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The bacteriological composition of biomass recovered by flushing an operational drinking water distribution system

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ABSTRACT

This study investigates the influence of pipe characteristics on the bacteriological composition of material mobilised from a drinking water distribution system (DWDS) and the impact of biofilm removal on water quality.

Hydrants in a single UK Distribution Management Area (DMA) with both polyethylene and cast iron pipe sections were subjected to incremental increases in flow to mobilise material from the pipe walls. Turbidity was monitored during these operations and water samples were collected for physico-chemical and bacteriological analysis. DNA was extracted from the material mobilised into the bulk water before and during flushing. Bacterial tag-encoded 454 pyrosequencing was then used to characterize the bacterial communities present in this material.

Turbidity values were high in the samples from cast iron pipes. Iron, aluminium, manganese and phosphate concentrations were found to correlate to observed turbidity. The bacterial community composition of the material mobilised from the pipes was significantly different between plastic and cast iron pipe sections ($p < 0.5$). High relative abundances of *Alphaproteobacteria* (23.3%), *Clostridia* (10.3%) and *Actinobacteria* (10.3%) were detected in the material removed from plastic pipes. Sequences related to *Alphaproteobacteria* (22.8%), *Bacilli* (16.6%), and *Gammaproteobacteria* (1.4%) were predominant in the samples obtained from cast iron pipes. The highest species richness and diversity were found in the samples from material mobilised from plastic pipes. *Spirochaeta* spp., *Methylobacterium* spp. *Clostridium* spp. and *Desulfobacterium* spp., were the most represented genera in the material obtained prior to and during the flushing of the plastic pipes. In cast iron pipes a high relative abundance of bacteria able to utilise different iron and manganese compounds were found such as *Lysinibacillus* spp., *Geobacillus* spp. and *Magnetobacterium* spp.

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1. Introduction

Drinking Water Distribution Systems (DWDS) are complicated engineering systems consisting of pipes, storage vessels, fittings, valves, etc. made of a variety of different materials such as cast iron, PVC and polyethylene that interact with the bulk water. The water that consumers drink at the tap has travelled potentially large distances taking significant durations through the distribution network and it is accepted that from leaving the treatment plant deterioration in quality might occur. This deterioration in water quality will be influenced by factors such as decay of disinfectant residual, temperature, hydraulic regime, water residence time and bacterial regrowth (Machell et al., 2010; Ramos et al., 2010).

Background concentrations of various dissolved substances and fine particles of different origin do enter DWDS within the bulk flow, and these substance and particles can accumulate forming layers of material attached to the internal surfaces of pipes (Husband and Boxall, 2011). There are different ways in which particles can enter in the system; suspended in the source water, in chemicals added at the treatment plant, ingress via pipe repairs, though cross-connections or fissures in the system, or released as products originated from the corrosion or erosion of pipes. Despite the maintenance of a disinfectant residual in most developed countries, microorganisms can proliferate and survive forming biofilms attached to pipes (LeChevallier et al., 1987; Szewzyk et al., 2000). While fungi, viruses and protozoa are also inhabitants of DWDS, we have focused our study on the characterization of bacteria since these microorganisms are dominant in drinking water biofilms due to their enhanced ability for producing extracellular polymeric substances, rapid growth and adaptability (Chaves Simoes and Simoes, 2013).

If the shear stress at the pipe wall exceeds the normal daily values, the material accumulated at the pipe wall and conditioned to the normal forces will be mobilised into the bulk water (Husband and Boxall, 2010). The mobilisation of material from the pipe wall does not only result in aesthetically unacceptable discoloured drinking water (Husband et al., 2008) but also releases microorganisms into the network (Lehtola et al., 2007). Typically discolouration events occur when there are significant changes in the hydraulic conditions, such as rezoning, changing seasonal demand or pipe bursts (Prince et al., 2003). Most of the particles associated with discolouration events in DWDS contain iron and manganese (Seth et al., 2004). Although it has been suggested that microbial biofilms may play an important role in discolouration, the role of biofilms in the accumulation of particles, such as iron and manganese in DWDS is not well understood.

It is commonly accepted that the material removed by flushing pipes is a combination of water, biofilms, suspended solids and what hydraulic engineers identify as soft or loose deposits (Zacheus et al., 2001; Batté et al., 2003a). The main aim of this study was to characterise the bacteriological composition of the material removed from operational pipes during a flushing scheme, independently of the phase or category of material/substratum that these bacteria might be associated to. We use the microbiological definition of biofilm to refer to this material when applicable. Biofilms consist of microorganisms attached to a surface and/or to each other and embedded in a polymeric matrix made of proteins, polysaccharides, extracellular DNA, etc. (Stoodley et al., 1997; Donlan, 2002; Hall-Stoodley et al., 2004). We use this accepted definition without specifying the type of surface or substratum where microorganisms are adhered to, this can comprise from a single suspended particle to a cluster of loose deposits.

Biofilms have long been known to exist and grow throughout distribution systems, even in the presence of disinfectant residuals (Block et al., 1995; Momba et al., 1998; Zhang and Lu, 2006). Previous research suggests that shear forces influence biofilm strength, with erosion associated to system shear stress (Rittmann, 1982; Choi and Morgenroth, 2003; Abe et al., 2012) and it is accepted that if the hydraulic conditions in the network change and overcome biofilm adhesive forces, they can de-attach from the pipe (Volk et al., 2000; Ginige et al., 2011). Given the association of discolouration with pipe surface accumulations, the development of biofilms on inner-pipe surfaces may be playing an essential role in the process of discolouration that still needs to be explored.

To reduce the risk of discolouration water companies can 'flush' the pipes in order to remove material (Slaats, 2002). Flushing typically involves the selective closure of valves and opening of fire hydrants or washouts to create aggressive hydraulic forces within selected pipes. Research has shown that this cleaning strategy only partially removes biofilms depending on the operating hydraulic regime (Douterelo et al., 2013) and biofilms can rapidly regenerate.

Previous research has examined the influence of substrate materials on biofilm development and microbial growth, with mixed findings. Niquette et al. (2000), showed that the densities of bacterial biomass that develop on plastic materials such as High and Medium polyethylene (HDPE/MDPE) or PVC were lowest in comparison with steel and iron material. In contrast Keevil (2003) reported that plastic substrates can promote biofilm growth due to the release of biodegradable compounds. However, most of the studies concerning the effect of different materials on biofilm development in drinking water networks are based on research done under bench top scale laboratory conditions which do not reproduce conditions in real DWDS and little is known of what occurs in real operational networks. In addition, most of the existing knowledge on the microbial ecology of drinking water networks is based on planktonic communities not in biofilms attached to pipes. Considering that biofilms have been associated with various problems in DWDS such as changes in water quality (e.g. discolouration), the hosting of opportunistic pathogens and the corrosion of pipes (Szewzyk et al., 2000; Beech and Sunner, 2004) further research is needed to fully understand these ecosystems.

The present research aims to characterize the bacterial composition of material accumulated within operational drinking water networks consisting of different pipe materials and mobilized by flushing. The new knowledge obtained from this research will lead to better understanding of how the accumulation and mobilization of material on pipes within DWDS occurs, and hence what the risks and impacts for the quality of drinking water are.

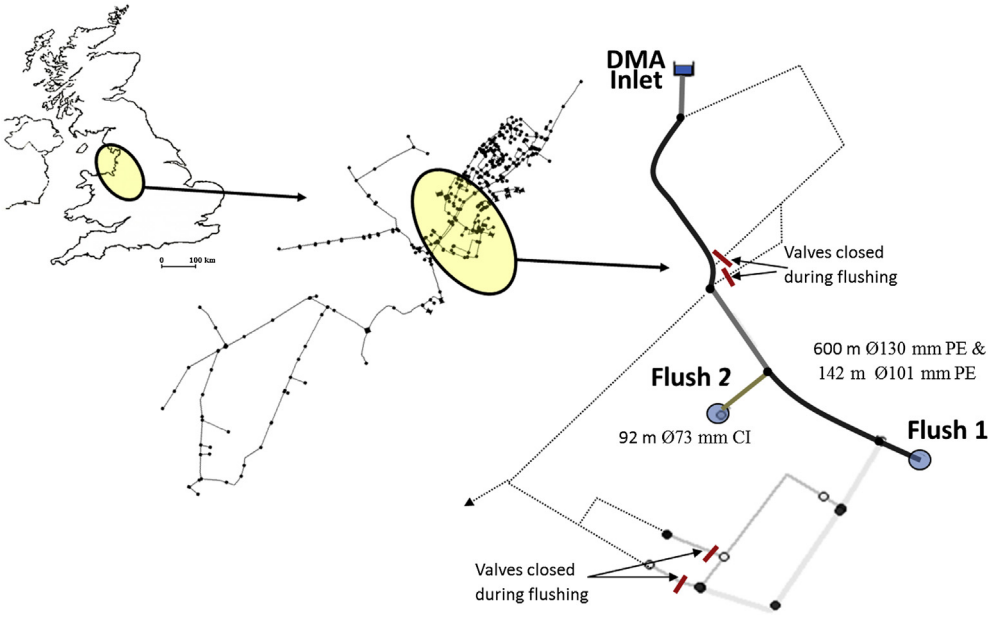


Fig. 1 – Location of the sampled hydrants in Northwest England, showing DMA layout and characteristics of the flushing sites.

1.1. *Objectives*

The objectives of this paper are to:

1. Characterise the bacterial communities in the material mobilised by flushing from pipes in operational drinking water distribution systems.
2. Assess the influence of pipe material on biofilm bacterial community structure.
3. Investigate the potential role of bacterial biofilms on water discolouration.

2. Materials and methods

2.1. *Field site*

The trial site was a Distribution Management Area (DMA) in the Northwest of England that has been previously used in discolouration trials (Husband and Boxall, 2011). It is supplied by abstracted river water with aluminium sulphate coagulation treatment and fed by large diameter lined trunk main. Asset records indicate that there are no upstream unlined cast iron pipes. Two sections were identified for the trials. The first totalled 742 m of polyethylene pipe (600 m, Ø 130 mm and 142 m, Ø 101 mm) linked to the DMA inlet. The second pipe section fed a small number of properties and was 92 m of Ø 73 mm cast iron that was supplied by the first section. Although the DMA formed a number of complex loops, during the trials strategic valve closures ensured a single flow route and meant flushing flows exceeded normal daily peak flows in all sections. Fig. 1 shows a schematic of the site.

2.2. *Online turbidity monitoring and flushing scheme*

The trial involved opening selected hydrants incrementally to achieve target flow rates. Flushing flow rates were measured using a Langham standpipe mag-flow meter, Fig. 2A. The flow

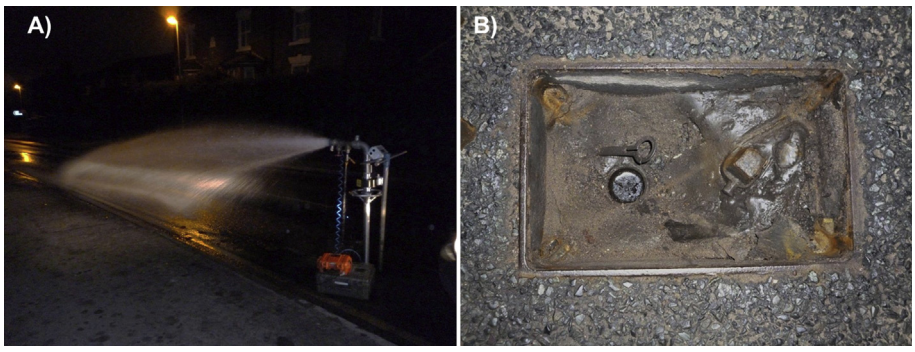


Fig. 2 – A) Equipment used during flushing; B) accumulation of debris and mud in the hydrant chamber from flushing site 1 (polyethylene pipes).

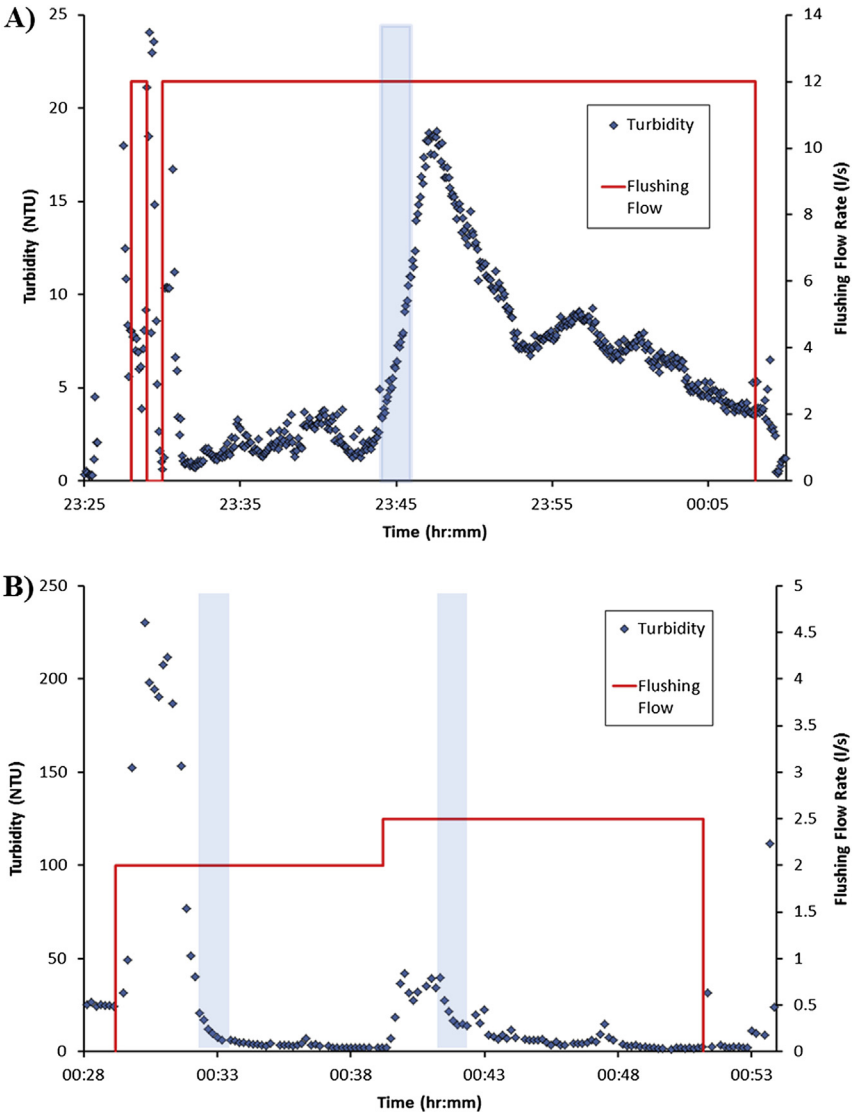


Fig. 3 – Flow increases during the experiment are shown with a red line and measured in l/s. Results of online turbidity meter measurements during the flushing event are showing NTUs and time and increase in flow applied. A) Polyethylene pipe 130/101 mm. B) Cast Iron pipe 73 mm. Light-blue bars indicate time of sampling. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rates were consistent with previous trials at this location and had been developed to investigate the relationship of hydraulic shear stress to material mobilisation (Husband and Boxall, 2011), flushing flow rates and durations can be seen in Fig. 3. Turbidity was measured and dual validated at 5 s polling using ATI A15/76 turbidity monitors. These had been adapted and tested extensively as mobile units for field studies at the University of Sheffield. Operations were undertaken at night to minimise customer impact. The last recorded interventions within this network were 42 months previously. It is therefore suggested that material mobilised is representative of mature biofilms.

2.3. *Discrete sampling and analysis*

Bulk water samples were taken directly from the outlet of each hydrant at pre-determined times, pre-flushing and during flushing as indicated on Fig. 3. The sampling times were at less than one complete turnover of the pipe volume. Sampling of water was planned to be at or around the time of the peak of material (turbidity) observed from previous studies at this site (Husband and Boxall, 2011). In total, 5 sample time points were obtained (2 pre-flushes and 3 during flushing).

2.3.1. *Physico-chemical analysis and heterotrophic plate counts (HPC)*

Several parameters were measured *in situ* at the times of discrete sample collection prior and during flushing of the pipes (Fig. 3). Free chlorine was measured using a Hanna Instrument HI 96711, temperature and pH using a Hanna portable meter and probe HI 991003. All other parameters were obtained by later laboratory analysis of the discrete samples.

Table 1 – Results from the physico-chemical analysis of water and colony plate counts.

	Inlet	Polyethylene		Cast iron		
		Pre-flush 1	Flush 1.1	Pre-flush 2	Flush 2.1	Flush 2.2
Temperature (°C)	6.40	6.10	6.30	6.00	6.40	6.20
pH	7.17	7.07	7.12	7.10	7.09	7.07
Free Cl_2 (mg/l)	0.45	0.33	0.44	0.42	0.43	0.40
Aluminium (μg/l)	10.9	340	1300	3120	1420	893
Iron (μg/l)	5.2	1380	2020	3570	2300	2360
Manganese (μg/l)	0.2	12.70	44.80	135.00	116.00	166.00
Nitrite as N (mg/l)	0.0029[a]	0.0029[a]	0.0029[a]	0.0029[a]	0.0029[a]	0.0029[a]
Nirate as N (mg/l)	2.95	3.06	2.90	2.88	2.91	2.94
TOC (mg/l)	1.66	1.87	1.71	1.84	1.73	1.68
PO_4 total (μg/l)	1460	1760	2690	5210	3460	3040
Sulphate as SO_4 (mg/l)	45.7	47.20	45.90	46.00	45.50	45.30
Colonies 2D 37 °C (No/ml)	0	10	0	0	0	0
Colonies 3D 22 °C (No/ml)	0	17	0	0	0	1

[a] Below detection limit.

All discrete sampling methods detailed in this section have been validated to meet Drinking Water Regulation requirements and were carried out by United Utilities Scientific Services, an UK accredited drinking water laboratory. Sulphate was measured using an ion chromatography system with conductivity detection (Cheeseman et al., 1989). NO_2^-–N and NO_3^-–N were determined using a SEAL AQUA 900 Discrete Analyzer (SEAL Analytical, Ltd., UK) with a cadmium coil reduction method followed by sulphanilamide reaction in the presence of N- (1-naphthylethylenediamine) dihydrochloride (U.S. EPA Method 353.2, version 2, 1993). The concentration of aluminium, iron and manganese was determined using acid digestion with nitric acid before analysis by Inductively Coupled Plasma-Atomic Emission Spectroscopy. Total Organic Carbon (TOC) concentration was assessed using persulphate oxidation by a non-dispersive infra-red detector method. The soluble reactive phosphate (SRP = PO_4^3–P) was measured by a modified molybdenum blue procedure (APHA, 1992).

Water samples were collected in designated containers and bacterial colony counts were determined by the pour-plate method by United Utilities Scientific Services. In brief, for the pour-plate method Yeast Extract Agar was used following the UK Environmental Agency recommendations (Environmental Agency, 2012). Cultures were incubated at 37 °C for 48 h (2-day colony) and 22 °C for 72 h (3-day colony) and colonies counted after that period of time.

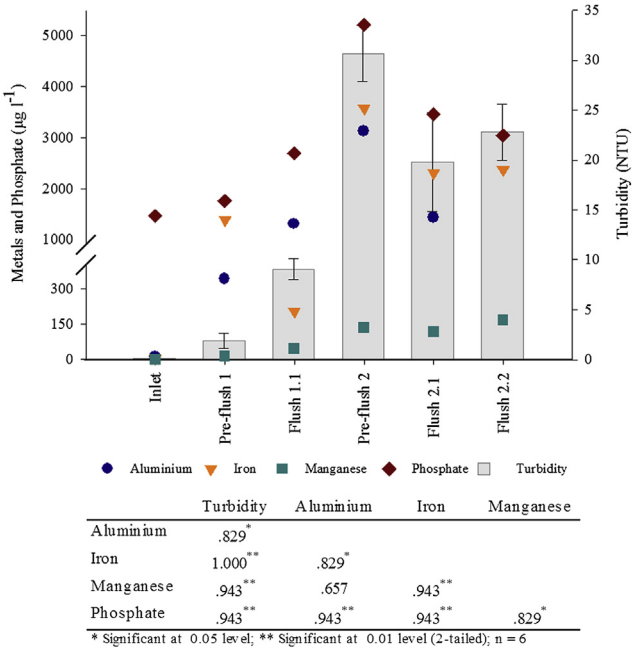
2.3.2. *DNA extraction and 454 pyrosequencing*

Four replicates of 1 L samples of bulk water were taken at the times of discrete sampling for DNA analysis (total 20 samples). These samples were each filtered through 0.22 μm nitrocellulose membrane filters (Millipore, Corp). Filters were preserved in the dark and at −80 °C for subsequent DNA extraction. DNA extraction from filters was carried out by a method based on proteinase K digestion followed by a standard phenol/chloroform-isoamyl alcohol extraction. The quantity and purity of the extracted DNA were assessed using Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, USA). A high-throughput sequencing method (454 pyrosequencing) was used to characterise bacterial communities and examine their relative abundance and diversity in the 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).

2.4. *Analysis of sequences*

2.4.1. *Taxonomic analysis*

In total 216123 16S rRNA gene sequences were obtained from the water samples from before and during flushing the two sampling sites. Sequences were cleaned by removing primers, tags and low quality sequence ends. Potential chimeric



	Turbidity	Aluminium	Iron	Manganese
Aluminium	.829*			
Iron	1.000**	.829*		
Manganese	.943**	.657	.943**	
Phosphate	.943**	.943**	.943**	.829*

* Significant at 0.05 level; ** Significant at 0.01 level (2-tailed); n = 6

Fig. 4 – Average turbidity data and metal levels in pre and during-flushing water samples. Error bars indicate standard error.

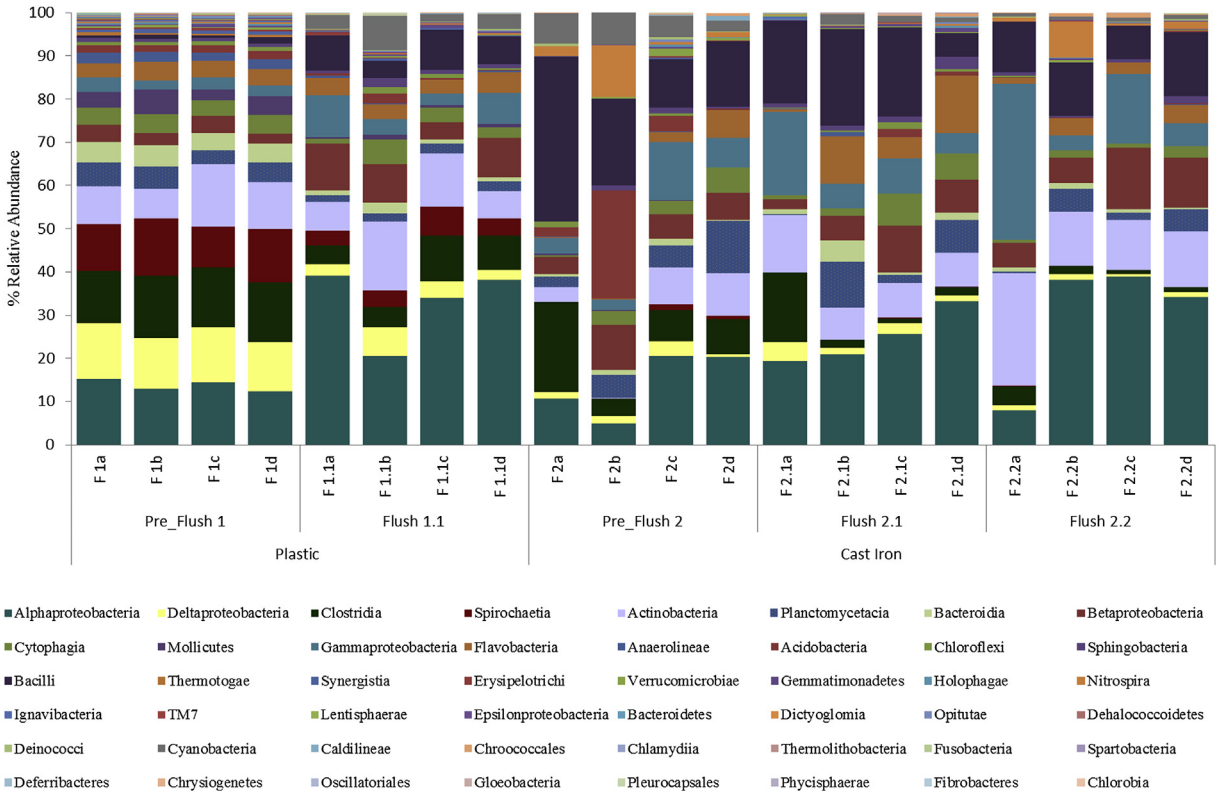


Fig. 5 – Comparison of the relative abundance of the major phylotypes found in the water collected from the network before and after flushing the internal pipe surfaces.

sequences were detected using Black Box Chimera Check software (B2C2) (Gontcharova et al., 2010) and excluded from the analysis. 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 bacterial database derived from the National Centre for Biotechnology Information (NCBI). The BLASTn outputs were compiled and validated using taxonomic distance methods as described previously (Dowd et al., 2008; Callaway et al., 2010).

Pyrosequencing data were deposited in the NCBI Sequence Read Archive (SRA) with the accession number SRA101471.

2.4.2. *Analysis of bacterial diversity: alpha and beta diversity*

The open source software package QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010b) was used to estimate the diversity within a sample (alpha-diversity) and to compare the diversity between samples (beta diversity). Sequences were quality filtered, aligned and clustered using QIIME pre-established parameters and community analysis pipeline (Caporaso et al., 2010b). Good quality sequences were clustered into Operational Taxonomic Units (OTUs) based on 0.95 (i.e. genus level) and 0.97 (i.e. species level) sequence similarity thresholds with the Uclust algorithm (Edgar, 2010). Representative OTUs were selected based on the most abundant sequences in the samples and the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) was used for taxonomic assignments. Sequences were then aligned using the Phyton Nearest Alignment Space Termination Tool (PyNAST) alignment algorithm (Caporaso et al., 2010a). Alpha-diversity was estimated using a rarefaction analysis (number of OTUs observed vs. number of sequences sampled) performed at 95% and 97% sequence similarity for each sample and the average was then calculated based on pipe material and flushing step analysed. Calculated collector's curves (Schloss and Handelsman, 2004) for Chao1 richness estimator (Chao, 1984) and Shannon diversity index (Shannon and Weaver, 1949) were obtained for all the sequences.

To compare bacterial diversity between samples (beta diversity), data was rarefacted to the smallest data set (1000 sequences) to reduce sequence heterogeneity and to allow for comparison of all samples at a similar sequencing depth. A phylogenetic tree was built using the FastTree algorithm (Price et al., 2009) for UniFrac distance matrix construction to calculate pairwise distances between communities in terms of their evolutionary history (Lozupone et al., 2011). Both unweighted (presence/absence information) and weighted (taking into account relative abundance of each OTU) UniFrac analysis were carried out and principal coordinate plots were generated.

2.5. *Statistical analysis*

Differences in bacterial community structure between samples depending on pipe material and flushing step were assessed using the relative sequence abundance at 95% (class

level) and 97% (species level) sequences similarities thresholds. The data was transformed by square root calculations and Bray–Curtis similarity matrixes were generated using the software Primer v6 (PRIMER-E, Plymouth, UK) and visualized using multidimensional scaling (MDS) diagrams. Analysis of similarity statistics (ANOSIM) was calculated using the same Bray–Curtis distance matrix to test the significance of differences between samples based on pipe material and flushing steps.

To investigate the relationships between water physico-chemical variables and biotic factors non-parametric Spearman's rank correlation coefficients (rho) were calculated using the software IBM SPSS 20.

3. Results

3.1. Turbidity results

Fig. 3 shows the measured turbidity and flow profile from both flushing operations. The first flush (polyethylene section) was a single flow operation (i.e. only one flushing step) due to the volume of water to be discharged with a minimum of 2 times turnover equating to 18.2 m^3. During this flush the hydrant was initially opened, then shut due to a leaking connection. It was then re-opened for 38 min at 12 l/s, at which point the turbidity had dropped below the regulatory 4 NTU (measured at hydrant). The turbidity peaked at 19 NTU. The second flush (cast iron section) incorporated a step increase in flow, intended to enable investigation of cohesive layer behaviour and composition. Hydraulic modelling prior to any fieldwork at this site had predicted higher flows than the 2.5 l/s achieved with the hydrant fully open. However, following the first test of this site in 2005, hydraulic calibration determined that the lower than expected peak flow was the result of a reduction in the effective pipe diameter (Boxall et al., 2004), most likely due to corrosion and tuberculation. The flushing flows imposed have since been kept consistent with this initial study (for long term analysis not reported here). In addition, although the increase is small, substantial further mobilisation of material is consistently obtained. This operation lasted for 25 min (total flush volume 3.5 m^3) with a peak turbidity of 230 NTU during the first step and 42 NTU following the 0.5 l/s flow increase.

3.2. Water physico-chemical analysis and HPC

Table 1 shows the data obtained from the physico-chemical analysis of the water and the colony counts after two and three days of growth. Several physico-chemical variables were similar among all the water samples; temperature average 6.2 ± 0.16 °C, pH near neutral 7.1 ± 0.03, free chlorine levels of 0.41 ± 0.04 mg/l, total organic carbon (TOC) 1.74 ± 0.08 and sulphate 45.98 ± 0.67 mg/l. This was to be expected from water obtained from the same supply and confirms the consistency of the bulk water irrespective of the local pipe material.

The variables that changed between samples followed similar trends to turbidity and significant positive correlations were obtained between turbidity, metals and phosphate (Fig. 4).

Colony counts were only high in the pre-flush samples from polyethylene pipes (Table 1). This is most likely attributable to contamination due to accumulated debris, mud and stagnant water in the hydrant chamber and bowl, as can be seen in Fig. 2B. It should be noted that while these samples are reported as pre-flush samples, what they represent is complex. The samples should not be considered as a measure of the normal bulk water throughout the system. This is because both pipes are dead-ends and prior to sample collection only a small volume of water was flushed through the hydrants, sufficient to remove chlorine residual from the standpipe and hydrant bowl disinfection. These samples are representative of bulk water at the dead end of pipes where mature biofilms have been growing for 42 months.

3.3. Differences in bacterial community structure with respect to pipe material

When the samples were analysed, taking into account the type of pipe material and independent of the flushed steps, clear differences in the structure of the bacterial community at different taxonomic levels were found between the samples from the polyethylene and cast iron pipes. At class level (Fig. 5), and calculating the average relative abundance for all the samples obtained from the material mobilised from the polyethylene pipes ($n = 8$), higher abundances of *Alphaproteobacteria* (23.30%), *Clostridia* (10.29%), *Actinobacteria* (10.26%), *Deltaproteobacteria* (8%) and *Spirochaetia* (8%) were detected. The bacterial community in the samples obtained from the cast iron pipes ($n = 12$) (Fig. 5) consisted mainly of sequences related to *Alphaproteobacteria* (22.85%), *Bacilli* (16.57%), *Gammaproteobacteria* (10.47%), *Actinobacteria* (10.15%) and *Betaproteobacteria* (7.53%).

At genera level, the average relative abundance of the most abundant genera for samples from the polyethylene and cast iron pipes (independent of flushing steps) was calculated. The results are displayed as two heat maps in Fig. 6. It can be observed that the most representative genera were different for the two pipe materials. The most abundant bacterial genera obtained from material mobilised in the samples from the polyethylene pipe were *Spirochaeta* spp., (7%), *Methylobacterium* spp. (7.04%), *Clostridium* spp. (4.37%), *Desulfobacterium* spp. (4.34%) and *Flavobacterium* spp. (2.62%). The same figure shows how *Lysinibacillus* spp. (7.45%), *Pseudomonas* spp. (4.23%), *Flavobacterium* spp. (3.89%), *Clostridium* spp. (3.69%) and *Bacillus* spp. (3.55%) were highly abundant in the samples from the cast iron pipe. However, the percentages of these bacterial groups varied depending on the flushing step, described in the next section.

Non-metric multidimensional scaling (MDS) plots of the relative abundance of bacteria show a clear separation of the bacterial community structure at class (Fig. 7A) and species level (Fig. 7B) between the samples from the cast iron and polyethylene pipes. The analysis of similarities (ANOSIM) confirmed that the bacterial composition of the material obtained from the two type of pipe materials was significantly different (class level; $R = 0.46$ and $p = 0.002$ and species level; $R = 0.45$ $p = 0.001$). It is notable that the pre-flush samples,

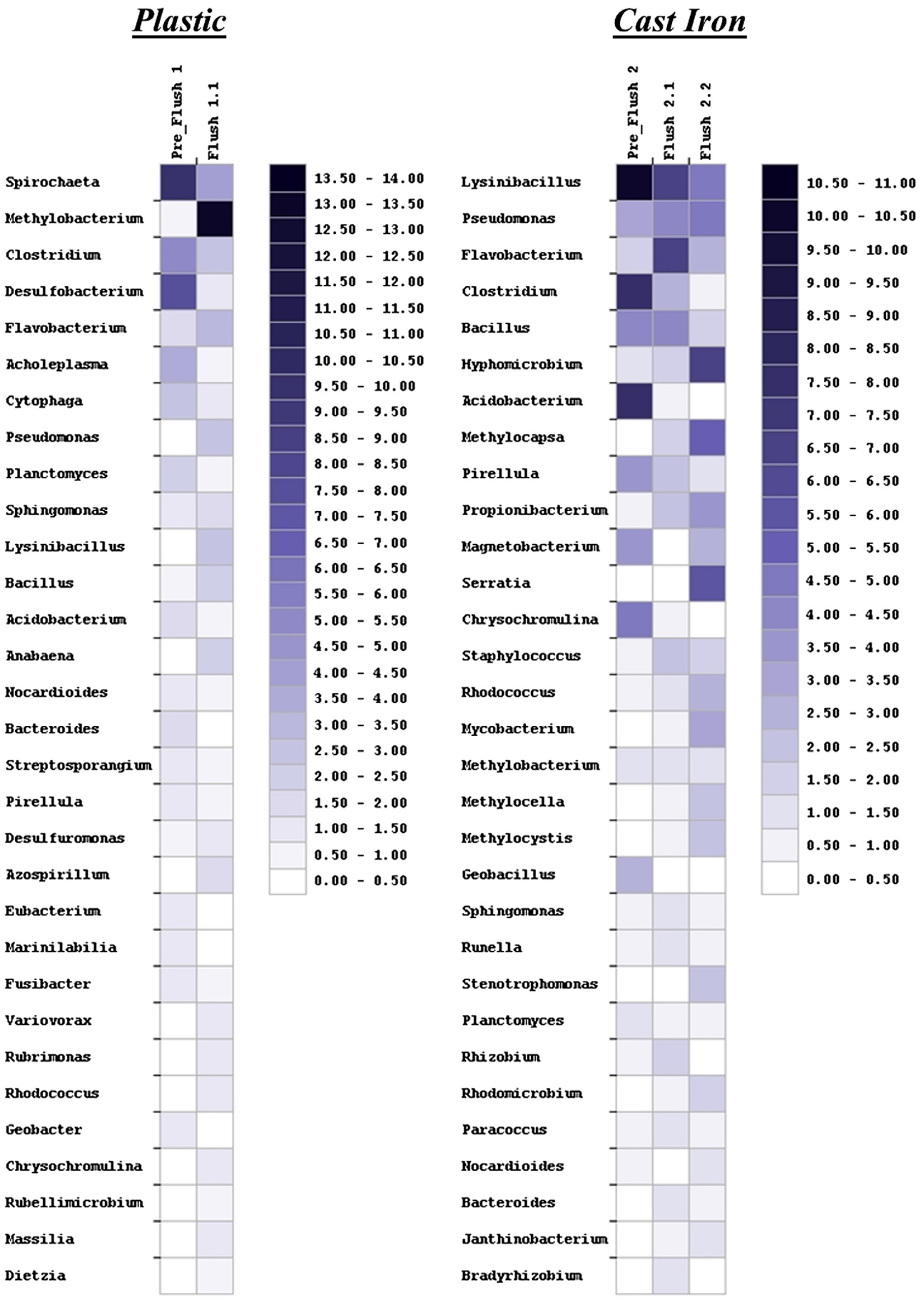


Fig. 6 – Heatmaps showing the percentages of the most abundant species at genus level within water samples from plastic and cast iron pipes. The relative abundance has been calculated as the average of 4 biological replicates for every sample.

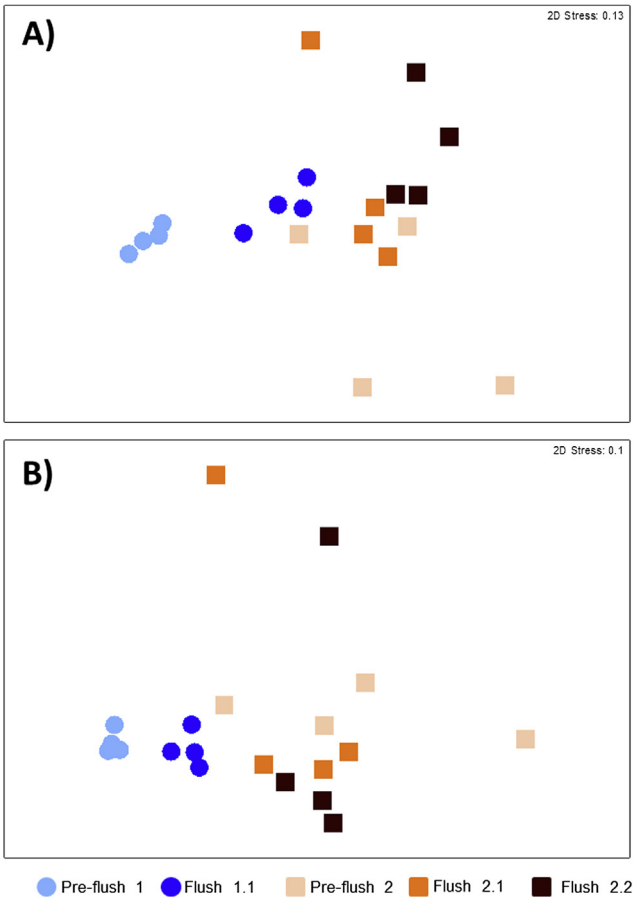


Fig. 7 – Two-dimensional plot of the multidimensional scaling (MDS) analysis based on Bray–Curtis similarities of the percentage sequence abundance (A) at class level and (B) at species level showing differences in the bacterial community structure between polyethylene and cast iron biofilms ($n = 20$). Symbols are representing individual samples and are coloured based on sample type.

prior to increasing flows to mobilise pipe wall materials, are different for the cast iron and polyethylene pipes.

Rarefaction calculated collector's curves obtained for 1000 sequences (Fig. 8) showed that the samples obtained from material mobilised from polyethylene pipes had higher observed OTUs than cast iron samples. The Chao1 richness estimator and the Shannon diversity index, estimated at 3% dissimilarity cut off, also showed higher richness and diversity within the samples obtained from polyethylene pipes. The results of the principal coordinate analysis from UniFrac metrics both weighted and un-weighted (Fig. 9), also distinguished between samples from polyethylene and cast iron pipes. Again it is notable that this difference in community with respect to pipe material is also evident in the pre-flush results.

3.4. *Differences in bacterial community structure with respect to flushing stage*

Different bacterial community composition was detected between pre and during-flushing water samples at class level (Fig. 5) and genera level (Fig. 6) from both plastic and cast iron pipe sections.

In the material removed from plastic pipes the relative abundance of some bacterial classes decreased in during flushing samples, for example *Deltaproteobacteria*, *Clostridia* and *Spirochaetia* decreased between 6 and 7% (Fig. 5). Other bacterial class increased due to flushing, particularly *Alphaproteobacteria* which increased an average of 19% in during-flushing samples and became the most represented bacterial class reaching an average abundance of 40% of the total bacterial community in one of the samples (F1.1a).

In the water samples obtained during flushing the cast iron pipes, the presence of *Alphaproteobacteria* also increased (10–15%), comprising between 20 and 40% of the total community of the removed material (Fig. 5). In samples from the cast iron pipe, increases in *Actinobacteria* (3–10%), *Gammaproteobacteria* (2–9%) and *Flavobacteria* (0.7–5%) were also detected during flushing. Conversely, other bacterial classes decreased in during flushing cast iron samples such as *Bacilli* (4.2–9%), *Acidobacteria* (7–7.5%), *Clostridia* (4.8–8%) and *Cyanobacteria* (4.1–4.5%) (Fig. 5).

At genus level compositional shifts in the bacterial community structure were also observed between pre and during flushing samples from the polyethylene pipe (Fig. 6). For example bacteria belonging to the genera *Spirochaeta*, *Desulfobacterium* and *Clostridium* decreased during flushing but *Methylobacterium*, *Bacillus*, and *Pseudomonas* increased in the material removed from polyethylene pipes due to flushing. In the samples from the cast iron pipe there were also differences among the flushing steps. *Lysinobacillus* spp., *Acidobacterium* spp. and *Clostridium* spp. were predominant in pre-flushing samples but their relative abundance decreased considerably during flushing. Interestingly, members of the genera *Hypomicrobium*, *Serratia* and *Methylocapsa* increased in the during-flushing samples, particularly in the second flushing step (Flush 2.2) suggesting that these bacteria are associated with the more strongly adhered material.

The MDS analysis showed a clear separation of pre- and during-flushing samples from the plastic pipe at class (Fig. 7A) and species level (Fig. 7B). This separation was also supported by the statistical ANOSIM analysis (class level; $R = 0.9$ and $p = 0.029$ and species level; $R = 0.75$ $p = 0.029$). Pre-flush samples from both flushing sites also showed significant differences between them (class level; $R = 0.79$ and $p = 0.029$). Despite visually observable differences at class and species levels between pre- and during-flushing samples from the cast iron pipe in the MDS plot (Fig. 7), when these are analysed using ANOSIM they are not statistically significant.

Collected rarefaction curves showing species richness and diversity indexes at 97% sequence similarity cut off, varied between pre- and during-flushing samples (Fig. 8). The number of observed OTUs was unexpectedly high in pre-flush samples from the plastic pipe (Fig. 8), most likely due to the complex nature of the collected samples. The pre-flush samples are mainly representative of bulk water samples at the dead end of pipes as has been explained in section 3.2. Less richness (Chao I estimator) and diversity (Shannon index) were detected for samples obtained during the last flushing step (Flush 2.2) of the cast iron pipe section.

Fig. 9 shows principal coordinate analysis based on UniFrac metrics (phylogenetic analysis), clearly separating pre- and

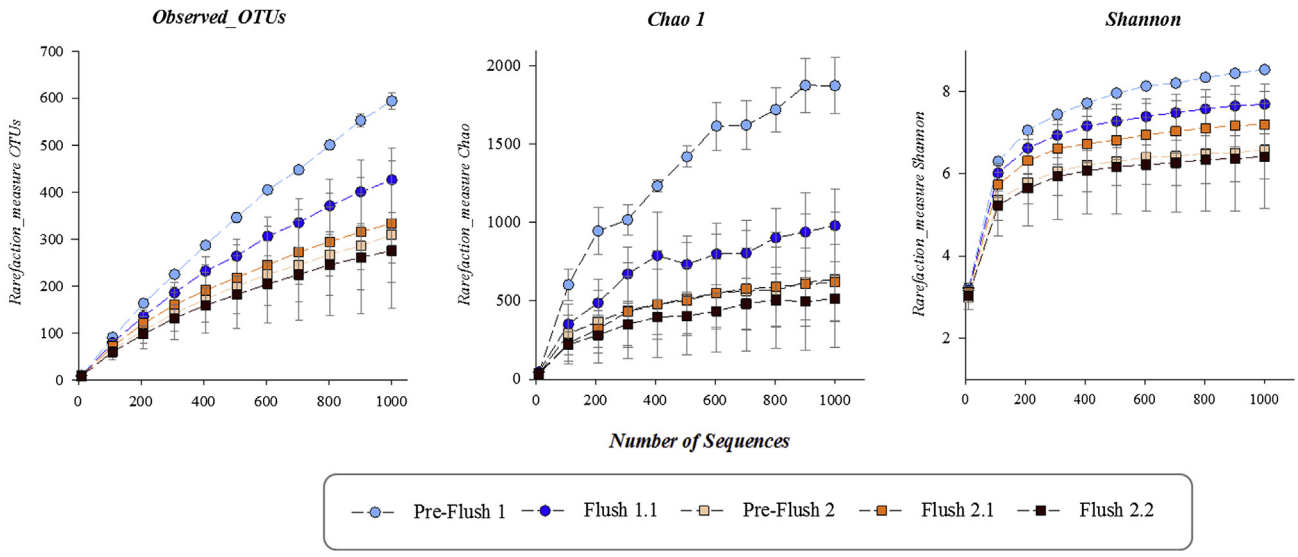


Fig. 8 – Rarefaction curves at 97% of sequence similarity for biofilm samples. Rarefaction curves were obtained for observed OTUS, Chao1 index richness estimator and Shannon diversity estimator using a rarefaction level of 1000 sequences to allow for comparison of all samples at a similar sequencing depth. Bars are indicating standard error.

during-flushing samples from polyethylene pipes. However, despite showing a certain degree of variance between samples from the cast iron pipe, these did not clearly cluster according to the flushing step analysed. This confirms the trend observed for the MDS analysis based on relative sequence abundance.

4. Discussion

4.1. *Water physico-chemical characteristics and effect of flushing on water quality*

The turbidity results indicate that both the polyethylene and cast iron pipe sections have accumulated material that was mobilised when the flows and corresponding shear forces on the pipe walls were increased. These data suggest that higher quantities of material are developed on cast iron pipes over time and that the material accumulated on this type of pipes appears to be more sensitive to flow changes when compared with polyethylene pipes (Fig. 3). This is in agreement with observations by Husband and Boxall (2011), adding weight to their suggestion that in both plastic and cast iron pipes there is ubiquitous material accumulation resulting from the background concentrations of particulate and soluble material within the bulk water, and that in cast iron pipes there are additional accumulation processes associated with corrosion.

The European Drinking Water Directive 98/83/EC on the quality of water intended for human consumption established

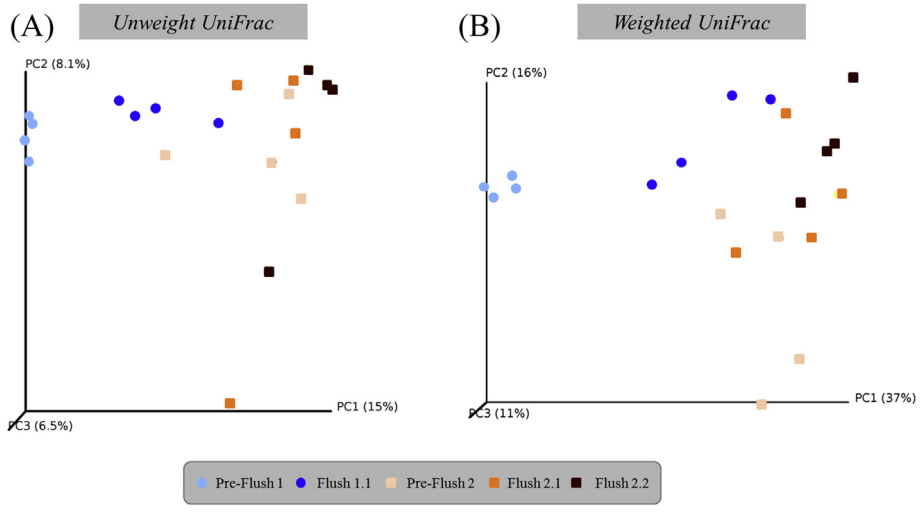


Fig. 9 – Three-dimensional principal coordinates plots of UniFrac analysis showing the phylogenetic clustering of the bacterial communities at 97% of sequence similarity. The axes are scaled according to the percentage of variance that they are explaining. (A) Un-weighted-UniFrac, (B) Weighted-UniFrac ($n = 20$). Symbols are representing individual samples and are coloured based on sample type.

that turbidity should be less than 1 NTU for an effective disinfection in the system. In addition, turbidity values above 5 NTUs can give colour to the water. When cast iron pipes were flushed turbidity reached values above 200 NTUs (Fig. 3), highly red coloured water. Metal concentrations were positively correlated with turbidity and increased when turbidity levels were higher (Fig. 4). Corrosion of cast iron pipes promotes the accumulation of iron particles in the system (Sarin et al., 2004; Vreeburg and Boxall, 2007). Furthermore, metals are used as coagulants in treatment plants to eliminate organic matter (Matilainen et al., 2010) and residual concentrations of these can enter the distribution system and deposit on the pipes (Twort et al., 2000). The excessive presence of these metals in potable water can result in discoloured water and/or changes in taste (Husband et al., 2008). In general, to avoid problems of discolouration, an adequate flushing scheme of the system can help to control the accumulation of metallic compounds. However, most of these flushing strategies only limit biofilm growth to a certain extent and do not completely eliminate microorganisms from the system (Douterelo et al., 2013).

The concentration of phosphate was higher in the water samples obtained from cast iron pipes and was positively correlated with turbidity and metals for both pipes (Fig. 4). Orthophosphate and polyphosphate are added to drinking water as corrosion inhibitors and to prevent the leaching and release of lead and copper from pipes (Volk et al., 2000). Orthophosphates react with dissolved metals forming a protective coating on inner-pipe surfaces and polyphosphates bind up with metals keeping them in solution (Sontheimer et al., 1985). Despite the beneficial effects of phosphorus addition, it is not clear how the addition of phosphate might affect microbial growth and diversity. Some authors suggest that phosphate promotes growth and/or increases bacterial diversity (Lehtola et al., 2002; Jang et al., 2012), while other authors suggest that phosphate does not influence total bacterial densities (Gouider et al., 2009) or biofilm growth (Batté et al., 2003b). Conversely, other studies reported that addition of phosphorus leads to a decrease in microbial diversity in drinking water treatment bioreactors (Li et al., 2010). Taking into account the results from this study further research is needed to clarify the effect that phosphate might have on microbial communities inhabiting operational DWDS and in the development of biofilms.

4.2. *General microbiological characteristics of the water samples containing material removed from distribution system*

In the samples obtained from both flushing sites we detected a high abundance of prosthecate and stalked bacteria such as *Prosthecomicrobium* spp., *Hypomicrobium* spp. and *Pirellula* spp. (Fig. 6). The formation of stalks and other cytoplasmic extrusions help bacteria to attach to surfaces and increase the total surface area of the cells, therefore improving their capability for nutrient uptake (Moore, 1981), particularly in nutrient-limited environments such as DWDS. Prosthecate bacteria were particularly abundant in samples obtained during flushing cast iron pipes. These were mainly bacteria belonging to the genus *Hypomicrobium* spp., which is generally involved in iron and manganese deposition (Moore, 1981). It has been observed using microscopy techniques that stalks recovered from such bacteria inhabiting water distributions systems were coated with insoluble ferric salt deposits (Ridgway and Olson, 1981).

The 16S rRNA libraries of all samples were dominated by *Alphaproteobacteria* (Fig. 5) which are habitually present in DWDS (Williams et al., 2004) and are particularly abundant in networks with chlorine as a disinfectant residual (Gomez-Alvarez et al., 2012). *Deltaproteobacteria* were also highly abundant in the material removed from both types of pipe material. Its abundance might be due to the capability of some of these bacteria to form disinfectant resistant spores (Stackebrandt et al., 1988). Such resistance would contribute to their survival in the system. *Clostridia* were also highly represented in all the studied samples. We presume that in order to survive in the distribution network, these obligate anaerobic bacteria must have been protected inside thick biofilms formed over 42 months. Species belonging to this genus can also survive in aquatic environments by forming endospores. For example, in running freshwater species of *Clostridia* spp. exist as endospores and when these spores find oxygen-depleted zones in a river they can germinate and become viable cells (Böckelmann et al., 2000). *Clostridium perfringens* is used as an indicator of the efficiency of drinking water treatment (Payment and Franco, 1993). However, we were not able to identify this particular species in our data, since most of the sequences were only classified at genus level.

Common genera found in all the samples, but with different relative abundance depending on the pipe material the sample analysed originated from included *Clostridium* spp., *Flavobacterium* spp., *Sphingomonas* spp., *Pseudomonas* spp., and *Methylobacterium* spp. All these bacteria have been previously found in drinking water systems as planktonic cells in the bulk water or forming part of biofilms in distribution systems simulators or water meters in distribution networks, (Williams et al., 2004; Gallego et al., 2005; Hong et al., 2010; Revetta et al., 2010). *Flavobacterium* spp. and *Pseudomonas* spp. are known to act as pioneers in the initial stages of biofilm formation in drinking water (Navarro-Noya et al., 2013). We can confirm, their presence in the material removed from pipes in an operational drinking water network and forming part of what can be considered mature biofilms.

4.3. *Influence of pipe material on bacterial community structure*

Clear differences in the bacterial community structure between the material removed from plastic and cast iron pipes was found at different taxonomic levels (Figs. 5 and 6). It is notable that *Spirochaeta* spp. was highly abundant in the samples from the polyethylene pipe but not in the ones from the cast iron pipe. Species belonging to this genus are anaerobic or facultative aerobic bacteria and they can only use carbohydrates as substrates to survive. These bacteria are good in competition for limited nutrients and their success in DWDS might be aided through mobility structures, enabling these bacteria to disperse and colonize new surfaces (Madigan et al., 2005). The symbiotic association of some of the *Spirochaeta* species with other

bacteria such as *Clostridium* spp. has been previously reported in other aquatic environments (Pohlschroeder et al., 1994) and this type of associations can also explain the high abundance of these genera in DWDS. The control and management of these bacteria could be important for managing water quality impacts of biofilms from plastic pipes.

In the samples obtained from the material removed from the cast iron pipe section we observed a high presence of bacteria specialised in using iron and manganese compounds (Fig. 6) such as *Lysinibacillus* spp. which is naturally present in soils and can oxidise manganese (Cerrato et al., 2010), *Geobacillus* spp., which can absorb several heavy metals including iron (Hetzer et al., 2006) and *Magnetobacterium* spp. which can accumulate magnetite (iron mineral, Fe3O4) inside their cells. *Magnetobacterium* spp. are aquatic bacteria habitually present in the oxic–anoxic transition zone of vertical chemical gradients in stratified environments (Flies et al., 2005; Cerrato et al., 2010). *Magnetobacterium* spp. was detected in pre and during-flushing samples from cast iron pipes (Fig. 6), suggesting that the material mobilised from the pipes was a mix of different biofilm layers, independently of the oxygen vertical gradient (i.e. oxic, anoxic and transition zone) existent in the material accumulated on the pipe walls. Significant positive non-parametric correlations ($p < 0.01$) between iron concentrations in the water samples and the relative abundance of some of the iron bacteria such as *Magnetobacterium* spp. and *Geobacillus* spp. was also detected (data not shown). Additionally, it has been suggested that the tubercles formed on corroded pipes serve as microenvironments for iron bacteria, promoting even further their accumulation on the pipes (Martin et al., 1982). The elimination of these bacteria in water networks is problematic since they are protected against the action of disinfectant within biofilms and to clean pipes, jetting or steam injection might be needed (Twort et al., 2000). In addition any cleaning strategy is only a temporary measure and the complete eradication of these bacteria in the network is unrealistic. Understanding of their behaviour and significance is therefore important to help inform future management strategies.

Previous research on microbial communities associated with drinking water networks suggests that cast iron pipe boundary surfaces favour microbial growth. For example, higher amounts of bacteria were detected in unlined cast iron pipes when compared with PVC in a model DWDS (Neden et al., 1992) and it has been reported that iron pipes can support 10–45 times more growth than plastic (Niquette et al., 2000). Whilst all conditions were not consistent such as pipe diameter and daily regime, the key factor affecting the bacteriological composition of the samples was type of pipe material. Pipe length and diameter are factors that primarily affect the amount and/or growth rate of biofilms and daily hydraulic regime influences the physical characteristics of biofilms such as for example the strength of attachment to the pipes but they do not seem to significantly affect their bacteriological composition per se as explained in Douterelo et al. (2013). Additionally, since the same water source supplied both sections of pipe, the high relative abundance of bacteria able to utilise iron and manganese in the material removed from the iron pipe and the correlation of the relative abundance of some of these bacteria with the concentration of iron corroborates that pipe material was the dominant factor in determining compositional differences between sites.

The results obtained in the present study, shown that in operational distribution networks, higher turbidity levels were detected in samples obtained from the cast iron pipe section, supporting the idea that more particles and microorganisms associated with these particles were detached from the cast iron pipes. However, bacterial communities in samples from the cast iron pipe were less diverse when compared with the polyethylene pipes (Fig. 8). It has been shown that polyethylene can release biodegradable organic compounds which can promote microbial growth (Yu et al., 2010). Several researches showed that plastic can stimulate biofilm growth, for example van der Kooij et al. (2005) demonstrated that cross-linked polyethylene (PEX) promoted biofilm development at higher rates than stainless steel and copper. Lehtola et al. (2004) suggested that microbial growth was promoted on polyethylene pipes by the release of phosphorus from this material. Similarly, laboratory studies on bacterial adherence have shown that bacteria adhered to a higher extent on polyethylene than to other materials (Simoes et al., 2007). It has also been shown that they can attach more rapidly to hydrophobic, nonpolar surfaces such as plastics than to hydrophilic materials such as metals (Donlan, 2002). From our results we can conclude that higher bacterial diversity was observed in material detached from polyethylene pipes but in terms of quantity, higher amounts of material (including particles and biofilm) were detached from cast iron pipes. This suggests that in cast iron pipes, a less diverse community dominated by iron and manganese bacteria was present and greater amounts of the biofilm and associated particles could be mobilised by hydraulic flushing forces. Conversely, a more diverse community was detected from the polyethylene pipes but less material was mobilised during flushing.

Cyanobacteria were present particularly in samples from the cast iron section. This is surprising as they normally depend on light for survival. Cyanobacteria have been previously found in drinking water (Revetta et al., 2010) and they might enter in the distribution system from the treatment plant but how they survive, if they are alive, in the dark attached to a biofilm is not clear. It is possible that some of the cyanobacterial populations detected in this study can temporarily survive in the darkness under anaerobic, reducing conditions (Richardson and Castenholz, 1987) but it is also possible that they are only trapped in the biofilms and are not metabolically active. Some species of cyanobacteria are considered contaminants in drinking water by the Environmental Protection Agency (EPA), since they can produce toxins. However, for the toxins to be detected, cyanobacteria must form blooms in the source water, a phenomenon that has only been observed in summer with high temperatures or when waters are highly eutrophic.

4.4. *Influence of flushing step on bacterial community structure*

Differences in the bacterial community composition of pre and during flushing samples from both pipe materials were observed at class and genera level (Figs. 5 and 6) but these

were most likely due to the particular characteristics of the pre-flush samples, which were collected after flushing a small volume of water through the hydrants and hence what they represent is the bacteriological composition of the bulk water in the dead end pipes.

The first increase in flow in the cast iron pipe (Flush 1.1) was able to remove more material than the second increase (Flush 2.2) as can be seen from the turbidity results (Fig. 3). This indicates that most of the material from the pipes is removed with the initial change in hydraulic conditions but that some is retained and only mobilised with further hydraulic increases. Clear differences in the relative abundance of different bacterial groups between samples from the different flushing steps in the cast iron pipe at class and general level were observed (Figs. 5 and 6). Members of the genera *Hypomicrobium*, *Serratia* and *Methylocapsa* increased in during-flushing samples particularly in the second flushing step suggesting that these bacteria are associated with the more strongly adhered material (Fig. 6). However the OTU-based analysis (Fig. 8) of the cast iron pipe samples did not show significant differences in bacterial richness and diversity with the increase of hydraulic shear stress. This result indicates that those layers of material that needed more force to be mobilised did not have a significantly different community structure when compared with those obtained in the first increase in flow, suggesting that the layers of material removed from the cast iron pipe walls have similar bacteriological composition independently of the shear force used. In this study, the smaller diameter of the cast iron pipe when compared to the plastic pipe might influence the observed results and the strength of material attachment to this type of pipes. However, due to technical constraints during the field trial it was not feasible to increase the flush velocity further in the network and consequently it was impossible to evaluate if a further hypothetical increase in velocity would have the potential to remove additional material of different bacteriological composition. This therefore indicates that under realistic flushing velocities the mobilising shear stress is not a dominant factor in driving the differences observed in bacterial community composition between sites.

5. Conclusion

This work has shown that the material mobilized from pipes of different material in close proximity within a single distribution management area and receiving the same source water had distinctly different bacterial communities. Although all conditions were not identical such as daily regime and pipe diameter the high relative abundance of iron and manganese bacteria in the samples obtained from the iron pipe and the correlation of the relative abundance of some of these bacteria with the concentration of iron suggests that pipe material was the main factor in driving bacterial community structure. The samples obtained during the flushing of the cast iron pipe had higher turbidity that the samples obtained from the plastic pipe section. This turbidity was positively correlated with iron, manganese and phosphate concentrations. While a lower turbidity response was observed resulting from the plastic pipes, there was greater bacterial diversity and richness in the samples from the plastic pipe in comparison to the cast iron pipe samples.

These findings clearly show the importance of local infrastructure on material accumulation and release processes occurring within DWDS. While source water may dictate overall levels of microbial activity and may provide the source of bacteria, it is important to understand and characterize local conditions to understand deterioration processes and hence evaluate possible associated levels of risk.

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