
Article:
Douterelo, I., Jackson, M., Solomon, C. et al. (1 more author) (2016) Spatial and temporal analogies in microbial communities in natural drinking water biofilms. Science of the Total Environment. ISSN 0048-9697

https://doi.org/10.1016/j.scitotenv.2016.12.118

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

I. Douterelo[1] M. Jackson[2] C. Solomon[2] and J. Boxall[1]

1. Pennine Water Group, Department of Civil and Structural Engineering, Mappin Street, University of Sheffield, Sheffield, S1 3JD, UK.

2. Wessex Water, Claverton Down Rd, Bath, Somerset BA2 7WW, UK.

Correspondent author: Isabel Douterelo

e-mail: i.douterelo@sheffield.ac.uk

Telephone: +44 (0)1142 225767

Fax: +44 (0) 114 222 5700

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.

Keywords: bacteria, biofilm, flow, succession, re-growth

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 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 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 96711(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 minute 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 hours at 30°C followed by an incubation period at 37°C for a minimum of 14 hours. 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 2L 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. Chimera 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 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 less than 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 et al., 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.

2.4 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

Figure 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 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 less than 0.1 Ml/day, after this the average flow increased and was stable ranging typically between 0.1 to 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 or E. coli were detected in any of the discrete samples analysed at the time of coupon collection. Temperature ranged from 5.3 ºC for SW and 8.5 ºC for GW in February 2014 to maximum values of 21.8 ºC for SW and 14.1 ºC GW in August 2014. 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 (8.3%) and Firmicutes (5 %) that was commonly found in SW planktonic communities. Within the Alphaproteobacteria the order Sphingomonadales accounted for 16.6% of the sequences recovered from all samples and the genus Sphingomonas (4.3%) was present in all biofilm samples and in SW planktonic samples. The genus Hypomicrobium

(4.7%) 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 more than 14% of those affiliated to the Pseudomonadales order. Actinobacteria were also abundant (>23%) in November with the genus Arthobacter (>7%) highly present in the samples. In February there was an increase again in Alphaproteobacteria (39 %) in the total community mainly represented by the order 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 the order 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 the 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 (less than 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 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

231 determined by several OTUs with less than 0.5 % relative abundance, marked in the heatmap
232 as “Others”.

233 **3.2.2 Shared OTUs and MDS**

234 Figure 4A shows Venn diagrams for each sampling season with the number of unique and
235 shared OTUs between bacterial communities. In August, all habitats shared a large number of
236 OTUs 87 between biofilm samples and 71 between planktonic communities. Conversely, a
237 very low number of OTUs were shared in autumn, where SW and GW biofilm samples only
238 shared 7 OTUs. The MDS analysis (Fig. 4B) using Bray-Curtis dissimilarity matrix (at 97%
239 sequence similarity cut off) showed high variability between planktonic samples and a more
240 stable community over time for biofilm samples from both SW and GW. Bacterial
241 community structure was highly variable for planktonic SW samples. Less variability
242 between biofilm samples was observed indicating that bacterial assemblages tend to re-
243 growth in the same way independently of the water source supplied and the operational
244 parameters. Differences in the community composition between samples were tested using
245 ANOSIM (Fig. 4C). However, no statistical significant differences were observed over time
246 (seasonality) between samples. Statistical differences were observed between habitats
247 (locations), ANOSIM showed significant differences between planktonic samples ($p<0.01$)
248 from SW and GW. Biofilm and planktonic samples also showed significant difference for
249 each sampling site SW ($p<0.01$) and GW ($p<0.01$). No significant differences were obtained
250 for biofilm samples at both sites (SW vs. GW).

251 **3.2.3 Alpha-diversity: diversity, richness and dominance**

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

260 Chao richness (Fig. 5B) for planktonic communities was higher for SW than GW sample,
261 with the exception of November 2013 samples, and similar for biofilm samples from both
262 sites. Dominance (Fig. 5C) was low for all water samples (< 0.3) 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)

3.3.1Taxonomic 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%).

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 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 increases 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 and negatively with Pseudomonas and 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 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 Fig. 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 (2011) 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 core community of

395 microorganisms in DWDS biofilm by using a high-throughput sequencing method and in
396 chlorinated systems supplied with different water sources and hydraulic regimes. The
397 methodology used here involved the insertion of relatively small areas of sterile, autoclaved,
398 pipe surface within a larger system that was not otherwise disturbed or impacted. This taken
399 with the lack of significant influence of the planktonic community on the biofilm structure
400 leads to the suggestion that this core community was predominately influenced by the
401 surrounding biofilm composition from the local or upstream pipes. It is interesting to note
402 that most likely internal regulatory factors were dominating rapidly such that the majority of
403 the biofilm communities change little over the 1 year of succession, although dominance does
404 drop for the SW 12 month-old samples (Fig. 8C). This is in agreement with Lyautey et al.
405 (2005) that if microbial succession is the predominant mechanism of temporal changes in
406 community structure, then these changes should be repeatable and predictable for a given
407 region. If the temporal changes observed in biofilm communities were autogenic this should
408 have led to comparable communities over the seasons, as seen here. It is interesting to
409 compare this observation with the findings of Douterelo et al (2016) where biofilm
410 community was shown to evolve over time in response to repeated flushing of an operational
411 system, the flushing was observed to exerting a selective pressure on the biofilms, and that
412 microbial dynamics were influenced by changes in water source parameters particularly
413 phosphate and metals. However, flushing does not remove all material and the remaining
414 biofilm left attached on the pipe walls will influence the regrowth of new biofilm. Conversely
415 this study, where sterile coupons were reinserted every three months, exhibits a rapidly re-
416 established community from the stable, undisturbed biofilm community of the surrounding
417 pipe surfaces. This again reinforces the influence of the local biofilm community rather than
418 the planktonic community or any other external selective pressure.
419 Biofilm communities studied here were specifically enriched with certain bacteria,
420 predominantly Pseudomonas. Pseudomonas was the main bacteria forming what can be
421 considered the dominant community of biofilm structures, likely sourced from the
422 surrounding undisturbed biofilm and hence independently of the water source studied. It is
423 known that mixed-species biofilm formation can depend on the presence of species with high
424 affinity and adherence to surfaces such as Pseudomonas facilitating the attachment of other
425 microorganisms (Dunne, 2002; Kostakioti et al., 2013). Pseudomonas species can easily
426 produce exopolysaccharides (Ghafoor et al., 2011; Irie et al., 2012) that can provide biofilms
427 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 (less than 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.

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.

Acknowledgments

The research reported here was supported by the UK Engineering and Physical Sciences Research Council (EPSRC) Challenging Engineering grant EP/G029946/1, the EPSRC Platform Grant Funding EP/1029346/1 and the EPSRC-LWEC Challenge Fellowship EP/N02950X/1. We would like to thank Wessex Water Services Ltd for sampling sites, field work and physico-chemical sample analysis.

REFERENCES

Bachmann, R.T., Edyvean, R.G.J., 2005. Biofouling: an historic and contemporary review of its causes, consequences and control in drinking water distribution systems. Biofilms 2, 197-227.

Boe-Hansen, R., Martiny, A.C., Arvin, E., Albrechtsen, H.J., 2003. Monitoring biofilm formation and activity in drinking water distribution networks under oligotrophic conditions. Water science and technology : a journal of the International Association on Water Pollution Research 47, 91-97.

Butterfield, P.W., Camper, A.K., Ellis, B.D., Jones, W.L., 2002. Chlorination of model drinking water biofilm: implications for growth and organic carbon removal. Water Res 36, 4391-4405.

Chandy, J.P., Angles, M.L., 2001. Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. Water Res 35, 2677-2682.

Deines, P., Sekar, R., Husband, P.S., Boxall, J.B., Osborn, A.M., Biggs, C.A., 2010. A new coupon design for simultaneous analysis of in situ microbial biofilm formation and community structure in drinking water distribution systems. Appl Microbiol Biot 87, 749-756.

Donohue, M.J., Mistry, J.H., Donohue, J.M., O'Connell, K., King, D., Byran, J., Covert, T., Pfaller, S., 2015. Increased Frequency of Nontuberculous Mycobacteria Detection at Potable Water Taps within the United States. Environmental Science & Technology 49, 6127-6133.

Douterelo, I., Husband, S., Boxall, J.B., 2014. The bacteriological composition of biomass recovered by flushing an operational drinking water distribution system. Water Res 54, 100-114.

Douterelo, I., Jackson, M., Solomon, C., Boxall, J., 2016. Microbial analysis of in situ biofilm formation in drinking water distribution systems: implications for monitoring and control of drinking water quality. Appl Microbiol Biot 100, 3301-3311.

Douterelo, I., Sharpe, R.L., Boxall, J.B., 2013. Influence of hydraulic regimes on bacterial community structure and composition in an experimental drinking water distribution system. Water Res 47, 503-516.

Dunne, W.M., 2002. Bacterial adhesion: Seen any good biofilms lately? Clin Microbiol Rev 15, 155-+.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10, 996-998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200.

Fish, K.E., Collins, R., Green, N.H., Sharpe, R.L., Douterelo, I., Osborn, A.M., Boxall, J.B., 2015. Characterisation of the Physical Composition and Microbial Community Structure of Biofilms within a Model Full-Scale Drinking Water Distribution System. PLoS ONE 10, e0115824.

Ghafoor, A., Hay, I.D., Rehm, B.H.A., 2011. Role of Exopolysaccharides in Pseudomonas aeruginosa Biofilm Formation and Architecture. Appl Environ Microb 77, 5238-5246.

Giao, M.S., Azevedo, N.F., Wilks, S.A., Vieira, M.J., Keevil, C.W., 2008. Persistence of Helicobacter pylori in heterotrophic drinking-water biofilms. Appl Environ Microb 74, 5898-5904.

Gomez-Alvarez, V., Humrighouse, B.W., Revetta, R.P., Santo Domingo, J.W., 2015. Bacterial composition in a metropolitan drinking water distribution system utilizing different source waters. Journal of Water and Health 13, 140-151.

Hammer, Ø. & Harper, D.A.T. 2006. Paleontological Data Analysis. Blackwell.

Hammer, Ø., Harper, D.A.T., and P. D. Ryan, 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4(1): 9pp.

Harper, D.A.T. (ed.). 1999. Numerical Palaeobiology. John Wiley & Sons.

Henne, K., Kahlisch, L., Brettar, I., Höfle, M.G., 2012. Analysis of Structure and Composition of Bacterial Core Communities in Mature Drinking Water Biofilms and Bulk Water of a Citywide Network in Germany. Appl Environ Microb 78, 3530-3538.

Holinger, E.P., Ross, K.A., Robertson, C.E., Stevens, M.J., Harris, J.K., Pace, N.R., 2014. Molecular analysis of point-of-use municipal drinking water microbiology. Water Research 49, 225-235.

Hwang, C., Ling, F., Andersen, G.L., LeChevallier, M.W., Liu, W.-T., 2012. Microbial Community Dynamics of an Urban Drinking Water Distribution System Subjected to Phases of Chloramination and Chlorination Treatments. Applied and Environmental Microbiology 78, 7856-7865.

Irie, Y., Borlee, B.R., O'Connor, J.R., Hill, P.J., Harwood, C.S., Wozniak, D.J., Parsek, M.R., 2012. Self-produced exopolysaccharide is a signal that stimulates biofilm formation in Pseudomonas aeruginosa. Proceedings of the National Academy of Sciences 109, 20632-20636.

Janjaroen, D., Ling, F., Monroy, G., Derlon, N., Mogenroth, E., Boppart, S.A., Liu, W.-T., Nguyen, T.H., 2013. Roles of ionic strength and biofilm roughness on adhesion kinetics of Escherichia coli onto groundwater biofilm grown on PVC surfaces. Water Res 47, 2531-2542.

Kasuga, I., Nakagaki, H., Kurisu, F., Furumai, H., 2010. Predominance of ammonia-oxidizing archaea on granular activated carbon used in a full-scale advanced drinking water treatment plant. Water Research 44, 5039-5049.

Kostakioti, M., Hadjifrangiskou, M., Hultgren, S.J., 2013. Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Postantibiotic Era. Cold Spring Harbor Perspectives in Medicine 3, a010306.

Lee, O.O., Wang, Y., Tian, R., Zhang, W., Shek, C.S., Bougouffa, S., Al-Suwailem, A., Batang, Z.B., Xu, W., Wang, G.C., Zhang, X., Lafi, F.F., Bajic, V.B., Qian, P.Y., 2014. In situ environment rather than substrate type dictates microbial community structure of biofilms in a cold seep system. Sci Rep 4.

Lehtola, M.J., Laxander, M., Miettinen, I.T., Hirvonen, A., Vartiainen, T., Martikainen, P.J., 2006. The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. Water Res 40, 2151-2160.

577 Ling, F., Hwang, C., LeChevallier, M.W., Andersen, G.L., Liu, W.-T., 2016. Core-satellite
578 populations and seasonality of water meter biofilms in a metropolitan drinking water
579 distribution system. ISME J 10, 582-595.

580 Liu, G., Ling, F.Q., Magic-Knezev, A., Liu, W.T., Verberk, J.Q.J.C., Van Dijk, J.C., 2013.
581 Quantification and identification of particle-associated bacteria in unchlorinated drinking
582 water from three treatment plants by cultivation-independent methods. Water Research 47,
583 3523-3533.

584 Lyautey, E., Jackson, C.R., Cayrou, J., Rols, J.-L., Garabétian, F., 2005. Bacterial
585 Community Succession in Natural River Biofilm Assemblages. Microbial Ecology 50, 589-
586 601.

587 Manuel, C.M., Nunes, O.C., Melo, L.F., 2007. Dynamics of drinking water biofilm in
588 flow/non-flow conditions. Water Res 41, 551-562.

589 Moritz, M.M., Flemming, H.-C., Wingender, J., 2010. Integration of Pseudomonas
590 aeruginosa and Legionella pneumophila in drinking water biofilms grown on domestic
591 plumbing materials. International Journal of Hygiene and Environmental Health 213, 190-
592 197.

593 Mukherjee, J., Karunakaran, E., Biggs, C.A., 2012. Using a multi-faceted approach to
594 determine the changes in bacterial cell surface properties influenced by a biofilm lifestyle.
595 Biofouling 28, 1-14.

596 Ndiongue, S., Huck, P.M., Slawson, R.M., 2005. Effects of temperature and biodegradable
597 organic matter on control of biofilms by free chlorine in a model drinking water distribution
598 system. Water Res 39, 953-964.

599 Neufeld, J.D., Vohra, J., Dumont, M.G., Lueders, T., Manefield, M., Friedrich, M.W.,
600 Murrell, J.C., 2007. DNA stable-isotope probing. Nat Protoc 2, 860-866.

601 Niquette, P., Servais, P., Savoir, R., 2000. Impacts of pipe materials on densities of fixed
602 bacterial biomass in a drinking water distribution system. Water Res 34, 1952-1956.

603 Pinto, A.J., Schroeder, J., Lunn, M., Sloan, W., Raskin, L., 2014. Spatial-Temporal Survey
604 and Occupancy-Abundance Modeling To Predict Bacterial Community Dynamics in the
605 Drinking Water Microbiome. Mbio 5.

606 Pinto, A.J., Xi, C., Raskin, L., 2012. Bacterial Community Structure in the Drinking Water
607 Microbiome Is Governed by Filtration Processes. Environmental Science & Technology 46,
608 8851-8859.

609 Ramalingam, B., Sekar, R., Boxall, J., Biggs, C., 2013. Aggregation and biofilm formation of
610 bacteria isolated from domestic drinking water. Water Science & Technology: Water Supply
611 13.

612 Robertson, C.E., Harris, J.K., Wagner, B.D., Granger, D., Browne, K., Tatem, B., Feazel,
613 L.M., Park, K., Pace, N.R., Frank, D.N., 2013. Explicet: graphical user interface software for
614 metadata-driven management, analysis and visualization of microbiome data. Bioinformatics
615 29, 3100-3101.

616 Simões, L.C., Simões, M., Vieira, M.J., 2007. Biofilm Interactions between Distinct Bacterial
617 Genera Isolated from Drinking Water. Applied and Environmental Microbiology 73, 6192-
618 6200.

619 Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. Nat Rev Micro 6,
620 199-210.

Table and Figures

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

Figure 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.

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

Figure 3: Heatmap showing the taxonomic distribution of quarterly water and biofilm samples studying biofilm re-growth with relative abundance > 0.5%. * indicates that only information from two samples was obtained. **Others** indicate the abundance of bacterial groups with < 0.5% of relative abundance.

Figure 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.

Figure 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

Figure 6: Heatmap showing the taxonomic distribution of samples used to study biofilm succession over time with relative abundance > 0.5%. **Others** indicate the abundance of bacterial groups with < 0.5% of relative abundance.

Figure 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.

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

653 **Supplementary material**: Table showing Spearman's rank correlations with significant
654 statistical values
655

656 Table 1

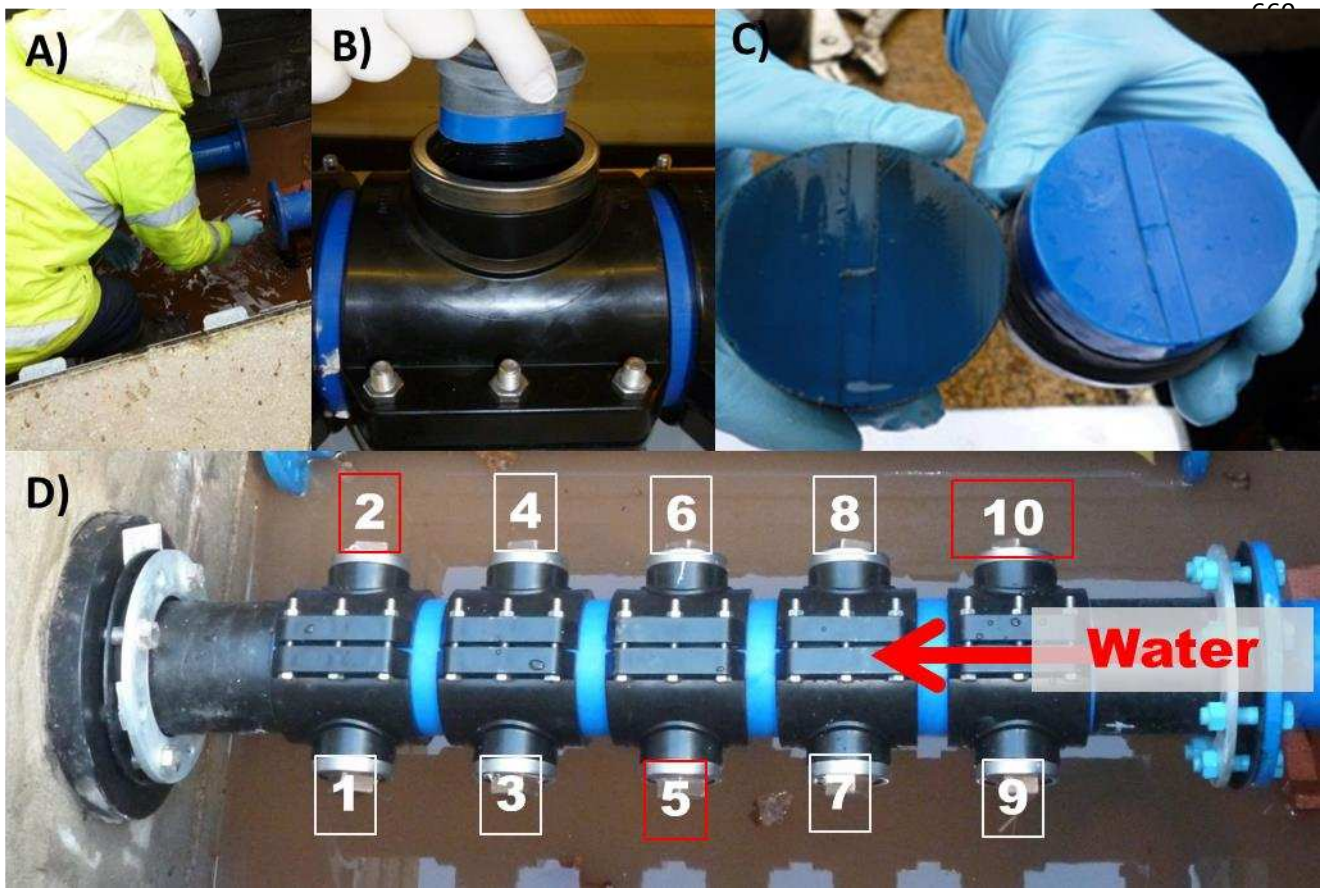
	Surface Water			*Ground Water*		
	Aug-13	**Nov-13**	**Feb-14**	**Aug-13**	**Nov-13**	**Feb-14**
HPC 37°C (cfu/ml)	0	0	0	0	0	0
HPC 22°C (cfu/ml)	0	0	0	0	1.5±0.3	0
E coli (cfu/100ml)	0	0	0	0	0	0
Temperature (°C)	21.8±0	7.3±0.1	5.3±0	14.1±0	7.45±0.3	8.05±0
Turbidity (NTU)	0.12±0.01	0.05± 0.1	0.2±0.03	0.12±0	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 CaCO3/L)	64.5±4.9	50±2.8	52.5±0.5	196.5±2.12	183.5±0.3	*
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 NH3/L)	0.01±0	0.07±0.1	<0.01	<0.01	<0.01	<0.01
Nitrite (mg NO2/L)	0.01±0	0.003±0	0.01	<0.01	<0.01	<0.01
Nitrate (mg NO3/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 SO4/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 (mgC/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

* No measured

657

658

659 Figure 1

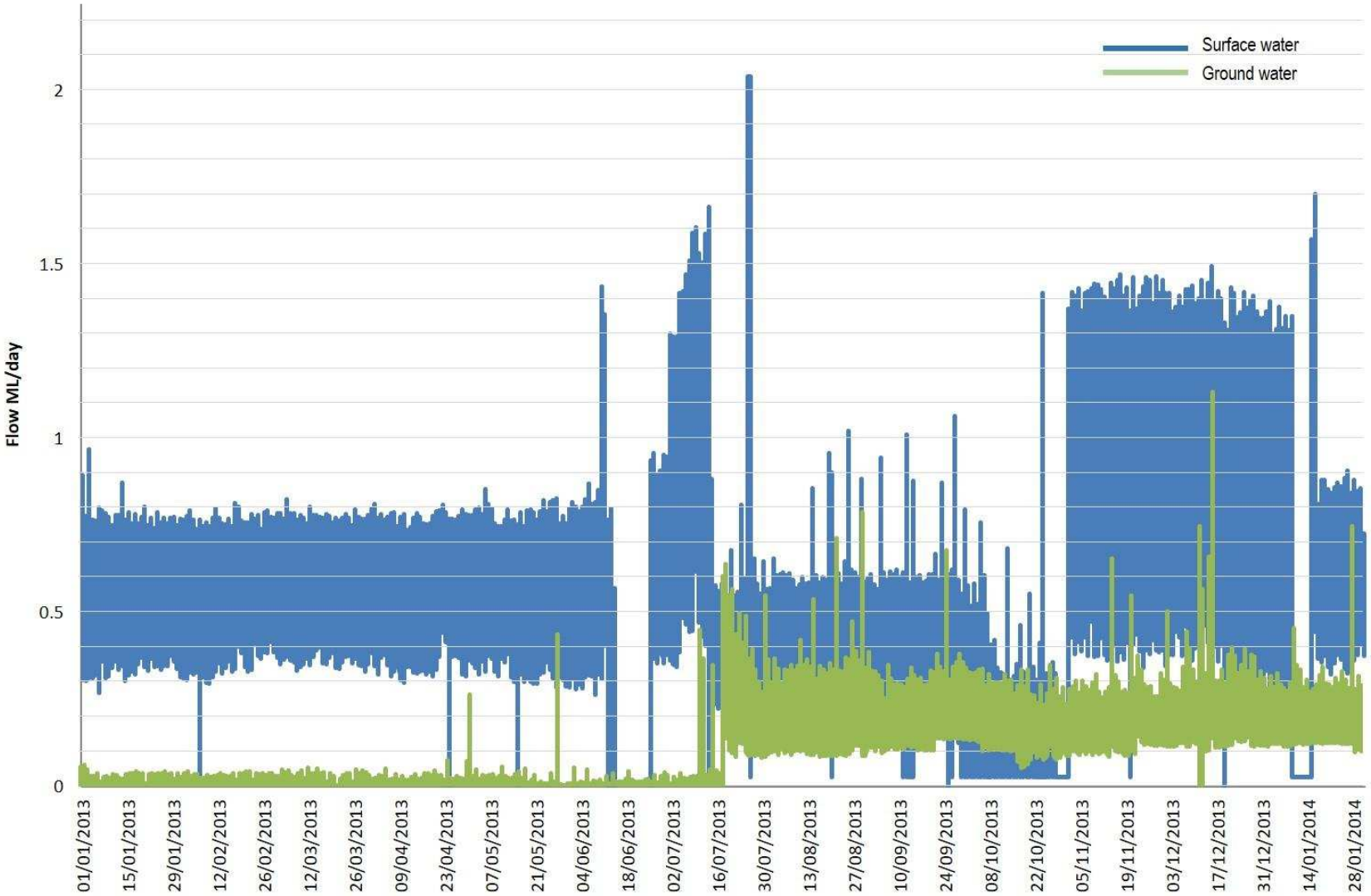


672

673

674

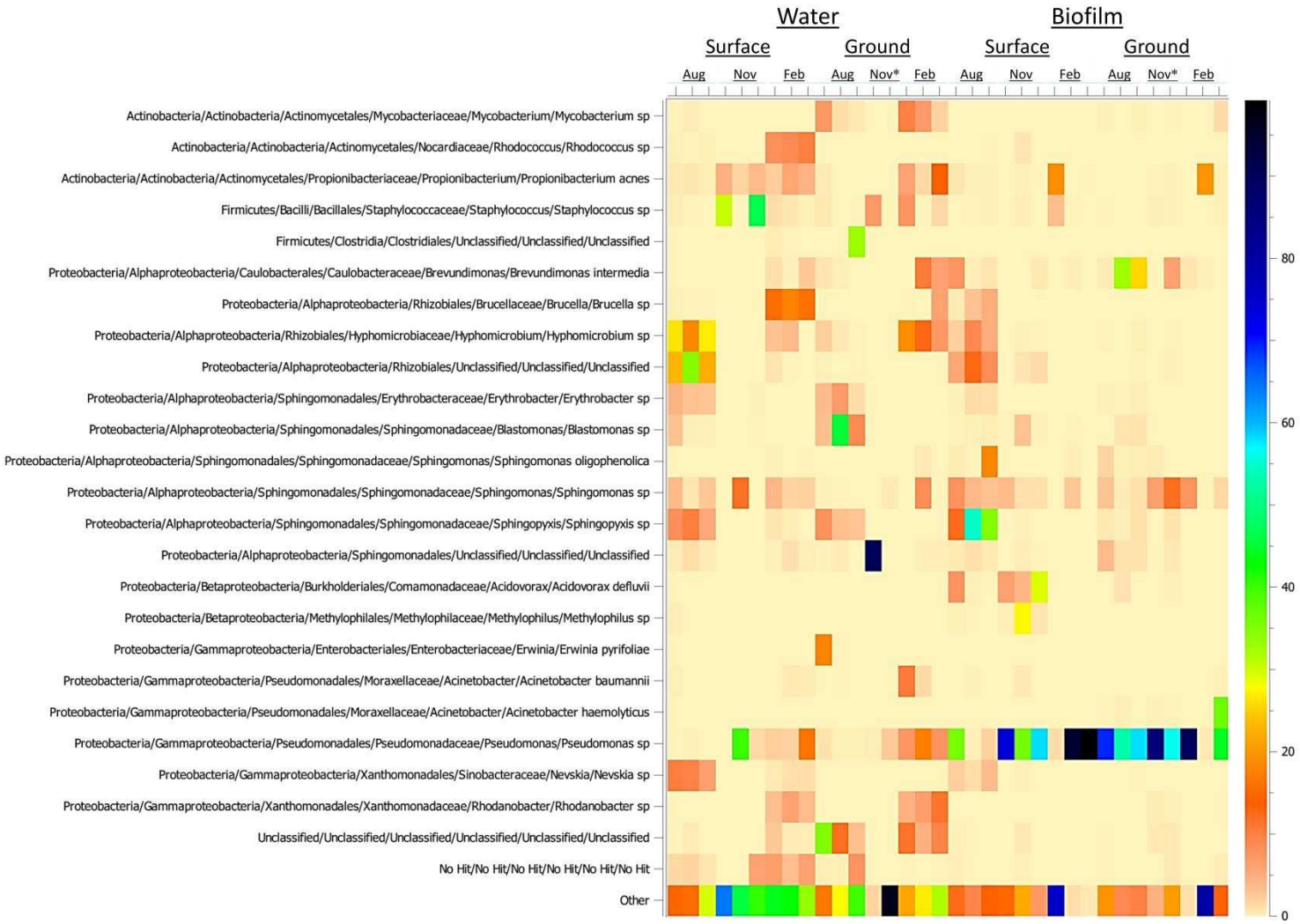
675 Figure 2



676

677

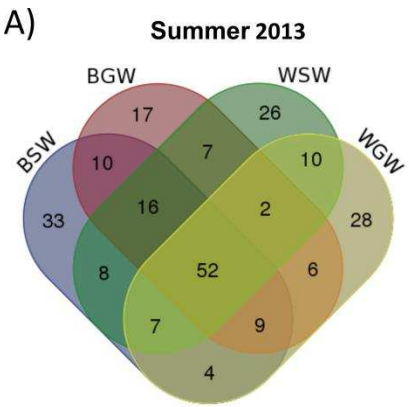
678 Figure 3

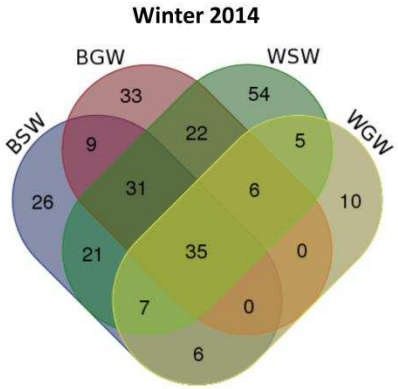


679

680 Figure 4

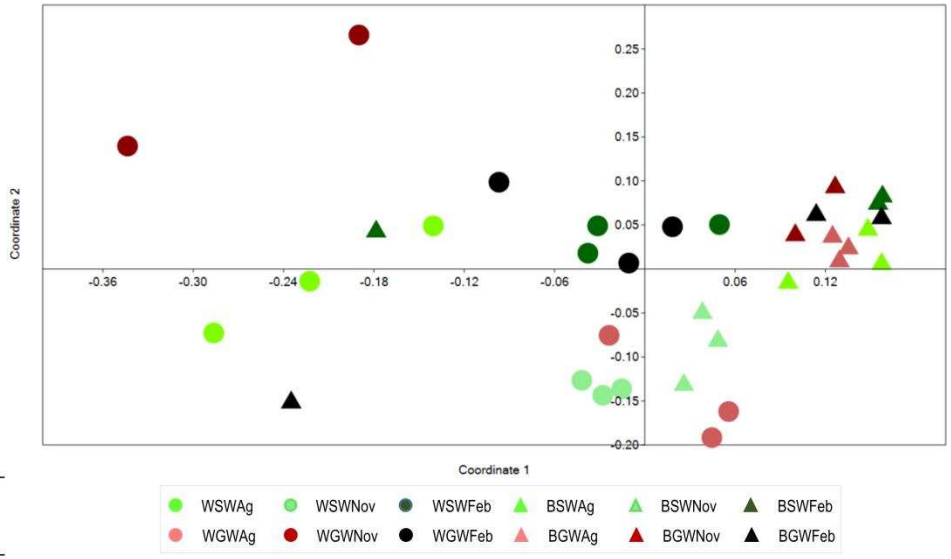
A)





		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

B)



C)

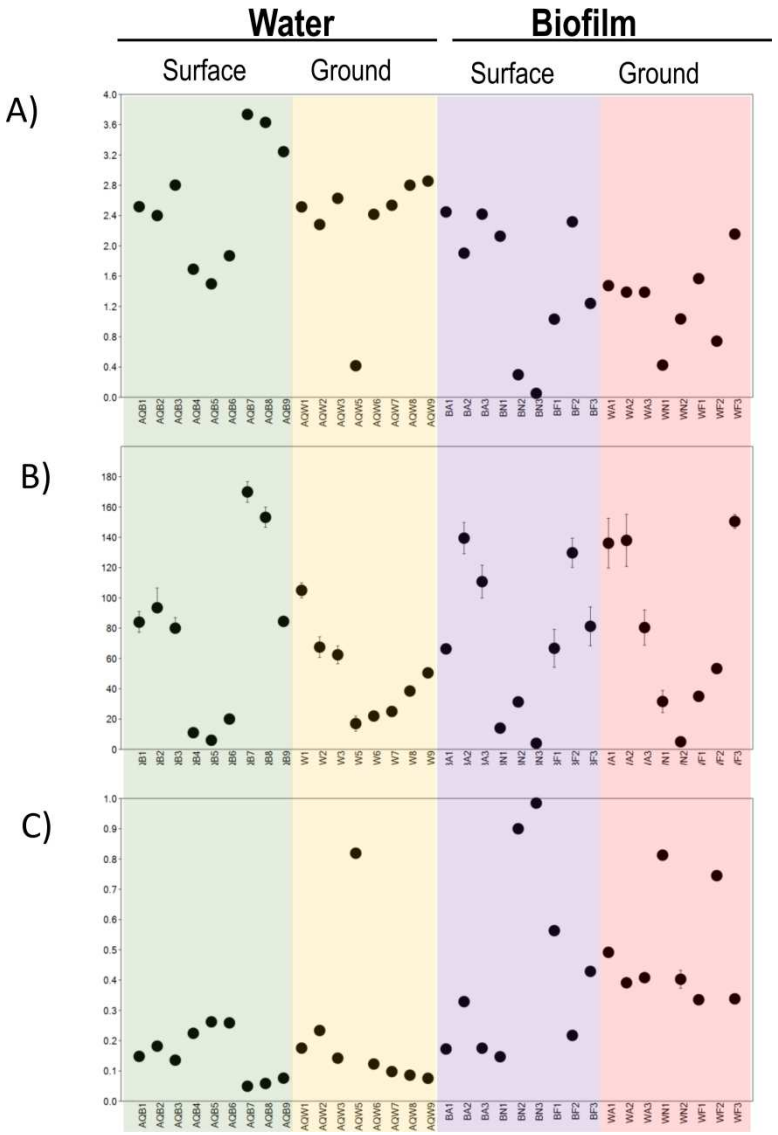
R values	WGW	BSW	BGW
WSW	0.1361*	0.2147*	0.4536**
WGW		0.2765**	0.3775**
BSW	0.2765**		0.04036
BGW	0.3775**	0.04036	

* p <0.05 ; **P<0.01

681

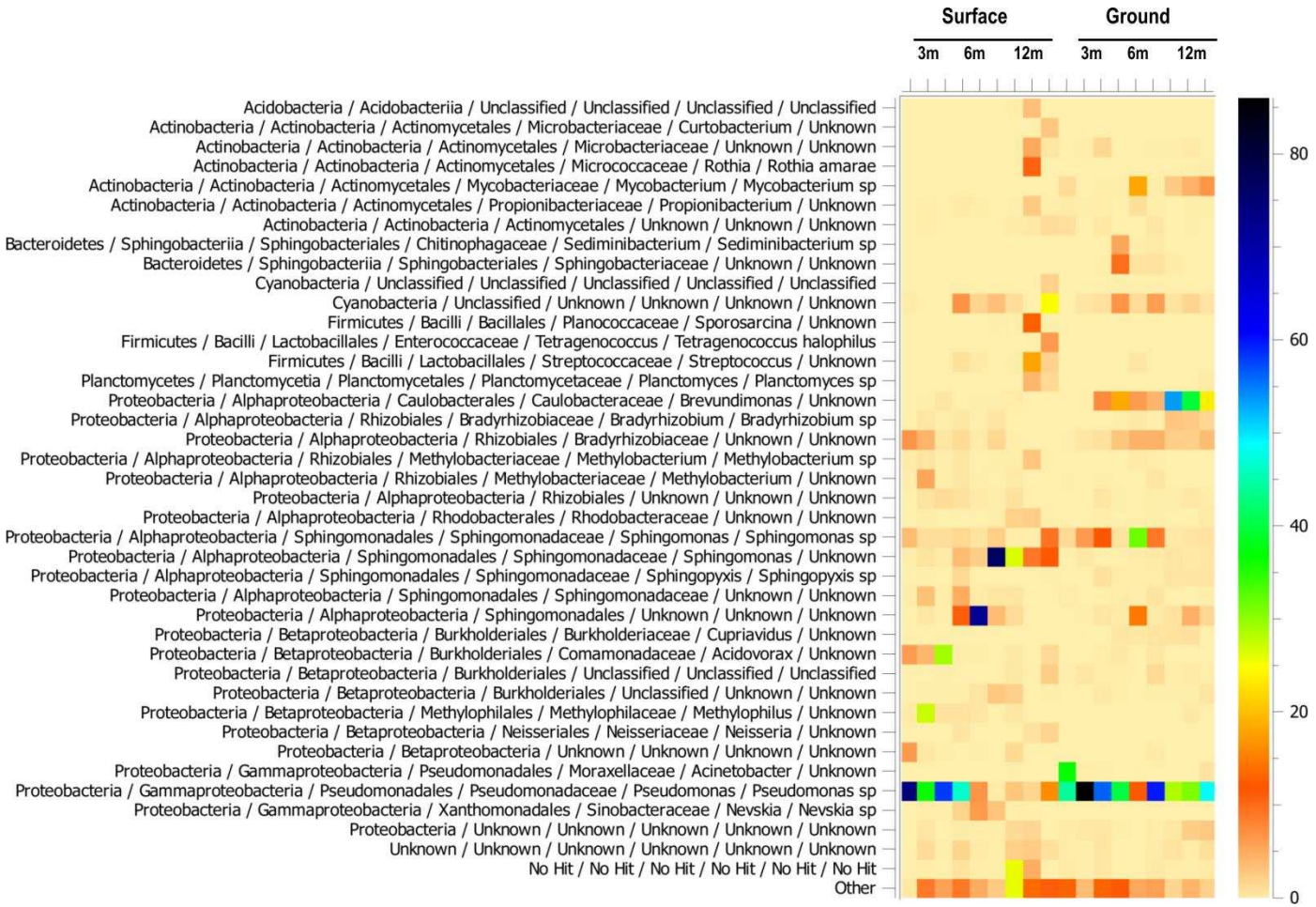
682

683 Figure 5



684

685 Figure 6

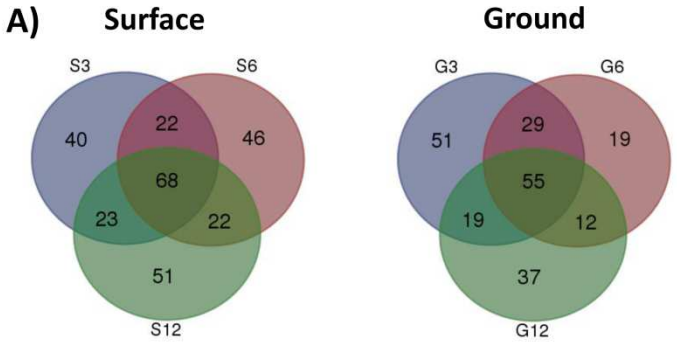


686

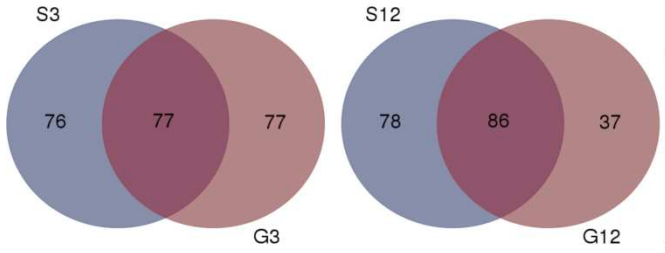
687

688

689 Figure 7

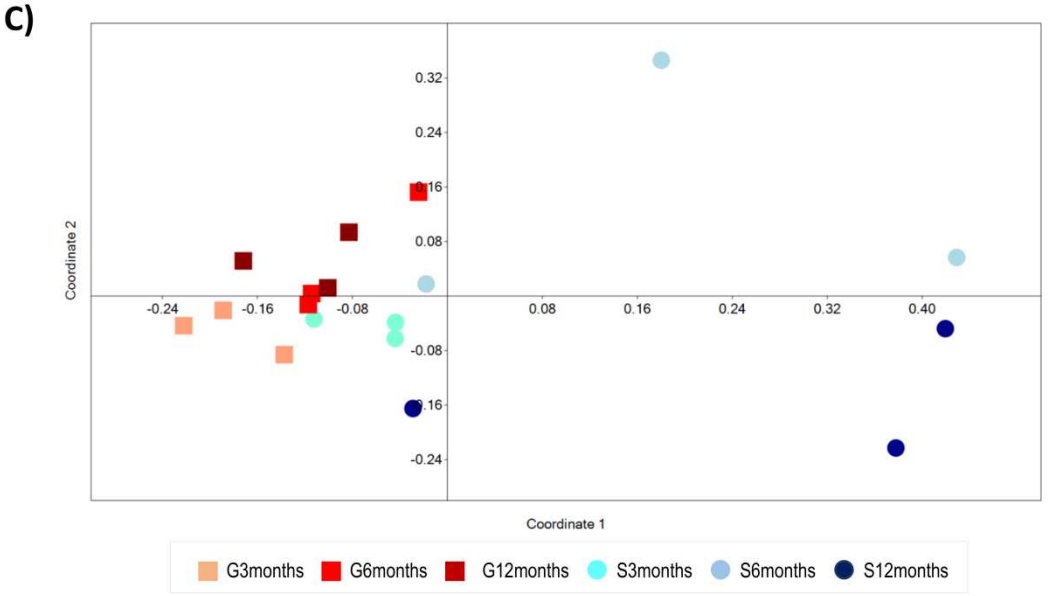


	Surface			Ground	
S3 vs. S6	Shared	90	G3 vs. G6	Shared	70
	Unique to S3	63		Unique to G3	31
	Unique to S6	68		Unique to G6	84
S3 vs. S12	Shared	91	G3 vs. G12	Shared	80
	Unique to S3	62		Unique to G3	49
	Unique to S12	73		Unique to G12	74
S6 vs. S12	Shared	90	G6 vs. G12	Shared	48
	Unique to S6	68		Unique 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	87
	Unique to S12	78
	Unique to G12	37

690



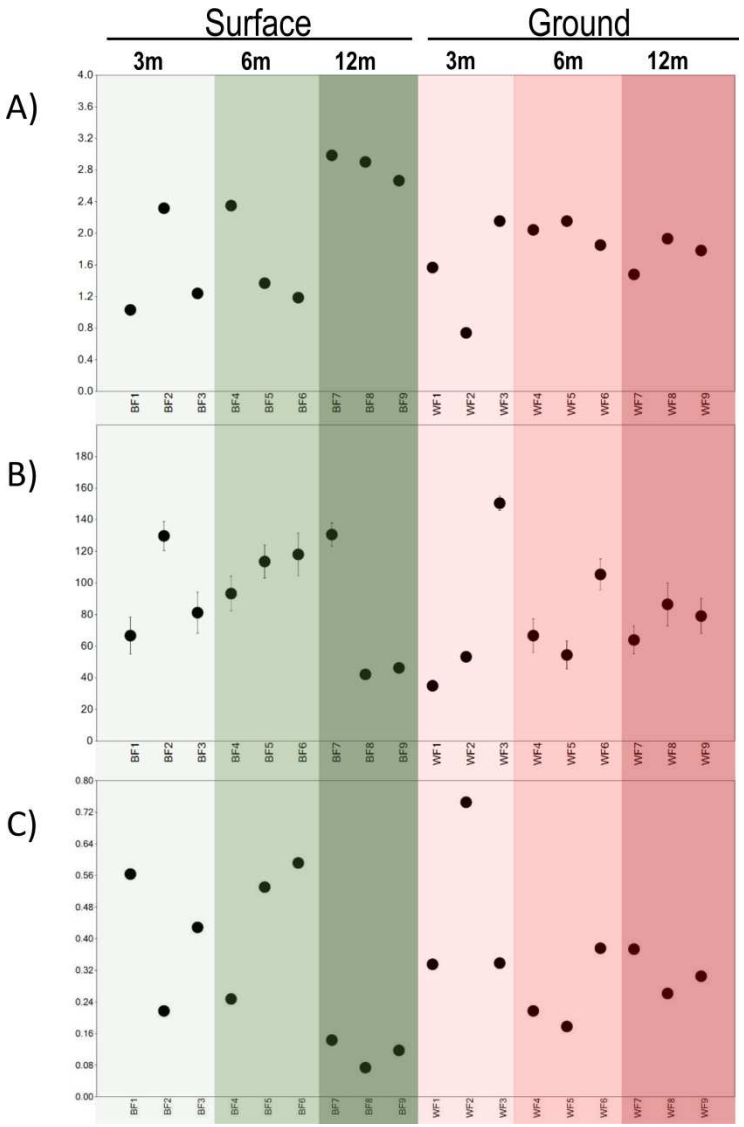
D)

R Values	WSWAg	WSWNov	WSWFeb	WGWAg	WGWNov	WGWFeb	BSWAg	BSWNov	BSWFeb	BGWAg	BGWNov
WSWAg											
WSWNov	0.8889										
WSWFeb	1	0.8889									
WGWAg	0.8889	0.9259	1								
WGWNov	0.75	0.4167	0.5833	0.9167							
WGWFeb	1	0.6296	0.4074	0.5926	0.75						
BSWAg	0.963	0.963	1	1	1	0.8148					
BSWNov	1	1	1	1	1	0.6667	1				
BSWFeb	0.4815	0.2963	0.3333	0.4444	0.5	0.1481	0.3333	0.2593			
BGWAg	1	1	1	1	0.9167	0.6296	1	0.4444	0.4815		
BGWNov	1	1	1	1	1	0.6667	1	0.8333	-0.25	1	
BGWFeb	0.3704	0.4444	0.3704	0.4815	1	0.1852	0.4074	0.1111	-0.0741	0.2963	0

** p <0.01 ; *P<0.05

691

692 Figure 8



693