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1 **Field assessment of bacterial communities and total trihalomethanes:**

2 **implications for drinking water networks**

3 Carolina Montoya-Pachongo*[a][1]

4 [a] Institute for Public Health and Environmental Engineering (iPHEE). School of Civil Engineering,

5 University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK

6 *Corresponding author's e-mail: cncm@leeds.ac.uk. Tel. +44 (0)113 34 39 137

7 Isabel Douterelo[b]

8 [b] Pennine Water Group, Department of Civil and Structural Engineering, Sir Frederick Mappin

9 Building, The University of Sheffield, Mappin St., Sheffield, S1 3JD, UK

10 Catherine Noakes[c]

11 [c] Institute for Public Health and Environmental Engineering (iPHEE). School of Civil Engineering,

12 University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK

13 Miller Alonso Camargo-Valero [d,e]

14 [d] Institute for Public Health and Environmental Engineering (iPHEE). School of Civil Engineering,

15 University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK

16 [e] Departamento de Ingeniería Química, Universidad Nacional de Colombia, Campus La Nubia,

17 Manizales, Colombia

18 Andrew Sleigh [f]

19 [f] Institute for Public Health and Environmental Engineering (iPHEE). School of Civil Engineering,

20 University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK

21 Juan-Carlos Escobar-Rivera [g]

22 [g] Departamento de Producción de Agua Potable, EMCALI EICE ESP. Calle 59 No. 12B–45, Cali,

23 Colombia

24 Patricia Torres-Lozada [h]

[1] Permanent e-mail address: caromoto@gmail.com

25   h Grupo de Investigación Estudio y Control de la Contaminación Ambiental (ECCA). Calle 13 No.

26   100-00, Cali, Colombia

27

28   **Highlights**

29   • Biofilms are not routinely monitored in drinking water networks

30   • The microbial ecology of a tropical water network was characterised by molecular methods

31   • In this study, key engineered factors and microbiological parameters correlated

32   • Bacterial communities in bulk water were different from those in biofilms

33   • Biofilms must be monitored and controlled to preserve drinking water quality

34

37

38   **Abstract**

39   Operation and maintenance (O&M) of drinking water distribution networks (DWDNs) in

40   tropical countries simultaneously face the control of acute and chronic risks due to the

41   presence of microorganisms and disinfection by-products, respectively. In this study,

42   results from a detailed field characterization of microbiological, chemical and

43   infrastructural parameters of a tropical-climate DWDN are presented. Water

44   physicochemical parameters and the characteristics of the network were assessed to

45   evaluate the relationship between abiotic and microbiological factors and their

46   association with the presence of total trihalomethanes (TTHMs). Illumina sequencing of

47   the bacterial 16s rRNA gene revealed significant differences in the composition of biofilm

48   and planktonic communities. The highly diverse biofilm communities showed the

49   presence of methylotrophic bacteria, which suggest the presence of methyl radicals such

50   as THMs within this habitat. Microbiological parameters correlated with water age, pH,

51   temperature and free residual chlorine. The results from this study are necessary to

52 increase the awareness of O&M practices in DWDNs required to reduce biofilm formation

53 and maintain appropriate microbiological and chemical water quality, in relation to biofilm

54 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 (DWDNs),

62 biofilms grow on any surface including pipes, valves, tanks, pumps and all the fittings of

63 the system. Biofilms are a major concern for water utilities. They can lead to corrosion

64 (Wang et al. 2011) and discoloured waters (Douterelo et al. 2014b), and pathogens may

65 be released to bulk water or detach and recolonize clean surfaces (WHO 2008). They

66 also act as precursors for the formation of disinfection by-products (DBPs), and

67 consequently, contribute to disinfectant decay (Wang et al. 2013a).

68

69 The control of microorganisms in DWDNs is predominantly conducted through chemical

70 disinfection. Chlorine was introduced to urban DWDNs at the beginning of the 20th

71 century and it has been used since then to control pathogenic bacteria in drinking water

72 systems around the world (Sadiq and Rodriguez 2004). Chlorine remains popular for its

73 ease of use, relatively low cost and relative appropriate effectiveness, and especially for

74 its residual effects (Sadiq and Rodriguez 2004). However, the formation of DBPs such

75 as trihalomethanes (THMs) by the chlorine oxidation of natural organic matter present in

76 water sources (Rook 1974) changed the perspective that drinking water safety was only

77 related to pathogens.

It is now widely accepted that DBPs are potentially carcinogenic, teratogenic and mutagenic substances (WHO 2008), and hence their control in water treatment works has improved considerably. THMs and haloacetic acids (HAAs) are regulated by most of the water authorities worldwide since they are the most persistent DBP species found in drinking water (Hrudey 2009, Bull et al. 2011). THMs and HAAs are now considered as largely unrelated to public health risks, but are currently considered primarily as surrogates or indicators for other DBPs (Hrudey 2009, Bull et al. 2011). Recently, emerging DBPs have increased with the changes of disinfection processes and some of them, for example haloacetonitriles, are substantially more toxic than THMs (Muellner et al. 2007). Consequently, the risk management associated with the control of DBP formation should be addressed to reduce the precursors of these substances, which may reduce other conceivable DBP formation and consequently should not create an alternative DBP risk (Hrudey 2009).

Although there is increasing research into biofilms in DWDNs, their analysis has not yet been included in routine operative and regulatory plans in the water industry. The majority of biofilm studies in DWDNs have been conducted in temperate climate geographic regions, with pipe materials and ages typical from industrialised countries (Holinger et al. 2014, Kelly et al. 2014, Sun et al. 2014, Wang et al. 2014). Studies coupling microbial, engineered and physicochemical factors together are very limited. Wang et al. (2014) evaluated the influence of three factors (disinfectant, water age and pipe material) on the microbial structure in a simulated drinking water network. Ji et al. (2015) also studied a simulated system to evaluate the influence of three factors (water chemistry, pipe material and stagnation) in plumbing systems, located at the outlet of five water treatment plants. To date, there is only one field study reported in a tropical, developing country (Ren et al. 2015) and therefore there is a clear need for further 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

107 explore the relationships between biotic and abiotic factors, and to further understand

108 the potential involvement of bacteria in DBP formation. Such relationships are important

109 to determine the dynamics occurring in a DWDN and to understand the complexity

110 present in a real-world system. Furthermore, the results reported here are needed to

111 inform operational strategies and to ultimately protect public health.

112

113 **2 Materials and Methods**

114 **2.1 Drinking water distribution network**

115 The study site was a DWDN in the city of Cali (Colombia), located at 995 meters above

116 sea level and with an annual average temperature of 24.5 °C (23.8-25.1 °C). The DWDN

117 comprises four sub-networks originated from four surface water sources and five

118 treatment facilities. These sub-networks operate by gravity, pumping, or by a

119 combination of both. In total, the entire distribution network includes 2,951 Km of

120 pipelines, 10 service reservoirs, 28 storage tanks, and 19 pumping stations in order to

121 deliver water to 2,946,245 people.

122

123 Sampling was carried out within the biggest sub-network that is fed with the Cauca River,

124 the second most important Colombian river, which is treated by conventional processes

125 including primary and secondary chlorine disinfection. The main treatment facility feeding

126 this sub-network has two open-air clarified-water reservoirs to be used as alternative

127 water source during events in which the turbidity of raw water is higher than 1,000 NTU.

128 Therefore, when turbidity readings from raw water exceed such threshold, the intake is

129 closed and the treatment work is fed from the two storage reservoirs until turbidity

130 readings drop below 1,000 NTU or for up to 9 hours. If turbidity readings do not drop

131　below 1,000 NTU after this time, drinking water supply is interrupted and affecting almost

132　80% of the served population.

133

134　**2.2　Sample collection**

135　Pipe sections were taken from nine sites reporting leakages over a 3-week period; one

136　site corresponded to a branch pipe (point 7) and the remaining eight to end of pipe

137　networks (Figure 1). It is important to highlight that sampling points 3 and 7 are two

138　different points but are closely located, and therefore they look overlapped in Figure 1.

139　Pipe sections were taken during leakage repairs to enable biofilm collection. In order to

140　preserve the biofilm and minimise any contamination from soil attached to external pipe

141　walls, each pipe section was rinsed after removal with sterile water, wrapped in polythene

142　and transported at 4 ºC for subsequent biofilm and DNA isolation. Bulk water samples

143　were collected at the same time from the nearest household. Households' taps were

144　flushed for 5 min, and then 6 L of drinking water were collected in sterile plastic bottles.

145　Each sampling point was characterized by water age and pipe characteristics (i.e., pipe

146　material, working age, and diameter).

147

148　Water age was determined from a hydraulic model applied to the sub-network and

149　provided by the local water company. This model was implemented in the software

150　Infowater 11.5 and EPANET 2.00.12. Raw water age data provided by the water

151　company were processed with the software ArcMap 10.2.2 to create Thiessen polygons

152　and then calculate water age zones classified in four ranges: low (<8.5 hours), medium

153　(8.5-13.0 hours), high (68.0-146.0 hours) and very high (>146.0 hours).
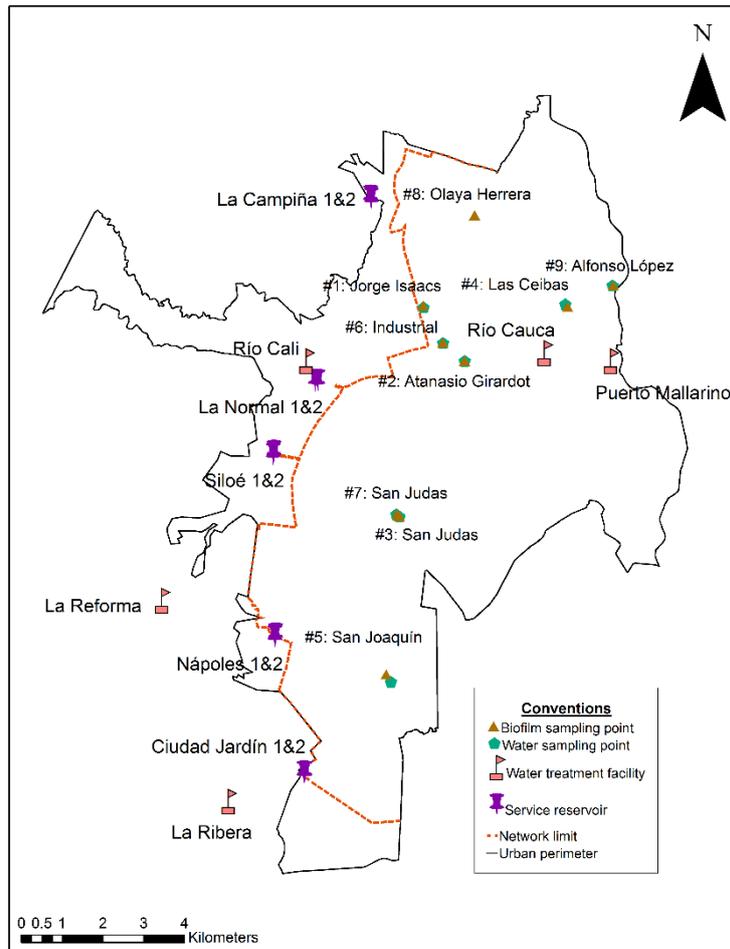
Figure 1. Location of sampling points

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

166 chromatograph (HP 5890, Wilmington, DE and Agilent Technologies 7890B, Santa

167 Clara, CA.), respectively.

168

169 TOC and dry-biomass were measured by scrapping a defined area on the pipe surface

170 of 75 cm^2 in triplicate. For TOC measurement in biofilms, scrapped biofilms were

171 resuspended in 250 mL of deionized water. For dry biomass, scrapped samples were

172 dried at 105 °C, for 24 hours and dry biomass per area (unit dry biomass) was calculated.

173 Due to the presence of a high amount of tubercles in the cast iron (CI) pipe of point 2, it

174 was not possible to calculate the unit dry biomass for this sample (Figure 2). On the

175 contrary, the surface of asbestos cement pipelines was flat, then scrapping biofilms from

176 them was a normal procedure. Detachment of asbestos fibres was observed during

177 scrapping biofilms.

178

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

180

181 **2.4 Molecular methods**

182 After rinsing the internal walls of the pipelines in the laboratory, biofilm samples were

183 collected by scrapping in triplicate using a sterile frame with area equal to 25 cm^2 and a

184 sterile spatula. DNA isolation was carried out using the Power Biofilm DNA Kit (MoBio,

185 USA) according to the manufacturer's instructions. In total 6 L of water were filtered for

186 every sampling point (2 L for each triplicate) through nitrocellulose filters (0.22 μm pore-

187 size); filters were further processed for DNA extraction using the Power Water DNA Kit

188 (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions.

189

190 Sequencing of DNA extracted from biofilm and water samples was performed by Illumina

191 MiSeq Technology using the Illumina PE MySeq reagent Kit v3 according to the

192 manufacturer's guidelines (Illumina, USA) and performed by the Molecular Research

193 DNA Lab (Shallowater, TX, USA). 2-5 ng/μL of DNA per sample (n=53) was used for

194 amplification (no replicates per sample were generated) and the V4 variable region of

195 the 16S rRNA gene was amplified using primers 515F/806R (Caporaso et al. 2011).

196 Sequence data were processed using Mr DNA analysis pipeline (www.mrdnalab.com,

197 MR DNA, Shallowater, TX). In summary, sequences were merged, depleted of barcodes

198 and primers, sequences < 150 bp and with ambiguous base calls were removed from

199 further analysis. Sequences were denoised and chimeras removed. Operational

200 Taxonomic Units (OTUs) were defined by clustering at 3% divergence (97% similarity)

201 and were taxonomically classified using BLASTn against a curated database derived

202 from Greengenes, RDPII and NCBI (DeSantis et al. 2006) (http://www.ncbi.nlm.nih.gov/,

203 http://rdp.cme.msu.edu).

204

205 The total number of reads generated per sample ranged between 7780-304912 and

206 between 13759-238406, for biofilm and bulk water samples, respectively. The number

207 of reads that passed quality scores ranged between 7240-256972 for biofilm and

208 between 10257-101379 for bulk water samples. The data set (number of reads per

209 sample) was not normalised or rarefacted to assess alpha-diversity, in order to avoid

210 losing information from potential important sequences (McMurdie and Holmes 2014).

211 **2.5   Data analysis**

212 The alpha-diversity of the samples at 97% sequence similarity cut off was analysed by

213 Margalef and Shannon community richness and diversity indices, respectively, which

214 were calculated with Primer6 software (PRIMER-E, Plymouth, UK). The medians and

215 means of such indices were statistically compared by t-test and Mann Whitney U test

216 using the software IBM SPSS Statistics 21. Statistical tests were carried out to assess

217 associations in both bulk water (species relative abundance (RA) and physicochemical

218 characteristics and water age) and biofilms (species RA and pipe characteristics, water

219 age, and unit dry biomass). The association of the RA of bacteria at species level and

220 the characteristics of the sampling points were determined by multi-dimensional scale

221 analysis (MDS), by means of Bray-Curtis similarity metrics, and analysis of similarities

222 (ANOSIM) using Primer6 (Clarke and Warwick 2001). Spearman correlations were

223 applied to determine the relationships between biofilm parameters and water

224 characteristics; Shapiro-Wilk tests were run in IBM SPSS Statistics 21 to determine

225 normal distribution of variables. All statistical results were contrasted with significance

226 level equal to 0.05.

227

228 **3   Results**

229 **3.1   Characterisation of the network, water quality and biotic parameters**

230 A summary of the network characteristics along with the corresponding water quality and

231 biotic parameters is presented in Table 1. The predominant pipe material was asbestos

232 cement (AC), with the exception of point 2, which corresponded to a CI pipeline. The

233 water age for biofilm and water samples are comparable with the exception of point 4,

234 where statistically significant differences were found. Since water samples were

235 collected from taps in households located as close as possible from leakage sites where

236 pipes were replaced to allow the collection of biofilm samples, water-related variables

237   were not associated with biofilm-related characteristics since the sampled pipeline was

238   not directly supplying the sampled household in every case.

239

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% **

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

242 Water quality characteristics including temperature, pH, free residual chlorine and TTHMs were

243 within expected ranges, except for the lowest concentration of chlorine (0.12 mg/L) that was

244 measured at point 4, which corresponded to the highest water age (146 h). Such concentration of

245 free residual chlorine is considered very low according to the recommended values set for drinking

246 water by local regulators in Colombia (0.3-2.0 mg Cl_2/L) (Ministerio de la Protección Social 2007).

247 TOC measured in biofilm samples presented a lower variation compared to the variation in biofilm

248 mass. All concentrations of TOC in bulk water were reported as lower than the detection limit (<0.8

249 mg/L). Regarding TTHMs, concentrations in all water samples were lower than 40 μg/L, which falls

250 below the maximum concentration of TTHMs allowed in drinking water according to Colombian and

251 UK regulations (100 and 200 μg TTHM/L, respectively) (Ministerio de la Protección Social 2007).

252

253 Several water physicochemical characteristics were correlated to identify the dynamics present in

254 the studied network; results are presented in Table 2. Significant negative correlations were found

255 between total residual chlorine and temperature (p=0.019), free residual chlorine and water age

256 (p=0.004) and free residual chlorine and TTHMs (p=0.017). Weak negative correlations were

257 identified between temperature and free residual chlorine (p=0.052, slightly higher than the level of

258 significance) and between pH and TTHMs (p=0.042). A positive correlation was observed between

259 temperature and TTHMs (p=0.003).

260

261 In relation to biotic factors, unit dry biomass presented the highest variation among all the variables

262 analysed. Although calculation of the unit dry biomass in the CI pipe sample (sampling point 2) was

263 not possible, the highest content of global dry biomass and TOC in the biofilm (233.7 - 3,664.8 mg)

264 (10.10 mg/L; Table 1) were found in this point. Concerning biofilms, correlations presented in Table

265 3 indicated that there is a strong positive relationship between unit dry biomass and pipe age

266 (p=0.008). Additionally, water age was negatively correlated with TOC in biofilms but no association

267 was identified between water age and unit dry biomass, possibly related to the influence of pipe

268 age/material over the later variable.

269

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%).
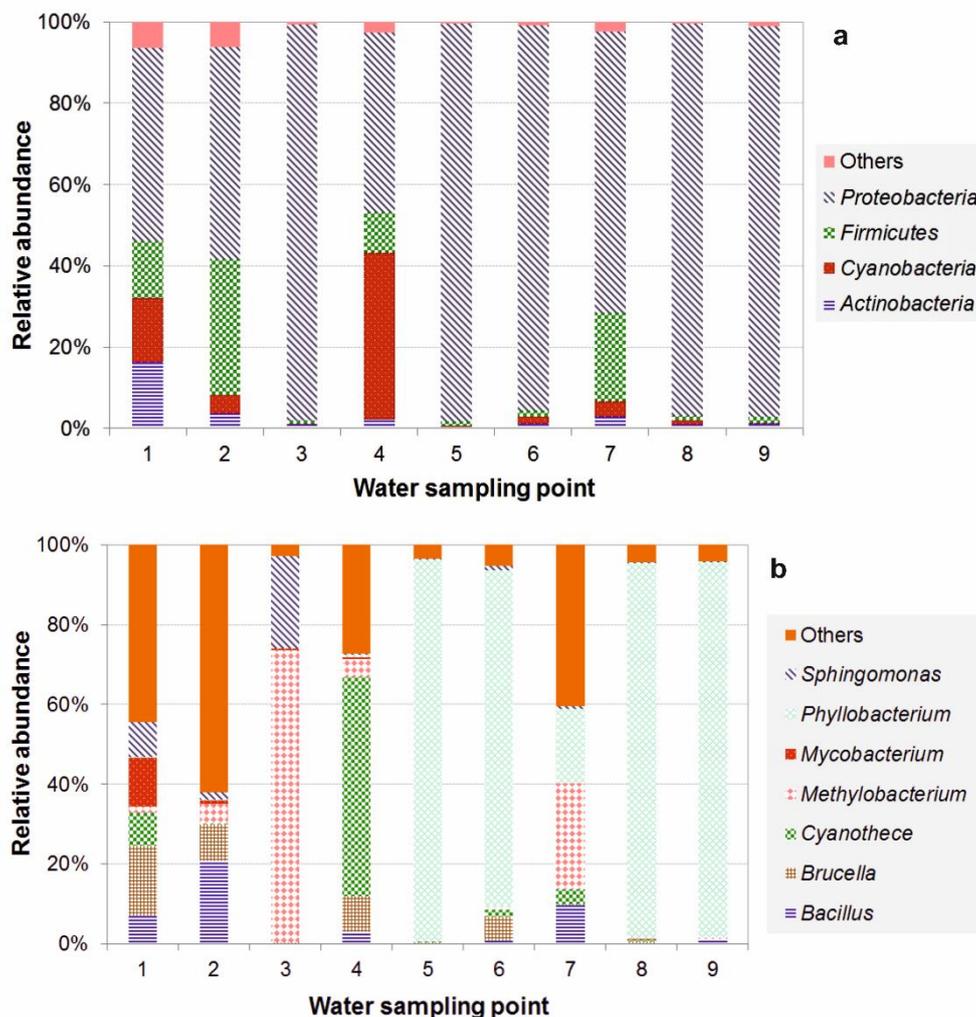
Figure 3. Relative abundance of bacterial to phylum level (a) and genus level (b) in water samples
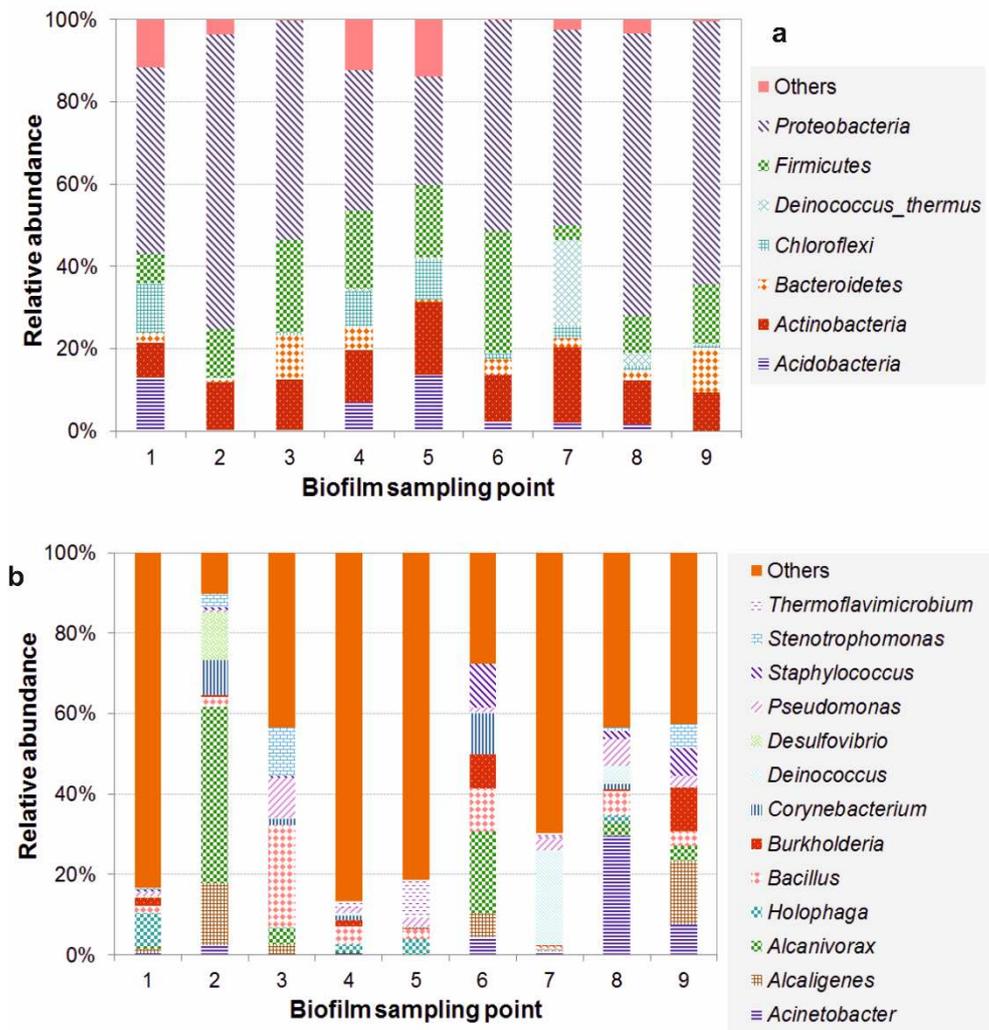
Figure 4. Relative abundance of bacterial groups to phylum (a) and genus level (b) in biofilm samples

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,

305 RA of bacteria is clearly grouped for pipe materials CI and asbestos cement. Importantly,

306 methanotrophic organisms were observed in biofilm samples such as *Methylobacterium* (RA=1.16%)

307 and *Methylosinus* (RA=3.34%). In bulk water, Spearman correlations with TTHMs were statistically

308 significant for the genus *Methylobacter* (ρ=0.437; p-value=0.023) and *Methylobacterium* (ρ=-0.417;

309 p-value=0.030).

310
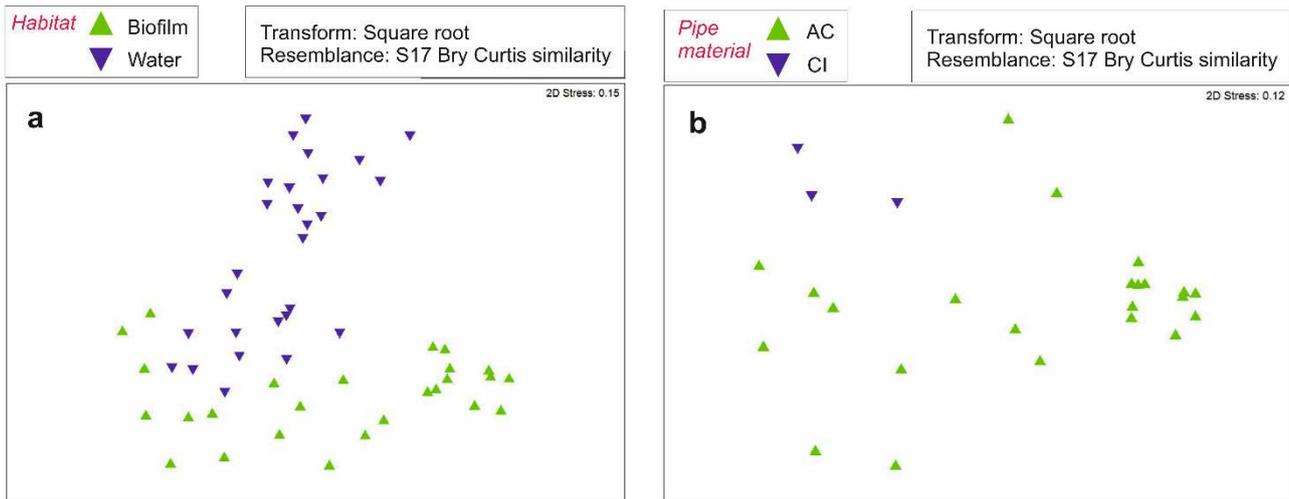
Figure 5. Non-metric MDS analysis of bacterial relative abundance. Factors Habitat (a) and pipe material -biofilm samples- (b)

3.3 Microbial richness and diversity

316 Richness and diversity were calculated (Table S2) according to factors such as habitat, pipe age,

317 and water age. Spearman correlations were applied to test the relationships between indices and

318 engineered factors, for both water (Table 2) and biofilm (Table 3) samples. Results from t-test

319 indicated that the means of richness and diversity indices of biofilm samples are higher than those

320 of water samples. Negative correlations were found between biofilm indices and pipe age and unit

321 dry biomass. Comparisons of medians indicated that the median of richness and diversity indices of

322 biofilm samples with high water age was higher than those with medium water age. In water samples,

323 median of richness index with very high water age was higher than those with low water age.

324 Richness index in bulk water negatively correlated with variable temperature, and both indices

325 positively correlated with pH. Positive correlations between water age and richness and diversity

326 indices were only found in biofilm samples.

327

328 **4 Discussion**

329 **4.1 Water quality, biotic parameters and their relationships with engineered factors**

330 DBP formation is influenced by parameters such as pH, temperature, TOC, chlorine dosage, and

331 water age. The interactions observed between these parameters and TTHMs confirm the dynamics

332 occurring in tropical DWDNs in relation to THM formation: increasing water age promotes decay of

333 free residual chlorine since the disinfectant is volatile and reacts with organic and inorganic matter,

334 likewise the concentrations of THMs were increasing. In temperate climates, Nescerecka et al.

335 (2014) and Wang et al. (2014) also identified depletion of disinfectant with higher water age in a real-

336 scale and simulated DWDNs, respectively. THM formation is directly influenced by pH and

337 temperature (Liang and Singer 2003), and such a relationship was evidenced by the current results,

338 which show a strong correlation between TTHMs and temperature. However, a negative relationship

339 between TTHMs and pH was found, which may be related to the narrow range of pH data evaluated

340 (Table 2); higher concentrations of THMs have been identified with higher pH in the range of 5-8 in

341 laboratory experiments (Liang and Singer 2003, Wang et al. 2012). The influence of pH on DBP

342 production remains unclear. Positive and negative correlations between pH and THMs have been

343 reported in other studies, such as in a Canadian DWDN by Rodriguez and Sérodes (2001).

344 Therefore, further research is needed to determine the actual influence of pH on the production of

345 DBPs particularly under tropical climate conditions.

346

347 **4.2 Characterization of the bacterial community structure of biofilms and bulk water**

348 *Actinobacteria*, *Firmicutes*, and *Proteobacteria* were the common phylotypes in the two habitats, with

349 the later community being the dominant group in the entire set of samples. Recent studies from other

350 geographic regions have reported that both water and biofilm samples were dominated by

351 *Proteobacteria* (Douterelo et al. 2013, Holinger et al. 2014, Kelly et al. 2014, Sun et al. 2014, Wang

352 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 genus comprises 11 species, 10 of them are associated with human infections (Scholz et al. 2010, Xavier

381   et al. 2010). *Staphylococcus* is an opportunistic pathogen detected in low percentages in bulk water

382   and biofilm samples here. This genus constitutes a major component of the human microflora

383   (Heilmann et al. 1996), and has been classified as a moderate biofilm former (Simões et al. 2007)

384   able to colonize hospital devices. The source of this opportunistic pathogen could be the surface raw

385   water, since the river basin was highly contaminated due to anthropogenic activities (Pérez-Vidal et

386   al. 2016). Mahapatra et al. (2015), by a laboratory study carried out in a subtropical region in India,

387   also identified *Staphylococcus aureus* in bulk water and 24-hour biofilms formed from incubation of

388   drinking water collected in kitchen taps.

389

390   The presence of some bacteria identified in this study could be related to soil sources. Particularly,

391   the genus *Holophaga* has been previously isolated from black anoxic freshwater mud from a ditch

392   in Germany (Liesack et al. 1994). Similarly, *Thermoflavimicrobium* and *Phyllobacterium* were highly

393   abundant in water samples at several sampling points and these organisms have been previously

394   reported in soil-related habitats (Rojas et al. 2001, Yoon et al. 2005).

395

396   Concerning the process of DBP production in DWDNs, several methylotrophic bacteria were

397   detected in most of the bulk water samples. *Methylobacterium* is methylotrophic organism, which are

398   ubiquitous in different environments including soil, freshwater, drinking water and lake sediments

399   (Leisinger et al. 1994). This genus is able to degrade DBPs such as HAAs (particularly dichloroacetic

400   acid) (Zhang et al. 2009), and they are biofilm formers with high resistance to sodium hypochlorite

401   disinfection in single-species biofilm (Simões et al. 2010). Importantly, *Methylobacterium* has not

402   been found yet in non-chlorinated DWDNs (Martiny et al. 2005, Liu et al. 2014). Therefore, the

403   presence of these microorganisms in DWDNs should be considered as a potential indicator of DBP

404   presence, despite of *Methylobacterium* presents facultative metabolism and it is able to use a wide

405   range of organic compounds as sources of carbon and oxygen (Gallego et al. 2005).

406

407   It has been observed previously the high structural and compositional variability within biofilms

408   growth under similar hydraulic conditions in chlorinated DWDS in the UK (Fish et al. 2015, Douterelo

409 et al. 2016); this study confirms the high natural heterogeneity of biofilms developed within the same

410 pipe material under tropical conditions. The way biofilm heterogeneity influences ecological

411 processes taking place in different DWDNs must be addressed, and should be considered when the

412 microbial structure of different pipe materials is assessed.

413

414 **4.3 Influence of network characteristics on bacteriological parameters**

415 Higher richness and diversity were found in biofilms when compared to bulk water samples, which

416 can be related to the favourable conditions offered by this micro-environment for bacteria survival

417 such as protection against disinfectant, bulk flow and higher availability of nutrients. Douterelo et al.

418 (2013) also found higher diversity and richness in 28 day old biofilms in a chlorinated DWDN,

419 indicating that only some bacteria in the bulk water have the ability to attach to pipe walls. For

420 instance, *Bacillus* was the only common genus detected in the two habitats in this study. *Bacillus*

421 can form spores that protect them from disinfection and when the environmental conditions are

422 favourable they start developing as active cells (Checinska et al. 2015). Conversely, Henne et al.

423 (2012), based on 16S r RNA fingerprints of extracted DNA and RNA, found that bacterial richness

424 (Margalef index) was higher in bulk water than biofilm samples from a 20-year old and chlorinated

425 DWDN. The authors hypothesized that only those bacteria that can actively contribute to the

426 succession of the biofilm were successful in colonising biofilms, while bacteria that cannot fill

427 perfectly the narrow niches in biofilms vanished over time. Identifying those bacteria more prone to

428 form biofilms can be used to inform control strategies to target specific microorganisms and avoid

429 further biofilm development.

430

431 The relationship found between pipe age and unit dry biomass may be related to the detachment of

432 some asbestos fibres, which was observed during biofilm scrapping from the sampled pipes and is

433 representative of the potential wear of the pipe material in time due to biological activity. The

434 influence of removal of such fibres was described by Wang et al. (2011), who tested the biological

435 activity in 53- 54-year old sections of asbestos pipes. By establishing microbial activity of iron-

436 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 °C (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).

465 With regard to water age, the effect of this factor on bacterial biofilms may be associated with the

466 relationship between this parameter and other water physicochemical characteristics as previously

467 discussed. In addition, low concentrations of chlorine, stagnation and low velocities conditions

468 associated with high water age lead to increase cells counts in bulk water (Nescerecka et al. 2014)

469 and favour biofilm formation (Fish et al. 2016). Water age is considered as a factor influencing the

470 biological stability of drinking water (Prest et al. 2016) and the microbial composition of building

471 plumbing materials (Ji et al. 2015). This was corroborated by Wang et al. (2014), who established in

472 simulated-DWDN biofilm samples that water age, disinfectant, and pipe material interact with each

473 other to create distinct physicochemical conditions and ecological niches, in which various microbes

474 can be selected and enriched. Spearman's correlations showed no associations between indices

475 and concentrations of free chlorine in this study.

476

477 The influence of other key water physicochemical factors, including pH and temperature, on the

478 microbial ecology of DWDN were also assessed here. Results indicated statistically significant

479 differences among bacterial species for pH, which was also correlated positively with both richness

480 and diversity. Due to the relationship between pH and alkalinity, and the governance of this factor

481 over the relative proportions of hypochlorous and hypochlorite, which present different disinfection

482 efficacies, pH is impacting the variability in the water bacterial community as was found by Sun et

483 al. (2014). Temperature and richness were negatively correlated; similar results were found by

484 Henne et al. (2013) by comparing microbial communities of cold and hot water ($\Delta T = 41$ °C

485 approximately); cold water presented higher community diversity and high stability over time. The

486 present study considered $\Delta T = 3$ °C, which corresponds to typical temperature values for tropical cities

487 with hot weather.

488

489 **5   Implications for O&M activities in DWDN**

490 This study approached the role of biofilms and bulk water bacterial communities in two key

491 processes: i) the relationship between them and DBPs and ii) their pathogenic significance.

492 Degradation and formation of DBPs has been previously associated with biofilms and the presence

23

493 of certain microorganisms such as methylotrophic bacteria (Fang et al. 2010a, Fang et al. 2010b,

494 Wang et al. 2012, Pu et al. 2013, Wang et al. 2013a, Wang et al. 2013b, Xie et al. 2013). However,

495 this study indicates that the formation of DBPs in the DWDNs is a complex process since precursory

496 and degradation biological reactions can simultaneously occur. Hence, TTHMs and HAAs modelling

497 efforts should consider the biological component on DBP chemistry, especially in the models where

498 the correlation coefficients are low, and then the predictability of these substance concentrations

499 may be improved. Recently, Abokifa et al. (2016) included reaction chlorine-biomass (biofilm and

500 planktonic cells) in a model to predict THMs in drinking water pipes under turbulent flow. Similarly, a

501 CFD model was developed by the authors of this study to simulate the chloroform and

502 dichloroacetonitrile formation potentials from biofilm chlorination, under laminar, transitional, and

503 turbulent flow. Manuscript is being prepared for further publication.

504

505 Prevention and removal of biofilms is a key concern for water utilities due mainly to their potential as

506 reservoirs of pathogens. Flushing water pipes has been proved as a suitable technique to remove

507 material attached to internal pipe surfaces but it is inefficient to completely detach biofilms (Abe et

508 al. 2012, Douterelo et al. 2013, Fish et al. 2016). Advanced water treatment processes such as

509 membrane filtration has been proved successful in highest reduction of number of microorganisms

510 in biofilms collected at the inlet of a DWDN (Shaw et al. 2014). However, a recent study argues that

511 is impossible to prevent biofilm accumulation but high flow variation could be used to promote young

512 biofilms, which are more vulnerable to disinfection (Fish et al. 2017). In the case of this studied

513 network, avoiding uncontrolled biofilm detachment and contamination of bulk water is particularly

514 difficult, as it exhibits specific O&M challenges associated with emptying of the network due to the

515 interruption of operation of the water treatment facilities, pumping operation, closing/opening valves

516 during leakages repairs and pipelines and accessories replacement. This may lead to favour the

517 formation of young biofilms, however it is important to consider that biofilms, planktonic cells, and

518 detached biofilm clusters are also DBP precursors, and then biofilm control must go beyond

519 disinfection.

520

521 Furthermore, CI pipes represent 10% of the total length of the pipelines and asbestos 30%; and

522 2,400 leakages were repaired in 2014. These O&M activities cause uncontrolled and partial removal

523 of sediments and biological material and allow the entrance of external particles, which all together

524 could be promoting microbial growth in the network. Future plans for pipeline replacements should

525 avoid the use of metal and cement pipes and instead promote the use of pipe materials with more

526 stable bio-chemical and physical conditions. It is also advisable to minimize the events that alter the

527 normal operation of the DWDN to reduce biofilm detachment; controlled cleaning procedures of

528 pipes such as flushing should be carried out to reduce the amount of nutrients available for

529 microorganisms in bulk water and biofilms and avoid alterations of the organoleptic conditions of

530 drinking water for the consumers. More importantly, the efforts carried out in protecting water sources

531 and improving water treatment could be useless if suitable O&M practices are not applied in the

532 DWDNs in order to preserve the safety of drinking water delivered to the customers.

533

534 **6 Conclusions**

535 To the authors' knowledge, this is the first study that characterised the bacterial community structure

536 in both water and biofilm habitats in a tropical-climate DWDN. It also explored the relationships

537 between biotic and engineered factors, with a specific focus on DBPs. The application of sequencing

538 analysis represents a step forward in the study of microbiological aspects of DWDNs in tropical-

539 climate countries. Most of the bacterial communities identified in this work have also been found in

540 temperate-weather water systems. This may indicate that some drinking water bacteria are

541 ubiquitous and that treatment and engineered environments shape the bacterial communities in a

542 specific way. This study found that, similarly to temperate-climate DWDNs, bacterial communities in

543 sampled biofilms are different from those in bulk water, with the former more diverse and richer. Pipe

544 age, water age, free chlorine, pH and temperature were associated with microbiological parameters

545 indicating that these are key to control microbial growth. Deeper analysis should be done in terms of

546 the influence of temperature variation in tropical-climate DWDNs. Pipe material also influenced the

547 microbial ecology of DWDNs; *Desulfovibrio* was identified exclusively in the CI pipe. Methylotrophic

548 bacteria were found in biofilms and bulk water; these microorganisms are known to be able to

degrade DBPs as haloacetic acids. Design and O&M of DWDNs should consider all the possible

procedures to minimise biofilm growth to manage both biological and chemical stability of drinking

water: to reduce nutrient concentrations in the water treatment, flushing dead end zones and after

repair and replacement activities, reduce water age, and use bio-stable pipe materials.

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