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Del Olmo, G., Husband, S., Sánchez Briones, C. et al. (4 more authors) (2020) The microbial ecology of a Mediterranean chlorinated drinking water distribution systems in the city of Valencia (Spain). Science of The Total Environment, 754. 142016. ISSN 0048-9697

https://doi.org/10.1016/j.scitotenv.2020.142016

The microbial ecology of a Mediterranean chlorinated drinking water distribution systems in the city of Valencia (Spain)

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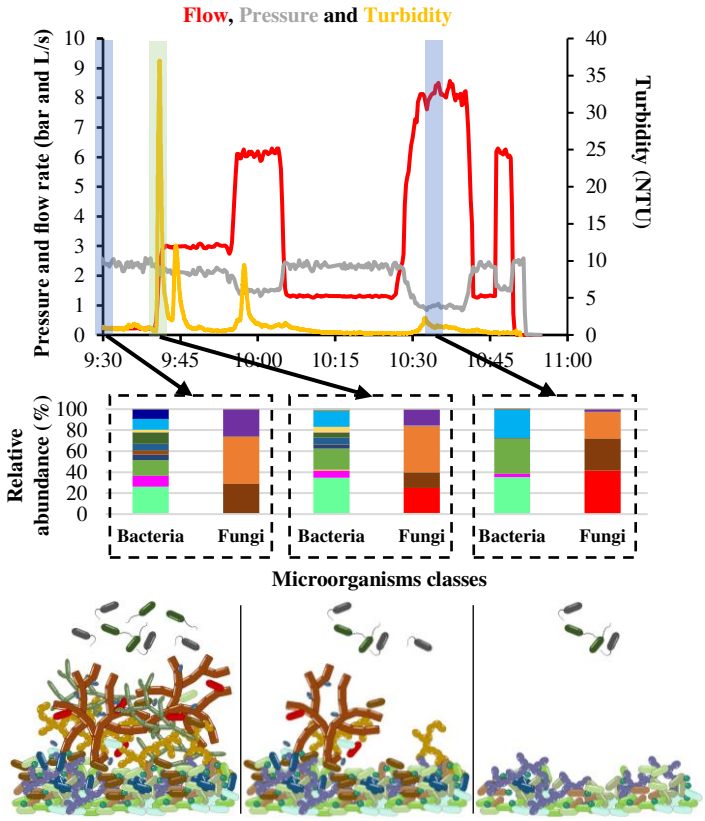
Flow, Pressure and Turbidity
Pressure and flow rate (bar and L/s)
Turbidity (NTU)
9:30
9:45
10:00
10:15
10:30
10:45
11:00
Relative abundance (%)
Bacteria
Fungi
Microorganisms classes

Highlights:

- Monitoring turbidity during flushing allows network performance analysis.
- Hydraulic strategies can be applied to control water quality issues from biofilms.
- Controlled flushing facilitated network and pipe biofilm community analysis.
- Core-community of microorganisms were present throughout the network.
- Bacteria showed more diversity with fungi more dominant and stable.

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Abstract

Drinking water distribution systems host extensive microbiomes with diverse biofilm communities regardless of treatment, disinfection, or operational practices. In Mediterranean countries higher temperatures can accelerate reactions and microbial growth that may increase aesthetic water quality issues, particularly where material deposits can develop as a result of net zero flows within looped urban networks. This study investigated the use of flow and turbidity monitoring to hydraulically manage mobilisation of pipe wall biofilms and associated material from the Mediterranean city of Valencia (Spain). Pipe sections of different properties were subjected to controlled incremental flushing with monitoring and sample collection for physico-chemical and DNA analysis with Illumina sequencing of bacterial and fungal communities. A core microbial community was detected throughout the network with microorganisms like *Pseudomonas*, *Aspergillus* or *Alternaria* increasing during flushing, indicating greater abundance in underlying and more consolidated material layers. Bacterial and fungal communities were found to be highly correlated, with bacteria more diverse and dynamic during flushing whilst fungi were more dominant and less variable between sampling sites. Results highlight that water quality management can be achieved through hydraulic strategies yet understanding community dynamics, including the fungal component, will be key to maintaining safe and ultimately beneficial microbiomes in drinking water distribution systems.

Keywords: biofilm, drinking water, pipe, turbidity, temperature.

1. INTRODUCTION

Drinking water distribution systems (DWDS) are engineered aquatic ecosystems naturally colonised and inhabited by microorganisms. The origin of microorganisms in DWDS is diverse, from treated water carry-through, *in situ* growth during distribution or in associated assets such as tanks and reservoirs, maintenance or repair operations and intrusion or contamination events (Gheisi *et al*., 2016; Medema *et al*., 2013). Most of the microbial biomass in DWDS is found attached to infrastructure as complex biofilms (Chan *et al*., 2019), composed by bacteria, archaea, fungi, viruses, and protists (Mathieu *et al*., 2019). Bacteria are the domain most studied, however understanding the need to study fungi is increasing due to associations that link its capacities to reduce the quality of supplied drinking water and its pathogenicity (Babič *et al*., 2017; Hageskal *et al*., 2009). Some fungal species have adapted to living in oligotrophic aquatic environments (E. G. Jones *et al*., 2014; Sonigo *et al*., 2011), and it has been shown they can form colonies with other organism like bacteria in DWDS biofilms (Douterelo *et al*., 2018, 2020; Douterelo, Jackson, *et al*., 2016).

Whilst the harsh oligotrophic conditions generally support a beneficial community that also promote a defensive barrier by excluding potentially opportunistic pathogens (Reuben *et al*., 2019), biofilms may become a source of technological problems for water companies. For example biofilms can promote corrosion, and in some circumstances communities may lead to the development of aesthetic changes to water odour, colour and taste (Batté *et al*., 2003; Simões & Simões, 2013). Even if delivered water is wholesome and safe to drink, service and quality is judged by consumers on the organoleptic properties and this can pose challenges to water companies. The importance given by the public to water aesthetics has been highlighted as critical for quality perception, service satisfaction and ultimately willingness to pay (De França Doria, 2010). Additionally, water microbiology can be affected by several factors, such as pipe material, nutrient loading, hydraulics, temperature, pH, and concentration of disinfectant (Chaves *et al*., 2020; Makris *et al*., 2014). As part of this challenge, monitoring and improved understanding of the impact of environmental conditions on the microbial communities within DWDS needs to be addressed to help inform maintenance strategies that can mitigate risks and promote a beneficial microbiome thereby safeguarding water quality and consumer confidence.

Extensive fieldwork, bench top trials and full scale laboratory pipe research have all highlighted particulate material continually present in the fluid phase accumulating on DWDS surfaces as layers with distinct shear strength characteristics (Choi & Morgenroth, 2003; Douterelo *et al*., 2013; Sunny *et al*., 2020). This DWDS phenomena is experienced worldwide, and a unifying feature facilitating wall attachment is the endemic presence of biofilms. Biofilm entrained material detachment from pipe walls is a primary cause of discolouration and the leading cause of consumer contacts regarding delivered water aesthetics (Husband *et al*., 2016). Due to the shear strength properties, in most cases the root cause is an increase in network hydraulics above a conditioned state, typically the peak daily demand. In many cases this may be attributable to water companies through planned activities, such as valving, re-zoning, or maintenance interventions. Other causes may include unplanned events such as bursts or third party connections, whilst some predictable scenarios may also precipitate mobilisation events, such as demand increases resulting from seasonal changes (e.g. tourist influx) or social events (e.g. sport or festivals). Changes in water chemistry (e.g. source changes or blending, changing disinfectant regimes) can also impact the established biofilm community, potentially leading to biofilm morphological changes with subsequent material erosion into the bulk flow. It is also considered likely rapid or excessive changes in temperature may play a role through community transitions, although this is yet to be established (Husband *et al*., 2016).

The development of layers with shear strength characteristics does facilitate pro-active maintenance as controlled flow increases can be used to safely remove accumulated material layers. Flow conditioning in trunk mains applies this concept to incrementally remove network material, mitigating risks and increasing resilience without any loss of water and at minimal cost (Husband & Boxall, 2016). The mobilisation of these layers does create short-term, but managed, low-level turbidity responses; however, flow conditioning has shown additional benefits including a reduction in material loading that reduces downstream asset deterioration and disinfection decay rates (Sunny *et al*., 2020). A consequence of the continual particulate loading in bulk flow, measurable as background turbidity, is that when flows approach quiescence, gravitational settling may occur. This is typically observed at dead-ends, as evidenced by flushing operations where short but high level turbidity responses are repeatedly witnessed (Blokker & Schaap, 2015). Harder to identify are tidal points in looped networks, where due to opposing hydraulic flows, pipe sections can have net zero flows. With material arriving from two directions in these tidal sections, significant and rapid deposits can develop. Crucially the location mid-network also increases risk impact and event likelihood as these

deposits are bounded by multiple consumers and sensitive to any localised hydraulic changes. Risks from hydraulic tidal points are common in residential networks where satisfying historic firefighting requirements meant highly interconnected systems. In addition to being easily disturbed, material deposits can support abnormal environmental conditions e.g. low disinfectant, low oxygen, high organic content, that when combined creates the potential for niche microbial communities and resulting organoleptic issues (Douterelo *et al*., 2020).

Changes in water organoleptic characteristics during distribution have also been associated to compounds leaching from plastic pipes, formation of disinfection by-products, metals originating from corroded pipes and biofilm metabolites (Zhou *et al*., 2017). In Mediterranean regions, distributed water temperatures are higher than the average range in Europe of between 3 and 25°C (Niquette *et al*., 2001; Preciado *et al*., 2019). Increased water temperatures can increase microbial growth and activity, potentially aggravating odour and taste problems whilst also selecting for different community compositions (Prest *et al*., 2016). In addition to microbial causes, another factor that has received wide attention with regards to aesthetic water quality is water hardness, the amount of dissolved calcium and magnesium in the water. Resulting scaling and residues are not dangerous but unsightly, causing wise-spread misconceptions about risks associated to drinking water (De França Doria, 2010).

Valencia on the Mediterranean coast is Spain's third largest metropolitan area. Despite significant investment in treatment technologies and continual testing to assure the highest water quality, there are repeating taste and odour reports from the supplied drinking water across the city. To help address this, a greater understanding of the microbial processes occurring within this higher temperature DWDS is required. A first step is to characterise the different communities present in the biofilm. This study proposes to achieve this though mobilisation of pipe-wall biofilms using controlled flushing operations and sample collection. By integrating with hydraulic and turbidity monitoring, discolouration responses can also be used to assess for material deposition behaviour. This could indicate possible zones with abnormal communities supporting niche conditions, whilst also investigating the possibility of hydraulic control strategies to mitigate future issues. By undertaking studies across a number of sites, this study could investigate potential differences due to pipe materials and hydraulic regimes. Findings can be used to understand the significance of temperature on the microbial ecology, that in turn can help inform future studies and operational strategies to manage biofilms in higher temperature DWDS.

2. MATERIALS AND METHODS

2.1. Site details and flushing plan

Six flushing locations as part of 3 daily campaigns were selected from within low-rise residential zones of Valencia for flushing analysis and to collect samples for microbial characterisation during November 2019. With reported organoleptic issues across the network, the sampled locations were carefully chosen following the recommendations of the supporting water supplier Aguas de Valencia and represented a range of pipe materials. All locations were supplied from the same treatment works to the south west of the city with flows generally in a north/north-east direction (**Figure 1.A**). The mean water temperatures recorded in the network during the cooler months (November-April) were in a range from 13 to 19 ºC, whilst in warmer months (May-October) temperatures went from 19 to 28 ºC (Aguas de Valencia supplied data). Chlorine is added to the drinking water at the outlet of the water treatment plant as hypochlorite at 1 ppm, and no more disinfection products are added in the rest of the network.

To study potential community differences in biofilms layers resulting from different shear strength characteristics, a standpipe with incorporated flow, pressure and turbidity monitoring was used to control applied flushing flows (www.langhamcontrols.com) with a secondary dual-validating Nephnet turbidity monitor for data confidence (www.ATIuk.com). Flow was controlled using a gate valve adapted via Greiner fittings (www.greiner.it) at the hydrant discharge point. This provides much greater control than the standard valves present within the hydrant, allows pressure measurement from within the standpipe and also prevents de-pressurisation within the standpipe that can create air bubbles known to impact flow and turbidity readings. To prevent discharge onto the street, hoses were used to divert the flushed water direct to local drains. Sample collection for microbial and chemical analysis was via tapping's in the standpipe. The experimental set-up is shown in **Figure 1.A** inset photograph.

Campaign 1 involved two hydraulically linked sites (flushing locations 1 and 2; sites are numbered in chronological order of testing), with the former downstream in the network. Campaign 2 involved 3 flushing locations (Sites 3, 4 and 5). Sites 4 and 5 were on the same 200 mm supply main with the latter an additional 750 m downstream after a number of consumer take-offs. Site 3 was part of a smaller sub-loop connected to the same 200 mm main and 250 m upstream of Site 4. Site 3 was unique compared to all the other locations in that it

only supplied a handful of properties so had considerably lower daily flow profiles. Campaign 3 was a single site (Site 6), hydraulically upstream of Campaign 2. The trial here planned to also investigate hydraulic resilience imparted by flushing operations as part of validating future hydraulic maintenance strategies.

With all flushing locations part of complex looped residential networks and no nearby flow monitors, it was not possible to accurately define flow profiles, including identifying possible flow reversals or tidal points in the test sections. In all cases (except Sites 4 and 5), valve operations were conducted in an effort to try and prevent potential abnormal flow directions being created in response to the additional demands created during flushing, whilst also trying to isolate distinct sections of main for investigation. Primary pipe lengths and construction material anticipated to be impacted by the flushing operations are shown in **Figure 1.B**. With the large number of inter-connections within these urban networks, these properties should only be considered as indicative, and for Site 6 the multiple possible links meant that no defining pipe property could be identified.

Due to the complex physical layout and limited hydraulic knowledge, the planning of pre-defined flow rates and flushing times was not practical. By monitoring turbidity responses at the flushing point, control however can be applied with inherent safety factors (Husband & Boxall, 2016). When an excess shear stress mobilising force is applied and maintained, the downstream turbidity response increases as material is mobilised. The peak turbidity corresponds to the travel time, defined by the flow velocity and the start of the pipe length effected, after which exponential turbidity decay is then observed. This however assumes the pipe under investigation has reasonably constant physical and hydraulic properties. In pipes with changing properties, including material, diameter or flow profiles, the response is more complex. An inherent safety feature for operational application is that the rising turbidity response can be curtailed at any point by reducing applied flow to previous conditioned values, i.e. removing the mobilising forces. Using the monitored standpipe, flushing impact was planned to be managed by a simple response feedback to flow increases. No turbidity response meant conditioning hydraulics had not been exceeded, so after a short delay (depending on anticipated turn-over time for pipe section being investigated), flow was increased again. The first response recorded indicated when conditioned hydraulics had been exceeded and this was maintained until response decay was observed. Further increases, or trial termination, could then be applied depending on time constraints. This approach however cannot account for

network deposition zones (e.g. tidal-points), that can create rapid and high magnitude responses. However, any response observed is evidence of risk mitigation as material is being removed from the network that could otherwise impact consumers or provide conditions for unfavourable microbial proliferation.

2.2. Standpipe connection and sample collection

Bulk water samples were taken from the hydrant standpipe during flushing operations for microbial and physico-chemical analysis. The standpipe was initially connected with the control valve shut but turbidity sample lines open to allow gradual filling. This prevents potential transients or rapid changes in flow when the hydrant valve is opened. Once connected a low flow was maintained (0.1-0.2 L/s) for a minimum of 15 minutes to allow system stabilisation prior to flushing commencement. For each campaign, an initial bulk water background sample was collected after stabilisation and prior to flushing. In-line with common practice, hydrants were initially opened prior to standpipe attachment to remove debris known to collect in hydrant connecting pipework with the potential to block monitoring sample lines (i.e. turbidity monitor). Without flow monitoring however this practice can also disturb test sections due to the imprecise control of hydrant valves. With flushing discharge direct to the local drainage system, a single combined sample was collected via tapping's in the standpipe that only had a low discharge rate (0.1-0.2 L/s). For scientific rigour, a minimum volume of 5 L was required to facilitate triplicate 1 L samples required per DNA extraction (conducted at The University of Sheffield after filtering at EMIVASA) plus physico-chemical analysis. This resulted in sample collection requiring typically in excess of 1 minute to complete. A consequence of ensuring triplicate DNA analysis was that only a limited number of samples could be filtered with available resources within hours of collection.

The water physio-chemical parameters were analysed in EMIVASA laboratories according to standard protocols. Metals were determined by inductively coupled plasma mass spectrometry, trihalomethanes (THM) compounds by gas chromatography-mass spectrometry, total organic carbon (TOC) by oxidation and infrared measurement, and organic compounds by colorimetric methods standardised by the company

2.3. DNA extraction and sequencing

Litre samples of bulk water were filtered via a sterile filtration unit using 0.22 µm pore-size nitrocellulose membrane filters (MCE Membrane MF-MILLIPORE, UK), and DNA was

extracted from that filter using a modified protocol described by Douterelo *et al*. (2013). Filters were placed into a 2 mL Eppendorf tubes with 740 µL of SET lysis buffer (40 mM EDTA, 50 mM Tris–HCl, pH 9, 0.75 M sucrose) and crushed with sterilized pestles. 90 µl of lysozyme (9 mg/ml) was added and the Eppendorf tubes were incubated at 37 °C for 30 min with shaking (100 rpm) in a Hybaid hybridisation oven (Thermo Scientific, UK). Afterwards, 90 µl of sodium dodecyl sulphate (SDS) and 25 µL of proteinase K (20 mg/ml) were added and the tubes were incubated at 55 °C for 2 h with shaking. The supernatant was transferred to another 2 mL Eppendorf tube. Subsequently, 137 µL 5M NaCl and 115 µl CTAB/NaCl solution (100:41 mg/ml) were added to the tubes and they were incubated at 65 °C for 30 min with shaking. The supernatant was extracted twice with 838 µl of chloroform:isoamyl alcohol (24:1) (SIGMA, UK). Finally, DNA was precipitated with 815 µl of isopropanol, then washed twice in 1 ml of 70% ethanol, dried for 30 min and re-dissolved in 50 µL DEP-treated sterile water. Quantity and purity of the extracted DNA were assessed using NanoDrop ND-1000 spectrophotometer (Nanodrop, Wilmington, USA).

2.4. Analysis of sequencing

Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) by Illumina HiSeq technology with the paired-end protocol following the manufacturer's guidelines. The bacterial 16S rRNA gene using primers 28F and 519R spanning the V1 to V3 hypervariable regions and the fungal specific primers targeting the ITS1-2 regions were used to perform the analysis. Sequence data were processed by MR DNA (MR DNA, Shallowater, TX, USA). In summary, sequences were depleted of barcodes and primers, then sequences <150bp, with ambiguous base calls and with homopolymer runs exceeding 6bp were removed from further analysis. Sequences were denoised and Operational Taxonomic Units (OTUs) generated whilst chimeras were removed. OTUs were defined by clustering at 3% divergence (i.e. 97% similarity cut off). Finally, OTUs were taxonomically classified using BLASTn against a database derived from RDPII (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov). The taxonomic analysis of data was provided in tab-delimited text format and excel sheets.

Alpha diversity indices like dominance (indicating the uniformity of the community, it ranges from 0, when all taxa are equally present, to 1, when one taxon dominates the community completely), Shannon Index (indicating diversity, relating to the number of different OTUs taking into account their relative abundance) and Chao-1 (a measure of richness, based on

number of different OTUs, as more species are detected the higher the richness) of bacterial and fungal communities at genus level, were calculated with the software PAST version 4.0 (Clarke & Warwick, 2005). To understand the relation between different parameters and microbial genera, Spearman`s rank non-parametric correlations were calculated using SPSS Statistics 26 (IBM, USA), and the data included were the relative abundance of the 43 most abundant genera of bacteria and fungi (total genera = 86), bacterial and fungal alpha diversity of every sample, the flow and turbidity registered in every flushing step, and the material and flushing steps codified (0 = absence of the parameter in the sample, 1 = presence of the parameter in the sample).

To infer the bacterial and fungal associations in the different pipe materials, microbial correlations at phylum level were constructed using significant Spearman correlation (p-value > 0,05) of the 48 most abundant bacterial and fungal OTUs at 97% cut off (n=96) (Chen *et al*., 2019). A heatmap was made based on the significant correlations (positives and negatives) between the bacterial and fungal phyla, to analyse the relationships between the two domains (bacteria and fungi) in every pipe material type. Also, ecological networks based on these correlations were visualized using the software Gephi (version 0.9.2) (Bastian *et al*., 2009) and to perform network-based analysis to determine the network connectivity. The analysis provided statistics of the network that Gephi offers, including density (D) which measures how close the graph is to being complete (with a density of 1 being a graph with all possible edges) and the clustering coefficient or transitivity (T) which shows the probability that the close nodes of a node are connected and shows the complexity of a structure (Kim *et al*., 2018). The ratio between D and T indicates the stability of the network with the lower this ratio the higher the network stability (Douterelo *et al*., 2020).

3. RESULTS

3.1. Turbidity analysis

The results of turbidity responses, applied flushing flow (hydrant discharge) and pressure for all 6 locations in the 3 daily campaigns are shown in **Figure 2**. The times samples were collected is highlighted. For each trial, turbidity responses were observed, highlighting beneficial cleaning effect as material is removed from the network. As turbidity responses could not all be anticipated, sample collection did not always capture significant mobilisation

events corresponding to deeper microbial layers or network deposits. A number of features common to flushing trials however can be identified from each site. These include initial short-lived turbidity responses that are associated with loose deposits that develop due to stagnant conditions in connecting pipework (i.e. between the supply pipe and the hydrant). Another feature is that where pipe associated turbidity responses are observed following a flow increase, further increases in flushing flow result in additional material mobilisation. This indicates material is not simply a loose deposit but must be subject to cohesive retention forces. Note that historically flushing operations tended to focus on rapidly removing material (the cleaning benefit), even if this involved producing high turbidities. With the advances in monitoring technology this practice may become no longer acceptable. This makes controlling, and evidencing, the cleaning benefits a challenge to operators as generally turbidity responses only start to become visible once exceeding 10 NTU, yet European standards for turbidity state that members should strive for a parametric value not exceeding 1 NTU in the water exiting treatment works (https://eur-lex.europa.eu/eli/dir/1998/83/2015-10-27).

Key features with respect to hydraulic characteristics and observed behaviour are noted for each operation: Site 1 is from a cast iron pipe deep in the network. It produced high turbidity responses, especially initially suggesting significant accumulations of weaker exposed material, although each subsequent increase also produced distinct responses as underlying material was eroded. This clear turbidity response when flushing at just 1 L/s, in addition to the unknown background demand at this time, indicates this pipe section does not experience daily demands in excess of this combined flow. As a consequence, it can be regarded as having a high potential sensitivity to small flow changes. Significant quantities of material were mobilised and this it can be proposed was due to the addition from this cast iron pipe of corrosion products. Due to a loose hose fitting, the trial initially commenced (initially mobilising material) and then had to be shut down before starting up again shortly after. A pipe length of around 210 m based on velocities in the pipe section is commensurate with the timing of the arrival of the peak turbidities observed following flow increases.

Site 2 was situated upstream of Site 1, and this location produced no turbidity responses from the 315 m of ductile iron/polyethylene test section during this work, indicating the pipe experienced normal peak flows in excess of the flushing 3.3 L/s, plus the unknown background demand. However, during the final stage, a significant and classic pipe mobilisation response can be observed (Husband & Boxall, 2016). If a combined pipe flow of 4 L/s is assumed (i.e.

315 3.3 L/s flushing and midday flow of 0.7 L/s, this equates to a velocity of 0.22 m/s) and the pipe
316 diameter remains around 150 mm, flushing results suggested the turbidity response has
317 propagated from approximately 200 m of pipe at 400 m from the hydrant. Analysis of the
318 network is required to identify this higher risk section.

319 Site 3 was a 90 m section of reportedly cast iron, supplying only a handful of properties. This
320 section demonstrated a very high sensitivity to flow with very significant turbidity responses
321 to each small increase. The results indicated the daily peak flow was less than 0.5 L/s. Even
322 connecting the standpipe and opening the hydrant valve prior to trial commencement caused
323 significant disturbance resulting in two turbidity responses that took time to clear due to the
324 low flow rate during the standpipe at this set-up stage. This unfortunately also resulted in the
325 background sample for this second campaign being unrepresentative of background water
326 quality. The sensitivity is likely linked to the low daily flows that can allow growth of large
327 biofilms and material retention, exacerbated by pipe corrosion. The reported small diameter
328 (60 mm) and possibility of significant material accumulation within this pipe is supported by
329 the large drop in pressure observed that meant the peak flushing flow obtained was less than 1
330 L/s. Travel times from flow increase to peak turbidity are however commensurate for a 60 mm
331 diameter pipe with a length of around 90 m.

332 In Site 4, even with a flushing flow of 10 L/s achieved through multiple 1 L/s incremental steps,
333 this site produced no discernible pipe associated turbidity response. This demonstrate how
334 monitoring turbidity can be used to help determine peak flows as it highlights how in this
335 strategic 200 mm cast iron they are in excess of this value (although it does not account for
336 addition of background flow at the time of flushing). One feature is that the hydrant fittings
337 were a source of significant material that was mobilised when initially connected. This
338 indicates this site may have been undisturbed for a long time, or it is exposed to a high flux of
339 material potentially travelling down this strategic main. If the latter, this could be a
340 consequence of cast irons contributing corrosion material, reducing water quality and
341 accelerating downstream accumulation rates. Within the trace however there are a number of
342 short duration turbidity spikes. These are not consistent with pipe length responses and are
343 typically associated with physical pipe features that create small hydraulically sheltered
344 localised deposits, in this case possibly associated with upstream valving disturbed during the
345 trial at Site 3.

346 Site 5 was further downstream but a continuation of the 200 mm cast iron pipe as Site 4. Initial
347 connection demonstrated the same material deposition issues and a first flushing flow of 5 L/s
348 returned negligible turbidity response from the pipe as experienced upstream. This had been
349 anticipated, and hence instead of incremental flow increases, larger 5 L/s steps were applied
350 based on the understanding developed at the previous site. Unlike Site 4 however, a turbidity
351 response was then observed once 10 L/s was applied, indicating daily flow conditioned values
352 were being exceeded. Although it could be argued this may be due to higher background
353 demand at the time of flushing (increasing combined total flow), more likely is that as further
354 downstream, daily demand was less following a number of consumer connections in the
355 interceding length between this site and Site 4.

356 The trial performed at Site 6 represented a section of network with unknown pipe properties
357 due to the convergence of multiple different sections. This campaign set out to show how the
358 hydraulic response behaviour and control principles, when combined with turbidity
359 monitoring, can be applied operationally as part of network maintenance and management. The
360 results demonstrated that for each increase in flow above a conditioned state there was a
361 turbidity response. This trial successfully showed that by controlling flow steps, mobilisation
362 can be limited even when actual pipe details are unknown. Analysis of the turbidity response
363 also indicated network features, for example a repeating short-duration turbidity response can
364 be observed. Based on assuming a diameter of 150 mm, the flushing flow rate and time between
365 the leading edge and response peak, this suggests material was originating from a 4 m section
366 of pipe. This turbidity response, highlighting an accumulation of material, could however be a
367 result of a number of factors, including different hydraulic properties in this section (a result
368 of the looped network), different diameter (hence different applied shear stress), different
369 material, e.g. cast iron, or a combination of these. A key operational finding at this site was
370 shown in the final phase when the flow, following initial flushing and reduction, was once
371 again increased. Where material had been previously mobilised, this second time there was no
372 response. This indicates the operation had successfully removed material and a new higher
373 conditioned value, or hydraulic resilience, had been imposed.

374 ### 3.2. Water physico-chemical analysis

375 **Supplementary Table 1** shows the data from physico-chemical analysis of samples obtained
376 during the flushing operations. Due to processing limitations only 5 of the 13 samples collected
377 could undergo detailed analysis, and 1 from each Site was selected as highlighted in Figure 2

(except Site 4 that with negligible turbidity response was omitted). As expected, all the parameters had generally similar measurements as the same water was supplied throughout the network. Water temperature was between 15.1-16.5 ºC, free and total chlorine were in a rage of 0.79-1.3 mg/L and 1.12-1.66 mg/L respectively, and the pH was near neutral, at 7.5-7.8. However, some variations can be highlighted in relation to metal levels, but care must be taken when interpreting these as values may reflect the time, or more specifically the turbidity and hence loading, when the samples were collected. For example, Sites 1 and 2 had higher aluminium content with 88.1 and 74.5 µg/L than elsewhere (the other sampling sites 48.0, 57.3 and 54.0 µg/L) which does support their hydraulic connectivity but leaves a question why this section returned higher aluminium. Sites with cast iron pipes unsurprisingly showed higher iron concentrations, with Sites 1, 5 and 6 at 17.1, 14.5 and 13.1 µg/L respectively). Site 3 however with only 2.3 µg/L is an anomaly as much higher iron levels would be anticipated if this is a cast iron pipe as reported and especially with the highest turbidity observed and recorded during sample collection. Low levels of mercury were detected in Site 6 (0.12 µg/L), while it was not detected in other sampling sites. Lead was only detected in Sites 3 and 6 (1.3 and 1.5 µg/L). TOC was lower in Site 3 (1.1 mg/L) compared with the other sampling sites (2.1, 2.2, 2.8 and 2.5 mg/L).

3.3. Microbial analysis

3.3.1. Taxonomical analysis of bacteria

Different bacterial community composition was detected in pre and during-flushing samples at class level and genera level in samples from the network sections studied. At class level (**Figure 3.A**), and showing the average for triple replicate samples, Bacilli, Actinobacteria, Gammaproteobacteria and Alphaproteobacteria were abundant in all cases. Bacilli had an average relative abundance higher than 25 % in Campaign 1, whilst Actinobacteria represented more than 25% of the total relative abundance in samples from Campaign 2 and 3. Several, bacterial classes decreased during flushing when compared with the background samples (pre-flush). The relative abundance of Gammaproteobacteria was particularly high after flushing at >25% in Step 2 samples of Sites 1 and 6 and Step 1 samples of Site 3, whilst Alphaproteobacteria (2-13%) had a similar relative abundance in all the samples, but in general, it was more abundant in background samples (8-11%). From Site 1, Betaprotebacteria decreased from 26% in pre-flush background samples to 19% and 9% in Step 1 and Step 2 samples, respectively, and it was no detected in post-flushing samples in Site 6.

When the genera data (**Figure 4**) was analysed (average of 3 replicates), several genera were common to all the samples, including *Propionibacterium* (6-24%), *Pseudomonas* (0-23%), *Staphylococcus* (0.5-22%) and *Sediminibacterium* (0-22%). At Site 1 *Aquabacterium* decreased from 21% in the pre-flush background samples to 16% in Step 1 and 6% in Step 2 samples. Other genus that decreased during flushing included *Anaerococcus*, from a relative abundance of 12% in Step 1 samples of Site 2 and was almost not present in Step 2 and 3 samples. In Site 3, *Staphylococcus* decreased from 15 % in the background samples to 8% in Step 1 and *Micrococcus* decreased from 8% in the background samples to almost not present in Step 1. In Site 6, *Halospirulina* relative abundance in the background samples went from 11% to not present in Step 1.

The opposite with relative abundance increasing during flushing was observed for *Pseudomonas*; in Site 1 it was present at very low relative abundance in background samples and increased with Step 1 (15%) and Step 2 (23%). In Site 6, *Pseudomonas* increased in Step 2 samples to 9%. In Site 2, *Flavobacterium* and *Staphylococcus* were not present in the first flushing steps, but they increased with successive flushes, 14% and 22% respectively.

3.3.2. Taxonomical analysis of fungi

At class level (**Figure 3.B**), Sordariomycetes was abundant in all samples, particularly from Site 2 during Step 3 (28%) and Site 6 in background samples (26%). Malassezyomecetes, Eurotiomycetes and Dothideomycetes were classes highly abundant in all samples. Malasseziomycetes was abundant in background samples from all the sites (> 20%), particularly in Site 6 (45 %). Eurotiomycetes was highly abundant in samples from Site 2 (16-40%), Campaign 2 (22-23%) and Campaign 3 (14-30%). Dothideomycetes represented 30-36% in background and Step 1 samples from Site1 and 42% and 35 % in Step 1 samples of Site 2 and background samples of Site 3 respectively. Agaricomycetes, was highly represented in the fungal communities of Site 1 during flushing samples (20-31%), Site 3 (10-57%), Site 5 (35%).

When the fungal genera were analysed (**Figure 5**), several fungi were found in all the samples; *Malassezia* (6-45%) was the genus most represented in all the samples together with *Aspergillus* (0-40%) and *Cladosporidium* (0-40%). Similarly, to what was observed for bacteria, several genera increased during flushes. *Aspergillus* tended to increase with flushes in samples from Site 1 from 6% in background samples to 19% in Step 2, Site 2 from 16% in

441 the Step 1 to 40 % in Step 2 and 19% in Step 3, and in Site 6 from 28% in background to 30 %
442 in Step 2. *Alternaria* also increased during flushes, in Campaign 1 was almost no present in
443 Background samples and then increased to 5% and 9%, and in Campaign 3 was not
444 representative in background samples whilst represented at 37% during flushing. The opposite
445 trend was observed for several genera, which decreased during flushes. *Malassezia* decreased
446 with flushes in all sites, for example in Site 1 went from 34% to 10% and in Site 6 from 45%
447 to 25% in Flush 2 samples. *Saccharomyces* (30%) only was clearly represented in samples
448 from flushing in Site 5.

449 *3.3.3. Alpha diversity for bacterial and fungal communities*

450 Alpha diversity for bacteria and fungi is detailed in **Figure 6**. Overall, richness and diversity
451 indices were higher for bacteria than for fungi, whilst dominance was higher for fungi.

452 No differences in these indices were observed for the type of materials and regarding flushes
453 for bacteria diversity, Campaign 1 and Site 3 increased with flushes, Site 2 and Site 6 increased
454 with the first flushing steps and then decreased. For bacteria richness: Campaign 1 decreased
455 with flushes (except the Step 2 in Site 1). Campaign 2 and 3 increased with the first steps of
456 flushing and then decreased.

457 For fungi, the dominance in Campaign 1 increased with the first steps of flushing and then
458 decreases, in Campaign 2 it increased during flushing and in Campaign 3, dominance deceases
459 and then increases. Fungal richness did not have high variation, and diversity tended to reduce
460 with flushes in Campaign 1 and 3 and stayed stable in Campaign 2.

461 *3.3.4. Physicochemical and microbial correlations*

462 The Spearman's correlations between physicochemical and microbial parameter are available
463 in **Supplementary Table 2**. Correlations with microorganisms varied along the flushing steps,
464 in the first flushing steps there were more correlations with fungus, while in the last flushing
465 steps bacteria correlations are dominant. In background samples, there were only negative
466 correlation with fungal genera like *Cladosporidium*, *Daedaleopsis*, *Gymnochlora*, *Pichia*,
467 *Podospora* and *Psathyrellathe*, also there were negative correlations with flow and turbidity.
468 However, in Step 1, the were only positive correlations with fungal genera (*Cladosporium*,
469 *Daedaleopsis*, *Lepiota*, *Lophotrichus* and *Pichia*). In the case of Step 2, there were positive
470 correlations with the flow, one bacterial genera (*Mycobacterium*) and one fungal genera

(*Alternaria*), and negative correlations with *Halospirulina*, *Lepiota*, *Lophotrichus* and *Penicillium*. In the last flushing step (Step 3), there were negative correlations with several bacterial genera (*Acinetobacter*, *Anaerococcus*, *Mesorhizobium*, *Rhodocista* and *Streptococcus*) and positive correlations with the bacteria *Streptococcus* and the fungi *Cercophora*. Also, turbidity was positively correlated with Step 3, as well as with the bacterial genus *Aquabacterium* and the fungal genera *Chaetomium* and *Rhodotorula*, but negative with background samples.

Regarding to material type, only the case of cast iron had significant positive correlations, where the bacterial genera *Acidovorax*, *Singulishaera* and *Variovorax*, and the fungal genera *Russula* and *Tricholadium* were included. Some of these genera were negatively correlated with the other material types; for example, *Acidovorax* and *Singulisphaera* were negatively correlated with iron-plastic material, whilst *Variovorax*, *Streptococcus*, *Aureobasidium* and *Russula* with asbestos cement.

Focusing on alpha diversity, several bacterial and fungal genera were correlated with the different diversity indices. For example, *Halospirulina* and *Propinibacterium* were positively correlated with bacterial dominance but negatively with bacterial diversity, whilst *Acinetobacter*, *Flavobacterium* and *Pseudoclavibacter* had a contrary behaviour. *Malassezia* was negatively correlated with fungal dominance but positively with fungal diversity and *Aspergillus* and *Daedaleopsis* were positively correlated with fungal richness. Also, bacterial genera had correlation with fungal alpha diversity as well as fungal genera with bacterial diversity indices, for example, *Pseudoclavibacter* was positively correlated with fungal dominance and *Anaerocucus* and *Micrococus* were negatively correlated with fungal richness, while, *Leptosphaeria* was positively correlated with bacterial dominance and negatively with bacterial diversity, and *Aspergillus* was negatively correlated with bacterial richness.

3.3.5. Microbial phyla correlations and networks

Based on the Spearman's correlations between most abundant bacterial and fungal OTUs in each material pipes (data not shown), it is noticed that, in all pipe materials, there are more significant correlations between OTUs belonging to different domains (bacteria-fungi) in a same proportion between positive (20, 29 and 24%) and negative (26, 24 and 22%) correlations (**Table 1**). Regarding to the correlations intra-domains, normally, there are more positives

501 (bacteria: 20, 12 and 13%, fungi: 21, 18 and 21%) than negatives (bacteria: 8, 9 and 11%, fungi:
502 5, 6 and 7%).

503 Analysing the heatmap (**Figure 7**), there were more representations of different bacterial phyla
504 than fungal. Proteobacteria, Ascomycota and Basidiomycota were the phyla with more
505 correlations in all the materials. Also, it is appreciated that depending on the material, the nature
506 of the correlations could vary; for example, the phylum Firmicutes had more proportion of
507 negative correlation between other bacterial phylum in the case of cast iron and asbestos
508 cement, but in the case of iron-plastic material the positive correlations with bacterial phylum
509 are more abundant, or the phyla of Fusobacteria and Gemmatimonadetes only have significant
510 correlations in iron-plastic pipe materials.

511 In relation with the networks (**Supplementary Figure 1**), it was observed that iron-plastic
512 materials (D = 0.066, T = 0.397, D/T = 0.166) were the most stable networks, followed by
513 asbestos cement (D = 0.070, T = 0.311, D/T = 0.225) and finally cast iron materials presented
514 the most fragile communities (D = 0.072, T = 0.231, D/T = 0.312).

515

516 ## 4. DISCUSSION

517 ### 4.1. Flushing and turbidity

518 All of the sites tested demonstrate common turbidity response behaviour that can be applied
519 when developing network maintenance plans. The trials highlight flow increases as a key cause
520 of material mobilisation, yet crucially how this can be controlled, especially with concurrent
521 flow and turbidity monitoring. A key aspect observed is that accumulated material displays
522 cohesive properties, that is for every increase in flow once a conditioned state is passed,
523 additional material was mobilised. This has implications in that flushing flows can focus on
524 removing material that is at risk of mobilisation. This is important as it recognises that the
525 concept of fully clean is not possible (especially when considered with continual ongoing
526 material accumulation processes) and it rejects the commonly held perception that very high
527 flows are required to be effective (Douterelo *et al*., 2013). This understanding that lower target
528 flows can be sufficient also has operational benefits, including reducing volumes of water to
529 be discharged (something that is increasingly critical as water scarcity issues increase) and
530 reduction in the risk of disturbing upstream sections. Analysing the turbidity response also
531 highlights many benefits, including the ability to assess peak daily demands based on initial

response observation, identify site specific discolouration risk sensitivity (including if material mobilised is from cohesive layers that are endemic to all pipe or deposits arising from hydraulically quiescent sections) and even the ability to determine locations and lengths of higher risk sections, as shown at Site 2 and 6.

Acknowledging material accumulation in conjunction with biofilms contributes to potential water quality issues (Husband *et al*., 2016; Makris *et al*., 2014), hydraulic maintenance demonstrated here offers a rapid, simple and effective mitigation strategy, whilst turbidity data provides multiple benefits in addition to evidence of the cleaning achieved. Coordinating trials with flow and turbidity monitoring has also shown how it can be used to prioritise maintenance schedules based on risk and sensitivity, and following initial trials such as these, target flows can be established, reducing future operation times. Site 6 also highlights that once an increased flow has been applied and the turbidity response passed, hydraulic resilience has been obtained such that flows can return to this value without impact. Of course, particulate material in the background flow will continue to accumulate (Husband & Boxall, 2016), so this resilience is temporally limited, although this deterioration rate can be investigated through repeat trials.

4.2. Microbiological characteristics of the water samples containing material removed from distribution system

This study represents the first detailed characterisation of mixed–species biofilm population in the DWDS of the Mediterranean city of Valencia. Sequencing results shows how several bacterial genera including *Propionibacterium*, *Pseudomonas*, *Staphylococcus* or *Sediminibacterium* and fungal genera such as *Malassezia*, *Aspergillus* and *Cladosporidium* were present across all the samples in the distribution network. This supports previous observations from UK DWDS, where a core bacterial community in biofilms was observed independently of the characteristics of the incoming water or localised conditions between sites (Douterelo *et al*., 2017, 2018, 2020). Similar observations were proposed by other researchers from different countries like Germany, USA Portugal or China studying mixed species biofilms (Henne *et al*., 2012; Kelly *et al*., 2014; Ling *et al*., 2016; Rickard *et al*., 2004; Simões *et al*., 2007, 2008; Tsagkari *et al*., 2017). In these studies, bacteria belonging to genera like *Acinetobacter, Burkholderia, Methylobacterium, Mycobacterium, Pseudomonas, Sphingomonas and Staphylococcus* played an important role in the formation of biofilms and aggregates.

Although more sampling is required to interpret the impact of discrete physico-chemical parameters on biofilm populations, the similarities observed between the samples studied here suggest that internal microbial factors (microbial interactions) are central in shaping biofilm formation and composition. As observed in this study, Henne *et al*. (2012), after studying mature biofilms (>20 year old water network) in DWDS, proposed that the initial microbial colonisation of pipes might depend on surface material, but then, the coexistence of the communities over the time influences the entire composition of the system by the exchange of microorganisms between the bulk water and the distribution network surfaces.

In this study, the 16S rRNA libraries of all samples were dominated by the four main classes of Actinobacteria, Bacilli, Gammaproteobacteria and Alphaproteobacteria. These have been found in chlorinated DWDS in the UK, where these taxonomical groups were particularly abundant in material mobilised from cast iron pipe sections (Douterelo *et al*., 2014). In others DWDS studies in the Netherlands, China and Germany, Proteobacteria had higher relative abundance than other phyla like Actinobacteria, with Alphaproteobacteria the most represented class (Henne *et al*., 2012; Lin *et al*., 2013; G. Liu *et al*., 2014; J. Liu *et al*., 2017).

Chlorination and shock chlorination are disinfection practices that water companies usually implement to prevent bacterial regrowth. In unchlorinated DWDS, like in the Netherlands, it is reported that usually bacterial diversity is higher when compared with chlorinated systems. However, fungal relative abundance is less in unchlorinated systems than in chlorinated ones (Bertelli *et al*., 2018; Nagy & Olson, 1982). Some authors suggest that this increase of bacterial diversity is due to the lack of chlorine (Roeselers *et al*., 2015), and propose that a higher diversity of certain beneficial microorganisms can protect against the proliferation of pathogens, what is known as the "protective biofilm" concept or the "probiotic approach" (Bertelli *et al*., 2018; Hong Wang *et al*., 2013). Actinobacteria and Alphaproteobacteria are widely reported in drinking water-related ecosystems such as in shower systems (Moat *et al*., 2016) and distribution systems (Douterelo, Husband, *et al*., 2016; Makris *et al*., 2014; Wolf-Baca & Piekarska, 2020; Yang *et al*., 2016; Zhou *et al*., 2017). Actinobacteria class was highly abundant in in this study and species belonging to this class have been found in DWDS and associated with organoleptic problems (Zacheus *et al*., 2001; Zaitlin & Watson, 2006). The presence of these iron pipe associated phylotypes suggests iron is a significant component within this network and could therefore be a precursor of aesthetic issues. Here we can confirm

the presence of these bacterial groups in this Spanish DWDS that operates with high temperatures and hard water (total hardness 42-43 ºF).

4.2.1. The importance of fungi communities and their interactions with bacteria

Along with the bacterial communities, fungi have been identified in this study as an important inhabitant of these ecosystems. Fungi can enter DWDS after treatment processes, by means of leaks, or from air in contact with water stored in reservoirs. They are known to have the ability to survive disinfection with chlorine (Gonçalves *et al*., 2006). Afonso *et al*. (2019) demonstrated that akin to bacteria, filamentous fungi in drinking water-ecosystems go through similar phases during stages of biofilm formation. It has been reported that fungi can colonise pre-establishment of bacterial biofilms, indicating a positive relationship between these two domains (Doggett, 2000). Furthermore, *in vitro* assessment of biofilm formation by microbial species (bacteria and filamentous fungi) isolated from a DWDS, showed that fungal stage development is important in the first 24 h of biofilm formation and may provide an advantage to the opportunistic bacteria like *Acinetobacter calcoaceticus*. As a result of this, it has been proposed that filamentous fungi could be indicators to determine biofilm formation in drinking water systems (Afonso *et al*., 2019).

This study identified more correlation inter-domains (bacteria-fungi) than intra-domains (bacteria-bacteria and fungi-fungi) (**Table 1**). Fungi are reported to support bacterial establishment in biofilms (Chaves *et al*., 2020; Lahaye *et al*., 2016), yet the exact mechanisms of interaction between these two organisms in biofilms still remains unknown. Fungi scavenge nutrients to support growth in oligotrophic environments and they have been considered likely secondary colonisers once biofilm has established on pipes (Gonçalves *et al*., 2006). The diverse community of fungi discovered in this Valencian drinking water network demonstrates a strong contribution to biofilm formation in DWDS, supporting concepts of mutually beneficial fungal and bacterial community interaction.

Many of the fungal genera identified in this study belong to the class Dothideomycetes, Eurotiomycetes and Malassezyomycetes. Eurotiomycetes (e.g. *Aspergillus*, *Penicillium*) and other classes present in the studied DWDS including Sordariomycetes (e.g. *Trichoderma*, *Fusarium*, *Acremoniun*) and Saccharomycetes such as *Saccharomyces*, are capable of transforming and removing organic contaminants from the environment such as toluene, polyaromatic hydrocarbons, synthetic dyes, polychlorinated biphenyl and pesticides (Harms *et*

625 *al*., 2011). Contrary to these beneficial activities, other fungal species detected in this study,
626 such as *Alternaria*, *Aspergillus*, *Cryptococcus* and *Cladosporium*, can cause infections through
627 mycotoxin production. Some species of the genus *Cladosporium* are linked with allergic
628 rhinitis and respiratory arrest in asthmatic patients (Assress *et al*., 2019). *Aspergillus*, present
629 in all the samples in this study, has been detected previously in DWDS and can cause allergies
630 if spores or hyphal fragments are aerosolized if contaminated water passes through shower-
631 heads, taps or toilet cisterns (Al-Gabr *et al*., 2013). The Valencian DWDS also presented high
632 relative abundance of sequences related to the genus *Malassezia*. Overall, members of the
633 *Malassezia* genus are commonly found in the environment and healthy human skin, but they
634 have been detected in shower-heads in the UK (Moat *et al*., 2016), where they have complex
635 interactions with other microorganisms. Moat *et al*. (2016), suggested that when the
636 interactions between *Malassezia* and other microorganisms in showerheads are disturbed, these
637 perturbations could cause health problems, particularly in immunocompromised individuals,
638 including superficial mycoses, dermatoses and allergic reactions. In DWDS, mycotoxins
639 concentrations tend to be low except in stored water, or stagnant zones such as dead ends, or
640 possibly tidal points, in pipes (Gonçalves *et al*., 2006). Fungi however are a known major
641 source of compounds that can yield taste and odour problems in drinking water systems. For
642 example, *Penicillium* has been also isolated from a Swedish distribution system and producing
643 a compound with an earthy flavour identified as 2,4,6-trichloroanisol (Nyström *et al*., 1992).

644 Reviewing these observations, precautions may need considering for biofilm mobilisation
645 effects, particularly if gross erosion occurs such as following hydraulic events, as demonstrated
646 possible in this work. Despite this key role, limited attention has been paid to fungi in DWDS.
647 This work confirms they form a key part of biofilms attached to pipe surfaces and as a result
648 they should be considered in surveillance methods to determine concentrations/biomass that
649 might trigger taste and odour problems or health-related complications. With this
650 understanding, more studies can now be applied to investigate associations and interactions
651 between bacteria and fungi, including accumulation and mobilisation effects on the community
652 dynamics and the impact on downstream consumers.

653 *4.2.2. Temperature as a potential factor*

654 Water temperature is a key factor influencing bacterial growth and high temperatures have been
655 associated with increased bacterial abundance in DWDS. In general, higher temperatures can
656 be associated with greater microbial growth and subsequently deterioration of aesthetic aspects

of water, sanitary risks, and malfunctioning of water installations (Prest *et al*., 2016). Previous studies in DWDS have shown that raw water quality and temperature can affect bacterial community characteristics in treated water including bacterial community composition, activity and abundance (G. Liu *et al*., 2013; Prest *et al*., 2016). Temperature can affect bacterial community composition (Preciado *et al*., 2019), by providing advantages to those species more competitive under higher temperatures (Prest *et al*., 2016). Donlan *et al*., (1994), showed that at warm temperatures in drinking water systems (15-25°C), biofilm accumulation rate increases, and this is associated with lowered disinfectant concentration and increased bulk water cell numbers. Comparing the microbial composition of material mobilised from the Valencian network (min. 13°C to max. 28°C) with that from a study of a UK network (min 3°C to max. 18°C), differences in community structure are observed. From UK studies, Alphaproteobacteria was observed as dominant, followed by Actinobacteria in all samples analysed, whilst in the Valencian network the dominant classes also include Actinobacteria, but in addition Bacilli and Gammaproteobacteria, with just a low relative abundance of Alphaproteobacteria. At genus level, the most abundant bacterial species in UK networks obtained from material mobilised in the samples from polyethylene pipes samples were *Spirochaeta*, *Methylobacterium* and *Clostridium*, while *Lysinibacillus*, *Pseudomonas* and *Flavobacterium* were highly abundant in the samples from the cast iron pipes (Douterelo *et al*., 2014). In the Valencia system, all the studied sites presented high abundance of *Propionibacterium*, *Stenotrophomonas* and *Staphylococcus*. Further sampling and analysis are needed to understand which factors are responsible for the difference in microbial communities between these two different networks. For example, not considered is water hardness, with the UK system studied of low hardness compared to the Valencian network which is regarded as high (42-44 ºF, **Table 1**). Regarding public health, water temperature has different effects on waterborne pathogens; higher water temperature can favour the inactivation of viruses, some parasites and fungi, whilst some bacteria, including pathogens may initially grow faster in water with higher temperatures (Hofstra, 2011). Further research would be needed to determine to which extent these changes affect bacterial competition processes within the drinking water distribution system.

4.2.3. Distribution of microbial communities

By collecting samples during the different flushing stages this study has been able to investigate differences in the bacterial community attachment strength. Samples taken pre-flushing typically represent the background composition, whilst as the flushing flow increases weakly

adhered, and most likely outer surface communities, are removed. With progressive flow increases raising the mobilising shear stress, more consolidated and less exposed (deeper lying) communities may be exposed and mobilised. Members of the genera *Pseudomonas*, *Flavobacterium* and *Staphylococcus* and the fungi *Aspergillus* and *Alternaria* increased during-flushing samples, suggesting that these microorganisms are associated with the deeper and more strongly adhered and consolidated pipe wall material. *Pseudomonas* and *Sphingomonas* have been described as initial coloniser and biofilm-forming organisms in water systems (Bereschenko *et al*., 2010; Douterelo *et al*., 2014). The abundance in the higher shear strength biofilm layers supports this involvement in initial biofilm formation within Valencian DWDS. However, alpha diversity analysis of the samples did not show significant differences in bacterial richness and diversity with the increase in hydraulic shear stress. Overall, the results from this study suggest that within the layers of material that needed more force to be mobilised, only certain types of microorganism, such as *Pseudomonas,* have different abundance when compared with those obtained in the initial flushing samples, whilst overall material removed from the pipe walls have similar microbiological composition independent of applied shear force.

Spearman´s correlation (**Supplementary Table 2**) allows another interpretation of how microbial genera are distributed among the different layers. Analysis suggests most of the fungal communities are located in the upper and less consolidated layers of the biofilm, since there were more positive correlations with the initial flushing steps before this correlation reduces with secondary flushing steps. This could be a biofilm adaptation as fungi are regarded as more disinfectant resistant (Pereira *et al*., 2013), and the presence in upper more mobile layers could help explain the relatively consistent fungal community observed. However, bacterial communities seem to be focussed in the inner layers of biofilms according to their positive correlations with the last steps of the flushing operations. With regards to specific microbial genera, *Halospirulina*, *Cladosporium*, *Daedaleopsis*, *Lepiota*, *Lophotrichus* and *Pichia* might dominate the most superficial layer of the biofilm, due to their strong positive correlations with Step 1 and, in the case of *Halospirulina*, because of its negative correlation with Step 2 and its high relative abundance in background samples. *Mycobacterium* and *Alternaria* could be highly located in middle layers according to their positive correlation with Step 2, while *Aquabacterium*, *Streptococcus*, *Cercophora*, *Chaetomium* and *Rhodotorula* might be occupying the deeper layers of the biofilm due to it positive correlation with Step 3 and turbidity.

723 Regarding *Acidovorax*, *Singulishaera*, *Variovorax*, *Russula* and *Trichocladium*, they might be
724 microbial genera with special affinity to cast iron pipes, due to positive correlation with this
725 kind of material and their negative correlation with the others (iron-plastic and asbestos
726 cement). There was not significant correlation between cast iron and turbidity, however, the
727 sites with cast iron pipes had the highest turbidity measures (**Figure 2**), and *Aquabacterium*
728 was positively related with turbidity. Indeed, *Acidovorax* and *Aquabacterium* have been
729 described as nitrate-reducing bacteria and reported to be corrosion-related microorganisms in
730 cast iron pipes, and believed to accelerate corrosion by oxidizing Fe(II) coupled with reducing
731 nitrate (Sun *et al*., 2014; Haibo Wang *et al*., 2015, 2017).

732 The complete elimination of biofilms in non-sterile environments such as DWDS is effectively
733 impossible. To facilitate optimum control, water utilities are now understanding they need to
734 comprehend the microbial dynamics to allow network management based on encouraging
735 environmental conditions that promote healthy microbial communities and limit potential
736 pathogens. As suggested by Flemming (2002), it is possible that drinking water biofilms can
737 inhibit the propagation of invading pathogens, thus helping to safeguard water quality.
738 Similarly, Hong Wang *et al*. (2013) suggested that a greater understanding of premise plumbing
739 buildings is needed to select for a desirable microbiome to limit proliferation of opportunistic
740 pathogens. This study is part of the first steps in understanding communities within DWDS,
741 and in particular those exposed to higher water temperatures. With this understanding future
742 studies can look into investigating how to sustain beneficial microbial communities and reduce
743 those associated with organoleptic issues.

744

745 **5. CONCLUSIONS**

746 Flow and turbidity monitoring have been used to demonstrate how hydraulic strategies can be
747 applied to control mobilisation of pipe wall material linked with aesthetic water quality issues.
748 Random pipe sections of different properties from within the Mediterranean city of Valencia
749 were subjected to controlled incremental flushing with sample collection for physico-chemical
750 and bacterial and fungal community analysis.

751 By monitoring turbidity response, it has been shown how hydraulics influences material
752 accumulation behaviour and how mobilisation (discolouration) impact can be controlled,

753 network resilience increased, unknown peak daily pipe flows assessed, site maintenance
754 prioritised and locations and lengths of higher risk sections identified.

755 A core microbial community was detected as present throughout the network with flushing at
756 different sites and with increasing applied shear stress yielding dynamic community
757 information. Microorganisms like *Pseudomonas*, *Aspergillus* or *Alternaria* increased during
758 flushing, indicating greater abundance in underlying and more consolidated layers. Bacterial
759 and fungal communities were found to be highly correlated, yet alpha diversity showed
760 bacterial communities more diverse between samples, while fungi showed more dominance
761 and stability. The results highlight that as part of water quality management, especially when
762 considering changing demand patterns and climate change, understanding community
763 dynamics, including the fungal component, will be key to supporting a beneficial and
764 ultimately safe microbiome.

765 This study was a first step highlighting techniques and approaches that can inform the design
766 of future experiments focussing on understanding the critical role of biofilms and factors such
767 as hydraulics and temperature.

768

769 **ACKNOWLEGMENTS**

770 Thanks to EMIVASA and Aguas de Valencia for their support and access to their drinking
771 water distribution network for study, sample preparation and analysis.

772

773 **AUTHOR CONTRIBUTIONS**

774 S.H, C.S.B, A.S, J.M and I.D were as involved in the design of the experiment with S.H, C.S.B,
775 A.S, C.C and J.M. running the trial operation sample collection. A.S performed the physico-
776 chemical analysis. S.H collected and analysed hydraulic and the turbidity data. G.D.O extracted
777 the DNA from samples. G.D.O and C.C analysed the microbial data. G.D.O, S.H and I.D
778 compiled and edited the manuscript.

779

ADDITIONAL INFORMATION

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

Funding: This work was supported by EMIVASA, Aguas de Valencia (Valencia, Spain).

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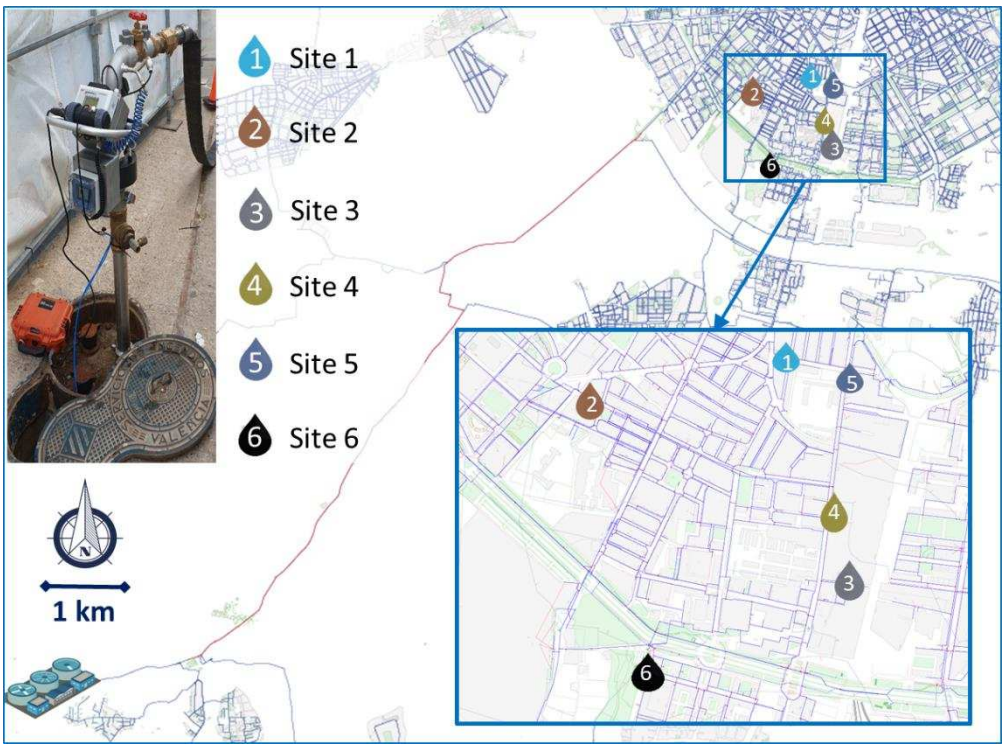
A)



B)

		CHARACTERISTICS		
		Diameter (mm)	**Length (m)**	**Pipe Material**
CAMPAIGN 1	**Site 1**	100	210	Cast Iron
	Site 2	150 160	210 105	Ductile Iron Plastic (Polyethylene)
CAMPAIGN 2	**Site 3**	60	90	Cast Iron
	Site 4	200	390	Cast Iron
	Site 5	200	450	Cast Iron
CAMPAIGN 3	**Site 6**	150	X	Asbestos Cement

Figure 1: A) Map of the location of network indicating sampling sites in Valencia city B) Table with the characteristics of the flushing site pipes. Site 6 length is not defined as multiple possible lengths effected.

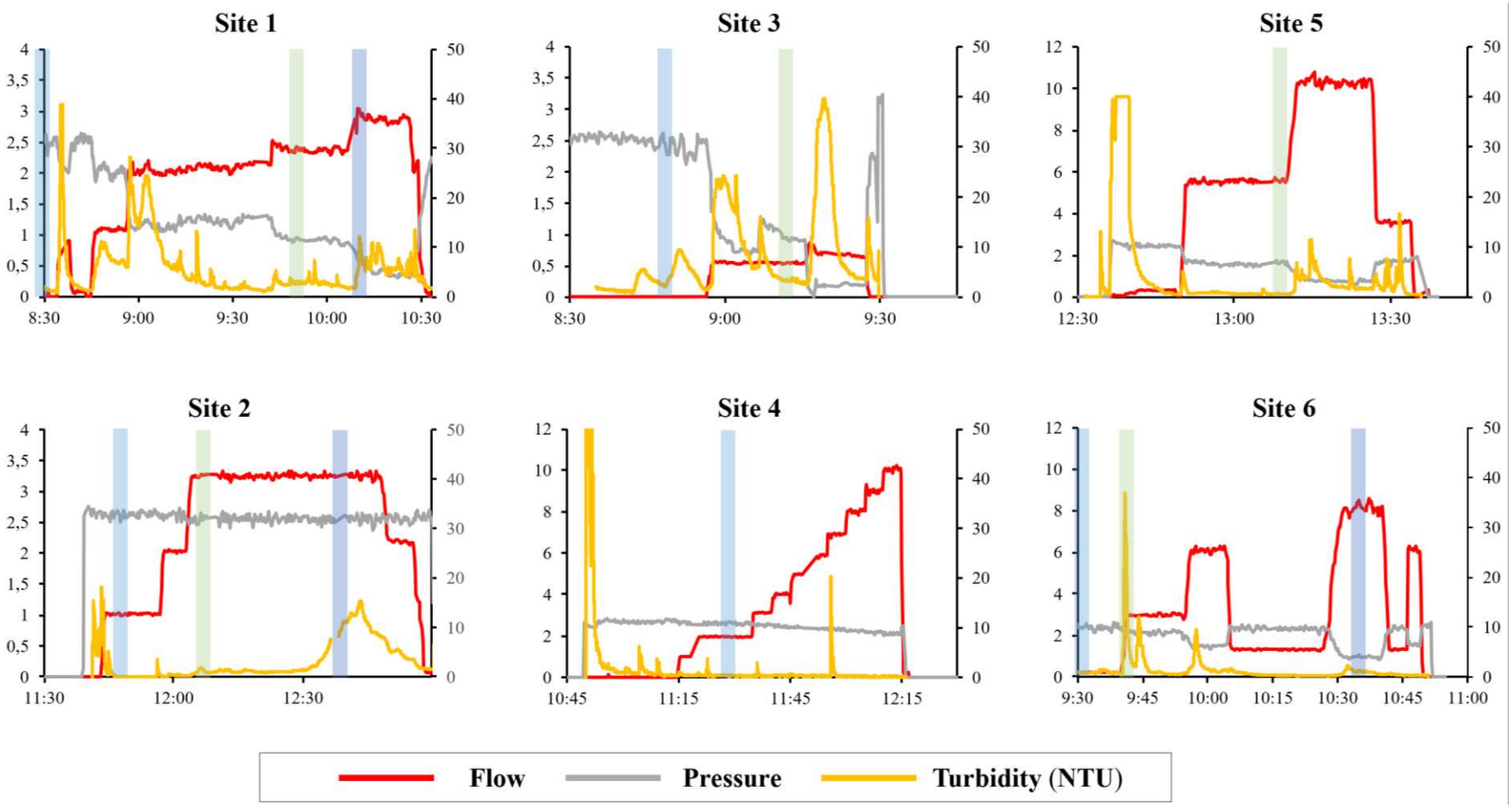


Figure 1: Time series results for pressure and hydrant discharge flow rate (bar and L/s, y-axis; note flows do not include unknown background flows in pipe) and turbidity (NTU, secondary y-axis) for the flushing operations. Highlight bars indicate time of sample collection for microbial analysis, with green shading noting samples also undergoing physico-chemical analysis (note axis scales not consistent).

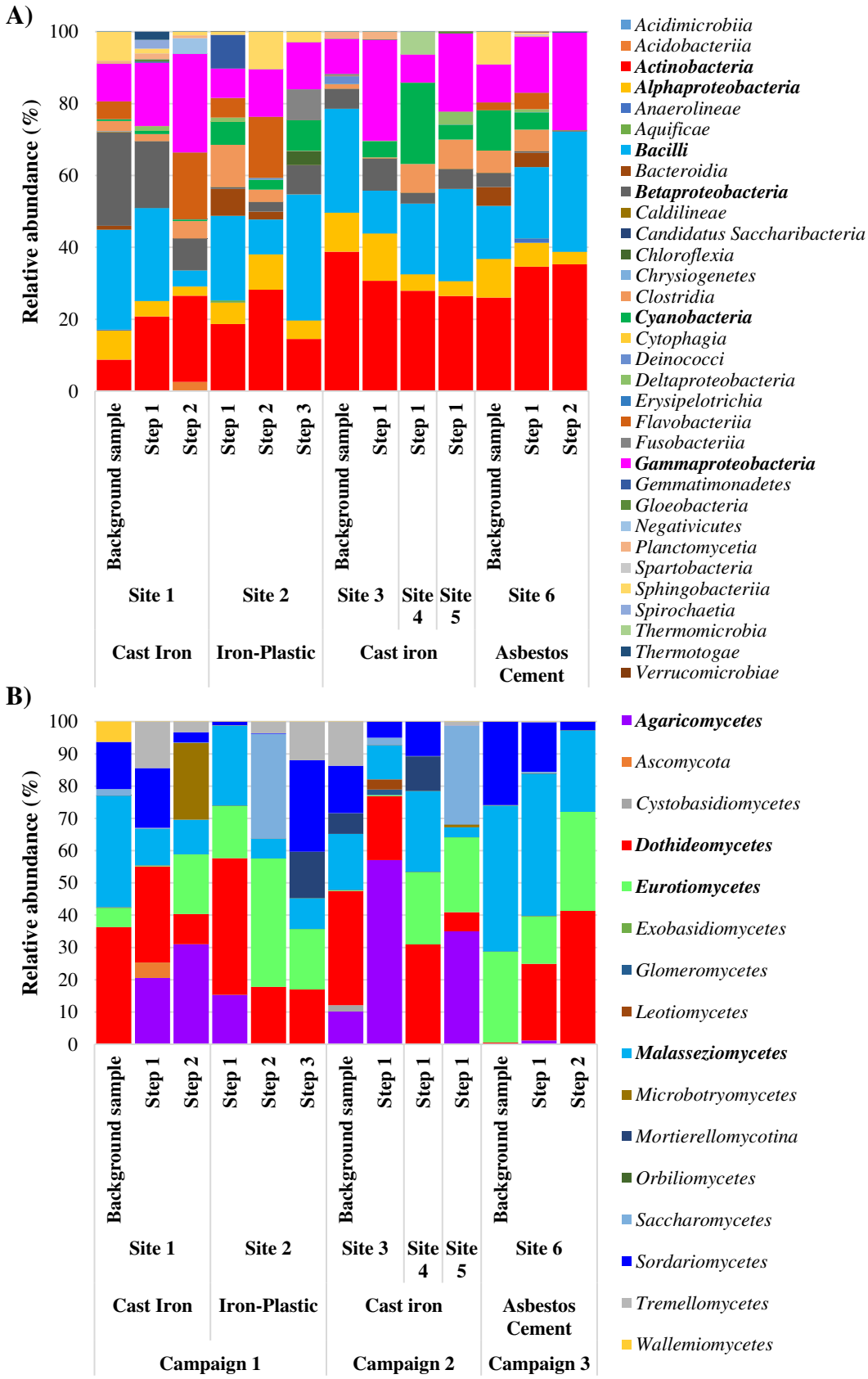


Figure 3: Comparison of the relative abundances of the major bacterial (A) and fungal (B) class found in the water collected from the network during flushing, showing differences between sampling sites and flushing steps. Each stacked bar has been calculated as an average of 3 replicates.

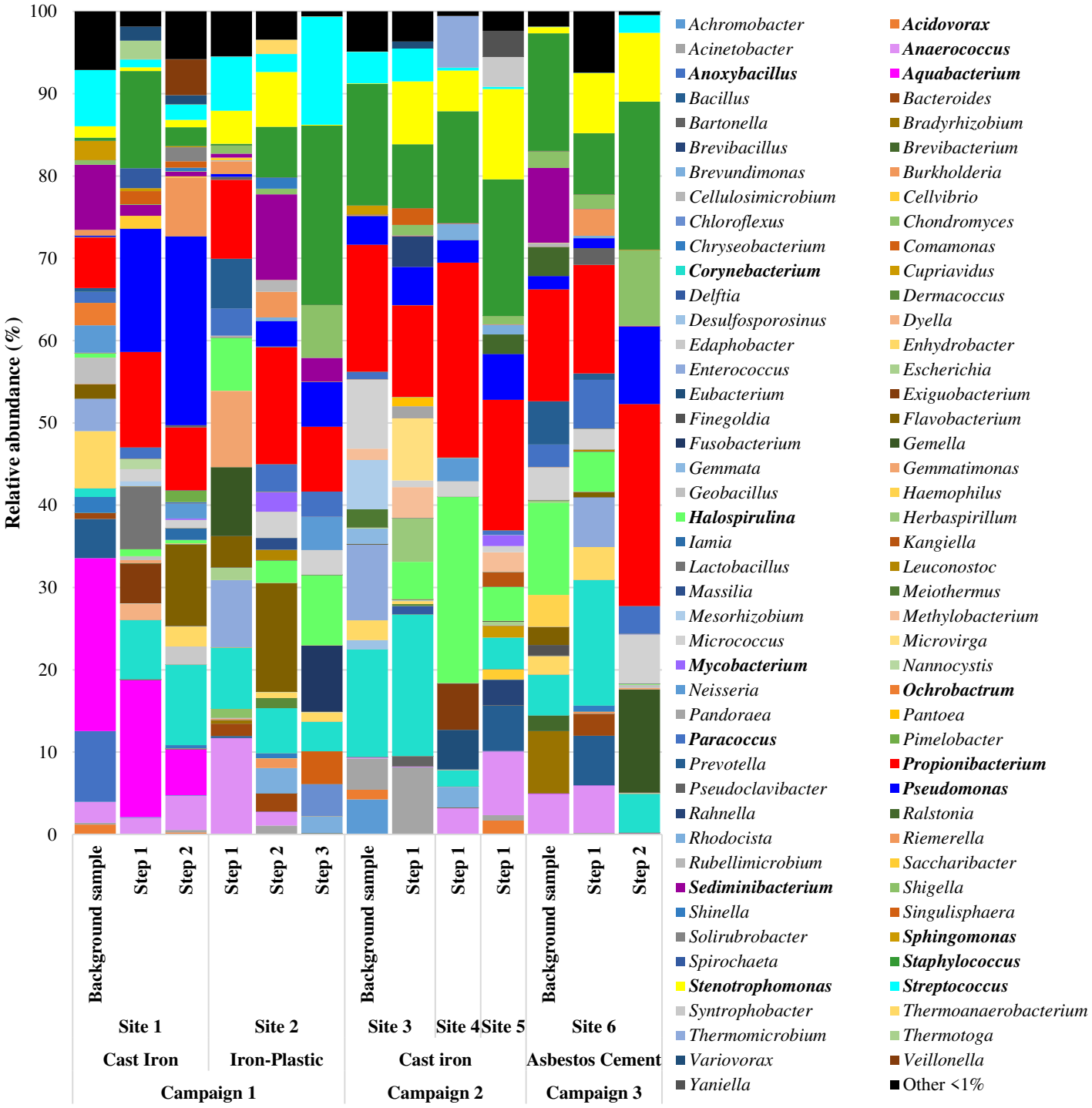


Figure 4: Comparison of the relative abundances of the most abundant bacterial genera found in the water collected from the network during flushing, showing differences between sampling sites and flushing steps. Each stacked bar has been calculated as an average of 3 replicates.

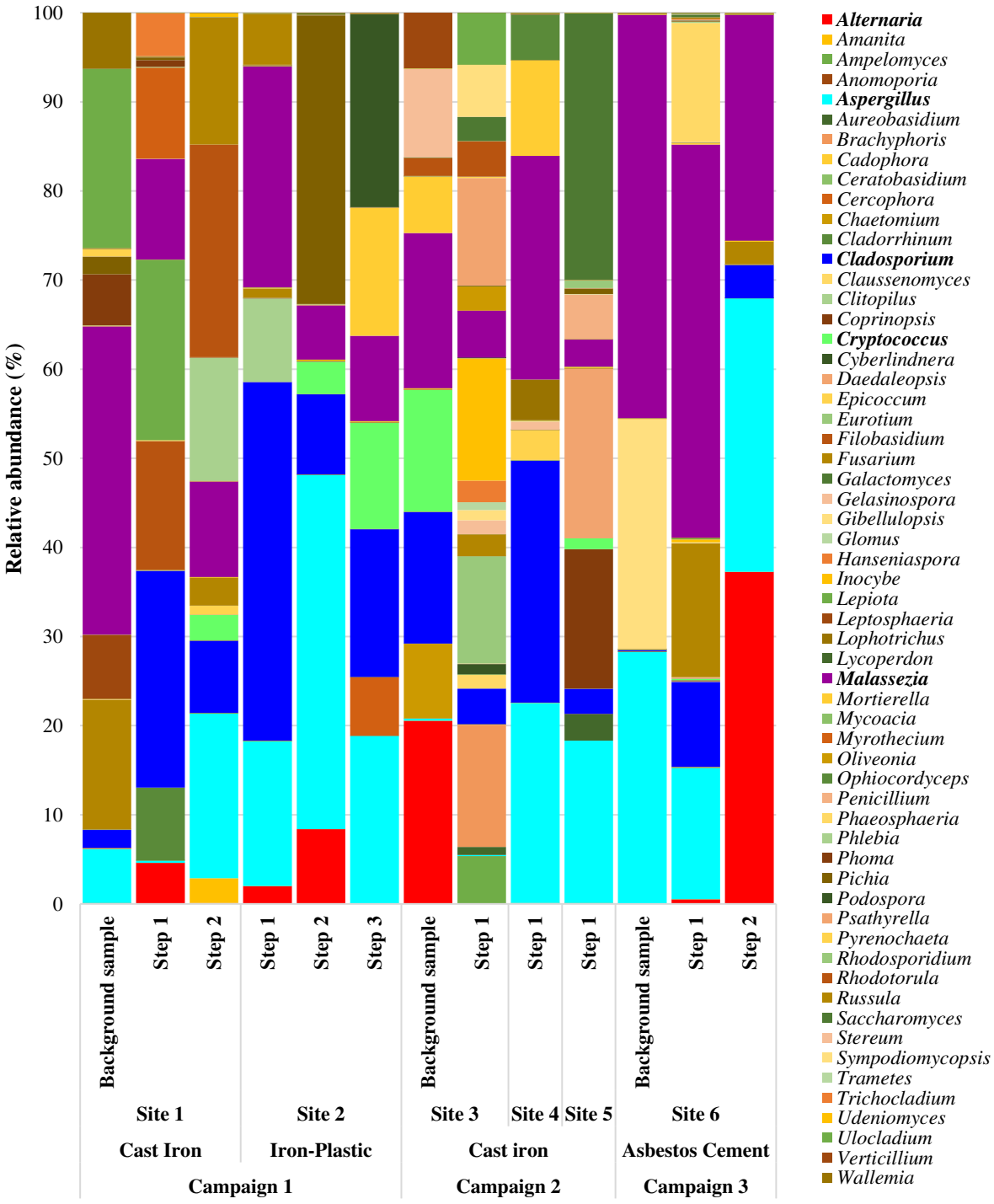


Figure 5: Comparison of the relative abundances of the most abundant fungal genera found in the water collected from the network during flushing, showing differences between sampling sites and flushing steps. Each stacked bar has been calculated as an average of 3 replicates.

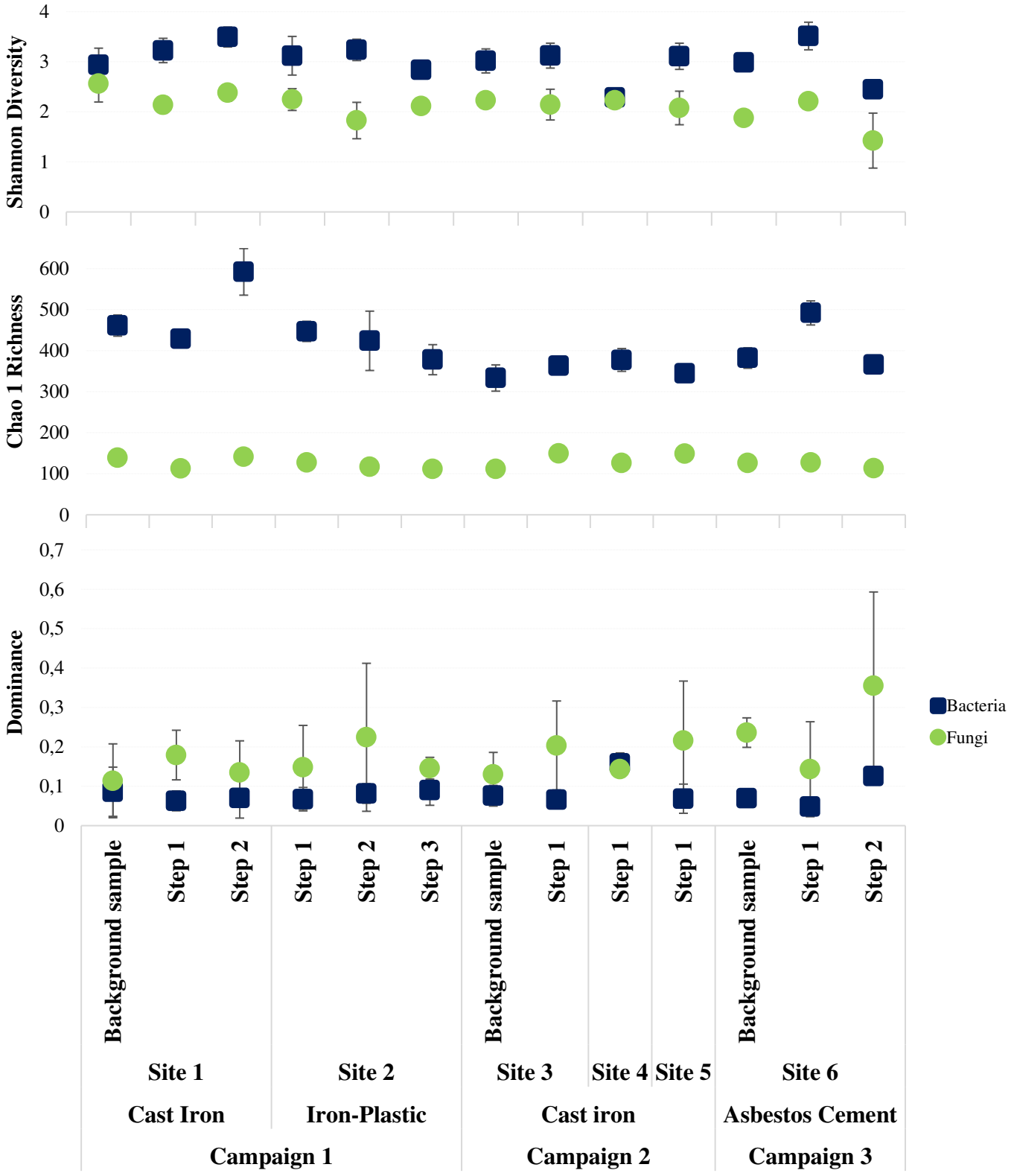


Figure 6: Graphs showing Alpha Diversity results: diversity (Shannon index) and richness (Chao1 index) indicators at a 95% sequence similarity cut-off from samples collected during flushing. Each point shows the average of 3 replicates with the standard errors as a bar.

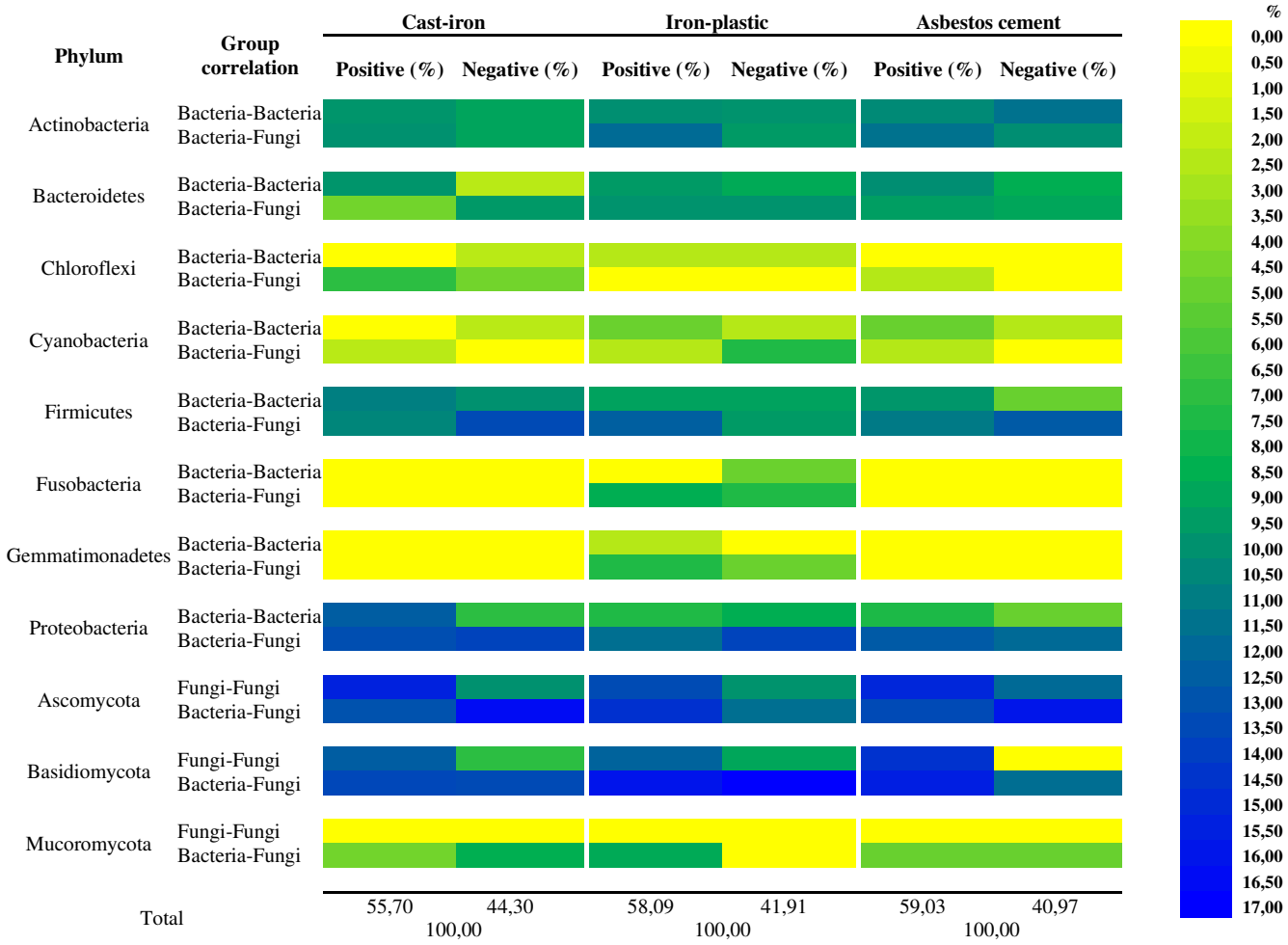


Figure 7: Heatmap of correlations between bacterial and fungal phyla. It is shown a heatmap based on the percentage of significant Spearman´s correlations (positives and negatives) of the bacterial and fungal phyla most abundant in every material type. Each specified phylum has been noted with the number of correlations (in percentage), both negative and positive, among the other bacterial and fungal phyla, in each pipe material studied.

Table 1: Count of positive and negative correlation between microbial kingdoms. The table shows the number of significant Spearman´s correlations (positives and negatives) of the 96 bacterial and fungal OTUs most abundant in every material type.

%	Cast-iron				Iron-plastic				Asbestos cement			
	Bacteria-Bacteria	**Bacteria-Fungi**	**Fungi-Fungi**	**Total**	**Bacteria-Bacteria**	**Bacteria-Fungi**	**Fungi-Fungi**	**Total**	**Bacteria-Bacteria**	**Bacteria-Fungi**	**Fungi-Fungi**	**Total**
Positives Corr.	20	20	21	61	12	29	18	60	13	24	25	62
Negatives Corr.	8	26	5	39	11	24	6	40	9	22	7	38
Total Corr.	28	46	26	100	23	53	24	100	23	45	32	100