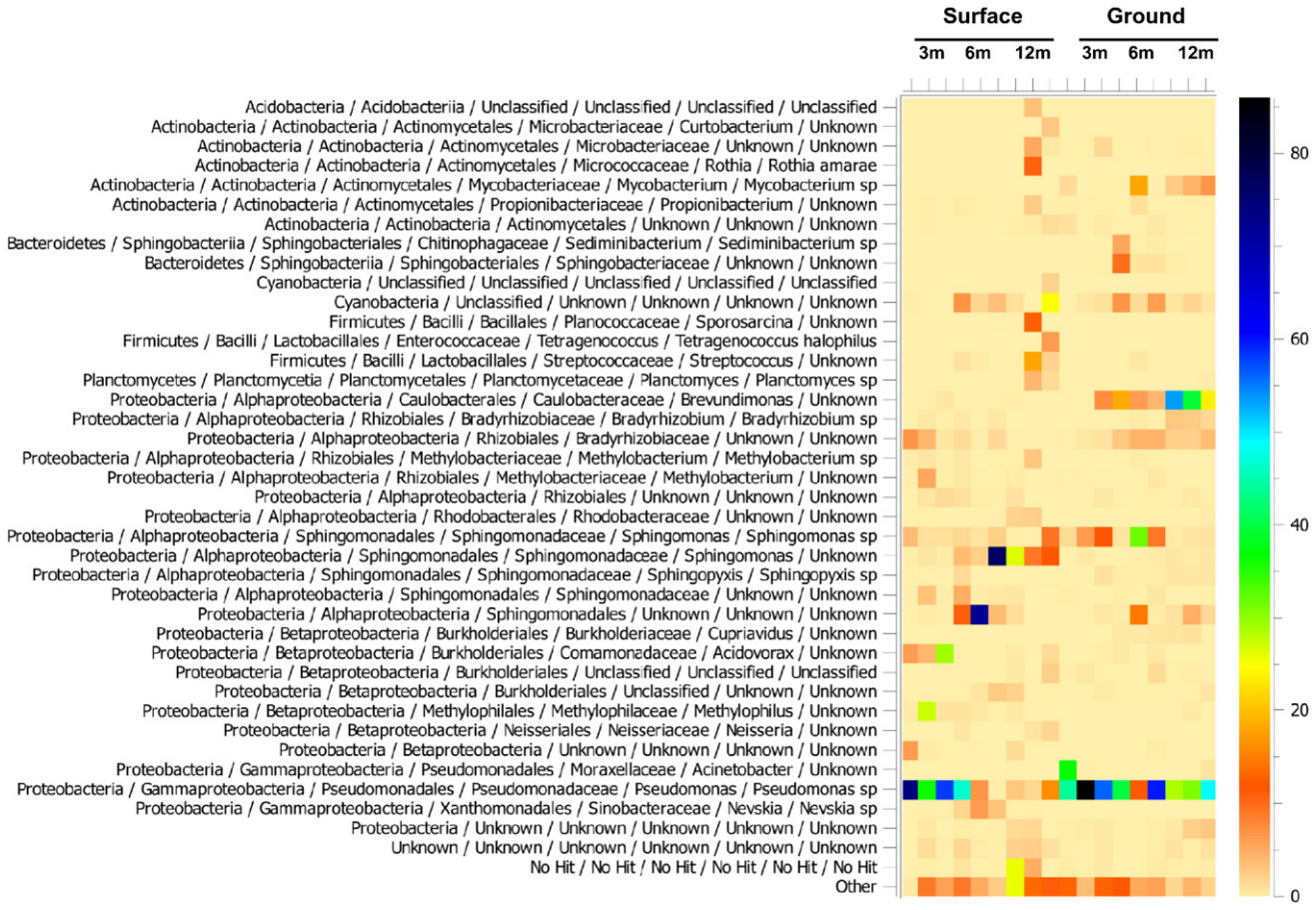


Fig. 6. Heatmap showing the taxonomic distribution of samples used to study biofilm succession over time with relative abundance >0.5%. "Other" indicates the abundance of bacterial groups with <0.5% of relative abundance.

between samples. The 3 month old SW samples and the GW samples from 3, 6 and 12 month-old biofilms showed a certain degree of clustering, while the SW 6 and 12 month-old samples were more scattered and dissimilar. The ANOSIM analysis showed that when each location was analysed independently non-significant statistical differences between months were observed (Fig. 7D). However, analysis of all samples for each habitat showed significant differences ($R = 0.122$ and $p = 0.0189$) between SW and GW samples.

3.3.3. Alpha-diversity: diversity, richness and dominance

Shannon diversity index presented similar levels for SW and GW biofilms for the 6 month- old biofilm samples and increased for 12 month-old samples particularly for SW (Fig. 8A). In the GW, the Shannon index was on average slightly lower in the 3 month-old samples, but was similar for the 6 and 12 month-old biofilm samples. Chao richness indicator (Fig. 8B) was higher for SW samples than for GW ones. In the SW samples the richness tended to increase from 3 to 6 month-old biofilms and then decreased for the 12 month-old samples. In GW, the Chao richness estimator was low for 3 month-old biofilm and then tended to increase from 3 to 6 to 12 month-old samples. In general, the dominance indicator (Fig. 8C) was slightly higher for SW samples for 3 and 6 month-old biofilms compared to GW, but decreased notably for the 12 month-old SW samples. GW samples had on average similar dominance levels between 3, 6 and 12 month-old biofilms.

3.4. Relation of physico-chemistry with microbiological parameters

Spearman's rank correlations were calculated to explore the correlation between different physico-chemical and microbiological parameters. These are shown in Supplementary material.

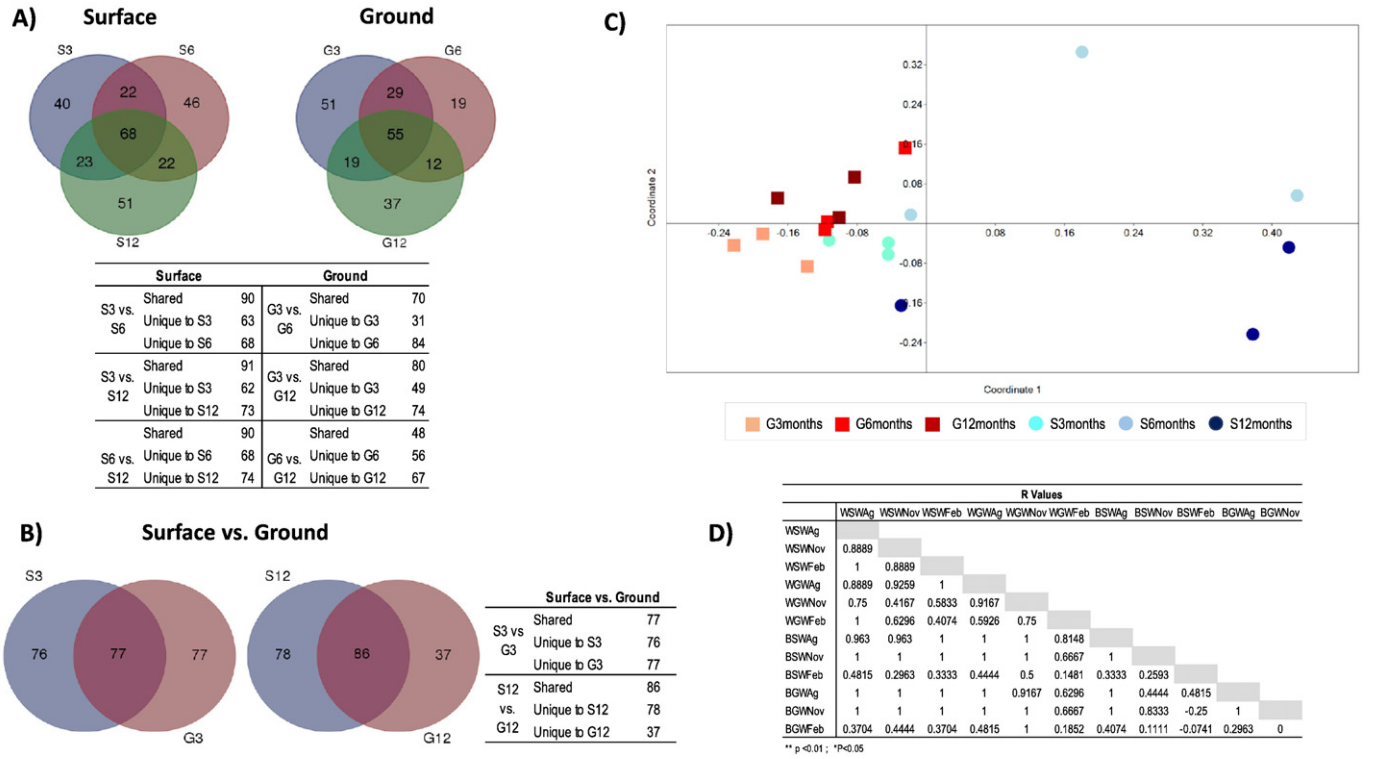
Flow was strongly positively correlated with pH and sulphate but negatively correlated with conductivity, alkalinity and nitrate. Flow was positively correlated with *Sphingomonas*, *Nevskia*, *Brucella*, *Rhodococcus* and *Propionibacterium* and negatively with *Mycobacterium*.

Significant positive correlations were detected between temperature and conductivity, alkalinity, TOC and pH. There was no correlation with chlorine, but levels where similar between sites and over time. There were also correlations with Chao and the relative abundance of several bacterial genera, including *Brevundimonas*, *Hypomicrobium*, *Erythrobacter*. Diversity was negatively correlated with *Pseudomonas* and dominance with *Rhodanobacter*. Turbidity was significantly positive correlated with levels of TOC, pH, Cl and the abundance of *Rhodococcus*, *Brucella* and *Hypomicrobium* among others. Conductivity and alkalinity and nitrate were also positively correlated.

Correlations between specific bacterial genera were also found. For example, the main OTU presented in biofilm samples, *Pseudomonas*, correlated positively with *Sphingomonas* and *Acidovorax* and was negatively correlated with *Propionibacterium*, *Staphylococcus* and *Erythrobacter*. *Nevskia* and *Sphingopyxis* were correlated with most of the other highly abundant OTUs in biofilms including *Rhodococcus*, *Propionibacterium*, *Brevundimonas*, *Erythrobacter* and *Sphingomonas*.

4. Discussion

Differences were seen in the taxonomic composition of SW and GW supplied systems, particularly in planktonic communities, with GW samples showing less marked changes over time when compared with SW. The variability in seasonal external factors had an effect particularly on SW planktonic communities. This was expected and similar observations were made in previous studies such as Gomez-Alvarez et



Surface			Ground		
S3 vs. S6	Shared	90	G3 vs. G6	Shared	70
	Unique to S3	63		Unique to G3	31
	Unique to S6	68		Unique to G6	84
S3 vs. S12	Shared	91	G3 vs. G12	Shared	80
	Unique to S3	62		Unique to G3	49
	Unique to S12	73		Unique to G12	74
S6 vs. S12	Shared	90	G6 vs. G12	Shared	48
	Unique to S6	68		Unique to G6	56
	Unique to S12	74		Unique to G12	67

Surface vs. Ground		
S3 vs G3	Shared	77
	Unique to S3	76
	Unique to G3	77
S12 vs. G12	Shared	86
	Unique to S12	78
	Unique to G12	37

Fig. 7. A) Venn diagram showing shared and unique OTUs for surface and ground water samples over time. B) Shared OTUs between both types of water habitats SW and GW. C) Metric multi-dimensional scaling (MDS) diagram. D) Table showing the results from the analysis of similarity statistics (ANOSIM). Labels: S: surface, G: ground, Ag: August, Nov: November, Feb: February, 3: 3 months, 6: 6 months, 12: 12 months.

al. (2015), which showed differences between GW and SW supplied drinking water-related systems in planktonic communities. Planktonic communities are commonly considered as the source of bacteria colonisers in DWDS biofilms (Henne et al., 2012). However, this study shows significant differences in the bacterial composition of these habitats (water *vs.* biofilm) and a lack of significant statistical influence of changes in the free-living organisms on the biofilm structure.

Ling et al. (2016) showed that seasonality was the main contributor to community structure variation by studying biofilm samples from household water meters and in tap water samples. Similarly, Bachmann and Edyvean (2005) reported that the origin of raw water had a great impact on the bacterial communities in DWDS and Pinto et al. (2014) found that the planktonic community was strongly correlated with the community found in water treatment works filters. However, unlikely these previous studies successional changes were detected in biofilm communities in the long term 1 year experiment (Fig. 7C) but no clear seasonal effect was observed on the quarterly samples (Fig. 4B). Consequently, changes in the bulk water communities did not exert a clear influence on the composition of the attached community.

A major result of this study is that the biofilm communities from both quarterly re-growth and succession over a year presented a similar core microbial community between both sites, and distinct from the planktonic community (as shown in Figs. 4B and 7C). The importance of external factors such as flow rate (Lehtola et al., 2006; Manuel et al., 2007), chlorine (Butterfield et al., 2002; Ndiongue et al., 2005), nutrient supply (Chandy and Angles, 2001; Boe-Hansen et al., 2003) and pipe material (Niquette et al., 2000) on biofilm development has been highlighted in other studies under controlled laboratory conditions. Here where biofilms were grown under natural, non-manipulated conditions, the main factors correlating with the distribution of certain bacterial groups were flow rate, TOC, temperature, pH and sulphate. The average flow rate was correlated with the relative abundance of several core bacteria consequently the presence or absence of some of these bacteria can be associated with the hydraulic conditions in the system. Previous work suggested that hydraulic conditions and shear forces have an influence on DWDS biofilms (Douterelo et al. 2013, 2016). Douterelo et al. (2016) showed such a difference across biofilm amount, strength and community composition for the same two sites as studied further here. However, it should be noted that the period covered in Douterelo et al. (2016) was exclusively from the near stagnant flow period at the GW site (Fig. 2). All 3 and 6 month-old samples reported here are from the flowing period of the GW site, and the 12 month-old samples were dominated by the flowing condition at this site. This suggests that while very low flow may lead to a different community and more and weaker biofilm material, flow rate might not be a central factor in shaping the dominant members of biofilm communities once a sustained regularly (daily) occurring turbulent flow regime is experienced. Douterelo et al. (2013) and (2014), both using a fully representative plastic pipe system under laboratory conditions, observed the influence of hydraulic regimes on biofilm structure, physical strength and discolouration risk. However, while representative of real system the conditions of these studies were controlled and repeated, hence it is possible that the unavoidable variations in daily conditions for the operational system studied here over such a long study period both at and between the sites obfuscated any such effects.

Based on the outcomes of this research, where a consistent core biofilm community was found independently of the sampling location, we can conclude that a group of bacteria that are adapted to DWDS are ubiquitous in these systems. We therefore hypothesise that there are internal factors related with the composition of the biofilm *per se* that are shaping the diversity of biofilms. The concept of a core community forming part of biofilms was first observed by Henne et al. (2012) using a molecular fingerprinting technique known as Single Strand Conformation Polymorphism (SSCP) in a non-chlorinated distribution system in Germany and has been corroborated by Ling et al. (2016) in water meters biofilms using pyrosequencing. The present study reinforces the existence of a universal

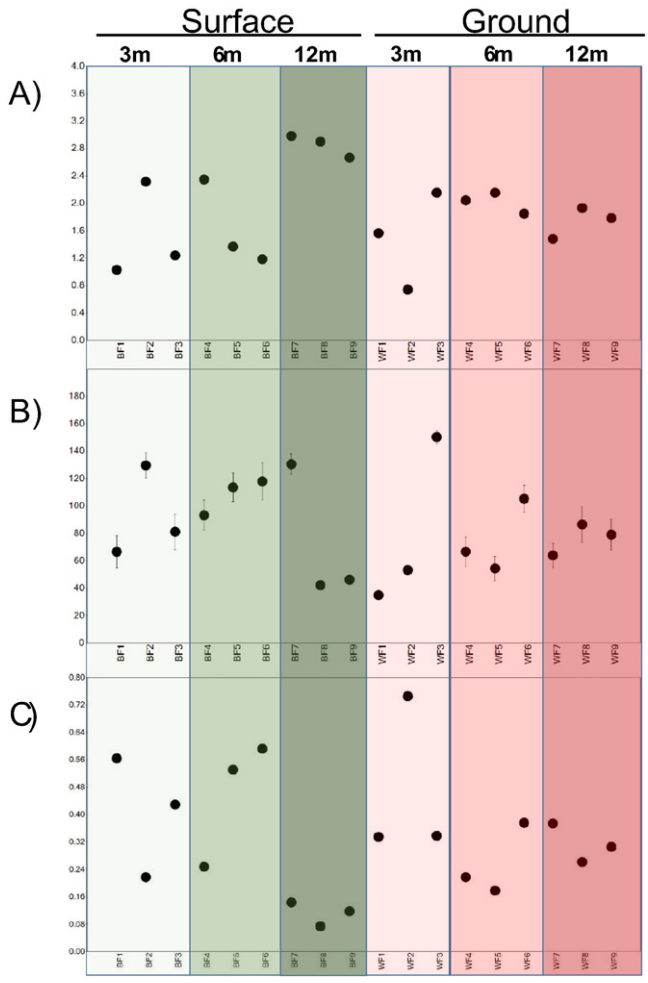


Fig. 8. Alpha diversity metrics for biofilm samples used to study biofilm succession. A) Shannon diversity index. B) Chao richness indicator. C) Dominance-H.

core community of microorganisms in DWDS biofilm by using a high-throughput sequencing method and in chlorinated systems supplied with different water sources and hydraulic regimes. The methodology used here involved the insertion of relatively small areas of sterile, autoclaved, pipe surface within a larger system that was not otherwise disturbed or impacted. This taken with the lack of significant influence of the planktonic community on the biofilm structure leads to the suggestion that this core community was predominately influenced by the surrounding biofilm composition from the local or upstream pipes. It is interesting to note that most likely internal regulatory factors were dominating rapidly such that the majority of the biofilm communities change little over the 1 year of succession, although dominance does drop for the SW 12 month-old samples (Fig. 8C). This is in agreement with Lyautey et al. (2005) that if microbial succession is the predominant mechanism of temporal changes in community structure, then these changes should be repeatable and predictable for a given region. If the temporal changes observed in biofilm communities were autogenic this should have led to comparable communities over the seasons, as seen here. It is interesting to compare this observation with the findings of Douterelo et al. (2016) where biofilm community was shown to evolve over time in response to repeated flushing of an operational system, the flushing was observed to exerting a selective pressure on the biofilms, and that microbial dynamics were influenced by changes in water source parameters particularly phosphate and metals. However, flushing does not remove all material and the remaining biofilm left attached on the pipe walls will influence the regrowth of new biofilm. Conversely this study, where sterile coupons were reinserted every three months, exhibits a rapidly re-established community from the stable, undisturbed biofilm community of the surrounding pipe surfaces. This again reinforces the influence of the local biofilm community rather than the planktonic community or any other external selective pressure.

Biofilm communities studied here were specifically enriched with certain bacteria, predominantly *Pseudomonas*. *Pseudomonas* was the main bacteria forming what can be considered the dominant community of biofilm structures, likely sourced from the surrounding undisturbed biofilm and hence independently of the water source studied. It is known that mixed-species biofilm formation can depend on the presence of species with high affinity and adherence to surfaces such as *Pseudomonas* facilitating the attachment of other microorganisms (Dunne, 2002; Kostakioti et al., 2013). *Pseudomonas* species can easily produce exopolysaccharides (Ghafoor et al., 2011; Irie et al., 2012) that can provide biofilms with a "stabilising effect". Thus the dominance of *Pseudomonas* independently of any external factor confirms that autogenic factors were shaping the biofilm community composition. The interaction of microorganisms in drinking water-related biofilms has been studied by selecting species isolated from drinking water systems and using dual combinations of them (Simões et al., 2007; Ramalingam et al., 2013). What remains unknown is the understanding of how natural mixed-species biofilm work and interact when there are also a combination of external factors that influence these communities. Here we confirm that in natural DWDS biofilms there is a clear tendency for particular bacteria to positively interact and form biofilms and this happens independently of external factors. These specific interactions can be exploited to exclude undesirable pathogens from healthy biofilm communities and to favour beneficial phenotypes. For example, in this study *Pseudomonas* was negatively correlated with the relative abundance of several bacteria including some potential pathogenic genera such as *Staphylococcus* and *Brucella*.

It is clear from looking at the 3, 6 and 12 month-old biofilms that once the core community has been establish changes over time are due to low abundance (rare) bacteria. Minor bacterial representatives (<0.5% relative abundance) were those that shaped the overall diversity over time and between sites. Similarly, Holinger et al. (2014) studying the bacteriological composition of tap water samples from different North American cities observed considerable variation among the rare phylotypes and that the most abundant taxa were similar from system to system, regardless of source water type. The author suggested that the similarity among the abundant taxa between systems was the consequence of the selective influence of chlorine-based disinfection and the local environment of the DWDS. In the present study chlorine was correlated with the distribution of a limited number of OTUs but was not the main factor affecting their distribution. However, what remains unknown is the relevance of these diverse minor representatives in the overall function of the system and whether if they play a central role in covering specific functions within biofilms that might change depending on the environment to adapt to different conditions.

Overall these experiments show that autogenic factors are important in shaping biofilm composition. In particular, it is surrounding biofilm community that is key in the processes of biofilm development, rather than the incoming bulk water community. Although it should be noted that only plastic pipes where studied here. This means that by simply adjusting or managing the physico-chemical characteristics of the water incoming the system it is not possible to fully control biofilm formation in DWDS. Most biofilm–related research focuses on how to eliminate biofilms but future control strategies might be better based on engineer biofilms to perform specific functions. Further consideration should be given to specific microorganisms able to colonise pipe surfaces and form biofilms, such that it might be possible to exclude adverse free-living organisms from colonising pipes protecting the supply of safe clean drinking water.

5. Conclusion

The dynamics of drinking water distribution systems biofilms were assessed over a one-year period and biofilm re-growth was analysed at quarterly intervals using sampling devices installed *in situ* in two operational networks. The two systems were supplied from surface and ground water sources, with only plastic pipework in and around the sampling locations. The planktonic communities clearly changed over time, particularly those supplied with surface water, influenced by seasonal changes. Independently of these and other characteristics of the incoming water between sites, the biofilm communities shared a high number of common and highly abundant operational taxonomic units (OTUs). The genus *Pseudomonas* was the main inhabitant of the biofilms independently of the network studied, forming part of a dominant core community ubiquitous to all biofilm samples, irrespective of if they were from succession over the one-year period or re-growth samples from quarterly intervals. The less abundant bacterial representatives (OTUs <0.5% relative abundance), rare OTUs, were responsible for most of the variability over time and between habitats. The main abiotic factors affecting the microbiology of the systems included flow rate, temperature and pH.

This research shows that while abiotic factors may influence the amount and strength of biofilm, its composition was strongly influenced by the biofilm community already present within the surrounding pipes. Such detailed understanding of the process of biofilm formation in DWDS is essential for the management of these systems for the delivery of safe clean drinking water, such as the potential to control or manipulate certain key bacteria to limit formation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.12.118.

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