ABSTRACT

Collecting biofilm samples from drinking water distribution systems (DWDSs) is challenging due to limited access to the pipes during regular operations. We report here the analysis of microbial communities in biofilm and water samples collected from sensors installed in a DWDS where monochloramine is used as a residual disinfectant. A total of 52 biofilm samples and 14 bulk water samples were collected from 17 pipe sections representing different water ages. Prokaryotic genome copies (bacterial and archaeal 16S rRNA genes, *Mycobacterium* spp., ammonia-oxidizing bacteria (AOB), and cyanobacteria) were quantified with droplet digital PCR, which revealed the abundance of these genes in both biofilm and water samples. Prokaryotic 16S rRNA gene sequencing analysis was carried out for a subset of the samples (12 samples from four sites). *Mycobacterium* and AOB species were dominant in the DWDS sections with low water age and sufficient residual monochloramine, whereas *Nitrospira* species (nitrite-oxidizing bacteria) dominated in the sections with higher water age and depleted monochloramine level, suggesting the occurrence of nitrification in the studied DWDS. The present study provides novel information on the abundance and identity of prokaryotes in biofilms and water in a full-scale operational DWDS.

Key words: Water distribution system; biofilm; water quality; 16S rRNA gene sequencing; nitrification

1. Introduction

Drinking water distribution systems (DWDSs) are an essential urban infrastructure that must be adequately managed in order to provide safe and high-quality drinking water to end-point consumers. However, water quality may deteriorate during distribution due to microbial processes in DWDS, including biofilm development (Liu et al., 2013). Biofilms occur universally in DWDS and are usually considered undesirable, because they are known to be the primary cause of many issues in drinking water quality, including nitrification, residual disinfectant decay, proliferation of pathogens, and aesthetic problems in color, odor, and taste (Liu et al., 2016; Zhang et al., 2009). Biofilms comprise communities of microorganisms that attach to surfaces through extra-cellular polymeric substances. Numerous factors can influence biofilm formation and growth in DWDS, including water characteristics (such as microbial numbers, nutrient concentration, temperature), pipe material, hydraulic conditions, and levels of residual disinfectant (which decays with water age) (Wang et al., 2012).

Many water utilities have switched from chlorine to chloramines for secondary disinfection of drinking water, primarily to reduce the formation of disinfection byproducts (Seidel et al., 2005). Chloramines also maintain disinfection residuals for a longer period throughout the distribution system (Norton and LeChevallier, 1997) and may penetrate biofilms more effectively (Lee et al., 2011). However, one major drawback of chloramination is nitrification where ammonia is sequentially oxidized to nitrite and nitrate. Ammonia-oxidizing bacteria (AOB) and/or archaea (AOA) oxidize ammonia to nitrite, while nitrite-oxidizing bacteria (NOB) convert nitrite to nitrate (Zhang et al., 2009). Residual ammonia can be present in chloraminated water from the reaction between chlorine and ammonia intended to produce monochloramine. Additional ammonia can be formed as a result of oxidization of the intermediate nitrite by chloramines, which in turn accelerates nitrification and residual chloramine decay (Zhang et al., 2009). Production of toxic nitrite and nitrate as well as the growth of heterotrophic bacteria,

45 (which may include opportunistic waterborne pathogens associated with loss of disinfectant) pose
46 risks to public health.
47 Nitrifiers (i.e., AOB, AOA, and NOB) are known to dwell in biofilms that provide them with
48 protection against disinfectants. Understanding the role and ecology of biofilms in DWDS is
49 therefore essential to develop effective strategies for management of water quality problems
50 including nitrification. The collection of biofilm samples from pipe walls within operational
51 DWDS presents a substantial challenge due to limitations in accessing the underground pipe
52 distribution network. Prior studies have used model systems (bench-top or pilot scale systems in
53 the laboratory) or have speculated on the development of biofilms based on tap water samples
54 and associated environmental factors (Abbaszadegan et al., 2015; Gomez-Alvarez et al., 2014;
55 Lee et al., 2011; Schwake et al., 2015; Wang et al., 2012). Although these studies have
56 contributed significantly to our understanding of biofilm growth within DWDS, model systems
57 inevitably differ from actual DWDS in terms of key hydraulic and environmental variable
58 including pressure, flow rate, water age, and local water quality. In addition, there are critical
59 limitations of using tap water samples containing only planktonic cells to infer biofilm
60 community, because of the distinction between planktonic and biofilm communities in DWDS
61 (Douterelo et al., 2013). To overcome these limitations, some efforts have been made to study
62 biofilms *in situ* in full-scale operational DWDS by collecting samples from a device inserted into
63 the pipe (Douterelo et al., 2014), water meters (Hong et al., 2010; Koskinen et al., 2000; Ling et
64 al., 2016; Lührig et al., 2015; Watson et al., 2004), or pipe samples (Cruz et al., 2020; Kelly et al.,
65 2014; Liu et al., 2020; Lührig et al., 2015)(Douterelo et al., 2020). Some previous studies also
66 employed full-scale experimental DWDS that accurately replicates the hydraulic and other
67 physical, chemical, and biological conditions of operational DWDSs (Douterelo et al., 2013; Fish
68 et al., 2015). However, relatively little is known about the spatial distribution of microbial species
69 and ecology across a full-scale operational DWDS.

70 The purpose of the present study was to investigate microbial abundance and composition in
71 a full-scale operational chloraminated DWDS by analyzing microbial communities colonizing
72 WaterWiSe sensors within a large DWDS. WaterWiSe is a wireless sensor network consisting of
73 in-pipe, online sensors (Allen et al., 2011), and was deployed to monitor the integrity of DWDS
74 by measuring hydraulic and basic water quality parameters including pressure/acoustics, flow
75 rate, pH, oxidation-reduction potential (ORP), conductivity, and fluorescent dissolved organic
76 matter. This system also provides a unique opportunity to study the microbiology within an
77 operational DWDS. The insertion probe for each sensor node provides a variety of substrate
78 materials (i.e., brass, stainless steel [SS], polyvinyl chloride [PVC], polyoxymethylene [POM])
79 for accumulation of biofilms and also allows collection of flowing bulk water from a local
80 sampling port (on the probe). Here, we report the analysis of microbial population and
81 composition in both biofilm and water phase samples to understand microbial ecology and
82 associated processes that may impact local water quality in a chloraminated DWDS.
83
84 **2. Materials and Methods**
85 *2.1. Sampling design*
86 A sampling campaign was designed to collect bulk water and biofilm samples from the
87 hydraulic and water quality sensors installed in a testbed network covering a 60-km^2 area. The
88 water source in this area is a blend of treated surface water and desalinated seawater, which is
89 supplied by a gravity-fed DWDS consisting of two service reservoirs. Monochloramine has been
90 used as a residual disinfectant in the system since 2005.
91 In February 2014, WaterWiSe sensors that had been operating at 17 sites (sampling site ID:
92 S1 to S17; actual locations of these sensors are indicated in Figure S1 in the Supplementary
93 Material) across the DWDS were replaced for periodic maintenance. The original sites were
94 chosen to optimize detection of hydraulic (bursts and leaks) and contamination events within the

pipe network. In-pipe water quality sensors were inserted in the center of the water pipe, and had been in service for periods ranging from 6 to 18 months at the time of sampling (biofilm age, Table 2). The studied DWDS can be sub-divided into two zones characterized by different water ages (retention time from the service reservoirs), with lower water ages in Zone 1 (3.1 to 20.1 h) compared to Zone 2 (35.9 to 45.1 h) based on EPANET simulations (Rossman, 2000), Table 1. Because Zone 1 covers a larger geographical area than Zone 2 (Figure S1), the testbed included higher number of sensor locations (sampling sites) in Zone 1 (15 sites) as compared to Zone 2 (2 sites). There were two versions of sensors that had been installed in the test bed: *A*) with a sampling tap on the top of the sensor unit that allowed collection of water sample from the middle of the water pipe through a tube in the insertion rod mechanism, and allowed collection of biofilm samples on four different types of sensor substrata, i.e., brass, SS, PVC, and POM (DuPont^{TM} Delrin$^{®}$) with sampled surface area of 77.4, 6.5-19.6, 113.0, and 28.3-53.4 cm^2, respectively; and *B*) without the bulk water sampling tap, and two different types of sensor surface material (i.e., SS and POM with sampled surface area of 185.3 and 78.5 cm^2, respectively) were available for biofilm sample collection (see Figure S2 for the photographs of these sensors). The version B sensors had been installed at two sites (i.e., S2 and S4), while the version A sensors had been installed at the remaining 15 sites.

2.2. Collection of bulk water and biofilm samples

A total of 52 biofilm samples (up to four samples from different types of sensor surface per site [brass, SS, PVC, POM]) and 14 bulk water samples were collected from 17 sensor installation sites. One biofilm sample (n = 1) was collected from each surface type of each sensor, and up to 5 L of bulk water was collected concomitantly from each site. Some samples were not available due to technical difficulties in sampling, which resulted in fewer samples than the expected maximum numbers (i.e., a total of 64 biofilm and 15 bulk water samples).

At each sampling site, bulk water samples were collected from the sampling tap (where

120 available) after flushing water from the tap for >5 mins, which was done before sensor
121 replacement. Physicochemical parameters, such as temperature, conductivity, total dissolved
122 solids (TDS), and salinity, were measured in the field immediately after sample collection using a
123 portable HI 9828 Multiparameter meter (Hanna Instruments, Inc., Woonsocket, RI). Free and
124 total chlorine were measured with a DPD colorimetric method using the Lovibond® Comparator
125 2000+ and tablet reagents (Tintometer Ltd., Amesbury, UK). Turbidity was measured using a
126 2100N Turbidimeter (HACH, Loveland, CO). Bulk water samples for microbiological analyses
127 (up to 5 L) were dechlorinated with sodium thiosulfate ($Na_2S_2O_3$) immediately after sample
128 collection and transported to the laboratory on ice.
129 After each sensor probe was dismounted from the water pipe, biofilms on the material
130 surfaces were collected by either scraping (for scaling) or swabbing using sterile cell scrapers or
131 cotton swabs, respectively. The sampled surface area (cm^2) was measured (see section 2.1 for
132 specific values) to normalize microbial counts and calculate microbial surface density
133 (copies/cm^2).
134 *2.3. Sample processing*
135 The dechlorinated bulk water samples (1 to 4 L of up to 5 L collected) were filtered through
136 the Isopore™ membrane filters (polycarbonate, pore size 0.2 μm, diameter 47 mm, cat. no.
137 GTTP04700; Millipore, Billerica, MA), and the filters were stored at -20ºC for DNA extraction.
138 Heterotrophic plate count (HPC) numbers in the dechlorinated bulk water samples were
139 determined using R2A agar plates with an incubation at 20ºC for 7 days (Reasoner, 2004). The
140 scrapings and swabs were suspended in sterile 1× phosphate-buffered saline (PBS), and biofilm
141 suspensions were prepared by vortexing. An aliquot of this biofilm suspension was used for
142 bacterial culture assays, and the rest of the suspension was stored at -20ºC for DNA extraction.
143 *2.4. DNA extraction*
144 Total DNA was extracted from the filters and biofilm suspensions using the PowerWater® and

PowerBiofilm® DNA Isolation Kits (MO BIO Laboratories, Carlsbad, CA), respectively, according to the manufacturer's instructions with slight modifications. Specifically, for the biofilm samples 15 µL of Proteinase K (Qiagen, Hilden, Germany) was added to the bead tube after the bead-beating step and incubated at 65ºC for 30 minutes to increase the yield of eukaryotic DNA from biofilms.

2.5. Droplet digital polymerase chain reaction (ddPCR)

TaqMan-based ddPCR assays for total bacteria, total archaea, *Mycobacterium* spp., AOB, *Nitrospira*-like NOB, *Gallionella* spp., cyanobacteria, and internal amplification control (murine norovirus plasmid DNA, pMNV) were performed with a QX200™ Droplet Digital™ PCR System (Bio-Rad, Pleasanton, CA). Reaction mixtures (20 µL) consisted of 10 µL of 1× ddPCR™ Supermix for Probes (Bio-Rad), forward and reverse primers and probe(s), and 2.0 µL of DNA template. The sequences of primers and probes are shown in Table S1 in the Supporting information. The reaction mixture was mixed with droplet generation oil (20 µL mixture and 70 µL oil) via microfluidics in the QX200™ Droplet Generator (Bio-Rad). The water-in-oil droplets were transferred to a standard 96-well PCR plate and subjected to PCR amplification (ramping speed at 2.5ºC s^{-1}) on a C1000 Touch™ Thermal Cycler (Bio-Rad). Upon completion of PCR, the plate was transferred to a QX200™ Droplet Reader (Bio-Rad) for automatic measurement of fluorescent reading in each droplet in each well. A clear separation in terms of fluorescent intensity was obtained between positive and negative droplets (Figure S3). Observed recovery efficiency of internal control pMNV was >90%, suggesting no substantial inhibition in any of the samples, except for S1 sample that showed 76% recovery (Figure S4). To minimize contamination during the DNA extraction and ddPCR processes, DNA extraction and ddPCR reagent preparation were performed in separate rooms. No template control (NTC) was included in all ddPCR runs, and no amplification was observed in any NTC reactions.

2.6. 16S rRNA gene amplicon sequencing and bioinformatics analysis

A subset of the samples (a total of 12 samples [eight biofilm samples and four water samples], collected from different substrata in S10, S11, S14, S15) was used for 16S rRNA gene amplicon sequencing analysis. DNA concentrations in DNA extracts were determined by Qubit® fluorometer (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. Both bacterial and archaeal 16S rRNA genes were PCR-amplified using the universal primers 926wF and 1392R targeting the V6-V8 regions (Mason et al., 2012). The PCR amplicons were sequenced with the Illumina MiSeq platform, a next-generation sequencer, with 300PE reads. The raw reads were quality trimmed, primers and adapters were removed using cutadapt-1.8.1. All data processing was conducted using QIIME 1.9.1 pipeline with Silva 16S rDNA database (>97% identity level). In order to account for observed differences in sequencing depth per sample, the operational taxonomic unit (OTU) abundance was rarefied to the lowest number of sequences in a sample.

2.7. Statistical analyses

Two-way analysis of variance (ANOVA) and Tukey-Kremer's post-hoc multiple comparison were performed within the R statistical computing environment (http://www.r-project.org) and Microsoft Excel for Mac 2018 (Microsoft Corp., Redmond, WA), respectively, to investigate whether the prokaryotic gene densities ($\log_{10}$ copy numbers/cm^2) were statistically different between sampling sites and substratum types. Differences were considered statistically different if the resultant *P*-value was 0.05 or lower. The statistical package PRIMER-PERMANOVA was used for multivariate statistical analysis. The OTU abundance matrix was square-root transformed and a Bray-Curtis resemblance matrix was used for further analysis.

2.8. Nucleotide sequence accession numbers

The raw sequencing reads were submitted to the sequence read archive (SRA) and can be retrieved via the DNA Data Bank of Japan (DDBJ) accession number DRA009881.

3. Results

3.1. Water quality

Bulk water samples were available at 14 of the 17 study sites across two zones of the DWDS. Water quality parameters measured in the bulk water samples are summarized in Table 1. The DWDS is located in a tropical area, with high ambient water temperature ranging from 28.7 ºC to 30.3 ºC, and pH ranged from 6.89 to 7.74 without a clear relationship with other parameters. There was a strong contrast in residual total chlorine levels and HPC between the two zones. Throughout Zone 1, adequate concentrations of total residual chlorine (1.4 – 2.0 mg/L: range for 10 sites) are maintained and there were correspondingly low HPC counts (2.5 CFU/mL or less: range for 11 sites), whereas Zone 2 samples showed much lower levels of total residual chlorine (0.2 – 0.35 mg/L: range for 2 sites) and relatively higher HPC counts (8.5 – 11.5 CFU/mL: range for 2 sites). Although the numbers of samples from each zone were limited, this result demonstrates the presence of higher levels of heterotrophic bacteria in DWDS sections with lower residual disinfectant levels associated with higher water age.

The abundance of prokaryotic genome copies in bulk water samples was determined by target-specific ddPCR (Table 1). Bacterial and archaeal 16S rRNA genes were detected with the highest copy numbers at S14 (1.51×10^6 and 2.79×10^4 copies/L, respectively). Bacterial 16S rRNA genes (mean 1.10×10^5 copies/L) were always more abundant than archaeal 16S rRNA genes (1.12×10^3 copies/L) with statistically significant difference ($P < 0.01$, *t*-test). *Mycobacterium* spp., AOB, and Cyanobacteria were also detected from all water samples with mean gene copy numbers of 2.69×10^4, 1.23×10^4, and 1.05×10^3 copies/L, respectively. The sampling locations did not seem to impact the absolute abundance of these bacterial members in bulk water samples as determined by ddPCR.

3.2. Prokaryotic genomes in biofilm samples

Abundance of prokaryotic genome copies in a total of 52 biofilm samples collected from four

different types of substratum (brass, SS, PVC, and POM) was determined by ddPCR (Table 2). Biofilm age (i.e., duration of sensor operation) varied from 6 to 18 months in both zones. Bacterial 16S rRNA gene was detected from all the biofilm samples with densities of up to 1.05×10^6 copies/cm^2. Statistical comparison of bacterial 16S rRNA gene densities ($\log_{10}$ copies/cm^2) between different substratum types demonstrated that bacterial 16S rRNA gene copy numbers on brass surface were significantly lower than those on other materials ($P < 0.05$). Whereas, the comparison of bacterial 16S rRNA gene copy numbers between different sites identified no statistically significant difference among sites ($P > 0.05$) despite the difference in biofilm age, suggesting that accumulation of bacteria on sensor surface reached equilibrium within 6 months of operation.

The archaeal 16S rRNA gene was always less abundant than the bacterial 16S rRNA gene, but exhibited similar tendencies in terms of differences between substratum types and sampling sites. For example, the archaeal 16S rRNA gene was less frequently detected on brass surface (3 out of 11, 27%) than on other substratum types (94% of SS, 100% of PVC, and 89% of POM samples). *Mycobacterium* spp. were also less frequently detected on brass surfaces (8 out of 11, 73%) than on other substratum types (100%). The density of *Mycobacterium* spp. was close to that of total bacteria based on 16S rRNA gene copy numbers, which was more notable in Zone 1 than in Zone 2 (Table 2). This result suggested that, in Zone 1, *Mycobacterium* spp. comprised a significant portion of the bacterial population in biofilms, but this was not the case in Zone 2. AOB were also detected in all biofilm samples at relatively high gene copy numbers, and at lowest densities on brass surfaces. Cyanobacteria was generally less abundant than *Mycobacterium* spp. and AOB.

Overall, densities of prokaryotic genome copies on brass surfaces tended to be lower than on other substratum types, while there was no clear relationship with biofilm age between 6 and 18 months. We also noted that the trends in microbial abundance in biofilm samples were similar to

those in bulk water, with bacterial 16S rRNA gene being the most abundant, followed by *Mycobacterium* spp. and AOB, and archaeal 16S rRNA and cyanobacteria being less abundant than other microbial groups.

3.3. 16S rRNA gene amplicon sequencing analysis

The prokaryotic community composition in the biofilm and water samples was determined for a total of 12 samples collected from four sites (S10, S11, S14, S15), based on 16S rRNA gene amplicon sequencing using the Illumina MiSeq platform. After quality filtering, 288,642 to 432,154 high quality reads were obtained per sample (Table 3). The rarefaction curves for all samples had reached plateaus (Figure S5), suggesting that the sequencing depth was adequate to capture most of the diversity within the microbial communities in each sample. There was no remarkable difference in richness and diversity indices between sample types or sampling sites (Table 3). Figure 1 shows relative abundances in total sequencing reads (%) of prokaryotic (including *Archaea* and *Bacteria*) 16S rRNA gene amplicons in biofilms on SS surfaces as well as in water samples. *Archaea* were much less abundant than *Bacteria* (relative abundance of up to 4.2 % in total reads; included in "Others"), which is consistent with the results of ddPCR absolute quantification (Table 2). In Zone 1 (S10 and S11), the genus *Mycobacterium* and the family *Nitrosomonadaceae* (genus unassigned) were abundant in both biofilm and local water samples. The family *Nitrosomonadaceae* comprises two genera, *Nitrosomonas* and *Nitrosospira*, both of whose cultivated representatives are chemolithoautotrophic ammonia oxidizers (Prosser et al., 2014). In contrast, the genus *Nitrospira*, which is represented by aerobic chemolithoautotrophic NOB (Daims and Wagner, 2018), was dominant in Zone 2 (S14 and S15) samples.

Non-metric multidimensional scaling (nMDS) plots, which produced an ordination based on the Bray-Curtis dissimilarity matrix, indicate a dissimilarity in microbial community structure in samples from Zone 1 (S10 and S11) and Zone 2 (S14 and S15) (Figure 2). These two zones had contrasting hydraulic and water quality characteristics, such as water age and residual disinfectant

levels, as described above (Table 1). Within each zone, the physical phase (biofilm vs bulk water) exerted greater influence on microbial communities than sampling locations (Figure 2).

4. Discussion

In the present study, we examined microbial communities in biofilm and water samples collected from the WaterWiSe sensors inserted in water pipes of a full-scale operational DWDS. Our strategy enabled collection of samples from the sensors installed at different locations within the DWDS with varying water age and residual chlorine.

The abundance of prokaryotic genome copies was determined by ddPCR. This allowed direct comparison of microbial abundance among different microbial groups in each sample. Bacteria were always more abundant than archaea in both bulk water and biofilm samples, which was also supported by 16S rRNA gene amplicon sequencing analysis. In addition to bacterial and archaeal 16S rRNA genes, *Mycobacterium* spp., AOB, and Cyanobacteria were selected as detection targets, because their presence in DWDS and significance to drinking water quality have been reported previously (Haig et al., 2018; Lipponen et al., 2004; Shaw et al., 2015; Zhang et al., 2017). In agreement with the previous studies, these bacterial groups were frequently detected at high abundance; for example, densities of *Mycobacterium* spp. as well as bacterial 16S rRNA genes in bulk water were comparable to those reported in a previous study based on quantitative PCR (Haig et al., 2018).

It has been reported that the characteristics of the substratum material may greatly influence formation and growth of biofilms in DWDS (Niquette et al., 2000; Wang et al., 2012). The WaterWiSe sensors were composed of multiple parts with different materials (i.e., brass, SS, PVC, and POM), which provided a unique opportunity to investigate the density of microorganisms depending on material types serving as a substrate for biofilms in real DWDS. One of the limitations of this study is that only one biofilm sample was collected from each

surface type of each sensor, although the density and composition of biofilms on surfaces can greatly vary due to heterogeneity in drinking water biofilms (Neu et al., 2019). The ddPCR results demonstrated that densities of microbial genome copies on brass were substantially lower than on other materials. This is probably because brass consists of copper and zinc, both of which exhibit antimicrobial properties (Espírito Santo et al., 2008; McDevitt et al., 2011). Other materials studied, especially SS and PVC, are frequently used as pipe material, and their ability to support drinking water biofilm has been investigated previously (Jang et al., 2011). Our results indicated that these materials support colonization and growth of biofilms in water pipes even in the DWDS sections where an adequate residual disinfectant level is maintained.

Prokaryotic 16S rRNA gene sequencing analysis using the Illumina MiSeq platform was performed to gain further insights into the impact of environmental factors to microbial composition. Due to resource constraints, biological and technical replicates could not be included in the sampling design (i.e., n = 1 for each sampling point) and only a subset of samples was subjected to 16S rRNA gene sequencing analysis, which is one of the major limitations of this study. In selecting the subset (12 out of a total of 66 samples), consideration was given to a comparison of microbial composition between different physical phases (water and biofilm), zones (S10 and S11 in zone 1 and S14 and S15 in zone 2), and substratum types (SS, PVC, and POM). The nMDS analysis showed that microbial composition was primarily impacted by zone, rather than physical phase or substratum type. Because the two zones were characterized by contrasting residual disinfectant levels and water age, these parameters could be the major factors affecting microbial composition in DWDS. Other parameters, such as age of biofilm on sensors (Table 2), pipe diameter, velocity, and pipe material (Table S2), differed among the studied sites, but similarities in microbial composition were observed within a zone rather than between zones (Figure 2). Although a number of previous studies reported the distinctions in microbial compositions between planktonic and biofilm communities in DWDSs (Douterelo et al., 2013;

320 Ling et al., 2016), our nMDS analysis indicated that microbial communities in water and biofilm
321 samples collected from the same site in the present study were similar. This inconsistency might
322 be derived from the age of biofilms and shear stress. Most of the previous studies examined
323 mature biofilms developed on pipe walls or water meters with presumably limited shear stress,
324 whereas our biofilm samples were relatively immature (i.e., 6 to 18 months old) and collected
325 from the surface of the sensors inserted in the center of water pipe with greater shear stress due to
326 higher water velocity.
327 Taxa identified in samples with high levels of a disinfectant like monochloramine include
328 species that are resistant to or tolerant of disinfectants. The genus *Mycobacterium* predominated
329 in Zone 1 where the residual disinfectant level was relatively high. Previous studies indicated that
330 chloramine is less effective than chlorine against *Mycobacterium* spp. and they are among the
331 most dominant members of the microbial community in chloraminated DWDS (Donohue et al.,
332 2015; Gomez-Smith et al., 2015). In Zone 2, relatively low residual disinfectant levels may have
333 allowed growth of other bacterial species including those susceptible to monochloramine. The
334 observed difference in microbial composition between the two zones could be primarily due to
335 different residual chloramine levels, because some previous studies suggested that the
336 disinfection pressure of chloramine substantially impacted microbial community structure in
337 DWDS (Cruz et al., 2020; Mi et al., 2015; Waak et al., 2019).
338 The other predominant taxon in Zone 1 was the family *Nitrosomonadaceae*, which is
339 represented by lithoautotrophic AOB that oxidize ammonia to nitrite (Prosser et al., 2014).
340 Although the concentration of ammonia was not measured in this study, free ammonia is
341 inevitably present in chloraminated drinking water as a consequence of the process to generate
342 monochloramine. The predominance of *Nitrosomonadaceae* in Zone 1 indicates biological
343 ammonia oxidation activities owing to the presence of free ammonia in the fresh chloraminated
344 water. The abundance of AOB was also demonstrated by ddPCR quantification where AOB 16S

345 rRNA genes were detected in all samples with high numbers of up to 1.55×10^5 copies/cm^2 in
346 biofilms and 9.07×10^4 copies/L in bulk water. Few studies have investigated the occurrence of
347 AOA in drinking water systems (Kasuga et al., 2010; Nagymáté et al., 2016; Van Der Wielen et
348 al., 2009), and it was reported that the number of AOA could exceed the number of AOB in
349 drinking water (Van Der Wielen et al., 2009). Our SYBR Green-based qPCR screening of AOB
350 and AOA *amoA* genes demonstrated that the AOB *amoA* gene is more widely distributed than
351 AOA *amoA* gene in this DWDS (Table S4). These results suggest that ammonia-oxidizing
352 activities of AOB contributing to nitrification were distributed across the DWDS.
353 There is a strong contrast in the predominance of the genus *Nitrospira* between the two zones
354 with higher relative abundance in Zone 2. *Nitrospira* is known as NOB and plays pivotal roles in
355 nitrification by oxidizing nitrite to nitrate (Daims and Wagner, 2018). The results suggest the
356 availability of nitrite produced as a result of ammonia oxidization and prominent nitrite
357 oxidization activities of *Nitrospira* in Zone 2, which was also implied in a recent study
358 investigating biofilm communities on pipe walls of a tropical DWDS (Cruz et al., 2020).
359 Nitrification, a biological oxidation of ammonia to nitrite by AOB and/or AOA and further to
360 nitrate by NOB, is a major issue for chloraminated DWDS (Zhang et al., 2009). This is because
361 the intermediate nitrite can also be oxidized by chloramine in drinking water, which consumes
362 chloramine and results in bacterial growth. In the present study, we observed the presence of
363 AOB across the DWDS as well as decreased total residual chlorine level and increased HPC
364 numbers in bulk water and abundance of *Nitrospira* in Zone 2, which collectively suggests the
365 occurrence of nitrification in the studied DWDS. Our observations on the distribution of nitrifiers
366 within the DWDS suggested that ammonia-oxidizers produce nitrite in Zone 1, which enhances
367 residual monochloramine decay, whereas in Zone 2, nitrite is oxidized by *Nitrospira* and
368 produces nitrate. One of the major limitations of this study is a lack of measurements of
369 ammonia, nitrite, and nitrate concentrations to confirm this process. Another limitation is that

very small numbers of samples were available from Zone 2 (i.e., 2 sites) due to limited sampling access within the operational DWDS. Nonetheless, our results are consistent at sites S14 and S15 in Zone 2 and the data from Zone 2 appear as outliers for the statistics on Zone 1. We are therefore quite confident of our findings, despite of the practical limitation on sampling access.

5. Conclusions

The present study provides novel information on the abundance and composition of prokaryotes present in biofilms and water in a full-scale operational DWDS. Our main conclusions are:

- The trends in ddPCR-based microbial abundance in biofilm samples were similar to those in bulk water, with bacterial 16S rRNA gene being the most abundant, followed by *Mycobacterium* spp. and AOB, and archaeal 16S rRNA and cyanobacteria being less abundant than other microbial groups.
- Densities of prokaryotic genome copies on brass surface tended to be lower than on other substrate types (SS, PVC, and POM).
- *Mycobacterium* and AOB species were dominant in Zone 1 with low water age and sufficient residual monochloramine, whereas *Nitrospira* species dominated in Zone 2 with higher water age and depleted monochloramine level. This result suggests the occurrence of nitrification in the studied DWDS.
- Microbial community structure was primarily affected by differences in zones characterized by contrasting hydraulic and water quality characteristics, such as water age and residual disinfectant levels. Within each zone, the physical phase (biofilm vs bulk water) had a greater influence on microbial communities than sampling location.

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Table 1. Water quality parameters and abundance of prokaryotic genome copies in bulk water[a].

			Water quality parameters					Prokaryotic genome copies (copies/L)				
Zone	Site ID	Water age (h)[b]	Temp. (°C)	pH	Total chlorine (mg/L)	Free chlorine (mg/L)	HPC[c] (CFU/ml)	*Bacteria*	*Archaea*	*Mycobacterium* spp.	AOB	Cyanobacteria
1	S1	16.0	28.7	7.36	2.0	<0.1	<1	5.30×10^{4}	4.75×10^{2}	1.83×10^{4}	1.41×10^{4}	2.10×10^{2}
	S2	12.3	NA	NA[c]	NA	NA	NA	NA	NA	NA	NA	NA
	S3	7.9	28.8	6.89	1.9	<0.1	<1	4.65×10^{5}	2.70×10^{3}	1.61×10^{5}	9.07×10^{4}	3.90×10^{3}
	S4	20.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	S5	10.0	29.0	7.39	1.9	<0.1	2	4.68×10^{4}	2.75×10^{2}	1.89×10^{4}	7.70×10^{3}	3.65×10^{2}
	S6	9.5	29.3	7.48	1.9	<0.1	1.5	6.12×10^{4}	5.70×10^{2}	1.91×10^{4}	7.15×10^{3}	7.00×10^{2}
	S7	11.0	29.3	7.57	1.9	<0.1	NT[d]	1.46×10^{5}	2.20×10^{2}	7.90×10^{4}	1.13×10^{4}	1.28×10^{4}
	S8	8.1	29.3	7.67	1.9	<0.1	1	3.12×10^{4}	1.10×10^{3}	9.15×10^{3}	6.50×10^{3}	2.55×10^{2}
	S9	5.0	29.2	7.48	1.8	<0.1	<1	1.20×10^{5}	4.73×10^{3}	4.87×10^{4}	1.14×10^{4}	6.67×10^{2}
	S10	3.1	29.1	7.51	1.9	<0.1	<1	3.04×10^{4}	3.00×10^{2}	1.89×10^{4}	3.57×10^{3}	2.98×10^{2}
	S11	11.1	29.1	7.65	1.9	<0.1	<1	3.93×10^{4}	3.43×10^{2}	1.24×10^{4}	6.03×10^{3}	9.67×10^{2}
	S12	7.8	29.2	7.43	1.4	<0.1	2	4.61×10^{4}	6.33×10^{2}	1.98×10^{4}	9.13×10^{3}	6.67×10^{2}
	S13	12.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	S16	13.9	30.0	7.53	NT	NT	2.5	2.47×10^{5}	1.07×10^{4}	4.73×10^{4}	2.24×10^{4}	1.43×10^{2}
	S17	17.2	29.1	7.27	NT	NT	1	4.67×10^{4}	6.67×10^{2}	2.05×10^{4}	1.02×10^{4}	1.40×10^{3}
2	S14	45.1	29.2	7.40	0.2	<0.1	8.5	1.51×10^{6}	2.79×10^{4}	2.46×10^{4}	4.23×10^{4}	1.57×10^{4}
	S15	35.9	30.3	7.74	0.35	<0.1	11.5	1.09×10^{6}	1.73×10^{3}	2.67×10^{4}	1.48×10^{4}	6.47×10^{3}
							Mean	1.20×10^{5}	1.12×10^{3}	2.69×10^{4}	1.23×10^{4}	1.05×10^{3}

[a] One bulk water sample (n = 1) was collected from each sampling site where the sample was available and analyzed once without technical replicate.

[b] 20-h average water age calculated with the EPANET hydraulic model.

[c] HPC, heterotrophic plate count; CFU, colony-forming units.

[d] NA, sample not available.

[e] NT, not tested.

Table 2. Abundance of prokaryotic genome copies in biofilm samples[a].

Zone	Site	Biofilm age (mo)	*Bacteria*				*Archaea*				*Mycobacterium* spp.				AOB				Cyanobacteria			
			Brass	SS[b]	PVC[b]	POM[b]	Brass	SS	PVC	POM	Brass	SS	PVC	POM	Brass	SS	PVC	POM	Brass	SS	PVC	POM
1	S1	12	1.90	3.98	3.35	NA[c]	<0.09	1.67	1.50	NA	0.24	4.14	2.69	NA	1.84	2.93	2.50	NA	0.96	0.97	1.06	NA
	S2	6	NA	3.47	NA	3.97	NA	0.06	NA	1.17	NA	3.19	NA	2.96	NA	2.47	NA	3.41	NA	1.44	NA	2.01
	S3	12	3.97	4.64	3.88	NA	1.93	2.60	1.15	NA	3.07	3.75	3.39	NA	3.95	4.61	2.91	NA	1.54	2.15	0.88	NA
	S4	10	NA	3.19	NA	4.89	NA	1.16	NA	0.94	NA	2.89	NA	3.46	NA	2.80	NA	3.42	NA	1.12	NA	2.17
	S5	12	2.29	4.21	4.41	NA	<-0.11	1.49	1.87	NA	<-0.11	4.30	4.60	NA	1.66	3.58	3.25	NA	0.03	2.22	1.48	NA
	S6	18	1.96	4.71	3.76	4.98	<0.12	2.56	1.48	3.26	<-0.12	4.78	3.48	4.38	1.72	3.57	3.16	3.83	0.78	2.22	1.72	2.50
	S7	6	4.45	4.37	4.60	NA	1.24	<0.61	0.32	NA	4.11	4.19	4.79	NA	3.33	4.04	2.69	NA	1.84	1.60	0.96	NA
	S8	18	NA	4.70	3.70	4.41	NA	2.71	1.52	2.57	NA	3.06	3.00	3.17	NA	4.01	2.77	3.60	NA	2.02	1.26	2.03
	S9	12	2.56	4.74	3.20	NA	<-0.02	1.81	0.34	NA	0.33	4.81	2.53	NA	1.14	3.42	2.39	NA	<-0.02	1.87	0.62	NA
	S10	12	1.78	4.15	3.39	4.88	<-0.01	1.50	0.80	2.98	<-0.01	4.29	3.18	4.53	1.24	3.44	2.95	3.63	<-0.01	2.18	1.08	2.46
	S11	6	2.03	4.71	3.98	3.87	<-0.04	1.85	1.14	0.49	1.26	3.88	4.24	3.88	1.52	4.70	2.73	2.70	<-0.04	2.56	0.72	0.76
	S12	6	NA	5.63	4.40	4.43	NA	2.25	0.21	<0.11	NA	4.87	4.23	4.03	NA	4.91	3.63	3.63	NA	1.54	0.95	1.22
	S13	6	NA	4.79	3.29	NA	NA	1.99	0.12	NA	NA	4.24	2.67	NA	NA	4.70	2.98	NA	NA	2.60	0.99	NA
	S16	12	2.72	6.02	4.04	5.06	<0.06	2.32	1.22	2.98	1.84	4.13	3.30	3.58	2.22	4.85	2.96	3.49	<0.06	3.09	1.80	2.86
	S17	6	NA	5.20	4.22	4.36	NA	1.69	1.25	2.09	NA	4.65	4.22	3.74	NA	5.19	3.56	3.89	NA	2.35	1.46	2.21
2	S14	18	4.09	5.62	3.98	NA	0.52	2.53	-0.07	NA	2.54	3.94	2.53	NA	2.91	4.30	2.49	NA	1.05	3.78	0.96	NA
	S15	12	2.48	5.19	4.88	NA	<0.01	1.33	0.86	NA	1.13	3.59	3.15	NA	1.98	3.48	4.35	NA	<0.01	3.37	1.40	NA
		Mean	2.66	4.67	3.94	4.54	1.23	1.85	0.91	2.06	1.81	4.04	3.47	3.75	2.14	3.94	3.02	3.51	1.03	2.18	1.16	2.02
		SD	1.02	0.75	0.50	0.44	0.70	0.68	0.60	1.06	1.36	0.60	0.77	0.52	0.90	0.81	0.52	0.35	0.63	0.76	0.35	0.65
		No. of positive	11	17	15	9	3	16	15	8	8	17	15	9	11	17	15	9	6	17	15	9
		(%)	(100)	(100)	(100)	(100)	(27)	(94)	(100)	(89)	(73)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(55)	(100)	(100)	(100)

[a] Values are expressed in $\log_{10}$ copies/cm^2. One biofilm sample (n = 1) was collected and analyzed from each surface type and analyzed once without technical replicate.

[b] SS, stainless steel; PVC, polyvinyl chloride; POM, polyoxymethylene.

[c] NA, not available.

Table 3. Alpha diversity of microbial communities in biofilms on various surface materials attached to sensors compared to bulk water communities.

Zone	Site	Sample type		DNA (ng/µL)	Richness (OTUs)	Shannon	Simpson
1	S10	Biofilm	Polyvinyl chloride	6.46	243	2.954	6.962
			Stainless steel	4.06	284	2.973	6.564
			Polyoxymethylene	12.44	267	3.051	10.743
		Water		3.21	360	3.508	0.893
	S11	Biofilm	Polyvinyl chloride	9.53	408	2.599	3.367
			Stainless steel	6.47	188	1.897	2.297
			Polyoxymethylene	5.71	264	2.808	5.625
		Water		3.63	281	4.030	0.962
2	S14	Biofilm	Stainless steel	3.59	244	3.275	9.955
		Water		5.81	173	2.366	0.756
	S15	Biofilm	Stainless steel	11.2	209	3.362	10.682
		Water		3.36	132	2.256	0.734

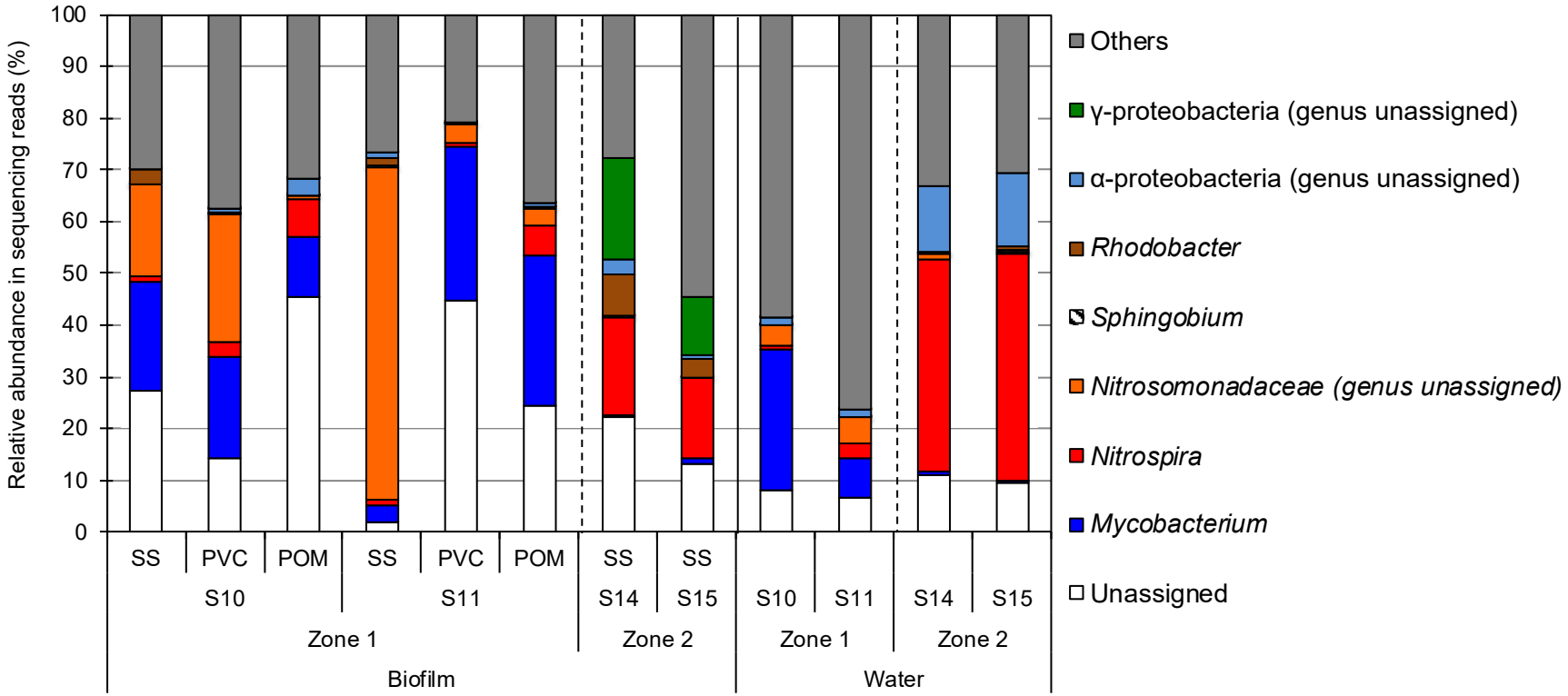

Figure 1. Comparison of microbial composition in biofilms on different types of surfaces (stainless steel ["SS"], polyvinyl chloride ["PVC"], and polyoxymethylene ["POM"]) and in bulk water collected from four sites. Biofilm and bulk water samples were analyzed without technical replicates. Results are expressed as relative abundance in total sequencing reads (%) of prokaryotic 16S rRNA genes (including *Archaea* and *Bacteria*).

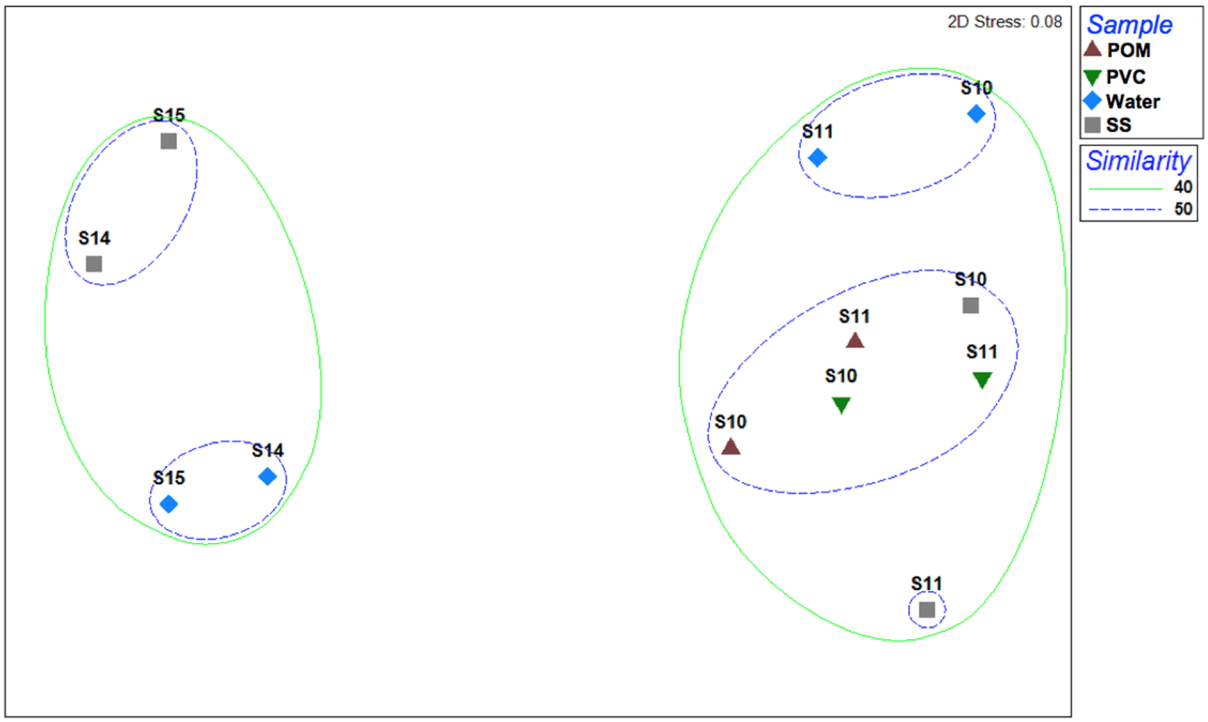

Figure 2. Non-metric multidimensional scaling (nMDS) analysis of microbial community composition (including *Archaea* and *Bacteria*) showing clear dissimilarity in community structure between Zone 1 (S10 and S11) and Zone 2 (S14 and S15) samples. The microbial community structure was profiled using the sequencing data of 16S rRNA gene amplicons at the OTU level for biofilm and water samples collected from four sites. Biofilm and bulk water samples were analyzed without technical replicates. POM, polyoxymethylene; PVC, polyvinyl chloride; SS, stainless steel.