Influence of hydraulic regimes on bacterial community structure and composition in an experimental drinking water distribution system

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Abstract

Microbial biofilms formed on the inner-pipe surfaces of drinking water distribution systems (DWDS) can alter drinking water quality, particularly if they are mechanically detached from the pipe wall to the bulk water, such as due to changes in hydraulic conditions. Results are presented here from applying 454 pyrosequencing of the 16S ribosomal RNA (rRNA) gene to investigate the influence of different hydrological regimes on bacterial community structure and to study the potential mobilisation of material from the pipe walls to the network using a full scale, temperature controlled experimental pipeline facility accurately representative of live DWDS.

Analysis of pyrosequencing and water physico-chemical data showed that habitat type (water vs. biofilm) and hydraulic conditions influenced bacterial community structure and composition in our experimental DWDS. Bacterial community composition clearly differed between biofilms and bulk water samples. *Gammaproteobacteria* and *Betaproteobacteria* were the most abundant phyla in biofilms while *Alphaproteobacteria* was predominant in bulk water samples. This suggests that bacteria inhabiting biofilms, predominantly species belonging to genera *Pseudomonas*, *Zooglea* and *Janthinobacterium*, have an enhanced ability to express extracellular polymeric substances to adhere to surfaces and to favour co-aggregation between cells than those found in the bulk water. Highest species richness and

diversity were detected in 28-days old biofilms with this being accentuated at highly varied flow conditions. Flushing altered the pipe-wall bacterial community structure but did not completely remove bacteria from the pipe-walls, particularly under highly varied flow conditions, suggesting that under these conditions more compact biofilms were generated.

This research brings new knowledge regarding the influence of different hydraulic regimes on the composition and structure of bacterial communities within DWDS and the implication that this might have on drinking water quality

Keywords: drinking water distribution systems, biofilms, discolouration, hydraulic regimes, pyrosequencing

Highlights

1. Pyrosequencing was applied to characterize bacterial communities within drinking water distribution systems under different hydraulic regimes
2. Bulk water and biofilm had markedly different bacterial community structure and composition
3. Higher species richness and diversity were detected within biofilms, particularly under highly varied flow conditions, than in bulk water samples
4. Under highly varied flow conditions more compact biofilms were generated

1. Introduction

Drinking water distribution systems (DWDS) are extreme environments with oligotrophic conditions where a disinfectant residual is commonly maintained. Despite this, microorganisms are able to survive within DWDS, in particular by attaching to the internal surfaces of pipes forming biofilms (Simoes *et al*., 2007a, b). Microbial biofilms have been conceptually, and under idealised test conditions, associated with various problems in DWDS

such as changes in water quality (e.g. discolouration, taste and odour), adsorption and trapping of materials from the bulk water, hosting opportunistic pathogens and promoting the deterioration of pipes (Szewzyk *et al*., 2000; Beech and Sunner, 2004).

Discolouration is the most common cause of water quality related customer contacts received by water companies in the UK. Discolouration is known to be associated with the mobilisation of accumulated particles, dominated by iron and manganese but with a significant organic content, from the inner-pipe walls into the bulk water due to increases in shear stress above conditioning values (Husband *et al*., 2008). Given the association of discolouration with pipe surface accumulations, the occurrence of biofilm on inner-pipe surfaces and the organic content of discolouration material samples it seems logical to speculate that biofilms and biological behaviour may be playing a role in discolouration processes. However, there is limited knowledge concerning the role of microbial biofilms in the process of discolouration and the biologically mediated accumulation of particulates, such as iron and manganese, in DWDS.

There are many different factors that might influence the formation and continual growth of biofilms on pipe surfaces such as flow regime, amount and type of disinfectant, concentration of organic carbon, etc. (LeChevallier *et al*., 1987). It has been previously suggested that normal (daily) hydraulic conditions within distribution systems are critical in determining the accumulation and subsequent detachment of biofilms (Rickard *et al*., 2004a; Manuel *et al*., 2007; Abe *et al*., 2012). Different research has focused on the study of how hydraulic regimes might influence biofilm formation (Liu *et al*., 2002; Cloete *et al*., 2003; Lehtola *et al*., 2005; Lehtola *et al*., 2006). However, these and similar studies generally employed idealised conditions such as bench top reactors, scaled pipeline and biological inoculation which do not realistically reproduce conditions in real DWDS (e.g. Schwartz *et al*., 1998; Murga *et al*., 2001; Batte *et al*., 2003). As a consequence, it is not well understood how conditioning shear

stress, and other factors, might affect formation of biofilms and its microbial composition within real DWDS and neither is there substantial information about how differences in biofilm composition might contribute to the process of material mobilization within such systems. To overcome these limitations the experimental work in this study has been carried out in a unique temperature controlled, full scale pipeline facility at the University of Sheffield (Fig. 1). This facility can fully recreate the hydraulic and other physical, chemical and biological conditions of real distribution systems. A particular technical advantage of the facility is the inclusion of PWG coupons (Deines *et al*., 2010). These can be fitted along lengths of the experimental system and enable DNA-based analysis of biofilms from the inner-pipe wall.

Molecular fingerprinting techniques such as DGGE and T-RFLPs have been previously used to evaluate microbial community structure in experimental or simulated water supply systems (Emtiazi *et al*., 2004; Schwartz *et al*., 2009; Yu *et al*., 2010; Sekar *et al*., 2012), but these techniques can only assess major changes in the composition of dominant microbial species in environmental samples (Forney *et al*., 2004). Pyrosequencing of the 16S ribosomal RNA (rRNA) is a recently developed molecular tool that provides a more precise characterization of bacterial communities since the diversity revealed within each sample is far larger than that detected by other molecular techniques such as fingerprinting. Recent studies have used pyrosequencing to characterize bacterial communities from impeller retrieved from customer water meters (Hong *et al*., 2010) and in membrane filtration systems from a drinking water treatment plant (Kwon *et al*., 2011). To date this technique has not been applied to the analysis of bacterial communities from internal pipe surfaces.

2. Aim and objectives

The aim of this study was to provide new knowledge of bacterial community structure and composition in DWDS. This was achieved by applying pyrosequencing to bulk water and biofilm samples obtained from the pipe wall of a full scale, temperature-controlled experimental facility representative of live DWDS. In particular we sought new understanding of the influence of different hydraulic regimes during biofilm development and the process of detachment from the inner-pipe surface. Such information is important to understand the role of biofilms within DWDS and any associations with risks to potable water quality, so that systems can be best operated and managed in the future.

3. Materials and methods

3.1 Experimental facility and operating conditions

The experimental facility consists of three recirculating loops of pipe fed by a common pump and returning to a common closed reservoir (Fig. 1). Flow in each loop is individually controlled to generate different hydraulic regimes. Each loop consist of 9.5 x 21.4 m long coils of 79.3 mm internal diameter High-Density Polyethylene (HDPE) pipe, thus each loop has a total length of 203 m such that pipe surface area is dominant over ancillaries. Polyethylene pipe was selected as it is a prevalent and representative current material used in distributions systems world-wide. The total volume of the system is 4.5 m^3. In order to provide representative water quality the facility is fitted with a trickle feed (and drain) from the local water distribution system. Data (not shown) from the treatment works supplying the local system indicated stable water quality throughout the duration of this experiment. The trickle feed was set to give a system residence time of 24 hours. The temperature of the facility was set to 16°C for all results reported here; this is representative of average spring and summer temperatures in UK DWDS, thus accurate for real systems but providing maximum representative levels of microbial activity.

Before experiments commenced, the facility was disinfected with 20 mg/l of RODOLITE H (RODOL Ltd, Liverpool, UK) which is a solution of sodium hypochlorite with less than 16 % free available chlorine. The system was flushed for 3 turnovers at maximum flow rate (4.2 l/s) and left standing for 24 hours. After that period the system was flushed again at the maximum flow rate with fresh water until the levels of chlorine were similar to those of the local tap water (average free chlorine 0.08 mg/l). After disinfecting the system, sterile PWG coupons (Deines *et al*., 2010) were arbitrarily fitted along and around the sample length of each pipe loop. The PWG coupon design (Fig. 1) allows direct insertion and close alignment with the internal pipe surface minimizing the distortion of boundary layer conditions that influence biofilm formation, such as boundary shear stress and turbulent driven exchange with the bulk water body. The facility thus allows the formation, growth, and detachment of biofilms to be captured under controlled but fully realistic conditions.

For the experiments reported here three different hydraulic regimes were applied based on daily patterns observed in real DWDS in the UK (Husband *et al*., 2008). The three regimes were: low varied flow, ranging from 0.2 to 0.5 l/s (loop 1), steady state 0.4 l/s flow (loop 2) and highly varied flow, ranging from 0.2 to 0.8 l/s (loop 3) (Fig. 2). These provide a range of representative conditions from steady state to highly varied, each have the same total net flow in every 24hour cycle. These daily regimes were repeated for a growth phase of 28 days.

After the growth phase, flushing of each loop was undertaken. Flushing is one of the simplest and most expedient methods used in practice to manage discolouration, typically achieved by opening fire hydrants to increasing hydraulic forces, shear stress, at the pipe wall to remove any loosely adhered material (Husband *et al*., 2011). Before flushing the facility, the flow of the growth phase was stopped and the bulk water sealed within each loop, the supply tank was emptied and refilled, this tank water was then combined with the water from one of the loops and circulated at 0.4 l/s for 3 turnovers to ensure thorough mixing. Biofilm and bulk

water samples were taken after this mixing, “pre-flush” samples. Flow was then increased in gradual steps from 0.4 l/s (shear = 0.2 N/m² and velocity = 0.05 m/s) to 4.5 l/s (shear = 3 N/m² and velocity = 0.57 m/s) to simulate a network flushing operation. Biofilm and bulk water samples were then taken, “post-flush” samples. This process was then repeated for the other two loops, including the refilling of the supply tank.

3.2 Sampling of biofilms and bulk water

To study the planktonic and biofilm communities within the system, water samples and PWG coupons were obtained as detailed above before and after the experimental flushing. Three replicates of 1L of bulk water were taken directly from the outlet of each of the three loops before and after flushing the system. In total 18 bulk water samples were collected for this experiment and filtered through 0.22 μm nitrocellulose membrane filters (Millipore, Corp). Three PWG coupons were removed before and three after flushing. However, for highly varied flow conditions before flushing only two coupons were obtained. In total 17 biofilm samples were removed from PWG coupons as described in Deines *et al*., (2010). Filters containing water and biofilms samples were kept in the dark and at 80°C for subsequent DNA extraction and pyrosequencing analysis.

3.3 Water physico-chemistry

Turbity was constantly measured by means of a turbidity meter (Chemtrac TM2200) installed in the system via tapping points towards the end of each loop. Several physico-chemical factors were analysed in pre- and post-flushing water samples. Every analysis was performed three times for each water sample (three subsamples) to increase the reliability of the measurements and the average of the three replicates was calculated. Free chlorine was measured using a HACH DR/2010 spectrophotometer. Measurements of temperature, pH and Oxidation-Reduction-Potential (ORP) were made using a Hanna H1991003 meter and

probes. Water samples for total iron and manganese were sent to AlControl Laboratories (Deeside, UK) for analysis.

3.4 DNA extraction and quantification

DNA was extracted and its quantity and quality determined for subsequent pyrosequencing analysis. DNA, from three different filters from each sampling point (bulk water and biofilm), was extracted using a phenol:chloroform based method and chemical lysis approach (Zhou *et al*., 1996). In brief, 800 µl of SET lysis buffer (40mM EDTA, 50mM Tris-HCl, pH9, 0.75M sucrose) and 90 µl of lysozyme (9 mg/ml) were added to the filters within 15 ml sterile tubes and incubated at 37°C for 30 min with rotation in a Hybaid hybridisation oven (Thermo Scientific, UK). Subsequently 100 µl of sodium dodecyl sulphate (SDS) and 27 µl of proteinase K (20 mg/ml) were added to the same tube and the sample incubated at 55°C for 2 h with rotation in a Hybaid oven. The supernatant (aqueous phase) was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), pH8 (Sigma, UK) and one of chloroform:isoamyl alcohol (24:1) (Sigma, UK). DNA was precipitated with 5M NaCl and isopropanol, then washed in 70 % ethanol, dried and re-dissolved in sterile water. Quantity and purity of the extracted DNA was assessed using Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). DNA in all the samples was normalized to a final concentration of 20 ng/µl and its quality (ratio of absorbance at 260/280 nm) was of ~1.7.

3.5 Massive parallel 16S rRNA gene pyrosequencing for characterising bacterial communities

A high-throughput sequencing method (pyrosequencing) was used to characterise bacterial communities and examine their relative abundance and diversity in water and biofilm samples. Extracted DNA was sent to the Research and Testing Laboratory (Lubbock, TX,

US) for bacterial 16S rRNA gene tag-encoded FLX amplicon pyrosequencing (bTEFAP). PCR amplification was performed using the primers Gray28F and Gray519r (Callaway *et al*., 2010). Sequencing reactions utilized a Roche 454 FLX instrument (Roche, Indianapolis, IN) with Titanium reagents, titanium procedures, a one-step PCR reaction (35 cycles), and 1 U of HotStar Highfidelity Polymerase was added to each reaction (Qiagen, Valencia, CA).

3.6 Sequence analysis

In total 181,709 16S rRNA gene sequences were obtained from biofilms and water samples. Two independent analyses were carried out with the sequences, one was performed by Research and Testing Laboratory (Lubbock, TX, US) in order to obtain taxonomical assignments from sequences reads and the other one was carried out using QIIME (Quantitative Insights into Microbial Ecology) to estimate alpha and beta diversity. The different terms used to measure diversity in an ecosystem were introduced by Whittaker (1960, 1972). Alpha diversity refers to the diversity within a particular sample (i.e. how many different bacteria are in a sample), and is usually expressed by the number of species or Operational Taxonomic Units (OTUs) when studying bacteria. Beta diversity measures differences in diversity between samples (i.e. comparison of bacterial distribution among samples).

3.6.1 Research and Testing Laboratory Taxonomic Analysis

In order to obtain taxonomy assignments from 16S rRNA sequences reads, low quality sequence ends, tags and primers were removed and sequences depleted of any non-bacterial ribosome sequences and chimeras using Black Box Chimera Check software (B2C2) (Gontcharova *et al*., 2010) as has been described previously (Dowd *et al*., 2008a,b). To determine the identity of bacteria in the remaining sequences, sequences were denoised, assembled into clusters and queried using a distributed BLASTn.NET algorithm (Dowd *et*

al., 2005) against a database of 16s bacterial sequences derived from the National Centre for Biotechnology Information (NCBI). Database sequences were characterized as high quality based upon similar criteria utilized by Ribosomal Database Project (RDP) ver 9 (Cole *et al*., 2009). Using a .NET and C# analysis pipeline the resulting BLASTn (Nucleotide Basic Local Alignment Search Tool) outputs were compiled, validated using taxonomic distance methods, and data reduction analysis performed as described previously (Dowd *et al*., 2008a,b; Callaway *et al*., 2010). Sequences with identity scores to known or well-characterized 16S sequences >97 % identity (<3 % divergence) were resolved at the species level, between 95 % and 97 % at the genus level, between 90 % and 95 % at the family level, and between 80 % and 90 % at the order level.

3.6.2 Alpha and beta diversity analysis with QIIME (Quantitative Insights into Microbial Ecology)

Prior to the estimation of alpha and beta diversity, sequences were filtered, clustered, taxonomically assigned and aligned using QIIME community analysis pipeline (Caporaso *et al*., 2010b). Sequences were filtering based on the sequencing quality file according to pre-established QIIME parameters (Caporaso *et al*., 2010b). To summarise, sequences shorter than 200 nucleotides, with one or more ambiguous bases and with quality score inferior to 25 were eliminated from the study. Sequences were clustered into Operational Taxonomic Units (OTUs) based on 0.97 and 0.95 sequence similarity with the Uclust algorithm (Edgar, 2010). Representative OTUs were selected based on the most abundant sequences and taxonomic assignment was conducted using the Ribosomal Database Project (RDP) classifier (Wang *et al*., 2007) then sequences were aligned using the Phyton Nearest Alignment Space Termination Tool (PyNAST) alignment algorithm (Caporaso *et al*., 2010a). A phylogenetic tree was build using the FastTree algorithm (Price *et al*., 2009) for UniFrac distance matrix construction.

To study alpha-diversity (diversity within samples) a rarefaction analysis (number of OTUs observed vs. number of sequences sampled) was performed at 95 and 97 % sequence similarity for each sample and the average was then calculated based on habitat type, hydraulic regime and sample description. Calculated collector's curves (Schloss and Handelsman, 2004) for different alpha-diversity metrics were included; Chao1 richness estimator (Chao 1984) and Shannon diversity index (Shannon and Weaver, 1949). Richness refers to the total number of OTUs in the samples and is calculated at different sequence similarity cut off. However, to estimate diversity the proportional abundance of a particular phylotype relative to the sum of all phylotypes is taking into account.

To compare bacterial diversity between samples (beta-diversity), samples were rarefacted to the smallest data set (2000 sequences) to reduce sequence heterogeneity and the UniFrac distance metric was applied (Lozupone *et al.*, 2011) to calculate pairwise distances between communities in terms of their evolutionary history. Both un-weighted (presence/absence information) and weighted (taking into account relative abundance of each OTU) UniFrac analysis were carried out and principal coordinate plots were generated.

3.7 Statistical analyses

To assess the similarity in bacterial community composition among samples, the relative sequence abundance at class and genus level (93 and 97 % sequence similarity cut offs respectively) for each sample was used to calculate pairwise similarities. All data were transformed by square root calculations and Bray-Curtis similarity matrixes were generated using the software Primer v6 (PRIMER-E, Plymouth, UK). Bray-Curtis similarity matrixes were visualised using multiple dimensional scaling (MDS) diagrams. Analysis of similarity statistics (ANOSIM) was calculated using the same Bray-Curtis distance matrix to test the significance of differences among samples based on hydraulic regimes and flushing. The

values for the ANOSIM R statistic ranges from 0 to 1, where R = 1 indicates that communities from different treatments are completely dissimilar.

To investigate the relationships between water physico-chemical variables and relative sequence abundance at species level (97 % sequence similarity cut off) within biofilm samples, non-parametric Spearman's rank correlation coefficients (rho) were calculated using PASW® Statistics 18.SPSS.

4. Results

4.1 Physico-chemical analysis

As shown in Table 1, pH values were near neutral (7.17-7.40) for all the samples. Temperature ranged between 15.53-16.23 °C for all samples, within the +/- 1°C control for the facility. Free chlorine levels were between 0.19-0.28 mg/l, with concentration slightly inferior at highly varied flow both before (0.12 mg/l) and after flushing (0.19 mg/l), probably an artefact of the supply water used to refill the tank prior to flushing this loop. High positive redox potential was found in the system at different hydraulic regimes and increased after flushing except for low varied flow conditions. Higher turbidity levels were found for highly varied flow loop before flushing compared to the other loops (Table 1), again likely to be an artefact of the supply water used to refill the tank prior to flushing this loop. After flushing, turbidity had increased considerably for steady state and low varied flow loops but only by a small amount under highly varied flow conditions. iron and manganese levels considerable increased after flushing and showed similar trends to turbidity.

4.2 Correlations between physico-chemical data and relative sequence abundance at species level

As shown in Table 2, turbidity levels, iron and manganese concentrations were strongly positively correlated between each other ($p < 0.01$). pH was positively correlated with temperature, redox potential and iron ($p < 0.05$). There were significant correlations between bulk water parameters and the relative sequence abundance at species level (97 % similarity cut off) of bacteria within pre and post-flushing biofilm samples. Turbidity, shear stress, iron and manganese concentrations in the bulk water were significantly negatively correlated ($p < 0.05$) with relative sequence abundance within biofilms.

4.3 Comparison of biofilm vs. bulk water bacterial diversity

As can be seen from Fig. 3 the dominant bacterial phyla within the biofilms, calculated as the average of the three biological replicates, were *Gammaproteobacteria* followed by *Betaproteobacteria*, *Alphaproteobacteria*, *Clostridia* and *Bacilli*. It was also observed that the position of the coupons along and around the pipe (i.e. crown, side and invert) did not significantly affect the microbiological characteristics of biofilms (data not shown). However, the percentages of each of these bacterial groups varied depending on particular hydraulic regimes and there was high variability between replicates for each sample. Within bulk water samples, *Alphaproteobacteria* clearly dominated the bacterial community composition (average of total number of samples up to 78 %) and to a much lesser extent *Betaproteobacteria* and *Clostridia* were also abundant (Fig. 3). At genus level *Pseudomonas*, *Zooglea*, *Janthinobacter* and *Sphingomonas* were predominant within biofilms and *Methylocystis*, *Methylocella*, *Sphingopyxis* and *Polaromonas* within bulk water samples (Fig. 4).

The rarefaction curves (Fig. 5) for each sample (observed OTUs) showed that the bulk water samples had less observed OTUs than biofilm samples. The Chao1 richness estimator and the

Shannon diversity index, estimated at 3 % (data not shown) and 5 % dissimilarity cut offs, also showed higher richness and diversity within biofilms than in bulk water samples (Fig. 5).

Non-metric Multi-Dimensional Scaling analysis (MDS) showed a clear separation among biofilm and bulk water samples at class and genus level (Fig. 6). The analysis of similarities (ANOSIM) confirmed that water and biofilm sample were significantly different (class level; R= 0.867 and p= 0.001 and genus level; R=0.98, p=0.001). The results of the principal coordinate analysis from UniFrac metrics (weighted and un-weighted) were similar to those based on Bray-Curtis similarity, clustering samples mainly by habitat type (Fig. 7).

4.4 Influence of hydraulic regimes on microbial communities

Gammaproteobacteria was the predominant group within the biofilms formed under low varied flow conditions (65 %, average of replicate samples) and in post-flushing steady state samples (total average up to 50 %) (Fig. 3). *Betaproteobacteria* were also abundant under steady state conditions (up to 56 % average total biofilm samples) in both pre and post-flushing samples and in highly varied flow post-flushing samples (up to 60 %). *Alphaproteobacteria*, *Clostridia* and *Bacillus* were the other main predominant phylogenetic groups under the three hydraulic conditions within biofilms (Fig. 3). In the bulk water, the different hydraulic regimes did not clearly influence the composition of the water samples, *Alphaproteobacteria* predominated in all the samples, followed by *Betaproteobacteria* and *Clostridia* under the three different hydraulic regimes. Despite this high similarity found in the distribution of bacterial groups in water samples, *Gammaproteobacteria* was only relatively abundant under highly varied flow conditions (up to 12 %) (Fig. 3). *Pseudomonas* was the genus predominant in the composition of biofilms, particularly at low varied flow conditions (total average up to 65 %). At steady state higher abundance of *Sphingomonas*, *Erytromicrobium* and *Methylophilus* was detected. Within biofilms at highly varied flow

conditions *Zooglea* and *Janthinobacterium* were more abundant when compared with other hydraulic regimes (Fig. 4). The percentage of these bacterial genera changed between hydraulic conditions but did not show a clear variation trend (Fig. 4). The hydraulic regimes did not significantly affect the community composition of bulk water samples at genus level. However, higher relative abundance of *Pseudomonas* and *Methylocella* were detected under steady state and low varied flow conditions.

Highest species richness (Chao1) and diversity index (Shannon) at genetic distances of 3 % (data not shown) and 5 % were observed at highly varied flow followed by steady state and low varied flow conditions, both with similar levels of observed OTUs and Chao estimator (Fig. 5).

The non-metric MDS based on relative sequence abundance at class and genus level did not show clear patterns in the distribution of samples (Fig. 8), probably due to the highly variability of bacterial diversity found in each of the biological replicates. ANOSIM did not show significant differences in the composition of bacterial communities according to hydraulic regimes. The results from unweighted-UniFrac metrics based on presence/absence of bacterial species in the samples tended to better cluster the biological replicates than the weighted-UniFrac metric where the proportion of species is taking into account (Fig. 7). Despite this, no statistically significant differences in the distribution of the samples according to hydrological regimes was detected.

4.5 Microbial communities within biofilms before and after flushing

There are changes in bacterial community composition between pre- and post-flushing biofilm samples, reflected in the different percentage of relative sequence abundance detected at different phylogenetic levels (Figs. 3 and 4). *Gammaproteobacteria* tended to decrease at low and highly varied flow conditions but increased at steady state conditions (Fig. 3). The

presence of *Betaproteobacteria* increased remarkably after flushing under highly varied flow conditions representing more than 50 % of the total community (Fig. 3). At genus level the abundance of certain bacteria also changed (Fig. 4), for example *Pseudomonas* decreased at low varied flow but increased at steady state conditions in post-flushing samples. At highly varied flow, *Janthinobacterium* and *Erythromicrobium* decreased below 2.5 % after flushing but species belonging to *Zooglea*, *Dechloromonas* and *Methylophylus* increased in post-flushing samples up to 30 % (Fig. 4). These compositional shifts in bacterial community composition between pre and post-flushing samples did not follow a clear trend, perhaps masked by the high variability found between the three biological replicates.

Species richness and diversity changed between pre- and post-flushing samples and it was surprisingly high at highly varied flow conditions (Fig. 5), but as observed above for relative sequence abundance, and for similar reasons, both richness and diversity varied without following a significant trend.

The MDS analysis did not clearly cluster pre- and post-flushing samples, however, differences in bacterial relative abundance before and after flushing according to hydrological regimes were detected (Fig. 8). ANOSIM analysis showed significant differences between pre- and post-flushing samples at highly varied flow (class level; $R = 0.583$ and $p = 0.01$ and genus level; $R = 0.667$, $p = 0.01$) and steady state conditions (class level; $R = 0.333$ and $p = 0.01$ and genus level; $R = 0.407$, $p = 0.01$). Confirming the trend observed above for the MDS analysis of relative sequence abundance, the principal coordinate analysis based on UniFrac metrics (phylogenetic analysis) did not clearly cluster pre- and post-flushing samples (Fig. 7).

5. Discussion

Significant differences in bacterial community composition were found between bulk water and biofilms under different hydraulic regimes ($p < 0.01$), with the highest bacterial richness and diversity detected within biofilms (Fig. 5). Previous research has observed that certain species, found here inhabiting the bulk water, have higher capacity to attach to surfaces and form biofilms than others due to their enhanced ability to express cell surface polymers that can increase cell hydrophobicity and favour processes such as co-aggregation (Rickard *et al*., 2003, Rickard *et al*., 2004b). In freshwater ecosystems, *Betaproteobacteria* can attach more easily to surfaces and they dominate the process of biofilm formation (Manz *et al*., 1999, Araya *et al*., 2003). This ability may then explain the predominance of this bacterial group within biofilms in this study (Fig. 3).

Biofilm is an advantageous way for microorganisms to live in environments such as DWDS. Within the Extracellular Polymeric Substance (EPS) matrix of biofilms, microorganisms are protected from the direct action of disinfectants and the availability of nutrients is higher (Emtiazi *et al*., 2004). Water companies in the UK are required to maintain disinfection residual in DWDS to protect against biological contamination, either intentional or accidental. The most common disinfectant in the UK is free chlorine, which due to it reactive nature decreases in concentration with time within the distribution system. The different sensitivity to chlorine of certain bacterial groups has been previously detected by McCoy *et al*., (2012). Our results are in agreement with this, with *Alphaproteobacteria* predominant in the bulk water due to their higher resistance to chlorine and *Betaproteobacteria* preferentially present in our biofilms. It is also interesting to note the high abundance of *Gammaproteobacteria* within biofilms detected in the data (Fig. 3), this bacterial group includes most of the known pathogens and opportunistic pathogens, confirming that biofilms are potential reservoirs for this kind of organisms (Mathieu *et al*., 2009).

405 At genus level, *Pseudomonas* has been considered as the most abundant bacterium in DWDS
406 independently of the water source and habitat type (Martiny *et al*., 2005). Our results
407 confirmed *Pseudomonas* prevalence in biofilms but not in bulk water samples where
408 *Methylocystis* and *Methylocella* were more abundant (Fig. 4). Methanotrophic degrading
409 bacteria such as *Methylocystis sp* can convert trichloroethylene into chloral hydrate, which is
410 a common by-product of disinfection when using chlorine in drinking water (Huhe *et al*.,
411 2011). The predominance in biofilms of species from genera such as *Pseudomonas*, *Zooglea*
412 and *Janthinobacterium* (Fig. 4) can be explained by the fact that they are able to produce high
413 amount of extracellular polymeric compounds which favour the formation of biofilms (Burns
414 and Stach, 2002; Bitton, 2011). *Zooglea* and *Hypomicrobium* have been previously observed
415 in drinking water samples and they are mainly abundant under oligotrophic conditions (Lee *et*
416 *al*., 2005).

417 The bacterial composition and community structure of biofilms changed between the three
418 different hydraulic regimes (Figs. 3 and 4). However, this variability was not statistically
419 significant. Bacterial communities from bulk water samples presented very similar
420 composition under the three hydraulic regimes and after flushing (Figs. 3 and 4). This result
421 was in contrast with the bacterial community composition observed within biofilms for a
422 particular hydrological regime which showed high variability between biological replicates
423 (Fig. 3). The observed consistency of the bulk water samples is to be expected due to the use
424 of a common reservoir that all pipe loops recirculated to. Given this common mixing and
425 cross contamination between loops any difference in the biofilm community is of note. It
426 could be of potential interest to utilise separate reservoirs in future, as we do not know the
427 extent to which the loops influenced each other. However, a common mixed source is
428 indicative of a real DWDS where various hydraulic conditions occur along any given flow
429 route and due to the presence of complex loops and interconnections in real DWDS. The

natural highly heterogeneous nature of drinking water biofilms has been previously observed, by Henne *et al*., (2012) using fingerprinting techniques; the authors observed that mature biofilms developed under similar conditions presented distinctive microbial communities. Based on this information further research into microbial succession on biofilms within our experimental system is needed to better understand the community structure of these microbial communities and the process of their accumulation over time.

Rarefaction curves of observed OTUs continued to increase with the number of sequences in the samples and did not reach a plateau indicating that further increases in sample size would yield more species. Despite this, the number of observed OTUs, species richness and diversity tended to be higher at highly varied flow (Fig. 5). Previous studies have suggested that biofilm growth might increase with higher flow velocity (Lehtola *et al*., 2005) and that rapid changes in water flow rates increase the concentration of bacteria in the water (Lehtola *et al*., 2006). On the other hand, Husband *et al*., (2008), suggested that less material is likely to accumulate at the pipe-wall under varied flow profiles compared to steady state regimes. However, the cited authors did not analyse the microbial composition of the mobilized material. Rochex *et al*., (2008), when studying the role of shear stress on the bacterial composition of biofilms reported a decrease in bacterial diversity at high shear stress and suggested that shear stress slows down the process of biofilm maturation. However, their study was carried out in an annular reactor, under nutrient-rich conditions, which are different from those in DWDS, and using a fingerprinting technique which can only detect the most abundant species in a microbial community.

We did not detect statistically significant changes in bacterial communities in bulk water samples in response to the flushing event (Figs. 7 and 8). Taking into account the relatively high bacterial abundance detected after flushing within biofilms (Fig. 5), only a limited amount of biological material was effectively removed from the pipe walls (and coupons) to

455 the bulk water which combined with the potential dilution of the biofilms in this 2 m^3
456 (approximate volume per loop and reservoir during flushing) facility did not allow the
457 detection of any significant changes. However, we did find that turbidity and iron in the water
458 were negative correlated with relative sequence abundance at 97 % similarity cut off in
459 biofilms (Table 2) suggesting that the contribution of bacterial cells in the process of material
460 mobilization. The data from this research highlighted the presence of bacteria such as
461 *Erythromicrobium*, *Leptothrix* and *Hypomicrobium* (Fig. 4) capable of metabolise iron and
462 manganese within biofilms (Ghiorse, 1984; Katsoyiannis *et al*., 2004; Ginige *et al*., 2011).
463 The relative abundance of these bacteria was positively correlated with turbidity, iron and
464 between each other (data not shown) indicating their potential involvement in discolouration.
465 Fe^{+2} and Mn^{+2} can be trapped within the exopolymeric matrix of biofilms and they can be
466 sources of energy for these microorganisms (Burns *et al*., 2002). Our results confirm previous
467 observations that bacterial mediated iron and manganese reduction and oxidation can take
468 place simultaneously in biofilms exposed to considerable concentrations of chlorine and
469 oxygen (Cerrato *et al*., 2010). Furthermore, the ability of some of these bacteria to form
470 dormant spores such as *Bacillus spp*. allows them to be resistant to disinfection (Bargar *et al*.,
471 2000; Cerrato *et al*., 2010).

472 We have detected differences in bacterial community composition within biofilms after
473 flushing between the three different hydraulic regimes (Figs. 7 and 8). However, as
474 mentioned above, the bacterial diversity detected after flushing is surprisingly high,
475 particularly at highly varied flow regime (Fig. 5). Using the same experimental facility and
476 similar hydraulic regimes, Sharpe *et al*., (2010) observed that higher conditioning shear stress
477 resulted in less material mobilised to bulk water and that a large amount of material remained
478 on the coupons after flushing. Vrouwenvelder *et al*., (2010), also observed that in drinking
479 water membrane systems, biofilms developed under low shear conditions were easily

removed. Generally, high shear stress and turbulent flow conditions favour the production of more dense and compact biofilms by the production of extracellular polymers (van Loosdrecht *et al*., 1995; Kwok *et al*., 1998; Pereira *et al*., 2002; Manuel *et al*., 2010). Biofilms with a more cohesive structure can be more resistant to external shear stress and detachment (Manuel *et al*., 2007; Abe *et al*., 2012). As a consequence, it is likely that thicker biofilms, which are mechanically more stable, are developed as an adaptative strategy in response to higher detachment forces (Rochex *et al*., 2008).

The data presented here was based on the assessment of 28-days old biofilms. This period is sufficient to generate detectable and quantifiable biofilm, but such biofilms are probably far from being mature. It has been suggested that the process of biofilm maturation can take several years (Martiny *et al*., 2003). Further research, using separate tanks in our experimental facility, during longer periods of time and with different source water will help to better assess the occurrence and role of bacteria in mature biofilms and within real DWDS.

6. Conclusions

This paper presents the results of application of pyrosequencing to DWDS which yields new and unique data about the influence of hydraulic conditions on bacterial community composition and structure in biofilms on inner-pipes surfaces and in the bulk water.

In particular this research highlighted that in our experimental system, which is a good representation of a full-scale DWDS;

- Bulk water and biofilms have different bacterial community structure and composition at different taxonomic levels. This habitat segregation suggests that despite the same origin, the bacteria predominant within biofilms are able to produce higher quantity of extracellular polymeric substance to initiate biofilm formation. On

the other hand, identified bacterial groups abundant in the bulk water are known to be more resistant to the influence of chlorine than those in the biofilms. *Gammaproteobacteria*, the bacterial group including most of the known pathogens and opportunistic pathogens, was highly abundant in biofilms, confirming that biofilms can act as reservoirs of this kind of microorganisms.

- Methanotrophic degrading bacteria such as *Methylocystis sp* were highly abundant in bulk water samples, suggesting that their capability of metabolising by-products of disinfection gives them an adaptative advantage over other species in this oligotrophic environment.
- High variability within biofilms growth under similar hydraulic conditions (biological replicates) confirms the high natural heterogeneity of these ecosystems.
- Different hydraulic regimes affect the composition and diversity of bacteria communities in 28-days old biofilms with a tendency for higher species richness and diversity detected at highly varied flow.
- The mechanical removal of biofilms by flushing did not completely remove bacteria from the pipe-walls, particularly under highly varied flow conditions, confirming that under these conditions more -compact biofilms are generated.

This research has generated important information regarding the contribution of microbial biofilms to material behaviour within DWDS. This information will assist in improving operation, control and management strategies to help safeguard drinking water quality.

Acknowledgements

The work reported here was supported by the UK Engineering and Physical Sciences Research Council Challenging Engineering grant EP/G029946/1. We would like also to thank Research and Testing Laboratory for the 454 FLX Pyrosequencing analysis.

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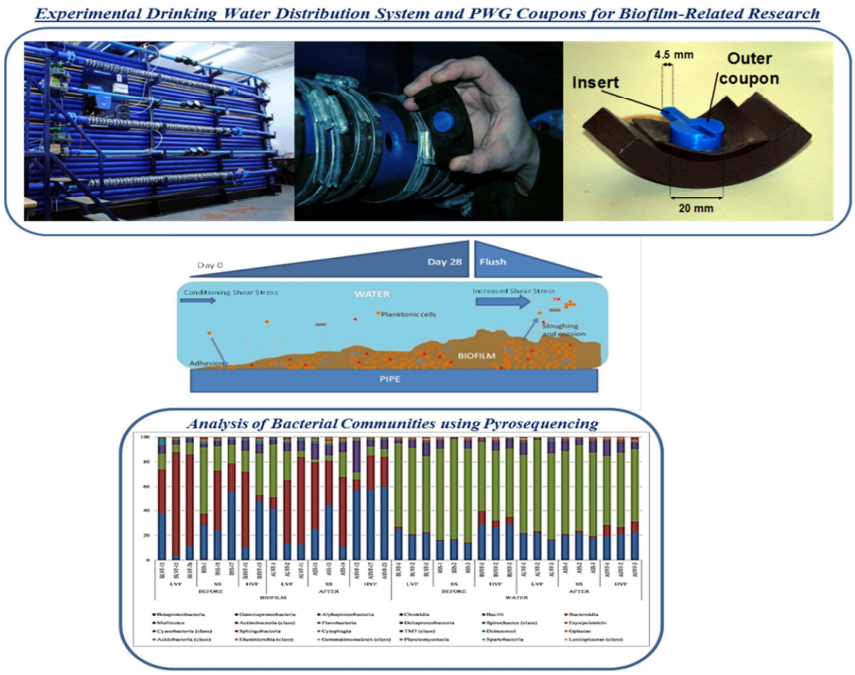


721

722 Graphical Abstract

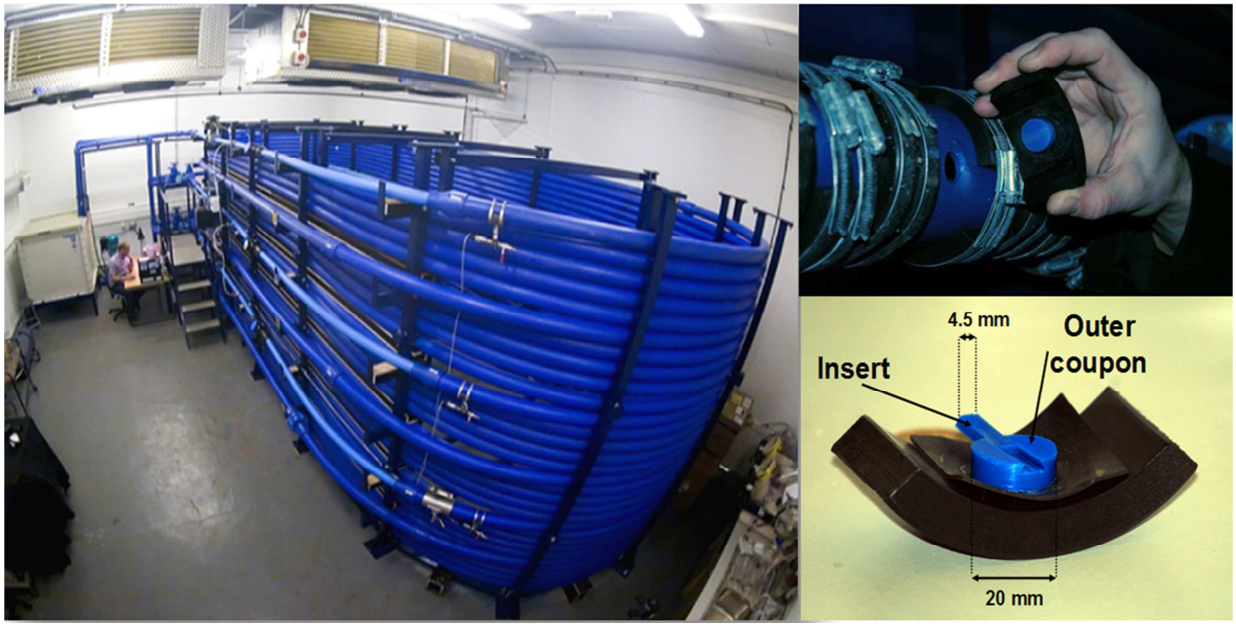
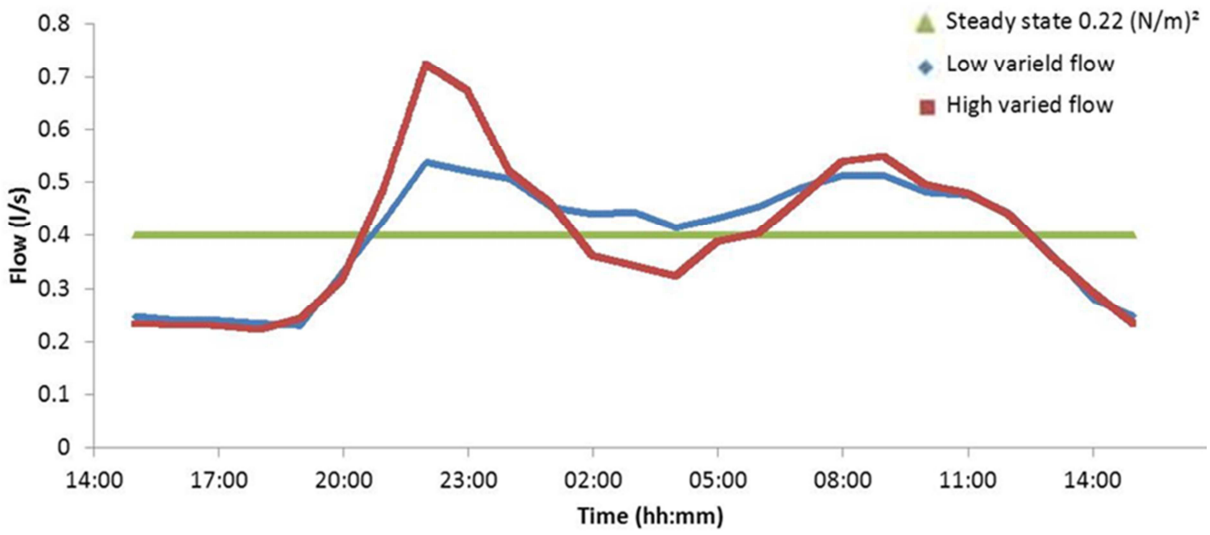


Figure 1: A) Full-scale laboratory pipe-loop experimental facility at the University of Sheffield. B) PWG coupons have the same internal diameter and curvature as the pipe and fit with the internal pipe surface. C) PWG coupon showing “outer coupon” (surface area 224 mm2) with l “insert” (surface area 90 mm2). Figures obtained from Deines et al., (2010).



728

729 **Figure 2**: Flow pattern used in the study representing three different hydraulic regimes based

730 on daily patterns observed in real DWDS in the UK.

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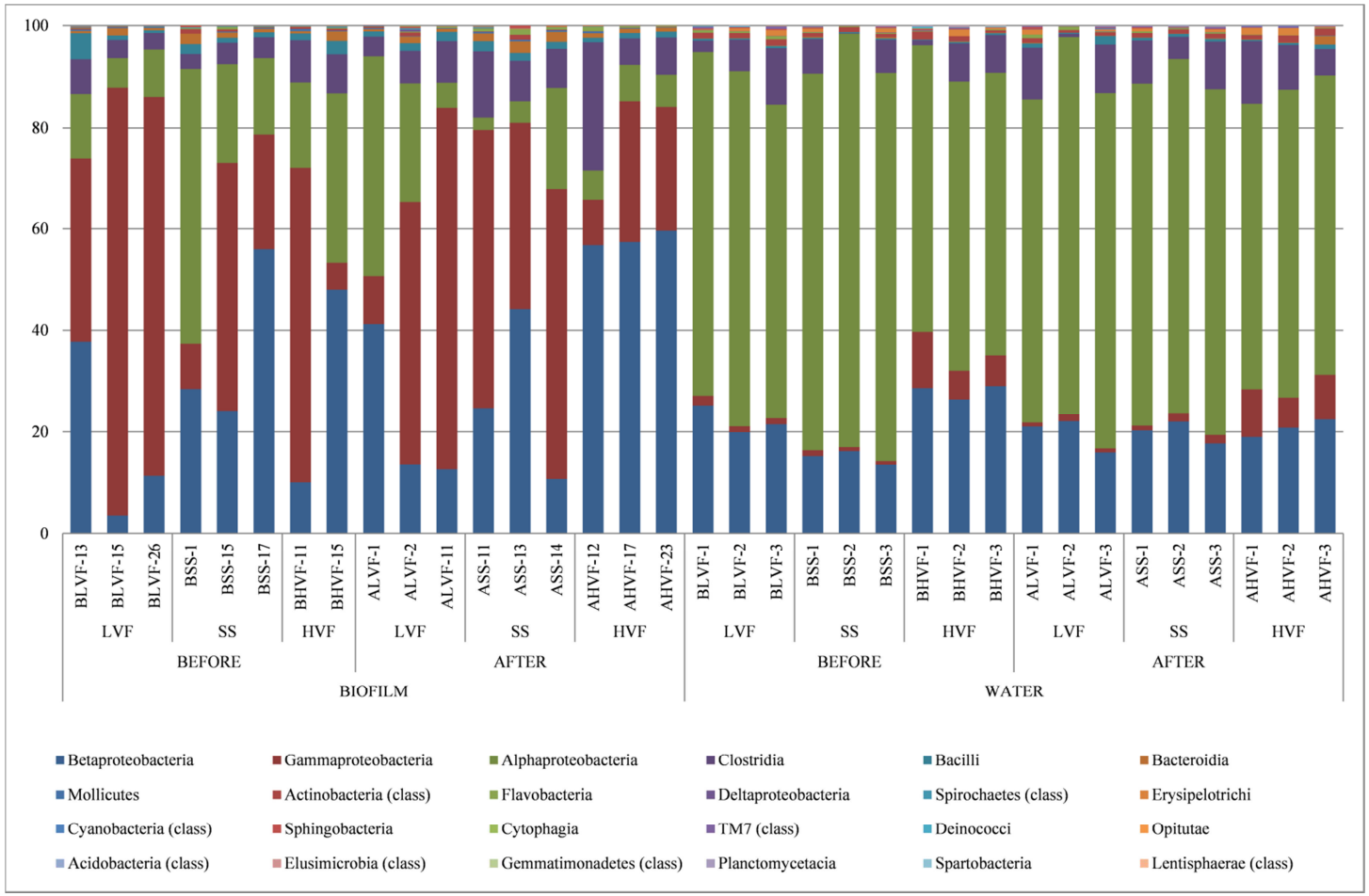


Figure 3: Comparison of the relative abundance of the major phylotypes found in biofilms and bulk water under the different hydrological regimes and before and after flushing the internal pipe surfaces. LVF (low varied flow); SS (steady state); HVF (highly varied flow); B (before) and A (after).

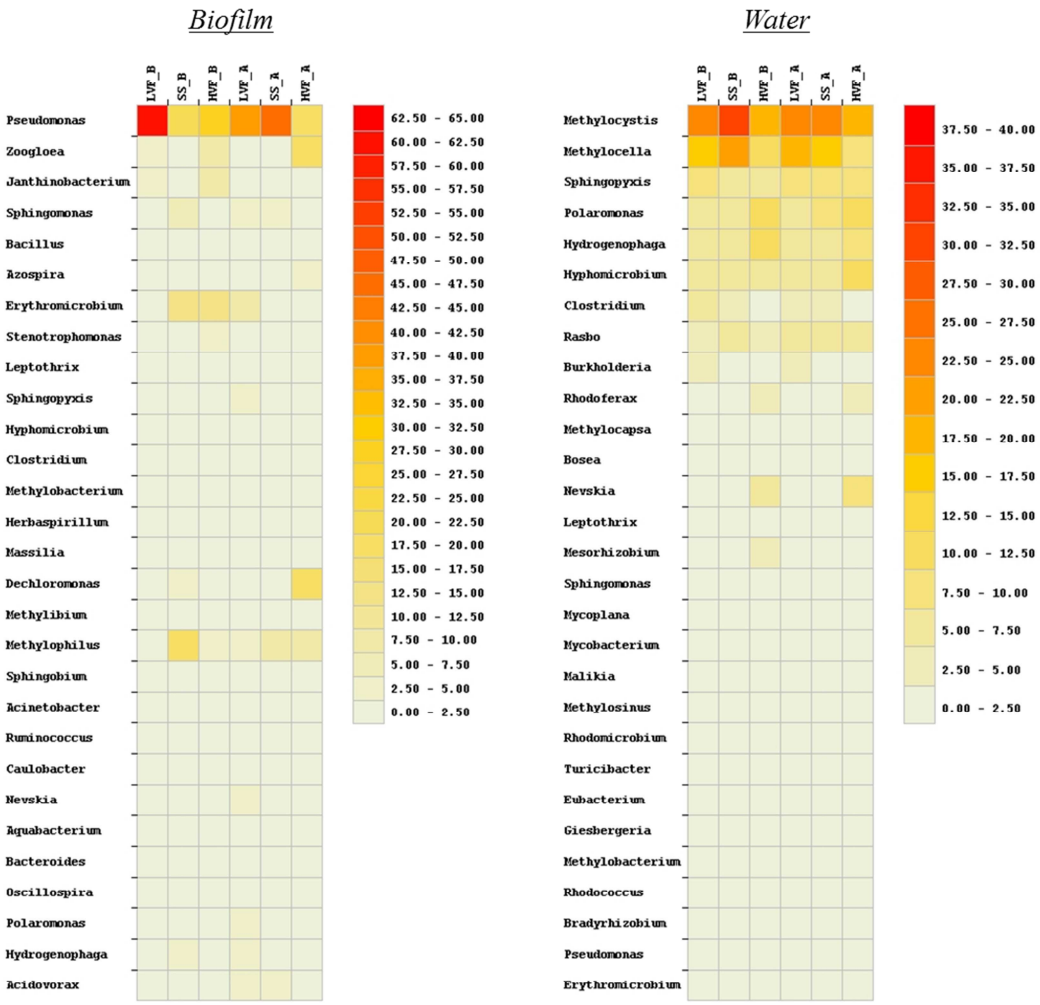
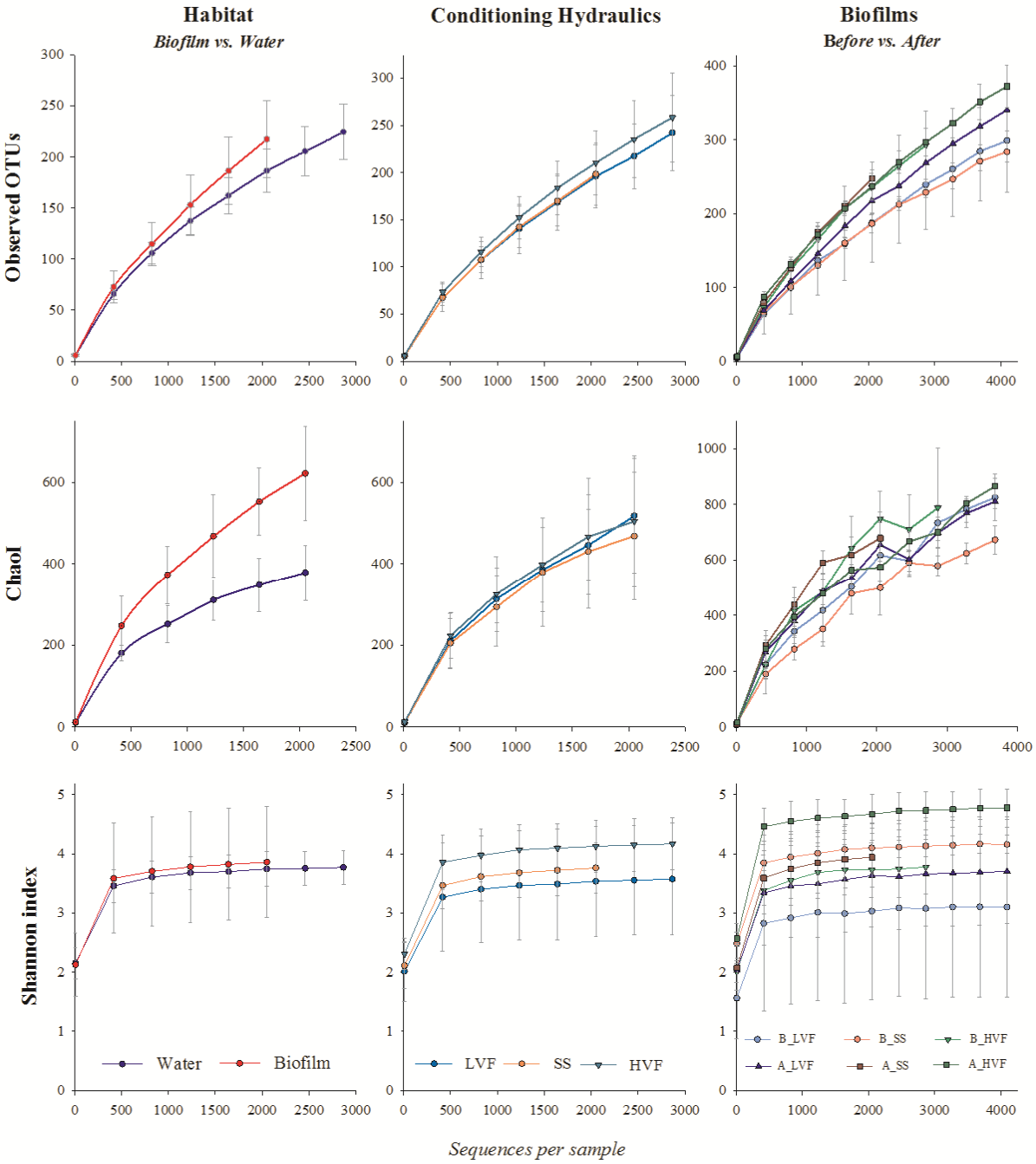


Figure 4: Heatmaps showing the percentages of the most abundant species at genus level within bulk water and biofilms. The relative abundance has been calculated as the average of the three (two for highly varied flow regime before flushing) biological replicates. LVF (low varied flow); SS (steady state); HVF (highly varied flow); B (before) and A (after).



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743 **Figure 5**: Rarefaction curves at 95 % of sequence similarity for water and biofilm samples.

744 Rarefaction curves were obtained for observed OTUS, Chao1 index richness estimator and

745 Shannon diversity estimator. Bars are indicating standard error.

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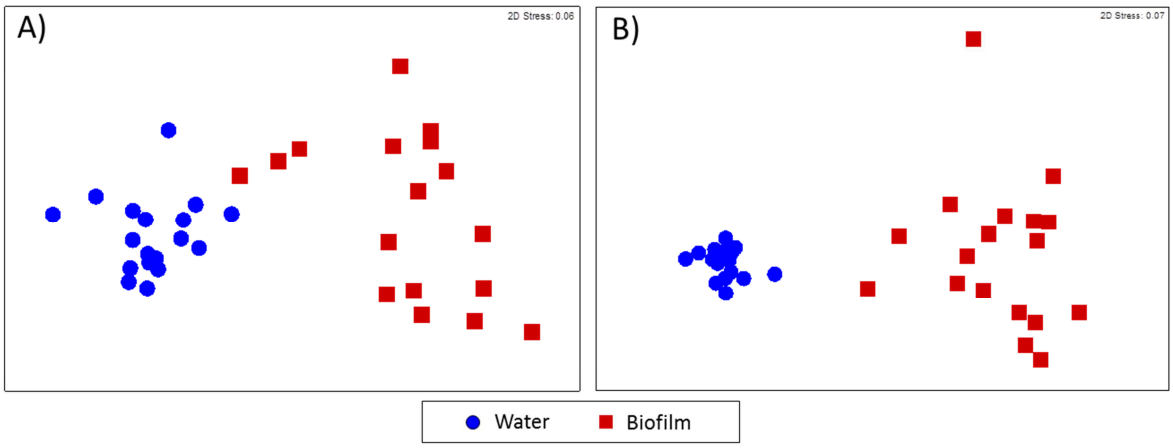
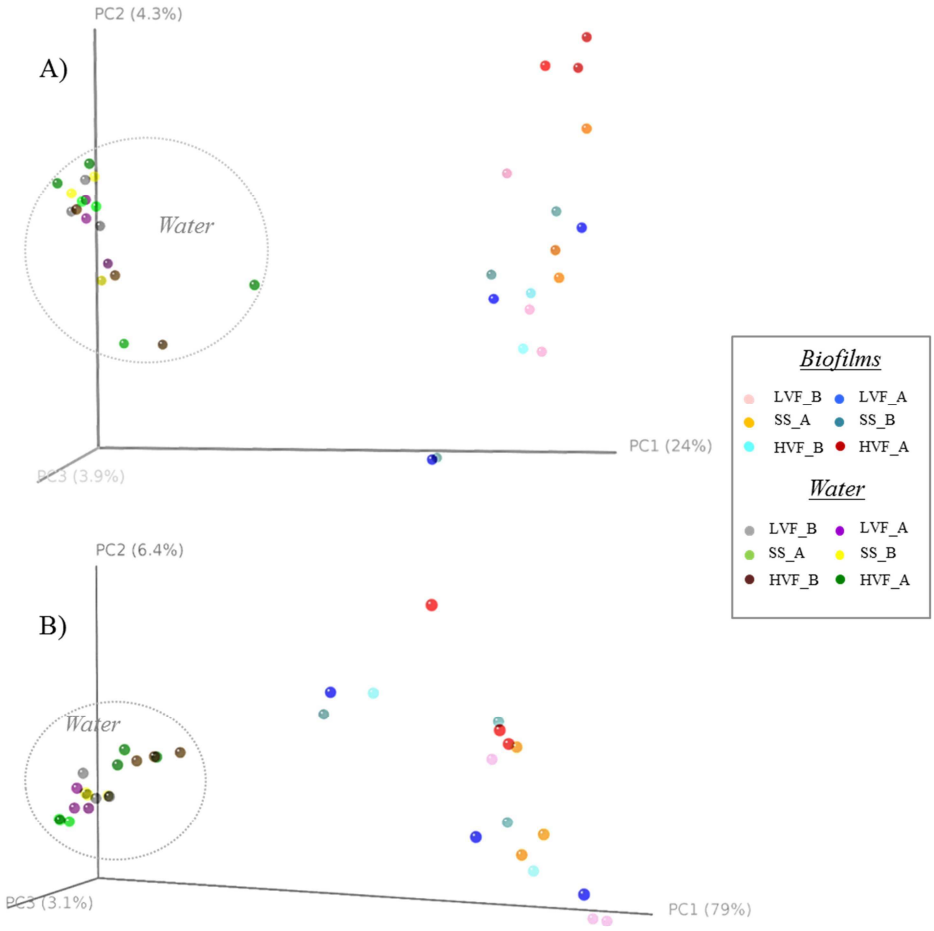


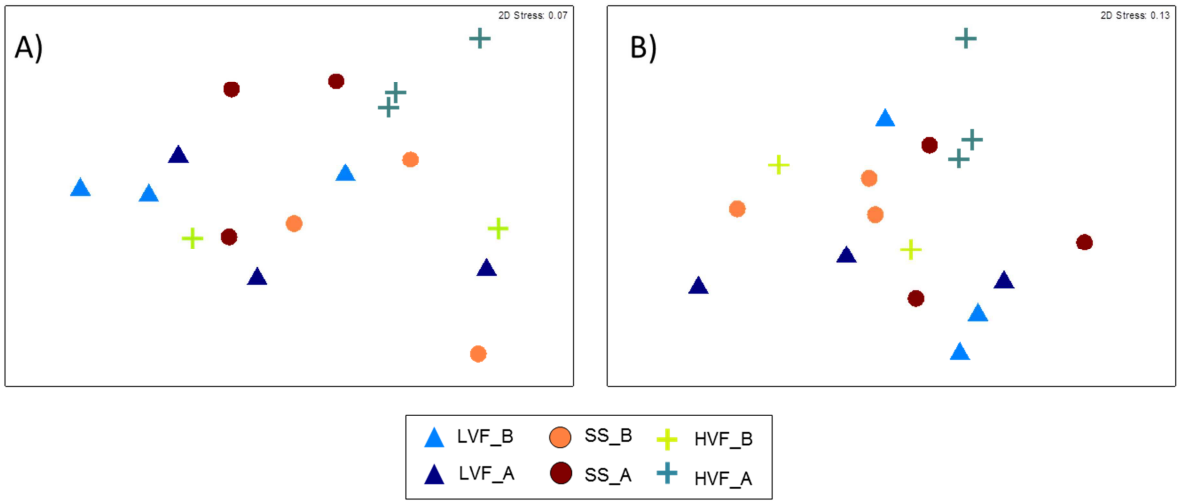
747

748 **Figure 6:** Two-dimensional plot of the Multi-Dimensional Scaling (MDS) analysis based on
749 Bray-Curtis similarities of the percentage sequence abundance A) at class level and B) at
750 genus level showing differences in the bacterial community structure between bulk water and
751 biofilms (n = 35). Symbols are representing individual samples and are coloured based on
752 sample type



753

754 **Figure 7**: Three-dimensional Principal Coordinates Plots of UniFrac analysis showing the
755 phylogenetic clustering of the bacterial communities within the test–loop facility at 97 % of
756 sequence similarity. The axes are scaled according to the percentage of variance that they are
757 explaining. A) Unweighted- UniFrac B) Weighted UniFrac (n = 35). Symbols are
758 representing individual samples and are coloured based on sample type. LVF (low varied
759 flow); SS (steady state); HVF (highly varied flow); B (before) and A (after).



760

761 **Figure 8:** Two-dimensional plot of the Multi-Dimensional Scaling (MDS) analysis based on
762 Bray-Curtis similarities of the percentage sequence abundance within biofilms A) at class
763 level and B) at genus level showing differences in the bacterial communities between
764 hydrological regimes before and after flushing (n = 17). Symbols are representing individual
765 samples and are coloured based on sample type. LVF (low varied flow); SS (steady state);
766 HVF (highly varied flow); B (before) and A (after)..

767

768 **Table 1**: Physico-chemical properties of bulk water from the test-loop facility before and after the flushing event.

	Flow Regime	Shear (l/s)	Turbidity (NTU)	pH	T (°C)	Redox (mV)	Fe (µg/l)	Mn (µg/l)	Free Chlorine (mg/l)
Before Flushing	LVF	0.5	0.029	7.21	15.53	275.00	36.00	4.93	0.23
	SS	0.5	0.014	7.30	16.07	247.33	26.00	4.77	0.28
	HVF	0.5	0.114	7.17	16.10	272.00	35.67	5.37	0.12
After Flushing	LVF	4.5	0.341	7.24	15.53	191.00	57.50	13.00	0.26
	SS	4.5	0.394	7.33	16.23	304.67	83.00	11.67	0.26
	HVF	4.5	0.179	7.40	16.10	357.33	67.00	11.00	0.19

LVF (low varied flow), SS (steady state), HVF (high varied flow)

769

770

771 **Table 2**: Spearman's correlation coefficients (rho) for water physico-chemical factors and the percentage of relative sequence abundance at 97

772 % similarity cut off within biofilms.

	BIOFILMS							
	SeqAb	**Shear**	**Turbidity**	**pH**	**T °C**	**Redox**	**Fe**	**Mn**
Shear	-.601*							
Turbidity	-.739**	.877**						
pH	NS	.658**	.758**					
T °C	NS	NS	NS	.554*				
Redox	NS	NS	NS	.585*	.644**			
Fe	-.622**	.877**	.894**	.600*	.500*	.600*		
Mn	-.657**	.877**	.932**	NS	NS	NS	.758**	
Cl	NS	NS	NS	NS	NS	-.499*	NS	NS

n=17; **= p < 0.01,*= p < 0.05, NS= p > 0.05; a two tailed test was used.

773