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Field assessment of bacterial communities and total trihalomethanes: implications for drinking water networks

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Highlights

- Biofilms are not routinely monitored in drinking water networks
- The microbial ecology of a tropical water network was characterised by molecular methods
- In this study, key engineered factors and microbiological parameters correlated
- Bacterial communities in bulk water were different from those in biofilms
- Biofilms must be monitored and controlled to preserve drinking water quality

Keywords: bacteria, biofilm, bulk water, chlorine, drinking water quality, disinfection by-products, operation and maintenance

Abstract

Operation and maintenance (O&M) of drinking water distribution networks (DWDNs) in tropical countries simultaneously face the control of acute and chronic risks due to the presence of microorganisms and disinfection by-products, respectively. In this study, results from a detailed field characterization of microbiological, chemical and infrastructural parameters of a tropical-climate DWDN are presented. Water physicochemical parameters and the characteristics of the network were assessed to evaluate the relationship between abiotic and microbiological factors and their association with the presence of total trihalomethanes (TTHMs). Illumina sequencing of the bacterial 16s rRNA gene revealed significant differences in the composition of biofilm and planktonic communities. The highly diverse biofilm communities showed the presence of methylotrophic bacteria, which suggest the presence of methyl radicals such as THMs within this habitat. Microbiological parameters correlated with water age, pH, temperature and free residual chlorine. The results from this study are necessary to increase the awareness of O&M practices in DWDNs required to reduce biofilm

53 formation and maintain appropriate microbiological and chemical water quality, in
54 relation to biofilm detachment and DBP formation.
55
56 **1 Introduction**
57 Biofilms are a group of microorganisms living as a consortium and attached to surfaces
58 due to the secretion of extracellular polymeric substances (EPS) (Srivastava and
59 Bhargava 2015). Biofilms are a successful survival strategy thanks to the presence of
60 EPS that protect cells against oxidant substances and improve availability of nutrients
61 as a result of organic matter retention. In drinking water distribution networks
62 (DWDNs), biofilms grow on any surface including pipes, valves, tanks, pumps and all
63 the fittings of the system. Biofilms are a major concern for water utilities. They can lead
64 to corrosion (Wang et al. 2011) and discoloured waters (Douterelo et al. 2014b), and
65 pathogens may be released to bulk water or detach and recolonize clean surfaces
66 (WHO 2008). They also act as precursors for the formation of disinfection by-products
67 (DBPs), and consequently, contribute to disinfectant decay (Wang et al. 2013a).
68
69 The control of microorganisms in DWDNs is predominantly conducted through
70 chemical disinfection. Chlorine was introduced to urban DWDNs at the beginning of the
71 20th century and it has been used since then to control pathogenic bacteria in drinking
72 water systems around the world (Sadiq and Rodriguez 2004). Chlorine remains popular
73 for its ease of use, relatively low cost and relative appropriate effectiveness, and
74 especially for its residual effects (Sadiq and Rodriguez 2004). However, the formation
75 of DBPs such as trihalomethanes (THMs) by the chlorine oxidation of natural organic
76 matter present in water sources (Rook 1974) changed the perspective that drinking
77 water safety was only related to pathogens.
78 It is now widely accepted that DBPs are potentially carcinogenic, teratogenic and
79 mutagenic substances (WHO 2008), and hence their control in water treatment works

80 has improved considerably. THMs and haloacetic acids (HAAs) are regulated by most
81 of the water authorities worldwide since they are the most persistent DBP species
82 found in drinking water (Hrudey 2009, Bull et al. 2011). THMs and HAAs are now
83 considered as largely unrelated to public health risks, but are currently considered
84 primarily as surrogates or indicators for other DBPs (Hrudey 2009, Bull et al. 2011).
85 Recently, emerging DBPs have increased with the changes of disinfection processes
86 and some of them, for example haloacetonitriles, are substantially more toxic than
87 THMs (Muellner et al. 2007). Consequently, the risk management associated with the
88 control of DBP formation should be addressed to reduce the precursors of these
89 substances, which may reduce other conceivable DBP formation and consequently
90 should not create an alternative DBP risk (Hrudey 2009).
91
92 Although there is increasing research into biofilms in DWDNs, their analysis has not yet
93 been included in routine operative and regulatory plans in the water industry. The
94 majority of biofilm studies in DWDNs have been conducted in temperate climate
95 geographic regions, with pipe materials and ages typical from industrialised countries
96 (Holinger et al. 2014, Kelly et al. 2014, Sun et al. 2014, Wang et al. 2014). Studies
97 coupling microbial, engineered and physicochemical factors together are very limited.
98 Wang et al. (2014) evaluated the influence of three factors (disinfectant, water age and
99 pipe material) on the microbial structure in a simulated drinking water network. Ji et al.
100 (2015) also studied a simulated system to evaluate the influence of three factors (water
101 chemistry, pipe material and stagnation) in plumbing systems, located at the outlet of
102 five water treatment plants. To date, there is only one field study reported in a tropical,
103 developing country (Ren et al. 2015) and therefore there is a clear need for further
104 studies in this area.
105 The current study characterised the physical properties, water chemistry and bacterial
106 communities of a DWDN located in a tropical-climate city. The aim of the study was to

explore the relationships between biotic and abiotic factors, and to further understand the potential involvement of bacteria in DBP formation. Such relationships are important to determine the dynamics occurring in a DWDN and to understand the complexity present in a real-world system. Furthermore, the results reported here are needed to inform operational strategies and to ultimately protect public health.

2 Materials and Methods

2.1 Drinking water distribution network

The study site was a DWDN in the city of Cali (Colombia), located at 995 meters above sea level and with an annual average temperature of 24.5 ℃ (23.8-25.1 ℃). The DWDN comprises four sub-networks originated from four surface water sources and five treatment facilities. These sub-networks operate by gravity, pumping, or by a combination of both. In total, the entire distribution network includes 2,951 Km of pipelines, 10 service reservoirs, 28 storage tanks, and 19 pumping stations in order to deliver water to 2,946,245 people.

Sampling was carried out within the biggest sub-network that is fed with the Cauca River, the second most important Colombian river, which is treated by conventional processes including primary and secondary chlorine disinfection. The main treatment facility feeding this sub-network has two open-air clarified-water reservoirs to be used as alternative water source during events in which the turbidity of raw water is higher than 1,000 NTU. Therefore, when turbidity readings from raw water exceed such threshold, the intake is closed and the treatment work is fed from the two storage reservoirs until turbidity readings drop below 1,000 NTU or for up to 9 hours. If turbidity readings do not drop below 1,000 NTU after this time, drinking water supply is interrupted and affecting almost 80% of the served population.

2.2 Sample collection

Pipe sections were taken from nine sites reporting leakages over a 3-week period; one site corresponded to a branch pipe (point 7) and the remaining eight to end of pipe networks (Figure 1). It is important to highlight that sampling points 3 and 7 are two different points but are closely located, and therefore they look overlapped in Figure 1. Pipe sections were taken during leakage repairs to enable biofilm collection. In order to preserve the biofilm and minimise any contamination from soil attached to external pipe walls, each pipe section was rinsed after removal with sterile water, wrapped in polythene and transported at 4 ºC for subsequent biofilm and DNA isolation. Bulk water samples were collected at the same time from the nearest household. Households' taps were flushed for 5 min, and then 6 L of drinking water were collected in sterile plastic bottles. Each sampling point was characterized by water age and pipe characteristics (i.e., pipe material, working age, and diameter).

Water age was determined from a hydraulic model applied to the sub-network and provided by the local water company. This model was implemented in the software Infowater 11.5 and EPANET 2.00.12. Raw water age data provided by the water company were processed with the software ArcMap 10.2.2 to create Thiessen polygons and then calculate water age zones classified in four ranges: low (<8.5 hours), medium (8.5-13.0 hours), high (68.0-146.0 hours) and very high (>146.0 hours).

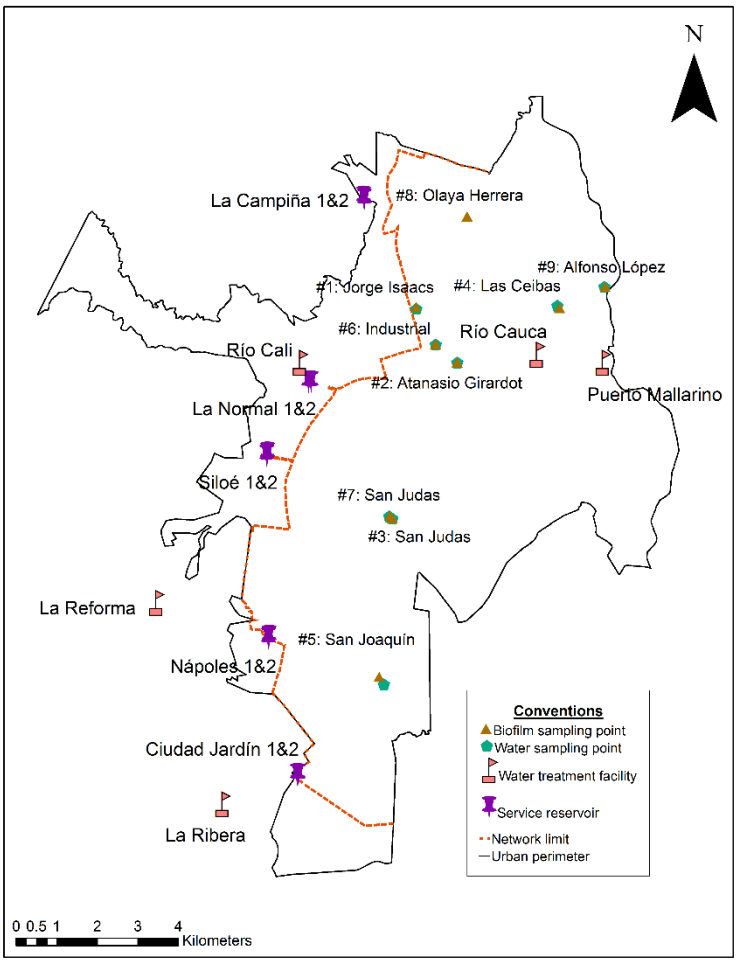

Figure 1. Location of sampling points

2.3 Physicochemical analysis

In-situ water parameters were measured as follows: (a) Temperature was measured by a bulb thermometer; (b) pH by portable meter kit (HQ40d HACH Cat. No. HQ40D53000000, Loveland, CO) coupled to a pH electrode; and (c) total and free chlorine by the DPD method using a HACH colorimeter II (Cat. No. 58700-00, Loveland, CO). Quantification of Total Organic Carbon (TOC) and total THMs (TTHMs) in bulk water was carried out by an accredited laboratory following standard methods (Eaton et al. 2005). Equipment for TOC and TTHMs analysis were total carbon analyser (Shimadzu TOC 5050A, article number 3750 K3-2, Columbia, MD) and gas

chromatograph (HP 5890, Wilmington, DE and Agilent Technologies 7890B, Santa Clara, CA.), respectively.

TOC and dry-biomass were measured by scrapping a defined area on the pipe surface of 75 cm^2 in triplicate. For TOC measurement in biofilms, scrapped biofilms were resuspended in 250 mL of deionized water. For dry biomass, scrapped samples were dried at 105 ℃, for 24 hours and dry biomass per area (unit dry biomass) was calculated. Due to the presence of a high amount of tubercles in the cast iron (CI) pipe of point 2, it was not possible to calculate the unit dry biomass for this sample (Figure 2). On the contrary, the surface of asbestos cement pipelines was flat, then scrapping biofilms from them was a normal procedure. Detachment of asbestos fibres was observed during scrapping biofilms.

Figure 2. Tubercles in cast iron pipe – Sampling Point 2

2.4 Molecular methods

After rinsing the internal walls of the pipelines in the laboratory, biofilm samples were collected by scrapping in triplicate using a sterile frame with area equal to 25 cm^2 and a sterile spatula. DNA isolation was carried out using the Power Biofilm DNA Kit (MoBio, USA) according to the manufacturer's instructions. In total 6 L of water were filtered for

every sampling point (2 L for each triplicate) through nitrocellulose filters (0.22 μm pore-size); filters were further processed for DNA extraction using the Power Water DNA Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions.

Sequencing of DNA extracted from biofilm and water samples was performed by Illumina MiSeq Technology using the Illumina PE MySeq reagent Kit v3 according to the manufacturer's guidelines (Illumina, USA) and performed by the Molecular Research DNA Lab (Shallowater, TX, USA). 2-5 ng/μL of DNA per sample (n=53) was used for amplification (no replicates per sample were generated) and the V4 variable region of the 16S rRNA gene was amplified using primers 515F/806R (Caporaso et al. 2011). Sequence data were processed using Mr DNA analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). In summary, sequences were merged, depleted of barcodes and primers, sequences < 150 bp and with ambiguous base calls were removed from further analysis. Sequences were denoised and chimeras removed. Operational Taxonomic Units (OTUs) were defined by clustering at 3% divergence (97% similarity) and were taxonomically classified using BLASTn against a curated database derived from Greengenes, RDPII and NCBI (DeSantis et al. 2006) (http://www.ncbi.nlm.nih.gov/, http://rdp.cme.msu.edu).

The total number of reads generated per sample ranged between 7780-304912 and between 13759-238406, for biofilm and bulk water samples, respectively. The number of reads that passed quality scores ranged between 7240-256972 for biofilm and between 10257-101379 for bulk water samples. The data set (number of reads per sample) was not normalised or rarefacted to assess alpha-diversity, in order to avoid losing information from potential important sequences (McMurdie and Holmes 2014).

2.5 Data analysis

The alpha-diversity of the samples at 97% sequence similarity cut off was analysed by Margalef and Shannon community richness and diversity indices, respectively, which were calculated with Primer6 software (PRIMER-E, Plymouth, UK). The medians and means of such indices were statistically compared by t-test and Mann Whitney U test using the software IBM SPSS Statistics 21. Statistical tests were carried out to assess associations in both bulk water (species relative abundance (RA) and physicochemical characteristics and water age) and biofilms (species RA and pipe characteristics, water age, and unit dry biomass). The association of the RA of bacteria at species level and the characteristics of the sampling points were determined by multi-dimensional scale analysis (MDS), by means of Bray-Curtis similarity metrics, and analysis of similarities (ANOSIM) using Primer6 (Clarke and Warwick 2001). Spearman correlations were applied to determine the relationships between biofilm parameters and water characteristics; Shapiro-Wilk tests were run in IBM SPSS Statistics 21 to determine normal distribution of variables. All statistical results were contrasted with significance level equal to 0.05.

3 Results

3.1 Characterisation of the network, water quality and biotic parameters

A summary of the network characteristics along with the corresponding water quality and biotic parameters is presented in Table 1. The predominant pipe material was asbestos cement (AC), with the exception of point 2, which corresponded to a CI pipeline. The water age for biofilm and water samples are comparable with the exception of point 4, where statistically significant differences were found. Since water samples were collected from taps in households located as close as possible from leakage sites where pipes were replaced to allow the collection of biofilm samples,

239 water-related variables were not associated with biofilm-related characteristics since
240 the sampled pipeline was not directly supplying the sampled household in every case.
241

Table 1. Network characteristics, water quality and biotic parameters and descriptive statistics

Sampling point No.	Network characteristics			Water age (hours)				Water quality						Biofilm characteristics
	Pipe material	Pipe age (Years)	Pipe diameter (Inches)	Water sampling point		Biofilm sampling point		Temperature (°C)	pH (Units)	Free res. chlorine (mg/L)	Total res. chlorine (mg/L)	TTHMs (μg/L)	TOC (mg/L)	Unit dry biomass (mg/cm^2) *
				Value	Classification	Value	Classification							
1	AC	56.45	4	13.95	High	13.99	High	26	7.32	1.20	1.35	30.3	0.819	1.41
2	CI	57.08	4	9.71	Medium	9.71	Medium	25	7.16	1.66	1.76	28.9	10.104	-
3	AC	33.88	3	12.37	Medium	12.37	Medium	25	7.35	1.28	1.43	23.5	1.210	1.45
4	AC	35.24	4	146.01	Very high	8.12	Low	-	7.04	0.12	1.61	36.7	1.453	0.29
5	AC	24.55	4	14.41	High	15.59	High	25	6.76	1.30	1.45	28.3	1.527	0.38
6	AC	42.81	8	10.06	Medium	10.06	Medium	26	7.01	1.12	1.33	35.5	1.739	3.23
7	AC	33.77	12	11.71	Medium	11.47	Medium	28	7.02	1.15	1.21	30.8	2.139	0.23
8	AC	52.85	4	13.23	High	13.23	High	26	6.86	0.86	1.02	38.6	1.849	2.09
9	AC	50.96	4	8.00	Low	8.26	Low	27	6.62	1.31	1.57	33.3	2.157	3.34
							Median	*26*	*7.02*	*1.20*	*1.43*	*30.80*	*1.739*	*1.41 ** *
							Mean	*26*	*7.02*	*1.11*	*1.41*	*31.76*	*2.555*	*5.20 ** *
							Standard deviation	*1*	*0.23*	*0.40*	*0.21*	*4.47*	*2.699*	*16.72 ** *
							Coefficient of variation (CV)	*3.85%*	*3.26%*	*36.25%*	*14.76%*	*14.09%*	*105.64%*	*321.53% ** *

** Average of replicates | ** Descriptive statistics of all data (including replicates) | AC: asbestos cement | CI: cast iron*

244 Water quality characteristics including temperature, pH, free residual chlorine and TTHMs were
245 within expected ranges, except for the lowest concentration of chlorine (0.12 mg/L) that was
246 measured at point 4, which corresponded to the highest water age (146 h). Such concentration of
247 free residual chlorine is considered very low according to the recommended values set for drinking
248 water by local regulators in Colombia (0.3-2.0 mg Cl_2/L) (Ministerio de la Protección Social 2007).
249 TOC measured in biofilm samples presented a lower variation compared to the variation in biofilm
250 mass. All concentrations of TOC in bulk water were reported as lower than the detection limit (<0.8
251 mg/L). Regarding TTHMs, concentrations in all water samples were lower than 40 μg/L, which falls
252 below the maximum concentration of TTHMs allowed in drinking water according to Colombian and
253 UK regulations (100 and 200 μg TTHM/L, respectively) (Ministerio de la Protección Social 2007).
254
255 Several water physicochemical characteristics were correlated to identify the dynamics present in
256 the studied network; results are presented in Table 2. Significant negative correlations were found
257 between total residual chlorine and temperature (p=0.019), free residual chlorine and water age
258 (p=0.004) and free residual chlorine and TTHMs (p=0.017). Weak negative correlations were
259 identified between temperature and free residual chlorine (p=0.052, slightly higher than the level of
260 significance) and between pH and TTHMs (p=0.042). A positive correlation was observed between
261 temperature and TTHMs (p=0.003).
262
263 In relation to biotic factors, unit dry biomass presented the highest variation among all the variables
264 analysed. Although calculation of the unit dry biomass in the CI pipe sample (sampling point 2) was
265 not possible, the highest content of global dry biomass and TOC in the biofilm (233.7 - 3,664.8 mg)
266 (10.10 mg/L; Table 1) were found in this point. Concerning biofilms, correlations presented in Table
267 3 indicated that there is a strong positive relationship between unit dry biomass and pipe age
268 (p=0.008). Additionally, water age was negatively correlated with TOC in biofilms but no
269 association was identified between water age and unit dry biomass, possibly related to the
270 influence of pipe age/material over the later variable.
271

Table 2. Spearman correlation coefficients for bulk water parameters

Variables ↓→	Richness index (Margalef)	Diversity index (Shannon)	Water age	pH	Temperature	Total residual chlorine	Free residual chlorine	TTHMs
Richness index (Margalef)	**-**							
Diversity index (Shannon)	*C.N.T*	**-**						
Water age	0.277	0.315	**-**					
pH	0.365 ***	**0.414 ***	*C.N.T*	**-**				
Temperature	-0.355 ***	-0.238	*C.N.T*	*C.N.T*	**-**			
Total residual chlorine	0.074	0.149	-0.067	0.117	**-0.476 ***	**-**		
Free residual chlorine	-0.251	-0.273	**-0.533 ****	-0.033	-0.401 ***	*C.N.T*	**-**	
TTHMs	-0.259	0.049	0.060	**-0.594 ***	**0.802 ****	*C.N.T*	**-0.671 ***	**-**

Correlation is significant at the 0.05 / 0.01** level (2-tailed)*

**** Correlation coefficient slightly higher than 0.05 → 0.052 ≤ p-value ≤ 0.089*

C.N.T: correlation not tested

Table 3. Spearman correlation coefficients for biofilm parameters

Variables ↓→	Richness index (Margalef)	Diversity index (Shannon)	Water age	Pipe age	Unit dry biomass	TOC
Richness index (Margalef)	**-**					
Diversity index (Shannon)	*C.N.T*	**-**				
Water age	0.364 ***	0.375 ***	**-**			
Pipe age	**-0.404 ***	**-0.512 ****	*C.N.T*	**-**		
Unit dry biomass	**-0.582 ****	**-0.733 ****	-0.196	**0.559 ****	**-**	
TOC - biofilm	-0.294	-0.357	-0.552 ***	0.334	0.259	**-**

Correlation is significant at the 0.05 / 0.01** level (2-tailed)*

**** Correlation coefficient slightly higher than 0.05 → 0.059 ≤ p-value ≤ 0.068*

C.N.T: correlation not tested

3.2 Characterization of the bacterial community structure of biofilms and bulk water

The RA to phylum and genera level for water and biofilm samples can be observed in Figure 3 and Figure 4, respectively; groups with RA lower than 10% were grouped in the category “Others”. Water samples were dominated by *Proteobacteria* (43-98%), followed by *Cyanobacteria* (0.05-41%), and *Firmicutes* (0.84–34%). Different genera were dominant in each water sample, but highly abundant genera in all samples were *Bacillus*, *Brucella*, *Cyanothece*, *Methylobacterium*, and *Phyllobacterium* (17.47-95.91%). Within the biofilm samples, the predominant phyla were *Proteobacteria* (26-72%), followed by *Firmicutes* (3–30%) and *Actinobacteria* (8-19%), and the most abundant genera in all samples were *Acinetobacter*, *Alcaligenes*, *Alcanivorax*, *Bacillus*, *Deinococcus, Holophaga*, and *Thermoflavimicrobium* (4.34–43.92%).

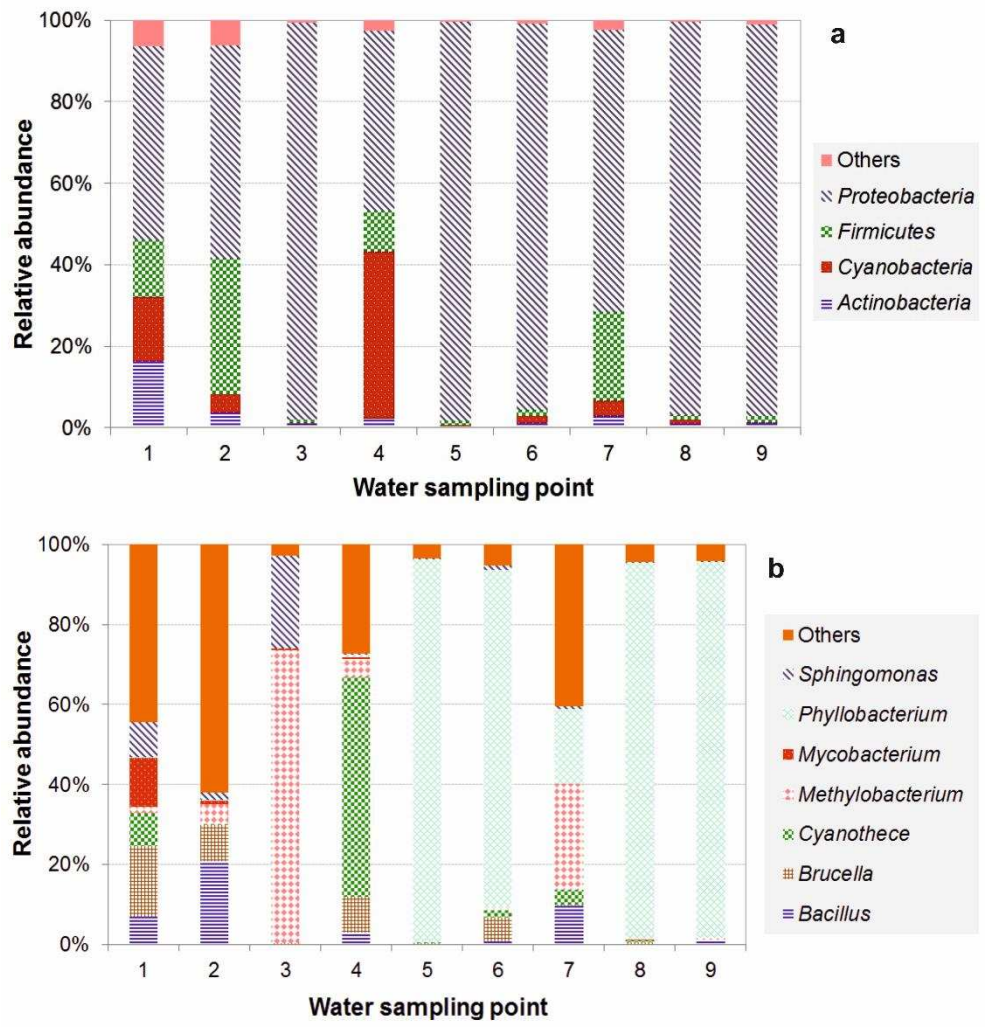

Figure 3. Relative abundance of bacterial to phylum level (a) and genus level (b) in water samples

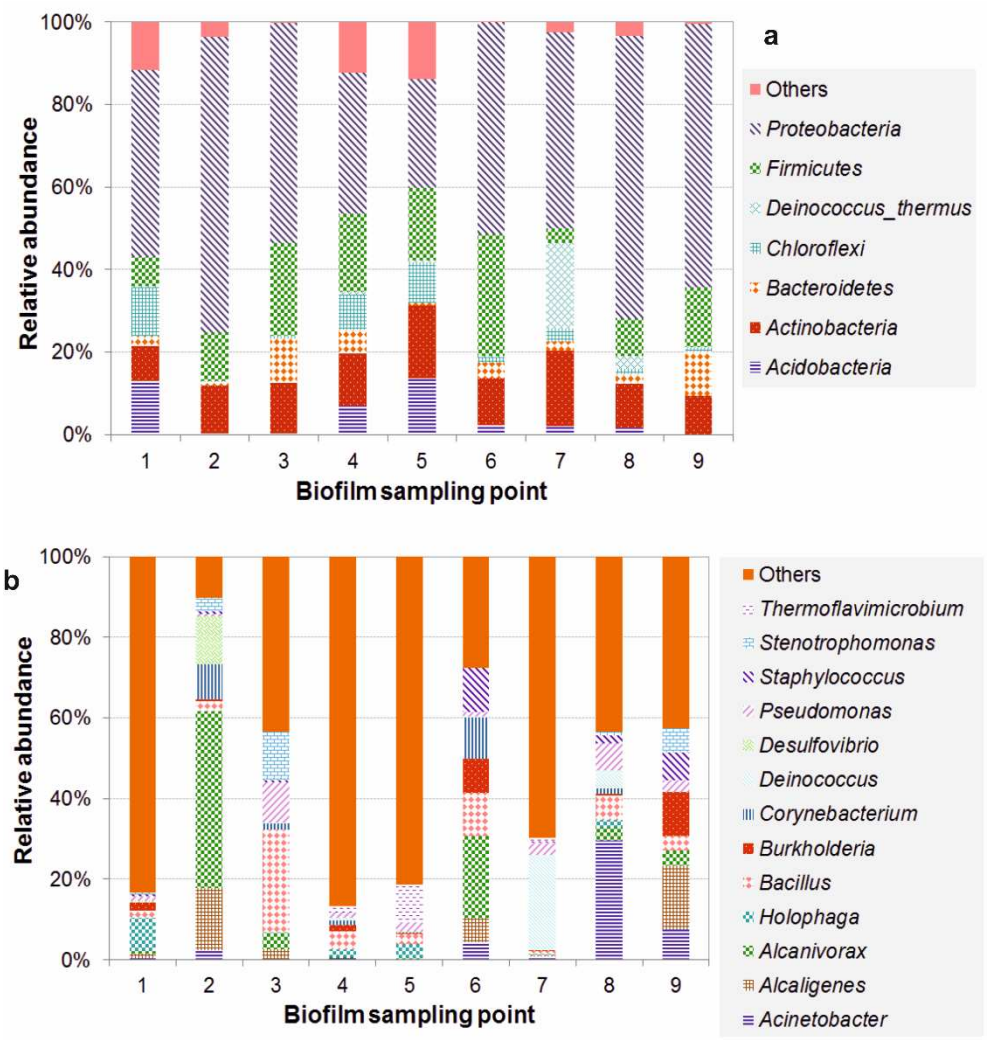

Figure 4. Relative abundance of bacterial groups to phylum (a) and genus level (b) in biofilm samples

The ANOSIM test was applied to assess the relationships between species RA and engineered factors (Table S1). With regard to water samples, relationships between species RA and factors water age, free chlorine, pH, and free chlorine and water age combined were statistically significant. For biofilm samples, the ANOSIM test results were statistically significant for the factors pipe age and water age, and unit dry biomass and pipe age combined. Factor “Pipe material” was not included in the statistic tests due to only one sample was collected from CI pipeline, then comparison between CI and asbestos cement would not be statistically strong. Habitat was also a factor influencing the RA of species. MDS analysis also revealed that habitat and pipe material were the factors which showed clear groups of bacteria RA by categories (Figure 5a and Figure 5b, respectively). This means that RA of bacteria is clearly differentiated between habitats biofilm

and bulk water. Similarly, RA of bacteria is clearly grouped for pipe materials CI and asbestos cement. Importantly, methanotrophic organisms were observed in biofilm samples such as *Methylobacterium* (RA=1.16%) and *Methylosinus* (RA=3.34%). In bulk water, Spearman correlations with TTHMs were statistically significant for the genus *Methylobacter* (ρ=0.437; p-value=0.023) and *Methylobacterium* (ρ=-0.417; p-value=0.030).

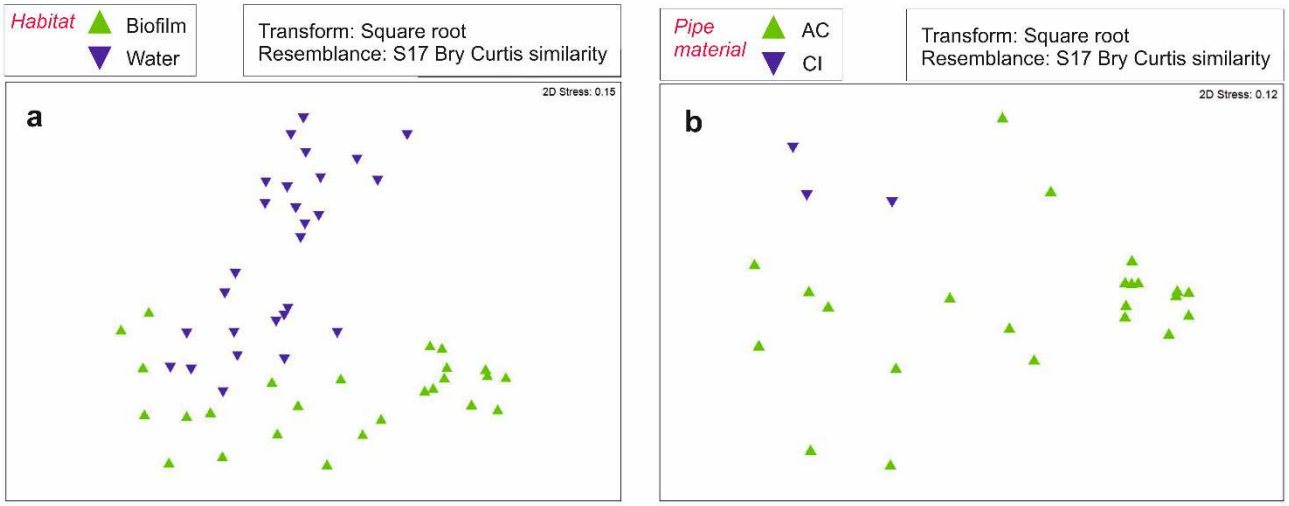

Figure 5. Non-metric MDS analysis of bacterial relative abundance. Factors Habitat (a) and pipe material -biofilm samples- (b)

3.3 Microbial richness and diversity

Richness and diversity were calculated (Table S2) according to factors such as habitat, pipe age, and water age. Spearman correlations were applied to test the relationships between indices and engineered factors, for both water (Table 2) and biofilm (Table 3) samples. Results from t-test indicated that the means of richness and diversity indices of biofilm samples are higher than those of water samples. Negative correlations were found between biofilm indices and pipe age and unit dry biomass. Comparisons of medians indicated that the median of richness and diversity indices of biofilm samples with high water age was higher than those with medium water age. In water samples, median of richness index with very high water age was higher than those with low water age. Richness index in bulk water negatively correlated with variable temperature, and both indices

positively correlated with pH. Positive correlations between water age and richness and diversity indices were only found in biofilm samples.

4 Discussion

4.1 Water quality, biotic parameters and their relationships with engineered factors

DBP formation is influenced by parameters such as pH, temperature, TOC, chlorine dosage, and water age. The interactions observed between these parameters and TTHMs confirm the dynamics occurring in tropical DWDNs in relation to THM formation: increasing water age promotes decay of free residual chlorine since the disinfectant is volatile and reacts with organic and inorganic matter, likewise the concentrations of THMs were increasing. In temperate climates, Nescerecka et al. (2014) and Wang et al. (2014) also identified depletion of disinfectant with higher water age in a real-scale and simulated DWDNs, respectively. THM formation is directly influenced by pH and temperature (Liang and Singer 2003), and such a relationship was evidenced by the current results, which show a strong correlation between TTHMs and temperature. However, a negative relationship between TTHMs and pH was found, which may be related to the narrow range of pH data evaluated (Table 2); higher concentrations of THMs have been identified with higher pH in the range of 5-8 in laboratory experiments (Liang and Singer 2003, Wang et al. 2012). The influence of pH on DBP production remains unclear. Positive and negative correlations between pH and THMs have been reported in other studies, such as in a Canadian DWDN by Rodriguez and Sérodes (2001). Therefore, further research is needed to determine the actual influence of pH on the production of DBPs particularly under tropical climate conditions.

4.2 Characterization of the bacterial community structure of biofilms and bulk water

Actinobacteria, *Firmicutes*, and *Proteobacteria* were the common phylotypes in the two habitats, with the later community being the dominant group in the entire set of samples. Recent studies from other geographic regions have reported that both water and biofilm samples were dominated by *Proteobacteria* (Douterelo et al. 2013, Holinger et al. 2014, Kelly et al. 2014, Sun et al. 2014, Wang et al. 2014, Mahapatra et al. 2015, Ren et al. 2015). This study also confirmed the

predominance of this phylum in the drinking water bacterial community. Several studies have reported the presence of microorganisms, which are ubiquitous in drinking water biofilms. In agreement with this observation, Henne et al. (2012) found that biofilm communities sampled at nearby points in a DWDN were similar, thus hypothesising that physically related biofilm communities will show similar community structures when developed over the years. In contrast, the spatial distribution of biofilms analysed in the current study revealed that dominant bacterial communities in the tropical DWDN (25-57 years old) were different in each sampling point. This may be related to the unstable hydraulic conditions of this water network, which may partially remove biofilm components, then altering the structure of bacterial communities. Similarly, in a laboratory-based full scale DWDN, high flow variations indicated the promotion of young biofilms with more cells and less EPS, by the potential cyclic removal of the first layers of the biofilms (Fish et al. 2017).

Other relevant microorganisms identified in this study due to their public health implications are *Cyanobacteria*. *Cyanobacteria* are a diverse group of photosynthetic microorganisms widespread in aquatic and terrestrial ecosystems. The main genus associated with *Cyanobacteria* in the current study was *Cyanothece*, which are not cytotoxin producers (Jakubowska and Szeląg-Wasielewska 2015). The source for the high presence of *Cyanobacteria* in the analysed samples is likely to be one of the reservoirs of clarified water located at one of the treatment facilities. Revetta et al. (2011), by analyzing 16S rRNA gene clone libraries derived from DNA extracts of 12 samples and comparing to clone libraries previously generated using RNA extracts from the same samples, found that these bacteria may be active in chlorinated drinking water. Since drinking water pipes are dark environments, how *Cyanobacteria* survive in these is not clear yet.

Furthermore, several potentially pathogenic and opportunistic microorganisms were also observed in biofilm and bulk water samples. For example, *Acinetobacter* was detected in biofilm (Mahapatra et al. 2015) and has been previously found in wastewater treatment reactors and contaminated clinical devices (Carr et al. 2003, Lin et al. 2003). *Brucella* was detected in water samples; this

383 genus comprises 11 species, 10 of them are associated with human infections (Scholz et al. 2010,
384 Xavier et al. 2010). *Staphylococcus* is an opportunistic pathogen detected in low percentages in
385 bulk water and biofilm samples here. This genus constitutes a major component of the human
386 microflora (Heilmann et al. 1996), and has been classified as a moderate biofilm former (Simões et
387 al. 2007) able to colonize hospital devices. The source of this opportunistic pathogen could be the
388 surface raw water, since the river basin was highly contaminated due to anthropogenic activities
389 (Pérez-Vidal et al. 2016). Mahapatra et al. (2015), by a laboratory study carried out in a subtropical
390 region in India, also identified *Staphylococcus aureus* in bulk water and 24-hour biofilms formed
391 from incubation of drinking water collected in kitchen taps.
392
393 The presence of some bacteria identified in this study could be related to soil sources. Particularly,
394 the genus *Holophaga* has been previously isolated from black anoxic freshwater mud from a ditch
395 in Germany (Liesack et al. 1994). Similarly, *Thermoflavimicrobium* and *Phyllobacterium* were
396 highly abundant in water samples at several sampling points and these organisms have been
397 previously reported in soil-related habitats (Rojas et al. 2001, Yoon et al. 2005).
398
399 Concerning the process of DBP production in DWDNs, several methylotrophic bacteria were
400 detected in most of the bulk water samples. *Methylobacterium* is methylotrophic organism, which
401 are ubiquitous in different environments including soil, freshwater, drinking water and lake
402 sediments (Leisinger et al. 1994). This genus is able to degrade DBPs such as HAAs (particularly
403 dichloroacetic acid) (Zhang et al. 2009), and they are biofilm formers with high resistance to
404 sodium hypochlorite disinfection in single-species biofilm (Simões et al. 2010). Importantly,
405 *Methylobacterium* has not been found yet in non-chlorinated DWDNs (Martiny et al. 2005, Liu et al.
406 2014). Therefore, the presence of these microorganisms in DWDNs should be considered as a
407 potential indicator of DBP presence, despite of *Methylobacterium* presents facultative metabolism
408 and it is able to use a wide range of organic compounds as sources of carbon and oxygen (Gallego
409 et al. 2005).
410

411 It has been observed previously the high structural and compositional variability within biofilms
412 growth under similar hydraulic conditions in chlorinated DWDS in the UK (Fish et al. 2015,
413 Douterelo et al. 2016); this study confirms the high natural heterogeneity of biofilms developed
414 within the same pipe material under tropical conditions. The way biofilm heterogeneity influences
415 ecological processes taking place in different DWDNs must be addressed, and should be
416 considered when the microbial structure of different pipe materials is assessed.
417
418 **4.3 Influence of network characteristics on bacteriological parameters**
419 Higher richness and diversity were found in biofilms when compared to bulk water samples, which
420 can be related to the favourable conditions offered by this micro-environment for bacteria survival
421 such as protection against disinfectant, bulk flow and higher availability of nutrients. Douterelo et
422 al. (2013) also found higher diversity and richness in 28 day old biofilms in a chlorinated DWDN,
423 indicating that only some bacteria in the bulk water have the ability to attach to pipe walls. For
424 instance, *Bacillus* was the only common genus detected in the two habitats in this study. *Bacillus*
425 can form spores that protect them from disinfection and when the environmental conditions are
426 favourable they start developing as active cells (Checinska et al. 2015). Conversely, Henne et al.
427 (2012), based on 16S r RNA fingerprints of extracted DNA and RNA, found that bacterial richness
428 (Margalef index) was higher in bulk water than biofilm samples from a 20-year old and chlorinated
429 DWDN. The authors hypothesized that only those bacteria that can actively contribute to the
430 succession of the biofilm were successful in colonising biofilms, while bacteria that cannot fill
431 perfectly the narrow niches in biofilms vanished over time. Identifying those bacteria more prone to
432 form biofilms can be used to inform control strategies to target specific microorganisms and avoid
433 further biofilm development.
434
435 The relationship found between pipe age and unit dry biomass may be related to the detachment
436 of some asbestos fibres, which was observed during biofilm scrapping from the sampled pipes and
437 is representative of the potential wear of the pipe material in time due to biological activity. The
438 influence of removal of such fibres was described by Wang et al. (2011), who tested the biological

activity in 53- 54-year old sections of asbestos pipes. By establishing microbial activity of iron-reducing bacteria (IRB), sulphate reducing bacteria (SRB) and biofilm-former bacteria in the patina layer (porous layer, mainly composed of microbial biomass along with interwoven asbestos fibres) of those pipes sections, they established that such microbial activity leads to deterioration of asbestos pipes and potential leakages (Wang et al. 2011). In this study, IRB including *Geobacter* were observed in biofilm samples, corresponding to 24-56-year old pipe sections and SRB such as *Desulforegula*, *Syntrophobacter* and *Clostridium* were also detected. Although these microbial groups were present with low RA, their presence may indicate the presence of an anoxic layer attached to asbestos pipes, which promotes the acidification of the media due to the production of organic acids from anaerobic metabolism, leading to local pH decrease. This facilitates the biodegradation of the pipe wall by the weathering and dissolution of the acid-receptive minerals in hydrated cement matrix, thus, creating pitting and voids (Wang et al. 2011). *Clostridium* was also identified in drinking water biofilms incubated, for 180 days, in rotating annular reactors, with continuous flowing water at average temperature of 25±1.5 ℃ (Chao et al. 2015).

The influence of pipe material on the bacteriological composition of biofilm samples is reflected on the presence of SRB such as *Desulfovibrio*, which was present exclusively in CI pipes. *Desulfovibrio* finds a favourable environment in this type of pipes, most likely promoting its corrosion and potentially leading to failure. Similar high abundance of this genus was detected by Ren et al. (2015) in 11-year old CI pipes however, Sun et al. (2014) reported low abundance of *Desulfovibrio* (0.01-0.19%) in 20-year old CI pipes. The tubercles found in the sampled piece of pipe (Figure 2) may create a favourable environment for the growth of these bacteria. Additionally, such tubercles can reduce the hydraulic capacity of the pipes due to the formation of scales and the accumulation of iron and manganese particles (Douterelo et al. 2014a). Several studies have confirmed the impact of pipe material over the structure of microbial communities in biofilm samples collected from simulated DWDNs (Wang et al. 2014), bench-scale pipe section reactors (Mi et al. 2015), real-scale DWDNs (Ren et al. 2015), and laboratory reactors (Chao et al. 2015).

Although there is not an absolute consensus about the best material to minimize biofilm growth, in general, plastics appear to be advisable over metals and cements (Fish et al. 2016).

With regard to water age, the effect of this factor on bacterial biofilms may be associated with the relationship between this parameter and other water physicochemical characteristics as previously discussed. In addition, low concentrations of chlorine, stagnation and low velocities conditions associated with high water age lead to increase cells counts in bulk water (Nescerecka et al. 2014) and favour biofilm formation (Fish et al. 2016). Water age is considered as a factor influencing the biological stability of drinking water (Prest et al. 2016) and the microbial composition of building plumbing materials (Ji et al. 2015). This was corroborated by Wang et al. (2014), who established in simulated-DWDN biofilm samples that water age, disinfectant, and pipe material interact with each other to create distinct physicochemical conditions and ecological niches, in which various microbes can be selected and enriched. Spearman's correlations showed no associations between indices and concentrations of free chlorine in this study.

The influence of other key water physicochemical factors, including pH and temperature, on the microbial ecology of DWDN were also assessed here. Results indicated statistically significant differences among bacterial species for pH, which was also correlated positively with both richness and diversity. Due to the relationship between pH and alkalinity, and the governance of this factor over the relative proportions of hypochlorous and hypochlorite, which present different disinfection efficacies, pH is impacting the variability in the water bacterial community as was found by Sun et al. (2014). Temperature and richness were negatively correlated; similar results were found by Henne et al. (2013) by comparing microbial communities of cold and hot water (ΔT=41 ℃ approximately); cold water presented higher community diversity and high stability over time. The present study considered ΔT=3 ℃, which corresponds to typical temperature values for tropical cities with hot weather.

5 Implications for O&M activities in DWDN

This study approached the role of biofilms and bulk water bacterial communities in two key processes: i) the relationship between them and DBPs and ii) their pathogenic significance. Degradation and formation of DBPs has been previously associated with biofilms and the presence of certain microorganisms such as methylotrophic bacteria (Fang et al. 2010a, Fang et al. 2010b, Wang et al. 2012, Pu et al. 2013, Wang et al. 2013a, Wang et al. 2013b, Xie et al. 2013). However, this study indicates that the formation of DBPs in the DWDNs is a complex process since precursory and degradation biological reactions can simultaneously occur. Hence, TTHMs and HAAs modelling efforts should consider the biological component on DBP chemistry, especially in the models where the correlation coefficients are low, and then the predictability of these substance concentrations may be improved. Recently, Abokifa et al. (2016) included reaction chlorine-biomass (biofilm and planktonic cells) in a model to predict THMs in drinking water pipes under turbulent flow. Similarly, a CFD model was developed by the authors of this study to simulate the chloroform and dichloroacetonitrile formation potentials from biofilm chlorination, under laminar, transitional, and turbulent flow. Manuscript is being prepared for further publication.

Prevention and removal of biofilms is a key concern for water utilities due mainly to their potential as reservoirs of pathogens. Flushing water pipes has been proved as a suitable technique to remove material attached to internal pipe surfaces but it is inefficient to completely detach biofilms (Abe et al. 2012, Douterelo et al. 2013, Fish et al. 2016). Advanced water treatment processes such as membrane filtration has been proved successful in highest reduction of number of microorganisms in biofilms collected at the inlet of a DWDN (Shaw et al. 2014). However, a recent study argues that is impossible to prevent biofilm accumulation but high flow variation could be used to promote young biofilms, which are more vulnerable to disinfection (Fish et al. 2017). In the case of this studied network, avoiding uncontrolled biofilm detachment and contamination of bulk water is particularly difficult, as it exhibits specific O&M challenges associated with emptying of the network due to the interruption of operation of the water treatment facilities, pumping operation, closing/opening valves during leakages repairs and pipelines and accessories replacement. This

521 may lead to favour the formation of young biofilms, however it is important to consider that biofilms,
522 planktonic cells, and detached biofilm clusters are also DBP precursors, and then biofilm control
523 must go beyond disinfection.
524
525 Furthermore, CI pipes represent 10% of the total length of the pipelines and asbestos 30%; and
526 2,400 leakages were repaired in 2014. These O&M activities cause uncontrolled and partial
527 removal of sediments and biological material and allow the entrance of external particles, which all
528 together could be promoting microbial growth in the network. Future plans for pipeline
529 replacements should avoid the use of metal and cement pipes and instead promote the use of pipe
530 materials with more stable bio-chemical and physical conditions. It is also advisable to minimize
531 the events that alter the normal operation of the DWDN to reduce biofilm detachment; controlled
532 cleaning procedures of pipes such as flushing should be carried out to reduce the amount of
533 nutrients available for microorganisms in bulk water and biofilms and avoid alterations of the
534 organoleptic conditions of drinking water for the consumers. More importantly, the efforts carried
535 out in protecting water sources and improving water treatment could be useless if suitable O&M
536 practices are not applied in the DWDNs in order to preserve the safety of drinking water delivered
537 to the customers.
538
539 **6 Conclusions**
540 To the authors' knowledge, this is the first study that characterised the bacterial community
541 structure in both water and biofilm habitats in a tropical-climate DWDN. It also explored the
542 relationships between biotic and engineered factors, with a specific focus on DBPs. The application
543 of sequencing analysis represents a step forward in the study of microbiological aspects of
544 DWDNs in tropical-climate countries. Most of the bacterial communities identified in this work have
545 also been found in temperate-weather water systems. This may indicate that some drinking water
546 bacteria are ubiquitous and that treatment and engineered environments shape the bacterial
547 communities in a specific way. This study found that, similarly to temperate-climate DWDNs,
548 bacterial communities in sampled biofilms are different from those in bulk water, with the former

549 more diverse and richer. Pipe age, water age, free chlorine, pH and temperature were associated
550 with microbiological parameters indicating that these are key to control microbial growth. Deeper
551 analysis should be done in terms of the influence of temperature variation in tropical-climate
552 DWDNs. Pipe material also influenced the microbial ecology of DWDNs; *Desulfovibrio* was
553 identified exclusively in the CI pipe. Methylotrophic bacteria were found in biofilms and bulk water;
554 these microorganisms are known to be able to degrade DBPs as haloacetic acids. Design and
555 O&M of DWDNs should consider all the possible procedures to minimise biofilm growth to manage
556 both biological and chemical stability of drinking water: to reduce nutrient concentrations in the
557 water treatment, flushing dead end zones and after repair and replacement activities, reduce water
558 age, and use bio-stable pipe materials.
559

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568

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570

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