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Abstract: Disentangling factors influencing suspended bacterial community structure across distribution system and building plumbing provides insight into microbial control strategies from source to tap. Water quality parameters (residence time, chlorine, and total cells) and bacterial community structure were investigated across a full-scale chlorinated drinking water distribution system. Sampling was conducted in treated water, in different areas of the distribution system and in hospital building plumbing. Bacterial community was evaluated using 16S rRNA gene sequencing. Bacterial community structure clearly differed between treated, distributed, and premise plumbing water samples. While Proteobacteria (60%), Planctomycetes (20%), and Bacteroidetes (10%) were the most abundant phyla in treated water, Proteobacteria largely dominated distribution system sites (98%) and taps (91%). Distributed and tap water differed in their Proteobacteria profile: Alphaproteobacteria was dominant in distributed water (92% vs. 65% in tap waters), whereas Betaproteobacteria was most abundant in tap water (18% vs. 2% in the distribution system). Finally, clustering of bacterial community profiles was largely explained by differences in chlorine residual concentration, total bacterial count, and water residence time. Residual disinfectant and hydraulic residence time were determinant factors of the community structure in main pipes and building plumbing, rather than treated water bacterial communities.

Keywords: drinking water distribution system; chlorine residual; residence time; high-throughput sequencing; building plumbing; bacterial community structure

1. Introduction

The use of residual disinfectants is a common practice in North America for limiting microbiological contamination in drinking water distribution systems (DWDS) and supplying safe water to consumers. Nevertheless, due to complex interactions between chemical, physical, and operational parameters, bacterial regrowth and proliferation of resistant pathogens in distribution systems can occur even in the presence of a residual disinfectant [1–3]. Bacterial growth occurs throughout DWDS, with significant increase in bacterial load observed in building plumbing due to unavoidable stagnation, low levels of disinfectant residual, and pipe material [4–7]. Opportunistic plumbing pathogens (OPPs), such as *Legionella* and *Mycobacterium avium* complex, are microbial inhabitants of drinking water systems and the primary cause of waterborne disease outbreaks in developed countries [8,9]. The presence of OPPs in hospital water plumbing is of special concern, leading to waterborne bacterial infections among susceptible patient populations [10–12].

The bacterial community structure has been examined in municipal DWDS with or without disinfectant, providing a better understanding of the planktonic microbial community structure in different locations throughout the systems [13–21]. In general, the composition of planktonic bacterial communities across full-scale DWDS remains relatively stable over time with the exception of minor changes at sites with longer water residence times [14,15,17–19]. In building plumbing, the importance of water treatment, distribution management choices, and stagnation in defining the composition of microbial communities has been investigated through pilot [22] and full-scale studies [23,24]. In their pilot study, Ji et al. observed that the utility source water and the water treatment were dominant factors in shaping the plumbing microbiome [22]. More recently, a full-scale study in an unchlorinated system also observed the water treatment process as a determinant of the bacterial community in treated and tap water; however, the source water did not have a detectable contribution to the treated and tap water community [24]. Furthermore, a study conducted in a building fed by chlorinated municipal water reported pipe diameter and stagnation within the building to greatly affect the community at the tap [23]. However, analyses of the microbial community structure across all subsystems of a full-scale chlorinated DWDS, from treated water, different distribution system sectors, and taps from premise plumbing sites are scarce [25].

In this study, the diversity and relative abundance of the planktonic bacterial communities in a chlorinated full-scale DWDS in Canada is reported. The bacterial profiles from water leaving two treatment plants, from different locations in five distribution system sectors, and from ten taps in a hospital were compared. The main objectives were to answer the following questions: (i) Does bacterial community composition vary between treated water, distributed water, and premise plumbing? (ii) Which microbial taxa are shared between these subsystems? (iii) Can cosmopolitan and endemic species be identified across the subsystems? (iv) Do water quality parameters influence the structure and diversity of microbial communities? Overall, this study delivers a better understanding of the factors influencing bacterial communities in the water leaving the treatment plant and across the distribution system pipes and premise plumbing, thus enabling better management of bacterial contaminants in DWDS.

2. Materials and Methods

2.1. Water Sampling

A total of 128 water samples were taken between 2012 to 2014 from two water treatment plants ($n = 8$), five sectors in the distribution system ($n = 110$), and taps in a hospital ($n = 10$) in Canada. Figure 1 presents the schematic diagram of the DWDS used for this study. This system serves approximately 1.5 million people distributed along nearly 4,000 km of pipes. Treated water samples were collected after chlorination from two treatment plants (TWA and TWB), both supplied by surface water. Samples were collected at four sampling events, at intervals of approximately three weeks between events (summer 2014). Water treatment consisted of flocculation, filtration, and chlorination. In addition, TWB had an ozonation step prior to chlorination. Sampling in the distribution system was performed twice from five sectors (DS1 through DS5) at intervals of about four weeks between the two sampling events. The pipes sampled in these sectors were made of cast iron or ductile iron with diameters ranging between 4 and 14 inches. For each distribution sector, samples were collected from multiple locations, at outdoor household taps after flushing until the water temperature was constant, confirming that the water from the main pipe had reached the tap. DS1 had a total length of 64.3 km and was sampled at ten different sites (two inlets, two dead-ends, and six sites along the sector). DS2 was 88.2 km long and was sampled at 12 different sites (three inlets, four dead-ends, and five sites along the sector). DS1 and DS2 were sampled in fall 2012. DS3 had a length of 99 km and was sampled at 13 sites (two inlets, six dead-ends, and five sites along the sector). DS4 was 77.5 km long and sampled at 10 sites (three inlets, four dead-ends, and three sites along the sector). DS3 and DS4 were sampled in the summer of 2013. DS5 was 52.8 km long and was sampled at ten sites (two inlets, four dead-ends, and four sites along

the sector) in fall 2013. In total, 55 sites across 5 sectors of the distribution system were sampled twice. Finally, samples from building plumbing were collected in a 60-year-old hospital with copper piping, during summer 2013. Samples were collected from conventional and foot-operated taps, located in five different wings and on various floors for a total of 10 taps (Table S1). Sampling was performed once at each tap (TAP1 through TAP10) collecting the first flush cold water after overnight stagnation. All samples were collected using sterile bottles, with sodium thiosulfate (1%).

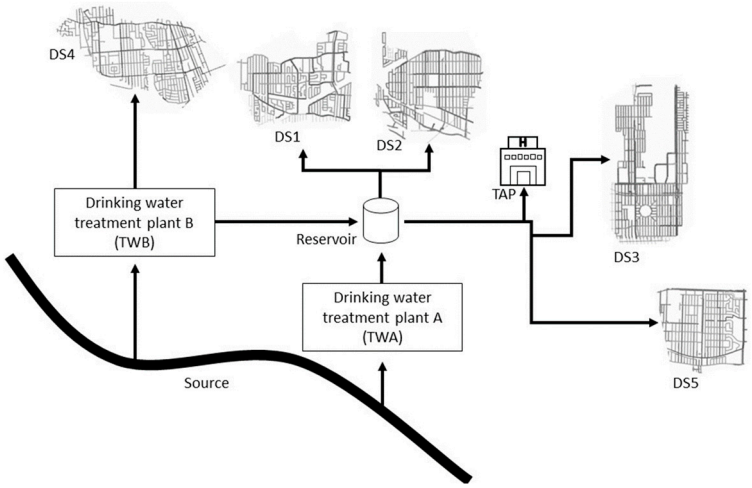

Figure 1. Schematic diagram of the drinking water distribution system sampled illustrating the subsystems: Treated water (TW), distribution system (DS), and building plumbing (TAP).

2.2. Water Quality Analysis

Hydraulic residence time at each sampled point was determined using calibrated models by WaterGEMS V8i software. All water samples were analyzed onsite for free chlorine using the N,N-diethyl-p-phenylenediamine method and a DR/2010 spectrophotometer (HACH Company, Loveland, CO, USA). Total cell counts were determined by fluorescence microscopy using DAPI for treatment plant and distribution system samples. Enumeration was done at 1000-fold magnification, with an epifluorescence microscope (Olympus, Tokyo, Japan). Total copper concentrations in tap water samples were measured by inductively coupled plasma mass spectrometry following the EPA 200.8 method with prior acid digestion in 0.5% HNO_3 for 24 h [26].

2.3. Filtration and DNA Extraction

Samples were filtered through a 47 mm diameter 0.45 µm pore sized mixed cellulose ester membrane. The choice of a 0.45 µm pore membrane may have excluded a fraction of bacteria [27], leading to a sub-representation of the absolute biodiversity. A volume of 10 L was filtered for treated water collected at the plant. For distribution system sectors, all samples collected from the different locations were pooled at the time of filtration using 500 mL of each sample. As an example, 500 mL of each sample collected from the ten sites of DS1 on the first sampling event were pooled together and thereafter named DS1-1. Pooling of samples was done to have a consolidated and representative sample of the diversity throughout the entire sector for each sampling event. In total, 10 DNA extracts were obtained for the five distribution system sectors sampled twice each. Finally, 500 mL of each tap water sample were individually filtered. Each filter was inserted into an extraction tube containing a garnet matrix and one 1/4 inch ceramic sphere (Lysing Matrix A, MP Biomedicals, Solon, OH, USA). DNA from the obtained biomass was extracted using the bead-beating protocol adapted as previously described [28]. Briefly, lysing buffer was added to each extraction tube prior to the bead-beating step performed on FastPrep-24 (MP Biomedicals, Solon, OH, USA), followed by ammonium acetate

precipitation, and successive cold 70% ethanol washes. Extracted DNA was stored at −25 °C until further analysis.

2.4. Bacterial 16S rRNA Gene PCR-Amplification and Sequencing

DNA extracts were amplified and sequenced by the Research and Testing Laboratories (Lubbock, TX, USA). The bacterial 16S rRNA gene was PCR-amplified for sequencing using a forward and reverse fusion primer as previously described [29]. PCR products were sequenced using paired-end Illumina MiSeq sequencing (Illumina, Inc., San Diego, CA, USA) 2 × 250 flow cell. Raw sequencing reads were processed with the software Mothur v.1.34.4 [30]. Quality filtering of reads was performed to remove low quality and chimeric sequences. Briefly, data were extracted from raw fastq files, and sequences containing ambiguous bases and/or longer than the expected fragment were discarded. Subsequently, sequences were aligned against the Silva reference alignment (Release 119) [31]. Potential chimeric sequences were identified using the UCHIME algorithm, and sequences not associated with bacteria were removed. Rare Operational Taxonomic Units (OTUs) identified at the 0.005% thresholds were removed [32]. A sample from treatment plant A (TWA-4) was removed prior to subsampling due to the small number of sequences obtained from that sample. Subsampling was performed to select 1013 sequences at random, corresponding to the sampling effort of the smallest library, to avoid downstream bias in statistical analysis due to varying sampling efforts. After quality filtering reads, eliminating rare sequences, and subsampling, the final OTU table consisted of 27,351 sequences and 762 OTUs that were assigned to different taxa levels using the RDP classifier. The taxonomic assignation of the OTU was based on the RDP classifier (Release 11) [33] with a minimum confidence threshold of 80% at the domain level and 50% for other taxonomic levels. Mothur was also used to calculate richness estimators and diversity indices and generate Venn diagrams displaying shared OTUs between sample groups.

Statistical analyses were performed using the software R (R Development Core Team, 2008). Ordination of the samples according to their bacterial community profile was first examined with a principal component analysis (PCA) using the package "vegan" [34]. The Hellinger transformation was applied to the 16S OTU relative abundance dataset before computing the Euclidean distance matrix used in the PCA to avoid unduly heavy weighting of rare OTUs [35]. The relationship between the water quality variable and the coordinates of the samples along the two first axes of the PCA was explored by computing Spearman correlation analyses. The Euclidean distance matrix was used to generate an unweighted pair group method with arithmetic mean (UPGMA) agglomerative clustering of the samples. The identification of statistically different clusters was done by performing 999 permutations of the OTU table separately across the samples and comparing the observed similarity score of each cluster against the expected values under the null hypothesis using the similarity profile tool (SIMPROF) implemented in the package "clustsig" [36]. Indicator OTU for significant clusters were identified using the indicator species analysis procedure implemented in the package "indicspecies" [37]. Minimal significance level (alpha) of the indicator OTU was 0.05, tested against 999 random permutations of samples among the clusters. For all statistical analysis, the significance level was defined as $p < 0.05$.

2.5. Raw High-Throughput Sequencing Data Accession Number

The raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP071223. In total, 28 sequences were deposited: 8 sequences for treated water samples (4 sampling events per water treatment plant); 10 sequences for distribution sectors, identified from 1 to 5 for each of the studied sectors (2 sampling events per sector); and 10 sequences for tap samples (1 sampling event per tap). Each sequence associated to distribution sectors is representative of the different locations sampled within the sector, as described in Section 2.1.

3. Results

3.1. Amplification of Total Bacterial Counts and Decrease in Disinfectant Residual Concentration through the DWDS

Free chlorine concentration decreased when the water residence time increased from water treatment plants (TW samples) to all sectors of the distribution system (DS samples) and even more so in taps from building plumbing (TAP samples) (Figure 2, $r^2 = -0.68$). The concentration of disinfectant residual ranged from 0.8 to 1.8 mg/L in TW samples, 0.01 to 1.1 mg/L in DS samples, and 0 to 0.10 mg/L in TAP samples from building plumbing. Total bacterial counts ranged in order of magnitude from 10^3 to 10^4 cells/mL in TW samples, 10^2 to 10^5 cells/mL in DS samples, and 10^4 to 10^5 cells/mL in TAP samples. Total bacterial counts increased while chlorine residuals decreased ($r^2 = -0.51$), mostly at stagnant sites and TAP samples. Large variations of disinfectant residual concentration, water residence time, and total bacterial count were observed over the five sectors in DS samples. The range of values observed for these parameters reflects the fact that samples within a sector were collected from various types of sampling points such as inlets (high disinfectant residuals, low levels of water residence time, and total bacteria), dead-ends (low disinfectant residuals and higher levels of water residence time and total bacteria), as well as points along the sectors encompassing intermediate values. It is important to consider that environmental conditions varied within a sector, representing real conditions occurring under normal operation.

3.2. Bacterial Community Diversity and Composition

Diversity analyses, including richness estimators and diversity indices, were used to assess the level of richness and evenness of bacterial communities (Table 1). The coverage of the sequence libraries was high, ranging from 97% to 98%, confirming that the core communities were covered in all samples. Diversity and richness were not significantly different between TW, DS, and TAP samples when performing an ANOVA statistical test ($p > 0.05$). Evenness, however, was found to be significantly different, with higher values in treated water vs. distribution sectors or tap samples.

Sequences were classified into 18 bacterial phyla, 37 classes, 64 orders, 95 families, and 149 genera. The relative abundance of different phyla and subclasses of Proteobacteria, as well as the hierarchical clustering of the samples is shown in Figure 3. The bacterial community structure changed noticeably among the different subsystems resulting in four clear clusters: One from each water treatment plant, one from distribution system sectors (9/10 samples), and one from taps of building plumbing (8/10 samples). Six phyla were identified in TW samples (Figure 3). All relative abundance numbers are presented with a standard deviation value within the analyzed group (treated, distributed, or tap water). Proteobacteria was the dominant phylum (60% ± 16%) and represented by Alphaproteobacteria (20% ± 17%), Gammaproteobacteria (10% ± 12%), and Betaproteobacteria (8% ± 14%). The remaining phyla were Planctomycetes (20% ± 12%), Bacteroidetes (10% ± 9%), Firmicutes (6% ± 6%), Cyanobacteria (2% ± 5%), and Actinobacteria (2% ± 2%). While the samples from both treatment plants (TWA and TWB) had the same groups of bacteria, they varied in the proportions in which these groups were present. Proteobacteria (76% ± 8% vs. 47% ± 3%) and Firmicutes (11% ± 7% vs. 3% ± 1%) were most abundant in TWA, whereas Planctomycetes (10% ± 12% vs. 27% ± 3%) and Bacteroidetes (2% ± 2% vs. 16% ± 6%) were most abundant in TWB. This variability likely reflects differences in treatment processes (presence of ozonation) and/or differences in chlorine dosing. TWB has a 70% higher median chlorine residual concentration than TWA). Furthermore, TWA samples exhibited greater variation in total bacterial counts (Figure 2).

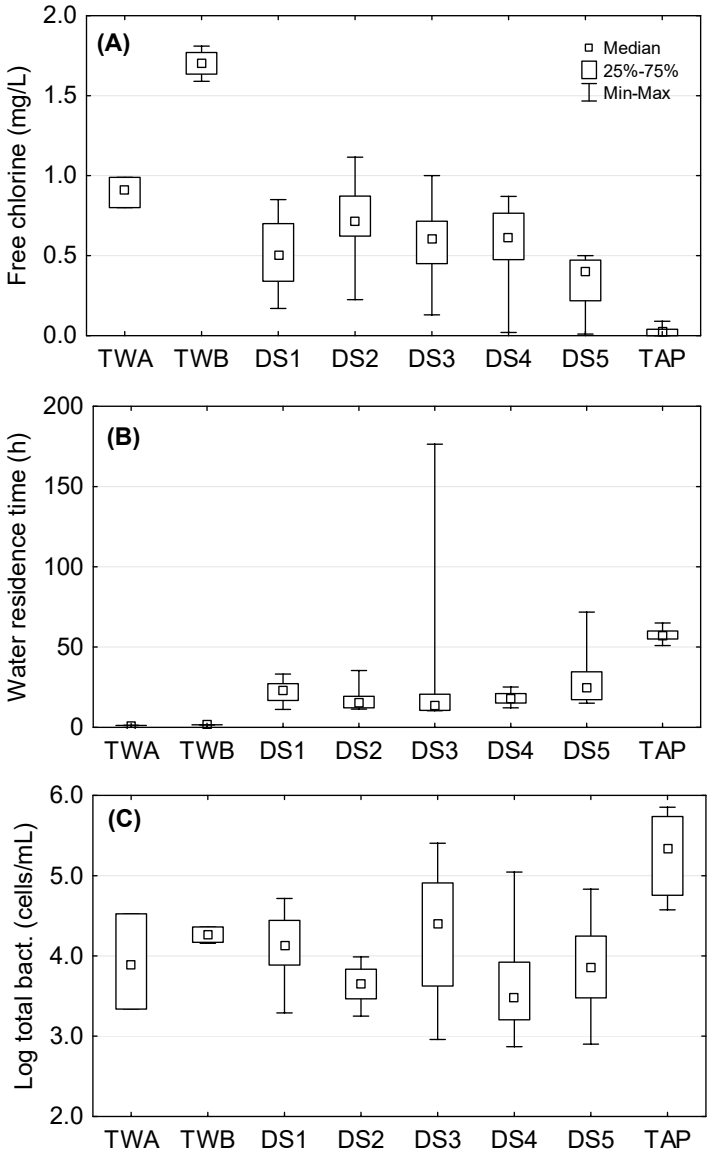

Figure 2. Box plots showing the water quality across the subsystems (treated water (TW), distribution system (DS), and building plumbing (TAP)): (**A**) Free chlorine, (**B**) water residence time, and (**C**) log of total bacterial counts.

Table 1. Average estimators and standard deviation of alpha-diversity for treated water (TW), distributed water (DS), and tap water (TAP).

Subsystem	Coverage [a]	Shannon	Ace	Chao	Evenness [b]
TW	0.98 ± 0.01	3.21 ± 0.26	105 ± 28	106 ± 28	0.72 ± 0.03
DS	0.97 ± 0.01	2.21 ± 1.18	138 ± 51	108 ± 30	0.51 ± 0.23
TAP	0.97 ± 0.01	2.64 ± 0.91	115 ± 50	101 ± 54	0.62 ± 0.14

[a] Good's coverage; [b] Shannon index-based measure of evenness.

Eight bacterial phyla were observed in the DS sectors (excluding sample DS3-2), with a clear dominance of Proteobacteria (98% ± 4%) mainly represented by Alphaproteobacteria (92% ± 8%). The following seven phyla accounted for 2% ± 5% of the sequences. Samples from the five different sectors displayed similar microbial populations (Figure 3), including DS4, which was exclusively supplied by TWB while the other sectors were supplied by a mix of TWA and TWB. Only DS3-2 exhibited a unique

bacterial community structure in comparison to the other DS samples. DS3 presented the highest levels of total bacteria and water residence time among the DS samples.

Analyses of TAP samples enabled identification of 11 bacterial phyla. Proteobacteria was once again the most abundant phylum (91% ± 9%), with Alphaproteobacteria (65% ± 20%), Betaproteobacteria (18% ± 15%) as the most abundant representative classes. The building tap samples presented very similar composition, with eight out of ten tap samples clustering together (Figure 3). Two different taps (TAP1 and TAP2) clustered separately from the others, closer to distribution system and treated water samples. These samples, which were taken from the intensive care department on the third floor of the hospital building used for our study, had the lowest total bacterial counts and water residence times. A larger proportion of Alphaproteobacteria was observed compared to the other taps. Moreover, TAP1 was located in a bathroom and TAP2 was near the entrance of the building, so these taps experienced less stagnation. The bacterial structures in the other taps were similar and grouped according to stagnation time and locations. TAP3 and TAP4 were taken from the transplantation department on the sixth floor. TAP9 was from the neonatology unit on the fourth floor. TAP3, TAP4, and TAP9 had the highest total bacterial counts and the lowest abundance of Proteobacteria (74–85%) and Alphaproteobacteria (44–52%). The remaining taps (TAP5, TAP6, TAP7, TAP8, and TAP10) were located in the departments of transplant and oncology on the second and third floors, respectively, and formed a significant cluster. These differences between taps suggest that specific conditions at each location (hydraulics, material, patterns of use, etc.) are likely drivers in the variability of the classes of Proteobacteria present.

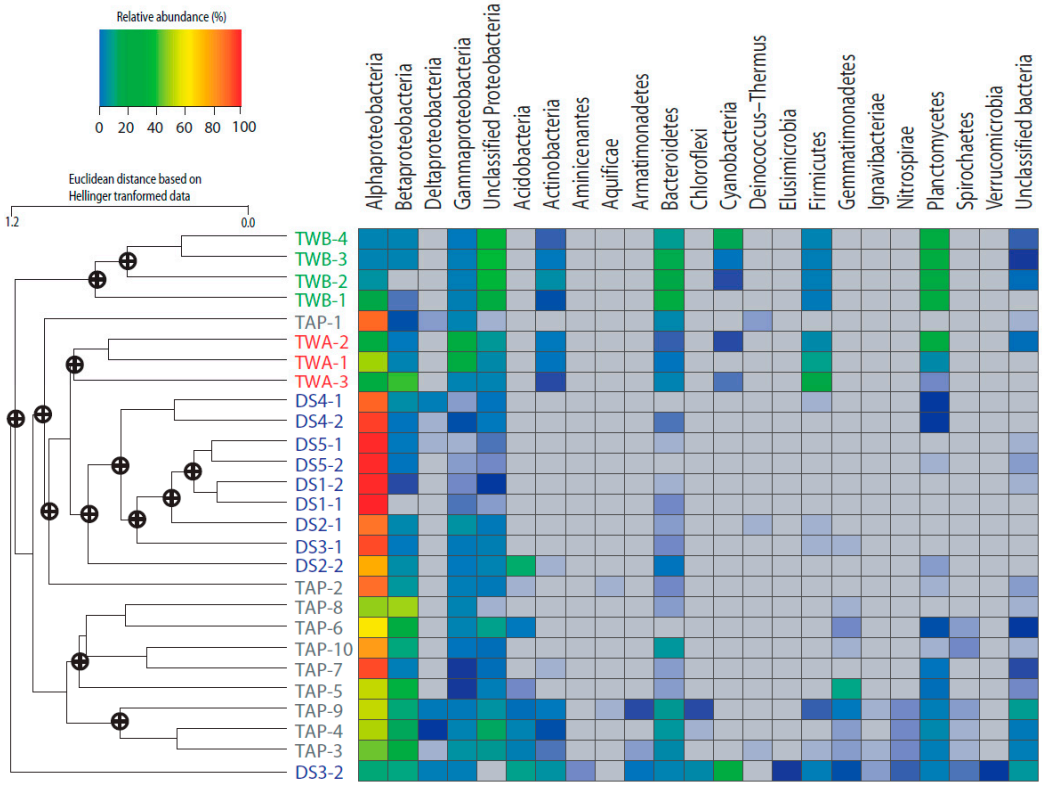

Figure 3. Heat map illustrates the relative abundance of different phyla and Proteobacteria classes in treated water (TW), distribution system (DS), and tap water (TAP) samples. Hierarchical clustering of samples is based on the similarity profile analysis of their bacterial community profiles (significant clusters at $\alpha = 0.05$). Samples with similar community structure cluster together, taking into account the relative abundance of each OTU.

3.3. Differences between Bacterial Communities over Time

The replicates for the treated water and distribution system sectors were collected at set time intervals. Thus, we analyzed differences between these replicates to detect any temporal variations occurring in the bacterial profiles at these locations. In general, the profiles from the treated water and distribution system replicates were stable within the sampling timeframe. As shown in the dendrogram (Figure 3), replicates from each location clustered very closely. Samples of treated water presented small variability in bacterial composition over time (samples were collected at three-week intervals). All samples from TWA and TWB clustered separately, reflecting differences in water source (presence of a 14 km canal) and treatment. TWB had a 70% higher median chlorine residual concentration than TWA, whereas TWA samples exhibited greater variation in total bacterial counts (Figure 2). The samples from sectors in the distribution system exhibited relative stability over the three-week sampling periods. DS1, DS4, and DS5 replicates clustered together, and presented very similar bacterial compositions, whereas DS2's profiles varied slightly between replicates. In contrast, DS3 replicates presented completely different profiles. As stated previously, DS3-2 displayed a unique profile compared to DS3-1 and other DS samples. This could be associated with the large variation in residence time observed between the DS3-1 and DS3-2 samplings (Figure 2).

3.4. Distribution of OTUs across the Subsystems and Their Relationship with Water Quality Parameters and Estimators of Diversity and Richness

A principal component analysis (PCA) was conducted to explore potential variables that could be influencing the taxonomic profile of bacterial communities in this study (Figure 4). The first two axes (PC1 and PC2) of this analysis explained 53% of the variability observed in the bacterial communities. Free chlorine, total bacteria, and water residence time were strongly correlated with PC2. Free chlorine was positively correlated (Spearman rho = 0.91), while total bacteria and water residence time were negatively correlated (Spearman rho = -0.71 and -0.81, respectively) with this axis. PC1 was positively correlated (Spearman rho = 0.63) with the number of viable bacteria in TAP samples. For these samples, a correlation was also found between axis PC2 and copper ions data (Spearman rho = -0.73). This correlation could not be evaluated for TW and DS samples since viable bacteria and copper data were not measured. With regards to the community diversity and richness of all samples combined (TW, DS, and TAP samples), no correlation was observed with the levels of chlorine residual, total bacteria, and water residence time (Spearman rho < 0.3).

3.5. Detection of Potential Opportunistic Pathogens, Cosmopolitan, and Endemic Species across the Specific Subsystems

The incidence of genera known to comprise potential opportunistic pathogens (OPPs) of interest was verified in the subsystems (see Table S2). *Pseudomonas* spp. and *Sphingomonas* spp. were detected at least once in all subgroups (14/27 and 23/27 samples, respectively). *Mycobacterium* spp. were detected in all TW samples and one TAP sample. *Legionella* spp. were only found in tap samples at a low frequency (3/10 samples).

To verify the taxonomic richness shared among the subsystems, and therefore identify ubiquitous species across the subsystems, the samples were categorized as treated water, distribution system, and taps to create a Venn diagram (Figure S1). Sample DS3-2 was excluded from the diagram because of the completely different bacterial composition found. The distribution system was the richest group, encompassing 39% exclusive OTUs, followed by the tap group (29%), and then the treated water group (18%). The distribution system shared 5% of OTUs with the treated water group and 5% of OTUs with the tap group. The treated water group shared 2% of OTUs with the tap group. The total shared richness between the three groups was 2%, including only members of Alphaproteobacteria (*Afipia* spp., *Sphingobium* spp., *Hyphomicrobium* spp., *Sphingorhabdus* spp., *Blastomonas* spp., *Methylobacterium* spp.) and Gammaproteobacteria (*Nevskia* spp. and *Pseudomonas* spp.).

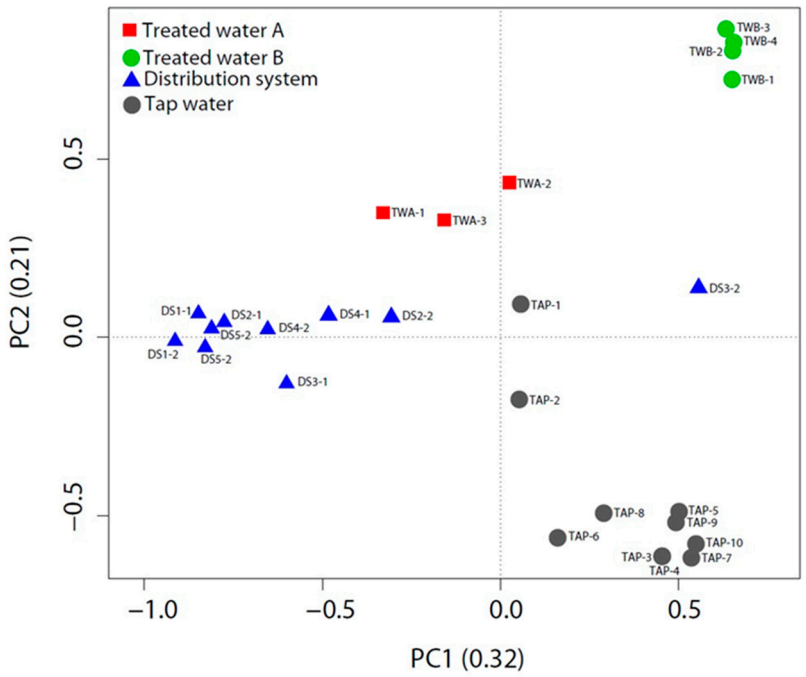

Figure 4. Ordination plot of principal component analysis (PCA) showing distribution of samples. Samples that cluster more closely together share a greater similarity structure. Axes PC1 and PC2 explained 53% of the variability in the data.

Indicator species analysis (Indval) was employed to highlight the OTU that best describes our three subsystems. This type of analysis reveals the most abundant indicator in a specific subsystem that is also present in the majority of samples from that group (33). The outcome of this investigation can be used to identify the presence of OPPs that may pose public health concerns, specifically in the building plumbing subgroup. Because of the completely different bacterial composition found in DS3-2, this sample was excluded from the indicator species analysis. The significant indicator OTUs with their respective number of sequences and statistically significant ($\alpha < 0.05$) indicator value for each subsystem are reported in Table 2. In this analysis, 46 OTUs were associated with one or two of our three subsystems. Thirty-eight of these OTUs were associated with only one group (TW, DS, or TAP) and eight with two groups (DS + TAP or TW + TAP). Treated water presented a great variety of bacterial indicators, including 23 exclusive indicators. The most abundant genera in this subgroup were *Arcticibacter* (8.4%), *Pirellula* (4.0%), *Planctomyces* (2.7%), *Bacillus* (2.6%), and *Aquisphaera* (2.4%). The *Mycobacterium* genus (0.9%) was also identified as an indicator in this group. Only five exclusive indicators were found in the distribution system group, all but one from the Alphaproteobacteria class: *Sphingorhabdus* (4.6%), *Brevundimonas* (0.7%), *Humitalea* (0.3%), and *Sphingopyxis* (0.2%). The non-Alphaproteobacteria indicator was from the Deltaproteobacteria class: *Peredibacter* (0.3%). Ten exclusive bacterial indicators were identified in the tap group, but all in relatively small abundance. The most abundant indicators in the TAP samples were also associated with DS samples: *Sphingomonas* (1.8% in DS and 17.7% in TAP), *Afipia* (0.6% in DS and 15% in TAP), *Hyphomicrobium* (3.4% in DS and 2.7% in TAP), and *Sphingobium* (1.7% in DS and 2.7% in TAP). One indicator belonging to the Planctomycetes (*Gemmata*) was associated with treated water and tap groups.

Table 2. Number of sequences of indicator species (at genus level) in treated water, distribution system, and taps from premise plumbing ($\alpha = 0.05$).

Phylum	Class	Order	Family	Genus	Indval	TW	DS	TAP
Acidobacteria	Acidobacteria_Gp4	Gp4	Gp4	*Gp4*	0.71	0	0	73
Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	*Mycobacterium*	1.00	64	0	1
			Propionibacteriaceae	*Propionibacterium*	0.62	6	1	0
Bacteroidetes	Bacteroidetes_incertae_sedis	Ohtaekwangia	Ohtaekwangia	*Ohtaekwangia*	0.71	0	0	16
	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Arcticibacter*	0.75	597	7	0
				Pedobacter	0.66	39	0	0
			Chitinophagaceae	*Flavisolibacter*	0.71	0	0	42
				Sediminibacterium	0.78	0	0	27
Cyanobacteria	Cyanobacteria	Family II	Family II	*GpIIa*	0.66	145	0	0
Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Bacillus*	0.93	181	0	0
	Clostridia	Clostridiales	Clostridiaceae 1	*Anaerobacter*	0.76	107	0	0
			Peptostreptococcaceae	*Clostridium XI*	0.85	26	0	0
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	0.83	0	1	127
Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	*Aquisphaera*	0.93	173	0	0
				Blastopirellula	0.66	13	0	0
				Gemmata	0.87	176	2	48
				Isosphaera	0.66	10	0	0
				Pirellula	0.92	287	0	9
				Planctomyces	0.90	192	12	0
				Rhodopirellula	0.85	20	0	0
				Singulisphaera	0.66	10	0	0
				Telmatocola	0.84	48	0	1
				Zavarzinella	0.91	65	0	3
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	0.67	0	62	0
		Rhizobiales	Beijerinckiaceae	*Beijerinckia*	0.76	0	24	54
			Bradyrhizobiaceae	*Afipia*	0.99	18	56	1523
				Bosea	0.91	23	144	69
			Hyphomicrobiaceae	*Hyphomicrobium*	0.97	23	311	274
			Phyllobacteriaceae	*Mesorhizobium*	0.76	24	0	0
		Rhodospirillales	Acetobacteraceae	*Humitalea*	0.58	0	25	0
			Rhodospirillaceae	*Dongia*	0.77	60	16	0
		Rickettsiales	Rickettsiaceae	*Orientia*	1.00	171	0	0
		Sphingomonadales	Erythrobacteraceae	*Altererythrobacter*	0.88	0	3	76
			Sphingomonadaceae	*Novosphingobium*	0.77	0	10	61
				Sphingobium	0.94	38	159	271
				Sphingomonas	0.99	22	164	1788
				Sphingopyxis	0.58	0	16	0
				Sphingorhabdus	0.94	41	415	1
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Chitinimonas*	0.66	465	0	0
			Burkholderiales_incertae_sedis	*Aquabacterium*	0.69	1	0	36
			Comamonadaceae	*Delftia*	0.60	8	2	0
		Ferrovales	Ferrovaceae	*Ferrovum*	0.71	0	0	11
	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	*Peredibacter*	0.58	0	29	0
	Gammaproteobacteria	Candidatus Carsonella	Candidatus Carsonella	*Candidatus Carsonella*	1.00	74	0	0
		Xanthomonadales	Sinobacteraceae	*Nevskia*	0.88	7	33	71
Spirochaetes	Spirochaetia	Spirochaetales	Leptospiraceae	*Leptospira*	0.63	0	0	7

Indicators in treated water
Indicators in distribution system
Indicators in premise plumbing
Indicators in distribution system and premise plumbing
Indicators in treated water and premise plumbing

4. Discussion

The relative influence of treated water quality on tap water quality and its hygienic implications has raised considerable attention and debate. The fundamental question raised is whether it is possible to determine the quality of water entering the distribution system that can ensure the maintenance of water quality throughout the primary and secondary distribution systems and, most importantly, in the building plumbing. If environmental conditions in building plumbing determine the abundance and diversity of fixed and suspended biomass, then local management and on-site treatment may be the most effective solutions to maintain water quality up to the tap. The impact of the primary distribution system on water quality has been documented in terms of loss of disinfectant residual, bacterial indicator compliance, and bacterial biomass [38]. Industry focus was placed on biological stability, not taking into account the hygienic implications of the diverse bacterial communities selected through that process [5,18]. The new frontier is complex building plumbing in which health-relevant water quality changes can occur [23,39]. Clear and evident changes in the planktonic bacterial community structures are expected to occur between the water's source and water after subsequent steps of treatment, as previously reported in chloraminated systems [14,40]. Nevertheless, after treatment, results from several studies using high-throughput sequencing to examine changes occurring in DWDS suggest that planktonic bacterial communities leaving the plant remain relatively stable throughout distribution up to the water meter [14,15,19], questioning the impact of water residence time and/or the presence of a disinfectant residual on microbiota. However, when it comes to building plumbing, stagnation and pipe material have been shown to be important factors influencing the bacterial composition [22,23,25].

The structure in the treated water phyla observed in this study was similar to that previously observed in the treated water from a Dutch plant [18], but the composition of the Proteobacteria classes differed. Predominance of Proteobacteria has been observed in other chlorinated and unchlorinated DWDS [3,13–15,17–19,41,42]. However, the predominant classes of Proteobacteria vary from one network to another, with Alphaproteobacteria usually predominant in chlorinated systems [20,41–43]. Cyanobacteria are also found in chlorinated DWDS samples, indicating that they can survive passage through different physical and chemical treatments and enter networks [13,20,43]. The community shifts observed between the water treatment plants most likely reflect the contribution of biofilms present in the distribution network and sporadic releases associated with hydraulic disturbances leading to biofilm detachment and resuspension of loose deposits and sediments. Among the ribotyping profiles we examined, DS3-2 was an outlier composed of Verrucomicrobia (0.7%), Aminicenantes (0.3%), and Elusimicrobia (0.8%). A small proportion of Verrucomicrobia (4%) has previously been reported in chlorinated drinking water [43]. Elusimicrobia and Verrucomicrobia have been reported in an unchlorinated DWDS [15].

Our results show that the specific environmental conditions (levels of chlorine, bacterial counts, pipe material, water residence time, and biofilm/water interface) of each subsystem are determinants of the bacterial community composition, promoting or inhibiting the growth of specific organisms. A 40% increase in Proteobacteria was observed in the distribution systems, followed by a 7% decrease in the building plumbing. Despite the superior proportion of Proteobacteria in both distribution system sites and taps, the distribution profile of OTUs encompassing this phylum differed between both locations. While DS samples were clearly dominated by Alphaproteobacteria (92%), in building plumbing the loss of disinfectant residual and building plumbing conditions resulted in a decrease in Alphaproteobacteria (65%) accompanied with an increase of the relative abundance of Betaproteobacteria (18% vs. 2% in DS), and other classes that were less abundant or absent in the distribution system. Similar responses of Alphaproteobacteria and Betaproteobacteria to disinfectant residuals have been previously reported [41]. Members of Alphaproteobacteria seem to exhibit a higher resistance to chlorine and are found in abundance in chlorinated networks. In contrast, the opposite is observed for Betaproteobacteria. The latter group grows more readily in low disinfectant conditions and has

been found to be more abundant in unchlorinated distribution systems [3,18] and in biofilms from chlorinated systems [42].

Other studies examining temporal variations in bacterial communities have also observed stable profiles over short and long periods of time in unchlorinated DWDS [15,18]. However, previous studies investigating variations in a chlorinated distribution system verified temporal variability in spring and summer samples, primarily due to changes in disinfectant levels in response to temperature fluctuations [41,44]. The percentages of shared richness found in this study were much lower than those found in an unchlorinated system [18], where 58–65% of OTUs were shared between treated water and the water from one location in the distribution system. This difference is probably explained by the larger variations in water quality in the systems, as influenced by the presence/absence of chlorine in the treated water at the plants, in the distribution system, and at the taps.

Several of the identified indicator OTUs in our study have been reported in previous studies. For example, the genus *Mycobacterium* includes opportunist pathogens that are generally highly resistant to disinfectants [45]. They have been detected as the most abundant genus in 89% of flushed tap samples from 16 cities in the United States using chlorine and monochloramine as disinfectants [16]. The presence of OTUs assigned to the genera *Bosea*, *Hyphomicrobium*, *Sphingomonas*, *Nevskia*, *Mycobacterium*, *Mesorhizobium*, *Aquabacterium*, and *Sphingopyxis* has been reported in bulk water and biofilm samples in a simulated distribution system using chlorine as a disinfectant [42]. *Nevskia* spp. were found to be more abundant in samples from a pipe break event [41] and in highly varied flow conditions [42]. The latter could explain its association with distributed water and building plumbing groups, where water usage patterns are highly variable between night and day, and between weekdays and weekends.

Sphingomonas spp. and *Afipia* spp. are both reported to have significant impact in healthcare settings [46–48]. A few species of *Sphingomonas* are considered to have clinical significance, with *Sphingomonas paucimobilis* being the most important. These bacteria can survive in low-nutrient environments and have the ability to form biofilms in water piping. This pathogen can cause various types of infections, often bacteremia/septicemia [48]. *Sphingomonas* spp. are resistant to disinfection, produce extracellular polymeric substances, and can rapidly colonize pipe surfaces [49]. A predominance of *Sphingomonas* spp. and *Sphingobium* spp. with antibiotic resistance have been previously detected in water samples from taps (household and hospital) and dental chairs in a university dental school clinic [50]. In addition, their tolerance to high levels of copper ions enables them to not only survive in copper piping but also to fully thrive, as well as, to promote copper corrosion [51]. In an unchlorinated system, *Sphingomonas* spp. were predominant in the pipe wall biofilm, in loose deposits, and suspended solids. *Sphingomonas* spp. are also the second most important genera detected in bulk water [52]. The predominance of *Sphingomonas* spp. in first flush tap samples from a large building could be attributed to the contribution of the biofilm in this section of the building plumbing, where smaller pipe diameters increase the proportion of biofilm released after short stagnation times, as is often encountered in hospital environments [7].

The second most important indicator species in taps were *Afipia* spp., a water-associated amoeba-resisting bacteria of public health significance. *Afipia* spp. have been isolated from a hospital water supply [46,53] through co-culture with amoeba. *Afipia* spp. are suspected to be involved in pneumonia since several patients with acute pneumonia had elevated levels of anti-*Afipia* antibodies [54]. *Gemmata massiliana*, found as an indicator in TW and TAP subsystems, has been isolated from two hospital water networks in France [55]. The frequent detection of this pathogen in two hospital networks suggested the potential exposure of patients and healthcare workers to this organism. Thus, the authors of that study suggest evaluating this organism as a potential opportunistic pathogen of public health significance.

The results from genera countaining OPPs and indicator analyses remain difficult to interpret, since these sequences may not be representative of organisms that cause relevant health effects. However, this can be investigated further through follow-up environmental sampling, helping clinicians identify sources of nosocomial pneumonia.

Overall, the comparative analysis of the diversity and relative abundance of the planktonic bacterial community in a full-scale DWDS using a high-throughput sequencing approach reveals three main findings. First, we have shown that the bacterial community profile in treated water, distribution system sectors, and taps in building plumbing are diverse and clearly distinct at different taxonomic levels. Second, we have linked noticeable variations in microbial communities to changes in the ranges of concentration of disinfectant residual, total bacterial counts, and water residence times. Finally, the use of indicators revealed the presence of important markers in different groups of DWDS samples. Although these findings provide relevant information, they are contingent upon the sensitivity of the methods used. More importantly, our results inform only on the diversity of suspended bacteria, not of the biofilm.

The combination of methods used in this study have a high potential for drinking water quality investigative monitoring to further our understanding of distribution system ecology. The generated information is needed to understand where utilities can act to prevent the growth of OPPs. Findings suggest focusing efforts on managing the distinct microbiome in building plumbing to prevent exposure to OPPs. Distribution conditions that may be linked with the prevalence of health-relevant specific bacterial groups or species could be identified using the methods described in this study. Additional research is needed to understand the drivers of the shifts of microbial communities in suspended and fixed microbiomes within a full-scale DWDS and building plumbing.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/11/3/627/s1, Figure S1: Venn diagram showing the shared OTUs across the subsystems, Table S1: Characteristics of the samples from premise plumbing, Table S2: Frequency of detection of genera containing opportunistic pathogens (OPs) across the subsystems.

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