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Intermittent Water Supply Impacts on Distribution System Biofilms and Water Quality

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ABSTRACT

Intermittent water supplies (IWS) are routinely experienced by drinking water distribution systems around the world, either due to ongoing operational practices or due to one off interruptions. During IWS events changing conditions may impact the endemic biofilms leading to hydraulic mobilisation of organic and inorganic materials attached to pipes walls with a resulting degradation in water quality. To study the impact of IWS on the microbiological and physico-chemical characteristics of drinking water, an experimental full-scale chlorinated pipe facility was operated over 60 days under realistic hydraulic conditions to allow for biofilm growth and to investigate flow resumption behaviour post-IWS events of 6, 48 and 144 hours.

Turbidity and metal concentrations showed significant responses to flow restarting, indicating biofilm changes, with events greater than 6 hours generating more turbidity responses and hence discolouration risk. The increase in pressure when the system was restarted showed a substantial increase in total cell counts, while the subsequent increases in flow led to elevated turbidity and metals concentrations. $SUVA_{254}$ monitoring indicated that shorter times of non-water supply increased the risk of aromatic organic compounds and hence risk of disinfection-by-products formation. DNA sequencing indicated that increasing IWS times resulted in increased relative abundance of potential pathogenic microorganisms, such as *Mycobacterium, Sphingomonas*, and the fungi *Penicillium* and *Cladosporium*.

Overall findings indicate that shorter IWS result in a higher proportion of aromatic organic compounds, which can potentially react with chlorine and increase risk of disinfection-by-products formation. However, by minimising IWS times, biofilm-associated impacts can be reduced, yet these are complex ecosystems and much remains to be understood about how microbial interactions can be managed to best ensure continued water safe supply.

1. Introduction

It has been demonstrated that the establishment of continuous water supply is preferential for public health, yet currently one-third of the world population is supplied via an Intermittent Water Supply (IWS) system (WHO, 2017). Routine IWS can be caused by different environmental and socio-economic factors including water stress, excessive water losses due to faulty distribution infrastructure, energy constraints and cost related to water pumping demands and rapid urbanisation (Lee and Schwab, 2005; Simukonda *et al.* 2018). Notwithstanding perceived safety concerns, the practise of IWS is expected to increase due to changing demographics, water scarcity and climate change (Vairavamoorthy *et al.*, 2008; Bivins *et al.*, 2017).

Every network, including those with continuous supply, experiences interruptions to supply, either planned (e.g., asset maintenance, repair or replacement) or unplanned (e.g., burst, valve failure) intervention. While such interruptions are generally one off, they have very similar impacts to ongoing IWS. The term IWS is used hereon to capture both scenarios.

Interruption to supply can disturb the extensive and endemic pipe wall biofilms within Drinking Water Distribution Systems (DWDS) leading to water quality deterioration. A diverse range of processes

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during IWS can affect biofilms including pressure loss, the complete or partial emptying of pipes, drying, and the flushing effect caused by the system refilling and the flow restarting. During IWS, partially filled pipes and stagnant water can change environmental conditions with the long residence times reducing oxygen and disinfectant (Agudelo-Vera, Blokker and Pieterse-Quirijns, 2014), thereby favouring the change of microbial communities and impacting proliferation, and this has been observed through increases in total cells and heterotrophic plate counts (HPC) (Mesquita *et al.*, 2013). Changes in biofilm composition or physical traits during IWS events may also reduce biofilm shear strengths rendering them less able to resist mobilising shear forces, akin to biofilms developed under low shear stress that have shown a less cohesive structure and detach readily when hydraulic conditions change (Manuel *et al.* 2007; Douterelo *et al.*, 2013). In addition, if pipes are completely drained, biofilms can experience air exposure and drying that can affect structural resilience (Timoner *et al.*, 2014). It is therefore considered likely that IWS will impact biofilm communities and resulting growth dynamics and alter physical structure. When the system is refilled and flow resumes, the increases in pressure and flow may result in greater biofilm and associated material mobilisation from the pipe walls into the bulk water (Kumpel and Nelson, 2016). System recommissioning, when the water supply is resumed, may therefore have significant water quality implications, such as discolouration, taste and odour issues and elevated metals, inorganics and pathogen concentrations.

Most IWS studies have focused on quantitative operational and hydraulic characteristics, disregarding the qualitative and microbial impact. Yet IWS can have important implications and consequences on water safety (Kumpel and Nelson, 2016; Bivins *et al.*, 2017). The impact of biofilm mobilisation into the bulk water has not been explored using microbial molecular methods. The aim of this research, by simulating realistic network conditions in a full-scale chlorinated pipe loop facility that allows laboratory control, monitoring and sampling, is to provide understanding of how different IWS drain down times impact the microbial ecology and water quality. DNA sequencing was used to investigate the effect of IWS on the characteristics and community behaviour, with cytometry, $SUVA_{254}$ ultraviolet absorbance, turbidity and multi-parameter monitoring tracking organic and inorganic water quality responses. Knowledge of IWS impact on microbial ecology and water quality is essential if network operators are to understand consequences associated with this practice and to develop guidelines and strategies aimed at minimising potential risks and promote delivery of safe water.

2. Materials and methods

2.1. Experimental pipe facility

A full-scale experimental chlorinated pipe facility capable of replicating DWDS conditions at the University of Sheffield (UK) was used to perform experiments investigating different IWS drain-down times. The facility is comprised of three identical flow and pressure-controlled independent loops 9.5 m wide x 21.4 m long coils of High-Density Polyethylene (HDPE) pipe with an internal diameter of 79.3 mm. For full details see Douterelo *et al.* (2013). To enable *in-situ* sampling of biofilms developed on the pipe walls, each loop had 6 specifically designed removable pipe sections of 0.5 m length (Fig. 1). Before the experiments, each loop was disinfected by adding 20 mg/L of RODOLITE H (RODOL Ltd, Liverpool, UK), a solution of sodium hypochlorite with less than 16% free available chlorine, following the protocol described by Douterelo *et al.* (2013).

2.2. Experimental design and conditions

To study the effect of IWS on microbial communities, biofilms were allowed to develop in the experimental pipe facility over 60 days at 20°C and a daily Low Varied Flow (LVF) regime ranging from 0.2 to 0.5 L/s (Husband *et al.* 2008). See **Supplementary Figure A** and **Supplementary Table 1** for more information on the LVF pattern used during the growth and flow rate conversions. After the 60-day biofilm growth phase the flow was stopped and the pipes were drained at a constant low flow of 0.06 L/s, selected as well below the minimum of the LVF profile to minimise likelihood of removing material from the pipe walls. In loop 1 the flow was stopped, and the pipe was left drained for 6 hours, loop 2 for 48 hours and loop 3 for 144 hours (¼, 2 and 6 days) (Fig. 2). Times were selected to simulate different IWS scenarios, since IWS duration can range from few hours for repair works or daily cycle to several days depending on location and constraints (Coelho *et al.*, 2003; Kumpel and Nelson, 2016).

After the designed IWS drain-down time, each loop was re-connected to the local supply and filled at a constant flow of 0.2 L/s. This minimised refill time whilst not surpassing the lowest flow of the LVF profile that had conditioned the shear strength of material attached to pipe wall during the development phase. Once the pipes were full of water, the flow was re-started following scheduled stages combining different values of pressure and flow (Fig. 2). First, the system was run with the peak values of pressure (2.0 bar) and flow (0.54 L/s) from the LVF development profile (stage 0). After this pressure only was increased to 2.9 bar (stage 1) and then higher flows (2.3 and 4.1 L/s) were applied (stages 2 and 3, respectively). Finally, an extra stage was performed to test the impact of very high flow (6.1 L/s) that resulted in a lower 2.3 bar of pressure to achieve (stage 4). **Supplementary Figure B** shows the theorical flow and pressure applied in each stage of the water supply restarting and **Supplementary Table A** provides more information on flow rate conversions of the water supply restarting for each stage. Transitions between the hydraulic states were performed slowly to avoid dynamic hydraulic conditions such that all forces can be approximated to steady state. Each stage was performed until flow and pressure values

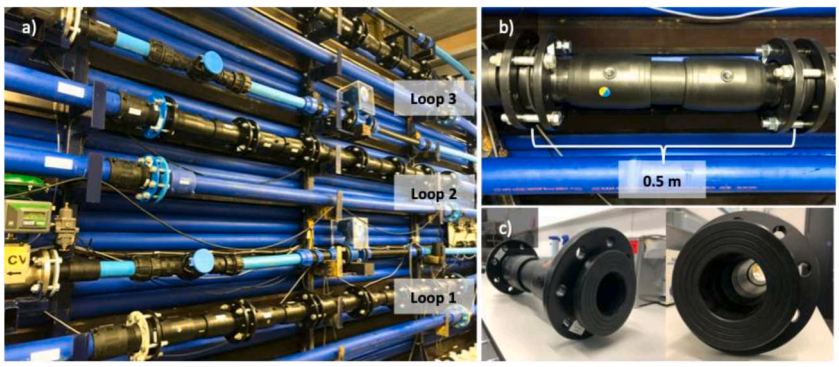

Fig. 1. a) Full scale experimental pipe loop facility composed by 3 individual loops; b) Sections used for biofilm sampling inserted and fixed into the system; c) Biofilm sampling removed showing detail of the inside of a section.

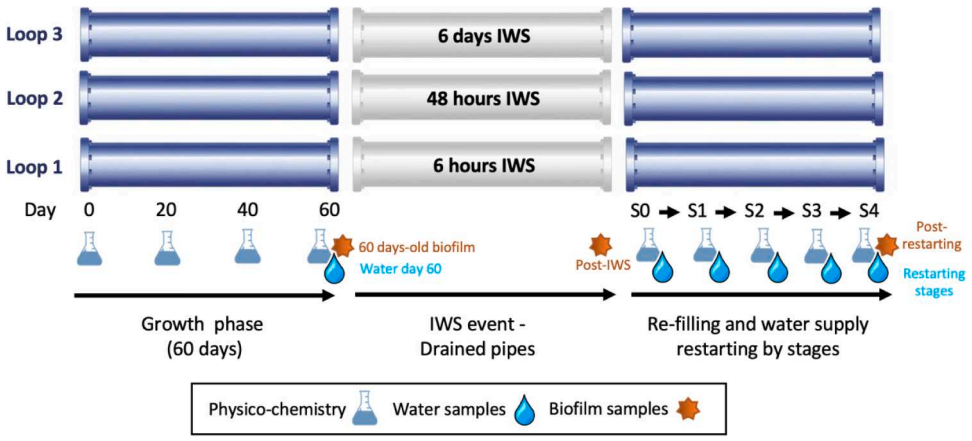

Fig. 2. Scheme of the experiment over time. The 3 loops were running under the same conditions for a growth phase of 60 days. Subsequently, each loop experienced a different IWS during which the supply was stopped, and the pipes drained. Finally, after each IWS, the loops were re-filled, and the supply was restarted by programmed steps (S). Sampling points for water physico-chemical analysis and water and biofilm samples for molecular analysis are indicated.

were stabilised and the water run for a minimum of 3 turnovers to provide enough time during the recycling for the water to be mixed and turbidity to stabilise (Sharpe *et al.*, 2010).

2.3. Water quality physico-chemical and biological analysis

Using discrete water samples (in triplicate), a range of water quality parameters were analysed at the beginning of the experiment, every 20 days during the biofilm growth phase and at each stage post restarting after the different IWS times (Fig. 2). Water temperature and pH were tested using a Hanna portable meter HI 991003 (Hanna Instruments, Leighton Buzzard, UK) and free and total chlorine concentrations were analysed with a Palintest CS100 chlorosense (Palintest, UK).

Total organic carbon (TOC) and dissolved organic carbon (DOC) were used to quantify the amount of natural organic matter (NOM), while the ultraviolet absorbance at 254 nm wavelength (UVA_{254}) and the specific ultraviolet absorbance (SUVA) were analysed to determine the nature of the NOM content. TOC and DOC were analysed by the analytical chemistry laboratories at KRI (The Kroto Research Institute, The University of Sheffield, UK) using a Shimadzu TOC-$V_{CPH/CPN}$ Analyzer (Shimadzu, Kyoto, Japan). The UVA_{254} of the water samples was quantified using a spectrophotometer (DR5000, Hach, USA). UVA_{254} together with DOC was used to calculate $SUVA_{254}$, which is the average absorptive capacity of DOC molecules of water samples, used as a measure of DOC aromaticity (Weishaar *et al.*, 2003). For each water sample $SUVA_{254}$ was calculated as (**Eq. 1**):

$$SUVA_{254}\left(\frac{L}{mg-m}\right) = \frac{UVA_{254}\ (cm^{-1})\ x\ 100}{DOC\ (mg/L)} \quad (1)$$

The concentration of iron (Fe) and manganese (Mn) was tested together with turbidity as indicators of discolouration (Seth *et al.*, 2004). Turbidity was measured continuously online by an ATi A15/76 turbidity monitor (ATi, Delph, UK) installed in the experimental facility. Fe and Mn concentrations were determined at the KRI laboratories (The University of Sheffield) by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Water samples were collected in 20mL vials containing 5M of nitric acid and then ions were monitored on a Perkin Elmer Elan DRC II (PerkinElmer, Inc., USA) (Sloetjes and Wittenburg, 2008).

Flow cytometry (FCM) was used to quantify the amount and viability of microbial cells in suspension in the water during each experimental phase following the protocol described by Prest *et al.* (2013) (Prest *et al.*, 2013). Water samples were dechlorinated adding 1% (w/v) sodium ascorbate solution to avoid the disinfectant affect the staining (Safford and Bischel, 2019). SYBR® Green I (10000x stock, Invitrogen, UK) was used to estimate the total cell counts (TCC). Propidium iodide (PI) (1.5mM, Life Technologies Ltd., Paisley, UK) was used together SYBR® Green I to estimate intact cell counts (ICC) (Douterelo *et al.*, 2016). Measurements were carried out using a BD Accuri™ C6 Cytometer (Becton Dickinson (BD) U.K. Ltd., Oxford, UK) equipped with a 50mW laser emitting at a fixed wavelength of 488 nm. The resulting flow cytometer data was processed and analysed using the BD Accuri™ C6 software (BD Biosciences, UK).

To study the impact of the different IWS times on planktonic communities, 3 biological replicates of 10L of bulk water were taken on day 60 and at each scheduled stage of pressure and flow changes after system re-start. For day 60, 1 biofilm/water replicate was obtained from each loop, since all the 3 loops were running under the same conditions during the growing phase. At each stage of the water supply restarting after the different IWS, water samples were collected when flow and pressure were stabilised after 3 turnovers (Sharpe, 2012). For microbial analysis, cells in the water samples were concentrated using a Tangential Flow Filtration (TFF) system (PALL Life Science, New York, USA) (Schwartz and Seeley, 2002) and then filtered through 0.22µm nitrocellulose membrane filters (Millipore, Corp). Filters were preserved at -20°C prior DNA extractions were performed.

To study the biofilm communities, 2 pipe sections were removed from each loop (0.25m^2 of biofilm surface area) on day 60, post-IWS times and post-restarting (Fig. 2). Biofilm was removed from pipe sections and suspended in 500mL of phosphate-buffered saline (PBS) (Gibco®, Thermo Fisher Scientific, UK) using a sterile nylon brush and a standardised brushing protocol (Deines *et al.*, 2010), applied to all samples. From each section, 2 × 250mL biofilm suspensions were obtained, then pooled and filtered through a 0.22µm nitrocellulose membrane filters (Millipore Corp., USA) and preserved at -20°C until DNA analysis.

2.4. DNA extraction and sequencing

DNA extraction of biofilm and water samples was carried out using a chemical lysis method with hexadecylmethylammonium bromide (CTAB) and proteinase K chemical, followed by DNA purification with phenol/isoamyl alcohol (Neufeld *et al.*, 2007). After the extractions, a Qubit 4 Fluorometer (Invitrogen by Thermo Fisher Scientific, Wilmington, USA) with a High Sensitivity dsDNA Assay kit was used for the quantification of DNA concentration from each sample.

Extracted DNA was sent to Mr DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) for Next Generation Sequencing (NGS). DNA was sequences on the Illumina MiSeq platform following the manufacturer's protocols for pair-end sequencing. The bacterial 16S

rRNA gene was amplified using the primers 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') spanning the V1 to V3 hypervariable regions. For fungal analysis, primers ITS1FBt1 (5'-CTTGGTCATTTAGAGGAAGTAA-3')/ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') targeting the ITS1-2 regions were selected for amplification. Sequencing data were deposited in the NCBI Sequence Read Archive (SRA) with the accession number PRJNA707019.

2.5. Bioinformatics and community analysis

A range of bioinformatic tools were used in order to study the community of microorganisms present after the different IWS times. An initial quality control of the sequencing raw data was carried out using the FastQC software v0.11.8 (Andrew, 2010). BBDuk software v37.95 was used to remove sequencing errors (Davis *et al.*, 2018) and to filter and trim sequences with an average quality phred score below 20 and/or a minimum length of 100bp (Cock *et al.*, 2009). Sequencing reads were demultiplexed and depleted of barcodes by applying the sabre software (Joshi, 2011) and imported into the Quantitative Insights Into Microbial Ecology 2 program v2019.7 (QIIME2) (Bolyen *et al.*, 2019). Then, pair-end sequences were joined and dereplicated, chimeric sequences were identified and filtered and *de-novo* clustering by 97% similarity was performed to obtain the Operational Taxonomic Units (OTUs) using the vsearch plug-ins in QIIME2. The taxonomic assignment of the final OTUs was carried out using the classify-consensus-vsearch method (Rognes *et al.*, 2016) of the feature-classifier plug-in in QIIME2 (Bokulich *et al.*, 2018). 16S rRNA sequences were compared against the SILVA SSU r132 database (Quast *et al.*, 2013) and ITS2 sequences against UNITE 8.0 (Kõljalg *et al.*, 2013).

Rarefied tables based on of the relative abundance of 97% OTUs for both bacteria and fungi, were used to calculate alpha- and beta-diversity (Morris *et al.*, 2014). Alpha-diversity, which measures the internal diversity of each sample, was calculated as a measurement of Chao 1 index (richness estimator), Simpson index (dominance), and Shannon index (diversity) (Morris *et al.*, 2014) using the q2-diversity plug-in in QIIME2. For beta-diversity, which estimates the degree of differentiation between samples, the rarefied OTU table was square-root transformed and then the Bray-Curtis method was applied to construct similarity matrices using the vegan package v2.5-6 in R (Oksanen *et al.*, 2019). Bray-Curtis resemblance matrices were visualized by non-metric multidimensional scaling (nMDS) plots with ggplot2 package v3.2.1 in R (Wickham and Chan, 2016).

2.6. Statistical analysis

All biological and physico-chemical parameters were measured in triplicate, and the mean and standard deviation were calculated. In the same way, the relative abundance of each OTU and the values of alpha diversity indices, were calculated as the mean of all replicates analysed for one sample. The normality of the data sets was tested before performing significance tests by Shapiro-Wilk test. Statistical differences between IWS times of all physico-chemical and biological parameters were tested via the non-parametric Kruskal–Wallis test by ranks. Then, if significant differences were observed, the non-parametric Mann-Whitney *U* test was used to compare samples pairwise.

For beta diversity, analysis of similarities (ANOSIM) was applied to Bray-Curtis distance matrices to detect significant differences in biofilm and water microbial communities between IWS times (p-value). To establish the impact of different IWS times on them the global-R statistics was calculated, which has values ranging from 0 to 1, where 1 indicates that communities are totally different (Anderson and Walsh, 2013). Differences were considered statistically significant when p-value was $\leq$0.05, and all statistical tests were carried out using R software version 3.6.1 (r-project.org) (Team, 2014).

3. Results

3.1. Water physico-chemical and biological analysis

Results for water quality parameters during the growth phases and after different IWS times are shown in **Supplementary Table B**. In general, the physico-chemical analysis showed results consistent between the 3 loops during the 60 days of growth phase with differences observed in several parameters after the different IWS times. Temperature was stable during the growth phase due to the temperature control within the facility, average of 20.3 $\pm$ 0.1°C (n = 12/per loop). However, after IWS when the supply was restarted with fresh supply water the temperature averaged of 17.6 $\pm$ 0.3°C (n = 15/loop). pH values showed limited changes during the test, ranging from 6.8 to 7.5 in the 3 loops. Water disinfectant concentrations were similar over the development phase with total chlorine of 0.37 $\pm$ 0.06 mg/L and free chlorine of 0.31 $\pm$ 0.04 mg/L. A marked difference can be observed both during initial filling and during stage 0, as the pipe loops are refilled, with total chlorine 0.5 $\pm$ 0.09 mg/l and free chlorine 0.46 $\pm$ 0.08 mg/L. Post refilling chorine concentrations decay during the test stages with final concentrations matching those sustained during the development phase.

For NOM water quantification, during the growth phase TOC and DOC showed values ranging from 1.23 to 1.87 mg/L and 1.03 to 1.5 mg/L respectively (**Supplementary Table B**), with no significant differences (p-values >0.05). After restarting the water supply, TOC and DOC showed values similar to those in the growth phase and with no significant differences registered in any stage between the 3 different IWS times (p-values >0.05). Water $SUVA_{254}$ showed an average of 1.44 $\pm$ 0.07 L/mg-m (n = 12/loop) during the growth phase (**Supplementary Table B** and Fig. 3). After the water supply restarting, the highest values for $SUVA_{254}$ were observed after 6 hours of IWS, followed by 48 hours and 6 days with average values across test stages 2 – 4 (when flow increased above peak conditioning value achieved during the 60-day growth phase) being 2.5, 1.9 and 1.6 L/mg-m respectively (Fig. 3). Statistical tests (**Supplementary Tables C** and **D**) confirmed that significant differences were only detected after 6 hours of IWS when compared to day 60 (p-value $\leq$0.05). When water $SUVA_{254}$ values after the 3 IWS times were compared, significant differences were observed in stages 1, 2 and 4 (p-values $\leq$0.05). Pairwise comparisons showed higher significant $SUVA_{254}$ values in stage 1 after 6 hours of IWS, than after 48 hours and 6 days (p-values $\leq$0.05). In stages 2 and 4, significant differences were observed between all IWS times (p-values $\leq$0.05), again showing highest values following 6 hours with reducing concentrations from 48 hours to 6 days.

3.2. Water discolouration

Iron and manganese concentrations showed similar values for the 3 loops through the biofilm growth phase, ranging from 28.19 - 39.90 µg/

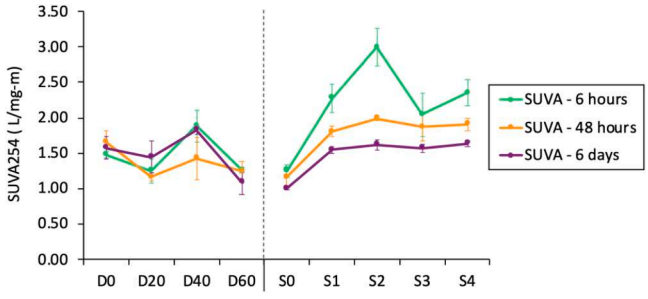

Fig. 3. $SUVA_{254}$ values every 20 days (D) during the growth phase and in each stage (S) after restarting the water supply after 6 hours, 48 hours and 6 days of IWS. All values represent an average of three water replicates analysis $\pm$ standard deviation.

L and 0.49 - 0.82μg/L respectively (**Supplementary Table B**), with no statistical difference between the 3 loops at any sampling point. However, when the water supply was restarted after the different IWS times, an increase in metal concentrations was observed. The highest Fe concentrations were observed after 6 days IWS, followed by 48 hours and 6 hours with an average across test stages 2-4 returning 144, 157 and 189 μg/L respectively. Statistical test (**Supplementary Tables C** and **E**) reported significant differences between IWS times for stages 2, 3 and 4 (p-values $\leq$0.05). When samples were compared pairwise, significant differences were found between 6 hours and 6 days on these stages (p-values $\leq$0.05). Mn concentrations across test stages 2-4 were 1.6, 2.9 and 2.9 μg/L respectively, statistically higher after 6 days than after 6 and 48 hours in all stages (p-values $\leq$0.05), and after 48 h than after 6h in stages 3 and 4 (p-values <0.05) (Supplementary Tables C and F).

Online turbidity measurements showed similar values in the 3 loops through the biofilm growth phase, average ranging from 0.049 to 0.051 NTU in the 3 loops. However, an increase in turbidity response when the water supply was restarted was observed after all the 3 IWS times (Fig. 4). Peaks in turbidity response were detected immediately after restarting the water supply, which can be associated to a loss of material from the pipe walls when hydraulic forces increase. Observed turbidity responses can be seen to cycle during each stage in response to imposed flow changes. With turbidity monitors situated at the end of the pipe loop (prior to a tank and then pump), the initial response is a well-defined peak and decay and represents material release and transport from the full pipe length. This is most clearly observed during stage 0 on start-up when velocities are least and is a well reported response observed during flushing events and anticipated based on PODDS modelling (Sharpe *et al.*, 2010; Husband *et al.*, 2016). As the water circulates around the pipe loop, the turbulent flows (for 79 mm pipe, transition to a turbulent regime when Re > 4000 occurs around 0.06 m/s or 0.3 L/s) facilitate effective mixing in the pipe and is sufficient to cause complete mixing within the tank, allowing the water and mobilised material to become well mixed. This can be observed with turbidity peaks decreasing and values flattening after 3 cycles, allowing comparison of overall responses between IWS events and imposed conditions.

Turbidity levels after 3 cycles of water turnover of each stage (**Supplementary Table B**) reported similar values for stages 0 and 1, while in stages 2, 3 and 4 when flows were increased higher turbidity levels were observed for all IWS periods. Statistical analysis (**Supplementary Tables G** and **H**) indicated that, in stages 0 and 1, turbidity values were significant lower after 6 hours than after 48 hours and 6 days of IWS (p-values $\leq$0.05), but there were not significant differences between 48 hours and 6 days (p-value >0.05). In stages 2 and 3, statistical differences between the 3 IWS times were observed (p-values $\leq$0.05), presenting after 6 hours the lowest values, followed by 6 days and then by 48 hours. In stage 4, turbidity levels after 6 hours of IWS were statistically lower (p-values $\leq$0.05), than after 48 hours and 6 days. However, turbidity response did not show statistical differences between 48 h and 6 days in this stage (p-value >0.05).

3.3. Flow cytometer counts

Results from FCM with TCC and ICC of planktonic cells during the growth phase and at each stage of the water supply restarting after different IWS times are shown in Fig. 5. Overall, TCC increases were observed in all stages after the 3 IWS periods than during the growth phase, except during stage 0. Highest TCC results are observed during stage 1 with decreasing counts during subsequent stages. When the different IWS times were compared (**Supplementary Tables I** and **J**), higher significant values of TTC and ICC in stages 1, 2, 3 and 4 were obtained after 6 days of IWS than after 6 hours and 48 hours (p-values $\leq$0.05). No significant changes were observed between 6 hours and 48 hours for TTC and ICC (p-value >0.05).

3.4. Microbial community structure

3.4.1. Alpha diversity

Chao 1, Simpson and Shannon indices were used to estimate the richness, dominance and diversity, respectively. **Supplementary Figure C** shows the results for these diversity indices at genus level for bacteria and fungi in all biofilm and water samples.

Statistical analysis (**Supplementary Tables K-N**) did not show significant differences in any indices for bacteria biofilm communities in post-IWS and post-restarting samples between the different IWS times

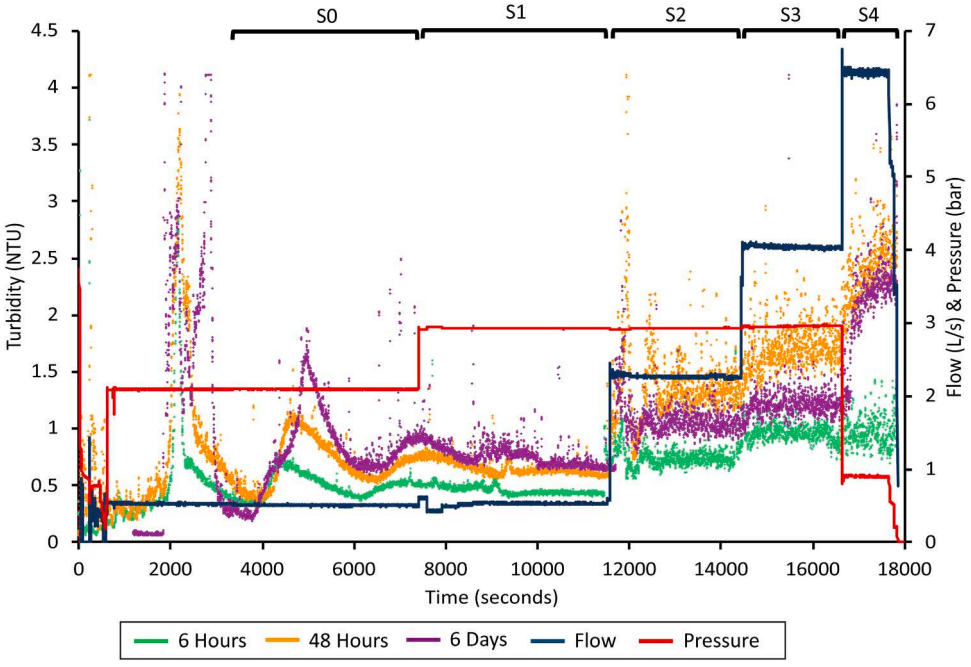

Fig. 4. Turbidity response, flow rate and pressure profile during the water supply restarting by stages after different IWS periods. S0 = Stage 0, S1 = Stage 1, S2 = Stage 2; S3 = Stage 3; S4 = Stage 4.

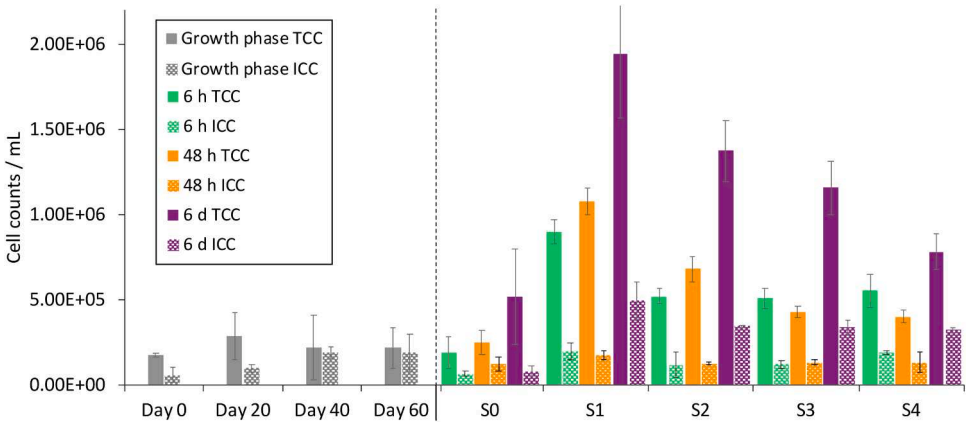

Fig. 5. Total cell counts (TCC) and intact cell counts (ICC) of planktonic cells in the bulk water during the growth phase and in each stage (S) after different IWS periods. All values represent an average of three water replicates ± standard deviation.

(p-values >0.05). For fungi biofilm communities, there were not significant differences between the 3 IWS times in samples post-IWS (p-value >0.05). Post-restarting the water supply, Chao 1 for fungi in biofilm samples did not show significant differences between IWS times, but Simpson was significant higher after 48 hours followed by 6 hours and 6 days (p-values ≤0.05). Consequently, Shannon index presented significant lower values after 48 hours than after 6 hours or 6 days (p-values ≤0.05).

Planktonic bacterial communities were not affected by IWS events, and no significant differences were observed in any index between water samples from the 3 IWS times at all the different stages of the water supply restarting (p-values >0.05). Planktonic fungal communities did not show significant differences for Chao 1 at any stage of water supply restarting between the different IWS times (p-value >0.05). Simpson and Shannon indices did not show significant differences in stages 0, 3 and 4 (p-value >0.05) during the water supply restarting, but significant changes were observed for these indices in stages 1 and 2. Overall, fungal communities after 6 hours presented lower significant values for Simpson, and consequently higher significant Shannon values (p-values ≤0.05) than fungal communities after 48 hours or 6 days.

3.4.2. *Beta diversity*

nMDS plots with the resemblance of bacteria and fungi communities at genus level in biofilm and water samples after the different IWS times are shown in Fig. 6. For biofilm samples, no clear separation of the bacterial and fungal communities was observed. The ANOSIM analysis (**Supplementary Table O**) confirmed that no significant differences were found for bacteria between 60-day old biofilms and after 6 hours of IWS. However, the bacterial community structure significantly changed after 48 hours and after 6 days of IWS. In addition, bacterial community structure showed significant differences between the different IWS times. For fungi in biofilm, ANOSIM (**Supplementary Table O**) showed significant differences between 60-day old samples and after 48 hours and 6 days of IWS. No significant differences were found between 60-day old biofilm and after 6 hours, as well as when biofilm fungal communities were compared between the different IWS times.

Regarding planktonic communities, samples clustered at bacterial genus level for the 3 IWS times analysed. The ANOSIM analysis (**Supplementary Table O**) confirmed that water samples from day 60 had a significant different bacterial community structure when compared with samples after all IWS times. Significant differences were also found when bacterial communities when compared after the 3 IWS times. For planktonic fungal communities no clear separation between samples was observed in the nMDS. ANOSIM confirmed the absence of significant differences between fungal communities of day 60 and after all IWS times, as well as between planktonic fungal communities after 48 hours compared to 6 hours 6 days. However, significant changes were observed for fungal planktonic samples after 6 hours and 6 days of IWS.

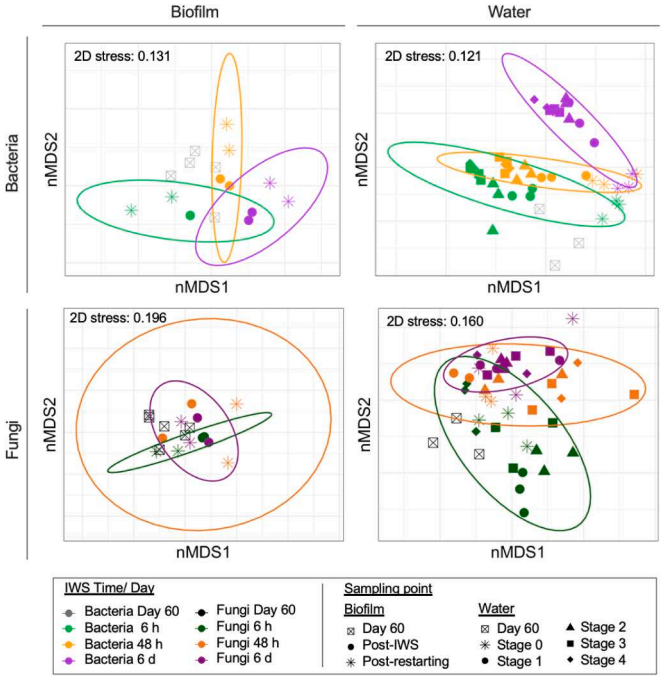

Fig. 6. Two-dimensional diagrams of the non-multidimensional scaling (nMDS) analysis based on Bray–Curtis similarities of the relative abundance of bacteria and fungi at 97% cut off in bulk water and biofilm samples from the different sampling points: day 60, post-IWS events and after restarting the water supply for biofilm samples; day 60 and each stage of water supply restarting for water samples. All replicates per sampling point are represented. Symbols are based the day/stage of sampling and different colours represent the different IWS times applied in this study.

3.5. *Microbial community composition*

3.5.1. *Taxonomic analysis of biofilm communities*

Differences in bacterial and fungal community composition at genus level were observed between 60-days old biofilm samples post-IWS and biofilm samples post-restarting the water supply after the different IWS times (Fig. 7 **A and B**).

Fig. 7 **A** shows the taxonomic analysis of bacteria in all biofilm samples. Overall, 60-days old biofilm samples in the 3 loops were dominated by *Phreatobacter* (33.5%), a not defined (NS) taxa belonging

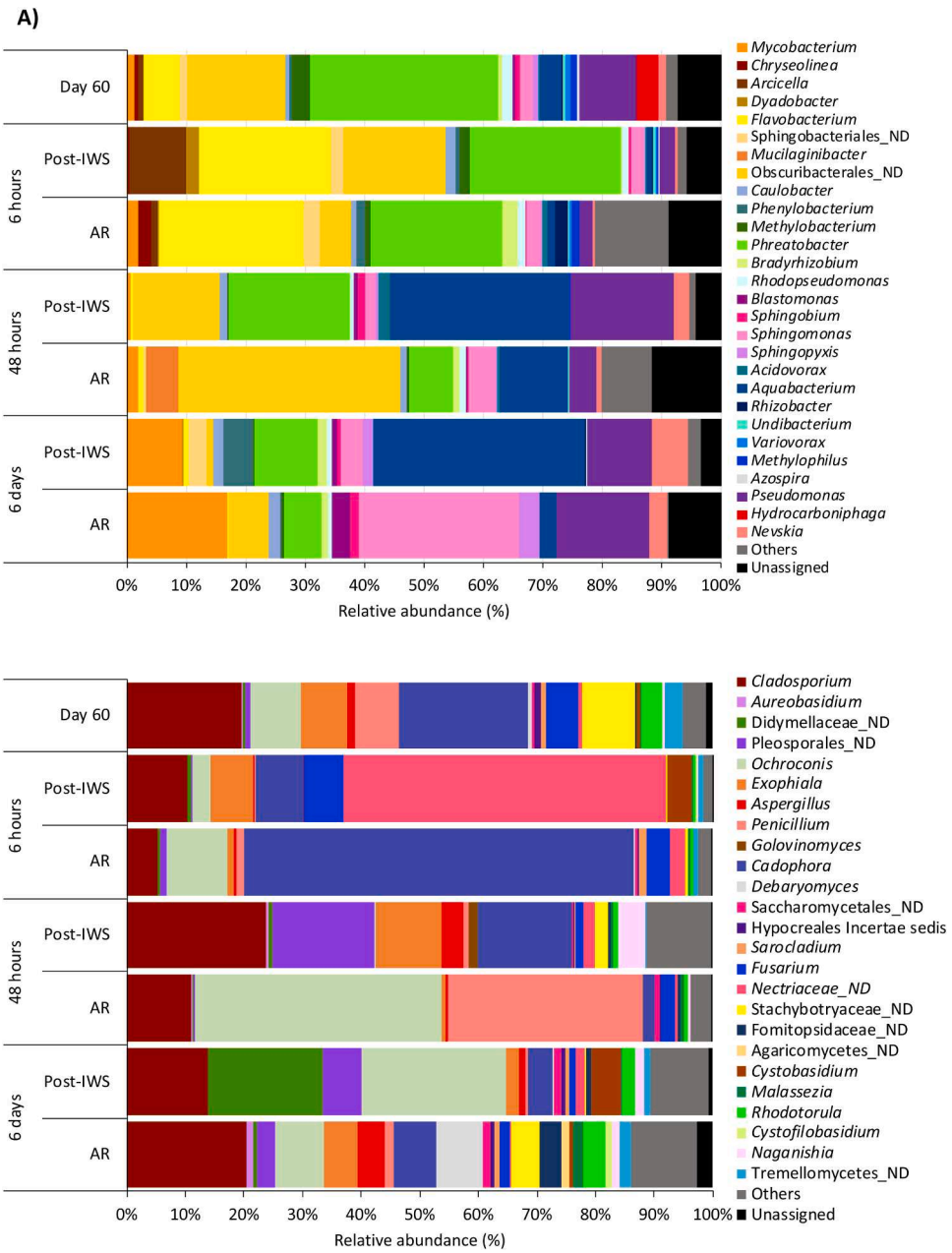

Fig. 7. Relative abundance of bacteria (A) and fungi (B) at genus level (>1% of the total sequences) of biofilm samples of day 60, post-IWS and after restarting the water supply (AR) after 6 hours, 48 hours and 6 days of IWS. The average of 2 biological replicates are represented. For day 60, R1 and R2 are the average of relative abundances from samples from the 3 loops (n = 3) since they were running at the same conditions. Remaining genera were combined in category "Others". Category "Unassigned" corresponds to unidentified OTUs and "ND" indicates not defined at that level.

to the order Obscuribacterales (17.5%) and *Pseudomonas* (10.0%). After 6 hours of IWS (both post-IWS and post-restarting) biofilm samples showed similar bacterial profiles to those of day 60: *Phreatobacter* (22.8% and 18.3%), *Flavobacterium* (19.9% and 20.1%), and a not defined (ND) taxa within the order Obscuribacterales (15.4% and 4.6%) presented high relative abundances. After 48 hours of IWS biofilm bacterial community composition changed when compared to day 60 or after 6 hours of IWS. In samples post-IWS, *Aquabacterium* (30.1%) dominated the community together with *Phreatobacter* (20.4%), *Pseudomonas* (17.5%) and Obscuribacterales ND (14.6%). Post-restarting the water supply after 48 hours of IWS biofilm communities showed that Obscuribacterales ND (37.3%) became the most abundant taxa, followed by *Aquabacterium* (15.5%) and *Phreatobacter* (7.3%). After 6 days of IWS, samples post-IWS showed that *Aquabacterium* (35.7%) was the most abundant genus together with *Pseudomonas* (11.0%), *Phreatobacter* (10.7%) and *Mycobacterium* (9.4%). In samples post-restarting the water supply after 6 days of IWS *Sphingomonas* (26.8%) was the most abundant genus, together with *Mycobacterium* (16.9%) and *Pseudomonas* (15.5%).

Fig. 7 **B** shows the taxonomic analysis of fungi in biofilm samples. In 60-days old biofilms the most abundant genera were *Cadophora* (22.2%) and *Cladosporium* (19.7%), followed by *Ochroconis* (8.5%), *Exophiala* (7.9%) and *Penicillium* (7.4%). After 6 hours of IWS, in samples post-IWS Nectriaceae ND was the dominant taxa (60.7%), followed by other taxonomic groups with lower relative abundance such as *Cladosporium* (11.5%). After restarting the water supply, after 6 hours the relative abundance of *Cadophora* (66.5%) increased becoming predominant in the community, followed by *Ochroconis* (10.5%). After 48 hours IWS and post-IWS, the fungal community was dominated by *Cladosporium* (23.6%), *Cadophora* (18.9%), Pleosporales ND (17.4%) and *Cadophora* (15.9%). Post-restarting the water supply after 48 hours of IWS, the fungal community was mainly dominated by *Ochroconis* (46.4%) and *Penicillium* (36.5%). After 6 days of IWS, in biofilms post-IWS the most abundant genera were again *Ochroconis* (24.7%), *Cladosporium* (13.8%) and Didymellaceae ND (19.6%). Post-restarting the water supply after 6 days *Cladosporium* (20.4%) became the most abundant genera, and other genera such as *Ochroconis* (8.3%) or *Debaryomyces* (8.0%) presented

high relative abundances.

3.5.2. Taxonomic analysis of planktonic communities

Differences in bacterial and fungal composition at genus level were observed in planktonic communities when the water supply was restarted after different IWS times (Fig. 8 **A and B**).

Taxonomic analysis of bacteria in water samples (Fig. 8 **A**) showed that on day 60 of the growth phase several genera such a *Reyranella, Phreatobacter, Nevskia, Sphingomonas, Methylobacterium* or *Cupriavidus,* were present with average relative abundances between 12.5% and 6.9%.

After 6 hours and 48 hours of IWS, bacterial planktonic communities followed a similar pattern of change through the stages of the water supply restarting. In stage 0, *Sphingomonas* (36.6 – 40.8%) and *Methylobacterium* (24.6 – 26.1%) were the most abundant genera. However, their relative abundance in samples from stage 1 to 4 decreased, whilst *Phreatobacter* became dominant (45.5 – 70.2%). A notable increase in the relative abundance of *Mycobacterium* (6.3 –11.6%) and the taxa belonging to the order Obscuribacterales (6.3 – 11.3%) was observed in stages 2, 3 and 4 after 48 hours of IWS. Similar results were observed after 6 days of IWS in stage 0, with high relative abundance of *Sphingomonas* (37.7%) and *Methylobacterium* (23.1%) in water samples. From stages 1 to 4 *Sphingomonas* (34.1 – 47.6%) continued to be the most abundant genus, followed by *Nevskia* (6.3 – 25.1%) and *Phreatobacter* (13.3 – 21.8%).

Fungal planktonic composition (Fig. 8 **B**) did not show significant changes after the water supply was restarted for the different IWS times. On day 60, the most abundant planktonic fungi in samples from the 3 loops were *Cadophora* (54.8%), Sordariomycetes ND (19.3%) and *Exophiala* (17.0%). After the 3 IWS, the taxonomic profile of planktonic fungi was similar: with high relative abundance of *Cadophora* (9.7 - 66.7%) *Exophiala* (5.1 - 31.6%) and *Cladosporium* (4.1 - 23.9%).

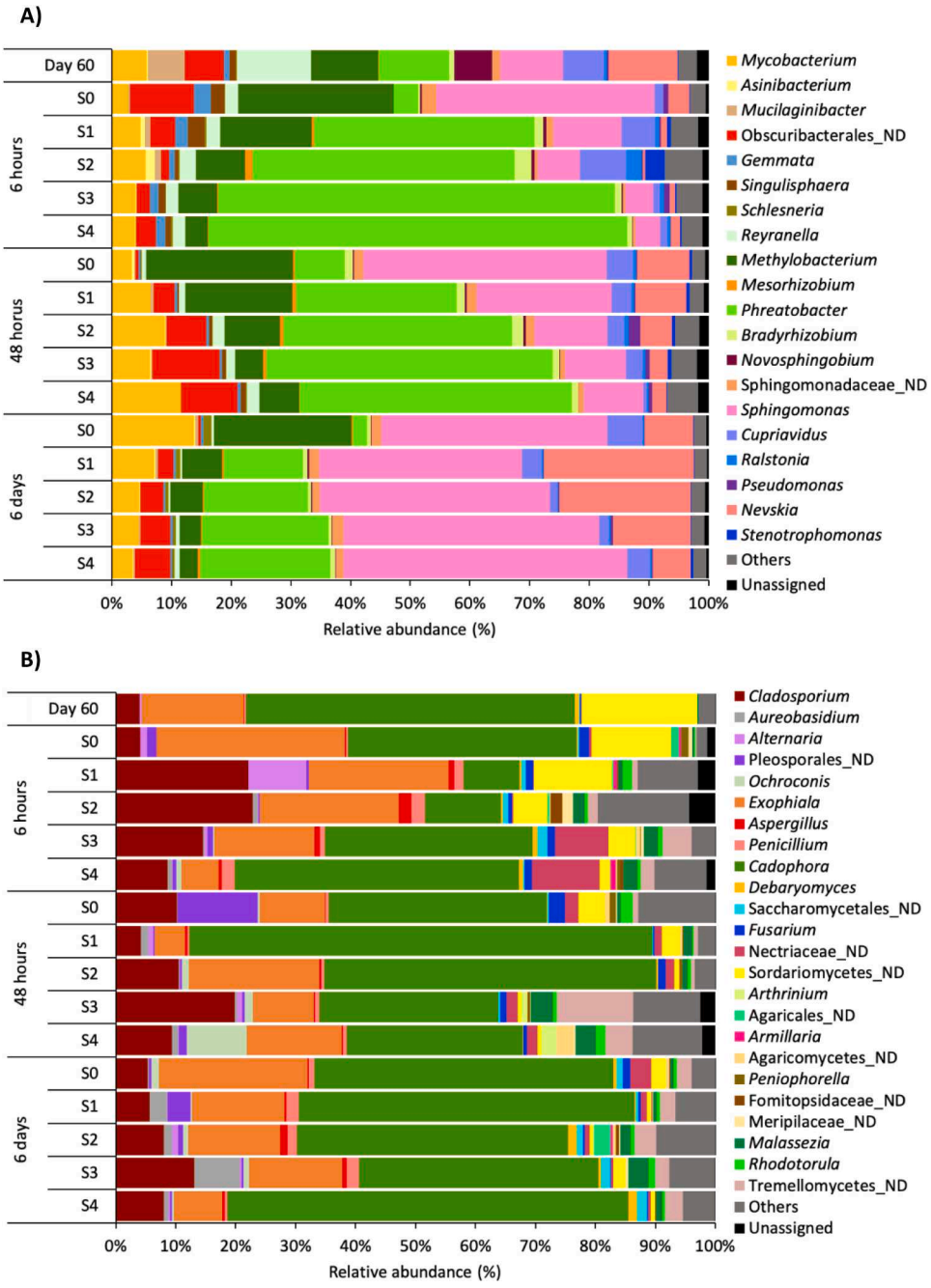

Fig. 8. Relative abundance of bacteria (A) and fungi (B) at genus level (>1% of the total sequences) of water samples from day 60 and each stage of water supply restarting after 6 hours, 48 hours and 6 days of IWS. The average of 3 biological replicates per sampling point are represented. For day 60, each replicate was taken from each loop since they ran under the same conditions during the 60 days. Remaining genera were combined in category "Others". Category "Unassigned" corresponds to unidentified OTUs and "ND" indicates 6 not defined at that level.

4. Discussion

This research investigated the unexplored impact of IWS on the combined hydrological, microbiological and physico-chemical characteristics of distributed drinking water with the aim of improving distribution system management. The study shows a number of findings that highlight considerations for future IWS operational practices. Primary amongst these are that microbial structural and compositional changes in biofilms occurred during IWS events, and those when combined with hydraulic changes impacted water quality. An experimental facility simulating DWDS was operated to understand the impact of IWS on biofilms, based on analysing changes within bulk water after imposing mobilising forces. A significant increase in turbidity and inorganic (Fe and Mn) concentrations were observed after recommissioning in all 3 IWS events. Under typical conditions mobilisation of biofilm and associated material occurs as shear forces increase with no response until the typical, or conditioned, state is exceeded (Chaves Simões and Simões, 2013; Douterelo et al., 2013; Husband, 2016; Husband et al., 2008; Sharpe, 2010). This is easily understood as no turbidity response was seen on a daily basis during the 60 days of biofilm growth phase, whereas a significant turbidity response was clearly linked to pipe wall material mobilisation on restart. The restarting flow created forces that eroded material previously conditioned to the same imposed hydraulic shear stress forces from the pipe wall into the bulk flow and subsequent increases also resulted in further discolouration responses, supporting validated concepts of cohesive material attachment (Husband *et al.*, 2016). This result indicates that changes occurred within the biofilm during IWS, with overall response magnitudes indicating least impact from the shortest IWS event. Similar to these observations, (Tokajian and Hashwa (2003) found higher turbidity levels when flow was restarted in a small IWS system supplied twice a week, and Cerrato *et al.* (2006) observed higher acute Fe and Mn concentrations during IWS conditions compared to the chronic concentrations found during continuous supply in PVC and iron pipes. Previous studies have shown that under stagnation biofilms can develop to a greater extent since they are not under hydraulic constraints (Chaves Simões and Simões, 2013). Coelho *et al.* (2003) likewise observed that HPC was higher when the flow was restarted after IWS events in different DWDS operating from 10-12 hours per 48 hours to twice per week in different locations; and Kumpel and Nelson (2013) reported a greater presence of total coliforms and *E. coli* in a chlorinated DWDS system performing under IWS (Kumpel and Nelson, 2013). In this study, higher turbidity and inorganics concentration values were observed with increased IWS duration. However, this distinct discolouration response was not always supported by significant increases in cell counts or SUVA, demonstrating that cell counts should not be considered linked to turbidity and different mobilisation processes between biofilm cells and entrapped material may occur.

It is hypothesized that longer times under the new changed environmental condition (i.e., drained pipe) could result in greater biofilm breakdown and cell death that can then be eroded (along with entrapped inorganics) by the imposed hydraulic shear forces. Two conflicting observations however are presented by the TCC results. The first is that the highest TCC counts occur during the pressure change stage when no significant turbidity response was observed and the second is the decreasing TCC values observed during the subsequent stages. The former does correspond to an increase in $SUVA_{254}$ (Fig. 3) and reinforces the hypothesis that there is not a clear correlation between cell counts and turbidity, an aspect of water quality monitoring that requires further investigation. The latter may be a consequence of exposure to the fresh water with higher incoming disinfectant concentrations, which could also help explain the reduction in cell viability observed (Nescerecka *et al.*, 2014). Based on the assumption that the established biofilms breakdown (i.e., changed their structure and compactness) during drain-down is proportional to time (for the IWS durations examined here) a greater cell release and higher discolouration response would be expected after 6 days than after 48 hours of IWS. Turbidity results from Stage 2 and 3, supported this for 6 to 48 hours but not for 48 hours to 144 hours suggesting material attached to the pipe walls after 144 hours of IWS was more difficult to remove. This increased resistance of particulate material release from biofilms after longer IWS periods could be explained by the resumption of biofilm development suggested by TCC and ICC results strengthening cohesive properties or potential impact of a drying process. It has been demonstrated that more than 90% of the wet weight of biofilms is water, and thus the absence of water can change the biofilm compactness (Schmitt and Flemming, 1999). When the biofilm dries out, its internal structure can change and the forces between the cells and the surface are strengthened. Cerrato *et al.* (2006) observed that biofilm morphology was affected, changing its texture and colour when plastic pipes were dried in a system operating under IWS. This drying process can make the biofilm more compact and increases the difficulty for water to penetrate (Melo, 2005), increasing shear strength properties and consequently higher shear stresses are required to erode them from the pipe. This research highlights that many interacting factors are at work and further investigations are needed to understand the combined action of hydraulic forces and pipe microbiome properties.

Of significance is that the large step increase in pressure (and not flow) during stage 1, from 2.0 to 2.9 bar, did not result in a measured turbidity response. It is noted that the pipe loop control algorithms were designed to prevent dynamic hydraulic conditions and avoid the shock loads associated with hydraulic transients, the change in pressure for each loop took between 6 and 10 seconds (far greater than the pressure wave transit time for the system, around 2 seconds), with no evidence of dynamic pressure transients. Therefore, this research demonstrates that operators are not constrained by water quality risks if undertaking controlled increases in network pressure. Furthermore, the findings highlighted material sensitivity to pipes recommissioning following a single IWS event, such as one-off maintenance activity in networks that are normally run under continuous supply. It is therefore likely that networks operated under regular IWS would develop biofilms adapted to these cycling conditions and not demonstrate the sensitivity highlighted here, although previous work (Nescerecka *et al.*, 2014) has indicated otherwise. Further experiments running repeating IWS events could investigate both community adaptions and subsequent water quality impact and test strategies to minimise responses thereby informing future start-up procedures.

Moreover, results shown here are based on 60-days old biofilm, enough time to detect and monitor discolouration events and changes in microbial communities according to previous studies performed in the same experimental facility (Douterelo, Sharpe and Boxall, 2014; Husband *et al.*, 2016; Fish and Boxall, 2018). However, disruptions in the water supply can happen periodically or for the first time after years of the pipe installation, thus affecting biofilms at different maturation stages. Future investigations on the impact of biofilms in DWDS under IWS, should consider conducting experiments in real networks to better understand microbial risks associated to different periods of IWS. Factors such as pipe age and/or material, type of disinfection, hydraulic regimes, and water source can influence biofilm structure and composition (Douterelo, Sharpe and Boxall, 2013; Gomez-Alvarez *et al.*, 2015; Ren *et al.*, 2015), hence can affect final water quality when biofilms are mobilised into the bulk water due to IWS.

The characteristics of NOM after different IWS times were analysed by determining TOC, DOC and SUVA. These parameters provide measurements of the concentration and type of NOM present in water, which can react with disinfectants and lead to the formation of harmful disinfection-by-products (DBPs) (Matilainen *et al.*, 2011). Specifically, the hydrophobic humic fraction of NOM is more reactive with oxidants like chlorine, favouring the formation of DBPs (Reckhow *et al.*, 1990; Weishaar *et al.*, 2003). TOC and DOC results showed typical concentrations previously reported for drinking water (Douterelo *et al.*, 2014; Li *et al.*, 2018) and no significant differences were observed in any stage

after restarting the water supply for the different IWS times. However, the highest SUVA values measured after 6 hours IWS when compared to 48 hours and 144 hours, indicated a higher proportion of aromatic organic compounds, which can potentially react with chlorine to form DBPs. The change in environment conditions (loss of water/-drying/oxygen levels etc.) would also impact material transport processes and hence organic material uptake and/or increase cell lysis. The lack of an evident increased risk for DBPs formation after 48 h or 144 hours IWS, when compared to 6 hours may be linked to insufficient time for the existing biofilm to adapt to the new conditions, limiting organic uptake. This change in environmental conditions will most likely have a higher impact on the outer (more exposed) layers of biofilms and could explain the higher SUVA observed after the initial increase in flow eroding these effected layers in the 6-hour IWS test. This is relevant as high concentrations of chlorine are commonly used post interventions in the pipe network as a protective measure against contamination that could further exacerbate DBP formation. Further research could investigate this phenomenon, to confirm results and then investigate how quickly increased NOM release occurs after draining down to indicate if a reduced operational window exists to minimise impact.

Microbial communities in IWS have undergone limited exploration using molecular methods (Melo, 2005). This knowledge gap has been addressed here, by analysing mixed microorganisms (bacteria and fungi) in both planktonic and biofilm communities. The compositional changes observed in the microbial communities indicates that environmental changes during IWS events affected biofilm communities. Sequencing results showed that some of bacterial phyla in biofilms enhanced by IWS belonged to alpha-, beta-, and gamma-Proteobacteria phylum, such as *Sphingomonas, Aquabacterium* and *Pseudomonas* respectively, as well as Gram positive bacteria, like *Mycobacterium*, and non-photosynthetic Cyanobacteria (Obscuribacterales ND), which have the ability to form endospores and be more resistant to stress conditions like desiccation (Sandle, 2016). Regarding planktonic bacterial communities, genera like Obscuribacteriales ND, *Methylobacterium, Nevskia, Phreatobacter, Mycobacterium* and *Sphingomonas* increased their relative abundance after IWS periods and when the water supply was restarted after IWS time (specially *Phreatobacter, Mycobacterium* and *Sphingomonas*). Similar communities were identified in both water and biofilm samples from an unchlorinated and chlorinated IWS system from Beirut (Lebanon) and Cali (Colombia), respectively (Tokajian *et al.*, 2005; Montoya-Pachongo *et al.*, 2018). This suggests that these genera could be specifically favoured by IWS events.

In terms of health and water quality, Ercumen *et al.*, (2015) showed that IWS can lead to waterborne illness and diarrheal diseases through contamination in the pipelines, limiting the availability of safe water during intermittencies. Our findings indicated that IWS times, especially after longer periods of IWS, favour the presence of potential pathogenic bacteria such as *Pseudomonas, Mycobacterium* and *Sphingomonas*, which presented high relative abundances compared to the 60-day old biofilms when the water supply was restarted. *Pseudomonas* and *Mycobacterium* genera include species of opportunistic pathogens and its presence in drinking water have been related to nosocomial infections (Vaerewijck *et al.*, 2005; Hilborn *et al.*, 2006; Costa *et al.*, 2015; Liu *et al.*, 2016; WHO, 2017). *Sphingomonas* is well recognized for its ability for extracellular polymeric substances (EPS) formation, thus contributing to biofilm development, and some species are involved in the infection of immunocompromised patients in hospitals (Johnsen *et al.*, 2000; Zhang *et al.*, 2012; Steinberg and Burd, 2015). The increase of the relative abundance of these genera in biofilms after longer IWS times could have important implications for public health if biofilm is mobilised into the bulk water. Similarly, the presence of *Methylobacterium* in water samples from this study indicates a potential risks since species belonging to this genus can cause opportunistic infections in immunocompromised patients in hospitals due to its ability to form biofilms and to exhibit tolerance to disinfecting agents, high temperatures and to drying (Kovaleva *et al.*, 2014).

Studies regarding fungi in DWDS have been gaining attention due to their biofilm formation ability and their interaction with bacteria in fungal-bacterial biofilms (Afonso *et al.* 2020), however, there are no IWS studies that include the analysis of fungal communities. The fungal analysis showed that these microorganisms, mainly represented by the Ascomycota phylum, were less affected by IWS events than bacterial communities, an indication of the key role fungal communities may play in biofilm stability. However, the dominance of certain fungi such as *Cadophora* increased with short IWS times. *Cadophora* is a phytopathogenic fungus, but it has not been related to human health problems (Travadon *et al.*, 2015). Other fungi such as *Ochroconis* and *Penicillium*, dominant in biofilm samples when the water supply was restarted, can contain pathogenic species associated to local human infections (Lyratzopoulos *et al.*, 2002; Novak Babič *et al.*, 2017). Therefore, it can be concluded that monitoring biofilm communities is proven essential since longer IWS can increase potential risks associated to pathogenic microorganisms.

These results provide a starting point for future research on the effects of supply disruption on the DWDS microbiome. More precise and efficient molecular methods, such as quantitative PCR (q-PCR), for the identification and quantification of specific microorganisms (Smith and Osborn, 2009; Nolan, Huggett and Sanchez, 2013), could help to accurately determine the presence of potential pathogens observed in this study trough metagenomics analysis. All this knowledge including infrastructure characteristics, turbidity thresholds and associated occurrence of potential pathogens could be used to establish an effective Microbial Risk Assessment Framework, to monitor and control the microbial hazards associated to IWS.

This novel research provides a number of findings that contribute to a better understanding of DWDS managed under IWS. The experiment was not replicated during this research because of the technical difficulties of repeating an experiment of this magnitude, in terms of water, energy, labour and economic expenditure. However, to the authors' knowledge, this is the first experiment under controlled conditions about different IWS times and their impact on biofilm mobilisation and water quality. Based on the findings from this research, the possible approaches that can be adopted to mitigate biofilm-associated risks caused by IWS include; routine pipe cleaning operations by for example unidirectional flushing (Slaats et al., 2002; Douterelo, Husband and Boxall, 2014) and air scouring (Pourcel and Duchesne, 2020), especially if IWS events are expected.

5. Conclusions

For the first-time this study shows how different IWS events impact distribution system microorganisms and biofilm mobilisation responses, and thus impact on water quality.

From this research several key findings were highlighted:

- Short IWS events (6 hours) resulted in higher concentrations of aromatic organic compounds, hence greater risk of potential DBP formation (in chlorinated systems).
- Pressure increases had no impact on network turbidity whilst flow increases promoted material release and discolouration risk for each subsequent increase.
- Turbidity changes do not reflect mobilisation of pipe wall total or intact cell counts.
- IWS events promoted changes in structure and taxonomic profiles of DWDS biofilm and planktonic communities, with: i) greatest compositional changes observed for longer IWS times, ii) fungal communities changed less than bacterial communities, iii) changes in planktonic communities observed after the different IWS can be associated to biofilm mobilisation and iv) relative abundance of potentially pathogenic and/or detrimental microorganisms favoured in biofilm samples after IWS with increasing incidence for longer IWS events. These include *Flavobacterium* after 6 hours of IWS, *Ochroconis*

and *Penicillium* after 48 hours of IWS, and *Mycobacterium, Sphingomonas, Ochroconis* and *Cladosporium* after 144 hours of IWS.

Author contributions

C. C. P., S. H., S. K. M., J. B and I. D. were involved in the design of the experiment. C. C. P., S.H. and I.D. carried out the experiment. C. C. P. performed the DNA extraction from samples and analysed the results. C. C. P. and V. S-C. were involved in the bioinformatic analysis. C. C. P., S. H., S. K. M., J. B, G. D. O. and I. D. contributed to the interpretation of results. C. C. P. wrote the manuscript and G. D. O., I. D. and C. C. P. were in charge of the adaptation of the manuscript for a journal publication. All authors participated in the corrections of the manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117372.

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