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Microbial Community Composition of Tap Water and Biofilms Treated with or without Copper−Silver Ionization

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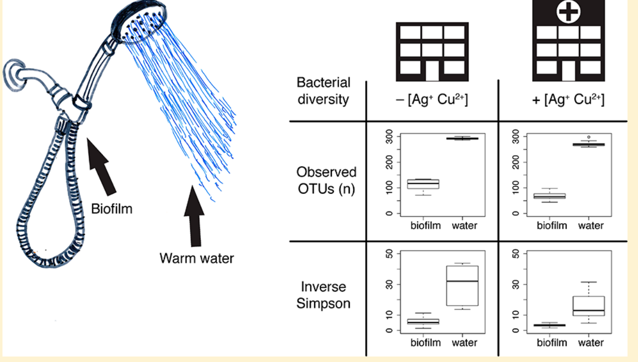
Supporting Information

ABSTRACT: Copper−silver ionization (CSI) is an in-house water disinfection method primarily installed to eradicate *Legionella* bacteria from drinking water distribution systems (DWDS). Its effect on the abundance of culturable *Legionella* and *Legionella* infections has been documented in several studies. However, the effect of CSI on other bacteria in DWDS is largely unknown. To investigate these effects, we characterized drinking water and biofilm communities in a hospital using CSI, in a neighboring building without CSI, and in treated drinking water at the local water treatment plant. We used 16S rDNA amplicon sequencing and *Legionella* culturing. The sequencing results revealed three distinct water groups: (1) cold-water samples (no CSI), (2) warm-water samples at the research institute (no CSI), and (3) warm-water samples at the hospital (after CSI; ANOSIM, $p < 0.001$). Differences between the biofilm communities exposed and not exposed to CSI were less clear (ANOSIM, $p = 0.022$). No *Legionella* were cultured, but limited numbers of *Legionella* sequences were recovered from all 25 water samples (0.2−1.4% relative abundance). The clustering pattern indicated local selection of *Legionella* types (Kruskal−Wallis, $p < 0.001$). Furthermore, one unclassified *Betaproteobacteria* OTU was highly enriched in CSI-treated warm water samples at the hospital (Kruskal−Wallis, $p < 0.001$).



INTRODUCTION

Treated drinking water contains a multitude of bacterial species.[1−3] The vast majority of bacteria present in drinking water do not cause a risk to human health. In fact, microbe-rich drinking water may be beneficial, for example by reducing the risk of allergic hypersensitivity reactions in children.[4] However, some bacteria frequently present in drinking water are opportunistic pathogens and can cause life-threatening infections in immunocompromised individuals. *Legionella* spp. are such opportunistic pathogens and are the most frequently reported cause of waterborne hospital-acquired infections worldwide.[5] *Legionella* spp. may cause Legionnaires' disease, an atypical form of pneumonia. The fatality rate of health-care associated Legionnaires' disease in Europe is almost 30%.[6]

Legionella bacteria are natural inhabitants of water and soils, can form biofilms, and thrive in multiple-species microbial communities.[7] They are heat-resistant and often present in warm-water distribution systems.[8] In fact, the most reported sources for infection are water heating systems,[5] but a range of other sources including cooling towers of air conditioning systems, decorative fountains, and spa pools have been reported.[5,9,10] As facultative intracellular pathogens, *Legionella* may survive within amoebae and other host cells, evading disinfection measures.[11] Furthermore, they are highly chlorine resistant.[12]

To eradicate these important opportunistic pathogens, many hospitals have installed additional in-house water disinfection systems. One such system is copper−silver ionization (CSI). CSI systems release positively charged copper and silver ions into the water flow. These bind to negatively charged cell walls. The resulting electrostatic stress causes bacterial cell walls to break down and the bacteria to die. Many hospitals that have installed a CSI system have experienced a drastic decrease in the number of *Legionella*-positive water samples[13−15] as well as *Legionella* infections.[14] Others, however, report an initial decrease of *Legionella*, followed by recurrent incidences.[16,17] These recurrences are thought to be due to too low levels of

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Table 1. Main Sample Details (for further information, see Supporting Information)

location/room	water sample	hose biofilm sample	sample type	CSI	filtration	water temp. °C	temp. category	water group
Oset DWTP								
laboratory	01AW	[a]	treated drinking water	no	no	9.7	cold	1
laboratory	01BW	[a]	treated drinking water	no	no	9.7	cold	1
research institute								
K637	FI06W	[a]	water intake	no	yes	9.6	cold	1
K637	FI23W	[a]	water intake	no	no	8.7	cold	1
U526	[b]	FS02H	staff shower	no	yes	37.6	warm	2
U526	FS03W	FS03H	staff shower	no	yes	37.8	warm	2
U526	FS04W	FS04H	staff shower	no	yes	37.8	warm	2
U526	FS05W	FS05H	staff shower	no	yes	37.1	warm	2
U630	FS08W	FS08H	staff shower	no	yes	38.2	warm	2
U630	FS09W	FS09H	staff shower	no	yes	34.4	warm	2
U630	FS10W	FS10H	staff shower	no	yes	39.1	warm	2
K358	FS11W	FS11H	staff shower	no	yes	10.0	cold	1
U526	FS12W	FS12H	staff shower	no	yes	37.6	warm	2
U526	FS13W	FS13H	staff shower	no	yes	35.0	warm	2
hospital								
technical room	LIA22W	[a]	water intake	no	no	8.2	cold	1
technical room	LIB21W	[a]	water intake	yes	yes	37.7	warm	3
430B	LS14W	[b]	patient shower	yes	yes	36.9	warm	3
439B	LS15W	[b]	patient shower	yes	yes	38.0	warm	3
315A	LS16W	LS16H	patient shower	yes	yes	36.7	warm	3
306B	LS17W	LS17H	patient shower	yes	yes	38.1	warm	3
337A	LS18W	LS18H	patient shower	yes	yes	37.6	warm	3
636C	LS19W	LS19H	staff shower	yes	yes	37.3	warm	3
643G	LS20W	LS20H	staff shower	yes	yes	37.5	warm	3
643C	LS24W	LS24H	staff shower	yes	yes	38.5	warm	3
636B	LS25W	LS25H	staff shower	yes	yes	37.5	warm	3
634B	LS26W	LS26H	staff shower	yes	yes	37.6	warm	3

[a]No hose biofilm samples taken at DWTP and water intakes. [b]Sample excluded from analyses due to too little DNA or number of sequenced reads.

silver and copper ions[16] or *Legionella* escaping the CSI treatment by survival in biofilms or within amoebae.[13,17−20]

All studies to date that have investigated the effect of CSI on *Legionella* in drinking water systems have been based on *Legionella* culture.[13,14,17,18,21] However, not all viable *Legionella* cells within a sample are culturable.[22,23] Furthermore, *Legionella* are slow growing bacteria, and culture plates may be overgrown with fast-growing species before *Legionella* can be detected. Thus, culture-based methods are likely to underestimate viable *Legionella* bacteria in samples. On the other hand, culture-independent methods such as quantitative polymerase chain reaction (qPCR) tend to overestimate viable *Legionella* populations.[24] These methods generally detect and enumerate dead cells and environmental DNA in addition to viable cells in a sample. Culture-independent sequence-based methods also indicate that a higher diversity of *Legionella* spp. are present in drinking water distribution systems[25,26] than recovered by culture-based methods.

Despite the complementary information that may be gained by using culture-dependent and -independent methods, no study has yet used a combined approach to study the effects of CSI on *Legionella* in drinking water systems. In addition, CSI is likely to also have an effect on other bacteria present in drinking water systems, but very few studies have addressed this aspect of CSI. One study investigated the effect of CSI on selected plankton- and biofilm-associated pathogens in a model tubing system.[21] The results indicate that very high amounts of copper and silver ions were necessary to reduce the number of *Pseudomonas aeruginosa*, double the dose usually applied to eradicate *Legionella*.[21] CSI also failed to eradicate *P. aeruginosa* present in faucets in intensive care units.[27] In addition, nontuberculous *Mycobacterium* spp. and other heterotrophic bacteria have been shown to be more tolerant than *Legionella* to CSI in a Finnish hospital.[18] Finally, the effect of CSI on the entire bacterial community has not yet been evaluated.

Here, we used 16S rDNA amplicon sequencing to characterize the bacterial drinking water and biofilm communities in a hospital with CSI and compared these to the communities present in a neighboring building without CSI and those present in the treated drinking water at the waterworks that supplies both buildings. 16S rDNA is a culture-independent method based on the amplification and sequencing of all 16S ribosomal genes present in an environmental sample. The analyses were complemented with *Legionella* culture and *Legionella*-specific enzyme-linked immunosorbent assay (ELISA).

■ METHODS

Sampling Sites. Samples were taken in November 2015 at Oset drinking water treatment plant (DWTP), a research institute (Norwegian Institute of Public Health), and a hospital (Lovisenberg Diakonale sykehus) with a copper−silver-ionization (CSI) system installed. All three are situated in Oslo, Norway.

Oset DWTP serves 92% of Oslo's residents.[28] It uses water from Lake Maridalsvannet, a freshwater lake situated north of the city. Routine water treatment at Oset consists of coagulation, sedimentation, filtration, UV irradiation, and pH

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adjustment. In addition, small amounts of sodium hypochlorite, typically 0.1 mg Cl_2 L^{-1}, are added to the treated water even when the UV system works satisfactorily to ensure that the back-up disinfection system is functioning.[29]

The research institute and the hospital are neighboring buildings and receive drinking water from Oset DWTP through the same main pipes. At the research institute, the incoming water passes a coarse filter (100 μm pore size) before it is distributed further throughout the building. A portion of this water is heated to 65 °C via heat exchange and circulated through the warm-water pipes. At the hospital, a copper−silver-ionization (CSI) system was installed in 2009.[30] The incoming water is filtered (100, 20, and 5 μm pore sizes) and then passes the CSI system that continuously adds copper (200−300 μg L^{-1}) and silver ions (approximately 30 μg L^{-1}) to the water. After CSI, a portion of the water is distributed through cold-water pipes; the remaining water is heated to 70 °C via heat exchange, stored in hot water tanks, and distributed through the warm-water pipes.

Sampling. Two rounds of sampling were performed: Water and biofilm samples were taken at Oset DWTP, the research institute, and the hospital during the main experiment in November 2015. In addition, complementary water samples were taken from the water intakes at the research institute and the hospital in April 2016. This additional sampling was carried out because several water quality parameters from samples taken in November 2015 differed substantially for the intake waters of the two buildings. All samples and water quality parameters are listed in Tables 1, S1, and S2.

For the main experiment, samples were taken from the "treated water" tap at the onsite laboratory at Oset DWTP. At the research institute, two samples were taken from the water intake (one after the water had passed the coarse filter, the second without this filtration step to mimic the situation at the hospital) and from 10 staff changing room showers throughout the building. At the hospital, samples were taken from the water intake before filtration and CSI, from a water tap within the same room after filtration and CSI, as well as from 10 showers throughout the building: five from patient bathrooms (1−2 patients per bathroom) and five from staff changing rooms.

The same sampling protocol was used at all sites. First, the water outlets were flushed for 1 min and, where possible, the water temperature adjusted to 35−38 °C prior to sampling. Temperature adjustment was not possible at Oset DWTP and at the water intakes at the hospital and the research institute; thus only cold water was sampled at these sites. First, samples for bacterial culturing, ATP, and ELISA analyses were taken in separate autoclaved glass bottles: 1 × 500 mL for ATP, 1 × 1 L for ELISA, and 2 × 1 L for *Legionella spp.* culture. The bottles were immediately transported to the laboratory and processed. A total of 0.5 L of water was in addition sampled at the hospital for copper and silver analyses. Second, for DNA analyses, 10 L of water were filtered through a Sterivex 0.22 μm filter unit (Millipore) using sterile silicone tubing and a peristaltic pump (Watson Marlow 120S/DV, 120 rpm, approximately flow 100 mL min^{-1}). The filter was aseptically removed, capped on both sides, transferred to a 50 mL Falcon tube, and placed on ice until arrival in the laboratory, where it was frozen at −20 °C and further processed within 2 weeks. Finally, biofilm samples were taken. For DNA analyses, the showerhead was removed and the inside of the shower hose thoroughly swabbed (FLOQSwab with 30 mm breakpoint; Copan Italia). The swab tip was put into a 2 mL Eppendorf tube filled with 1 mL of autoclaved and sterile filtered 1 × phosphate buffered saline (PBS) solution. Then, the shower hose was dismounted and a second biofilm sample taken in the same manner from the faucet to which the hose had been connected. For intake water samples taken directly from faucets, faucet insides were thoroughly swapped as described for shower faucets. The swabs were stored on ice until arrival at the laboratory. Swabs were centrifuged for 30 min (4 °C, 2000*g*). Most of the supernatant was carefully removed, and the tube containing the swab tip and biofilm pellet was frozen at −20 °C until DNA isolation. For bacterial culture, one Copan eSwab (Copan Italia) was used to thoroughly swab the end of the shower hose that had been attached to the faucet. The swab was placed in 1 mL of liquid Amies medium, transported to the laboratory on ice, and immediately processed.

Water and Biofilm Analyses. For water chemistry analyses, 1 L of cold water was sampled in PE bottles at selected sampling points after flushing for 1 min, stored in opaque cool bags, and transported to the laboratories within 2 h. Water chemistry analyses were carried out by Oslo's Water and Wastewater authorities (VAV) according to accredited ISO methods.

Copper and silver analyses were performed by Eurofins Environment Testing Norway AS following method NS EN ISO 17294-2.

Adenosine triphosphate (ATP) was quantified using the Quench-Gone Aqueous (QGA) test kit (LuminUltra) according to the manufacturer's instructions in combination with the PhotonMaster luminometer (LuminUltra).

Legionella-specific ELISAs were carried out with the HybriScanD Legionella kit (Sigma-Aldrich) according to the manufacturer's instructions and results read on a Multiskan EX (Labsystems) plate reader.

Cultivation of *Legionella* from water samples was carried out according to standard methods.[31] In addition, 1 L of water was filtered through a second filter. The filter was cut into small pieces, placed in a tube containing 10 mL of saline solution (0.9%), and gently shaken for 2 min. From this solution, 0.1 mL was inoculated on GVPC-agar and BCYE-agar. For cultivation from biofilms, 0.1 mL of the liquid Amies medium was inoculated on GVPC-agar and BCYE-agar and incubated at 36 ± 1 °C for up to 10 days.

DNA Extraction. Sample order was randomized prior to DNA extraction. Water samples were isolated with the PowerWater Sterivex DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's protocol using a PowerVac Manifold Mini System. Biofilm samples were isolated with the FastDNA SPIN KIT for Soil (MP Bio). All biofilm samples were isolated according to the manufacturer's protocol Rev # 116560200-201411 with the following adjustments: sodium phosphate buffer was added directly to the swab samples, was pipetted up and down, and then added to the Lysis Matrix E-tube (step 2 in protocol). Optional step 16 (incubate at 55 °C for 5 min prior to elution to increase yield) was included in the protocol. Both biofilm and water samples were eluted in 100 μL of the provided elution buffers. The Qubit dsDNA High Sensitivity assay (ThermoFisher Scientific) was used to quantify DNA concentrations (Table S3). All samples with concentrations ≥ 5 ng μL^{-1} were reanalyzed with the Qubit dsDNA Broad Range assay. Samples with concentrations > 4 ng μL^{-1} were diluted to 2 ng μL^{-1} with molecular grade water. All samples were frozen in small aliquots.

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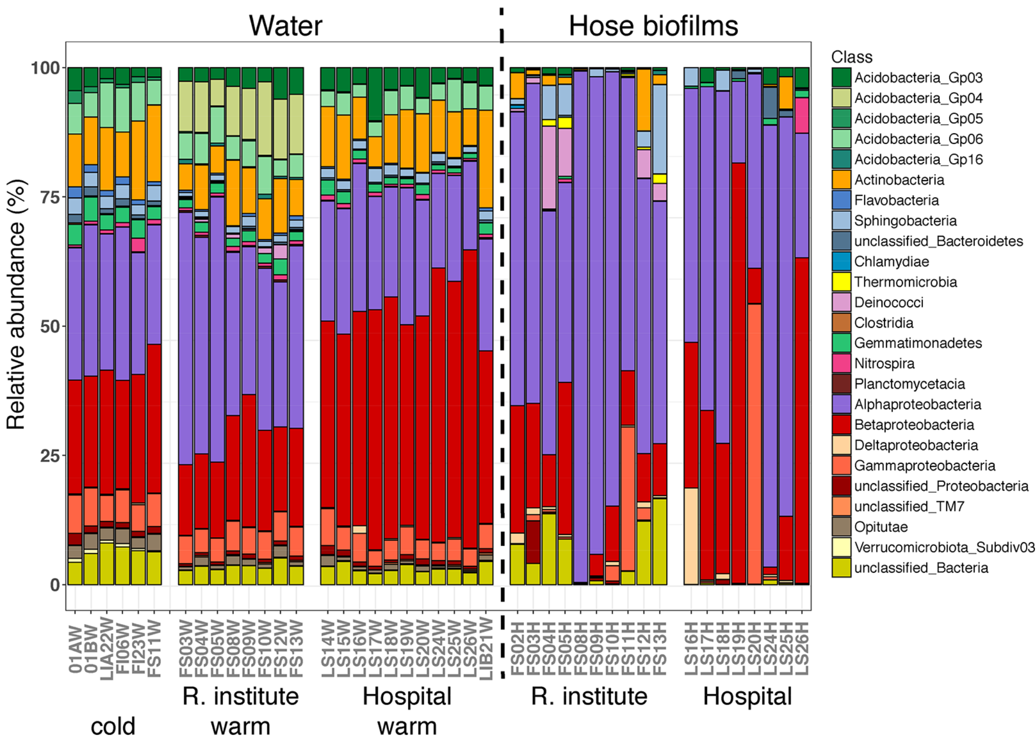

Figure 1. Relative abundance of bacterial classes in water and hose biofilm samples. Water samples: 01AW−FS11W, group 1; FS03W−FS13W, group 2; LS14W−LIB21W, group 3. See Diversity and Taxonomy of Water Samples section for discussion of water groups and Table 1 for all sample details.

16S PCR, Normalization, Amplicon Pooling, and Sequencing. The dual-index PCR protocol published in ref 32 was used with small amendments. PCR reactions were run in triplicate (the same reaction on three different PCR plates). Each 25 μL PCR reaction contained 12.5 μL of 2× Phusion Hot-start II High-Fidelity MasterMix, 2.5 μL of forward and 2.5 μL of reverse primer (1 μM each), 0.75 μL of DMSO, 1.75 μL of PCR-grade water, and 5 μL of template (controls or DNA, max. 4 ng/μL). The PCRs were run on a Bio-Rad S1000 Thermal Cycler using the following program: 1*[98 °C/30s], 33*[98 °C/15s, 54 °C/15s, 72 °C/15s], 1*[72 °C/60s], 4 °C/hold. Primers were HPLC purified and contained two phosphorothioate bonds at the 3′ end. PCR reactions were visualized on Lonza FlashGels, triplicates pooled, and normalized with a SequalPrep Plate (Invitrogen) according to the manufacturer's protocol using 25 μL of PCR product and 25 μL of binding buffer as input. The library was purified and concentrated using Agencourt AMPure XP beads (Beckman Coulter) with a 1:0.9 sample:beads ratio and eluted in 60 μL of 10 mM TRIS buffer. The following controls were included and treated in the same way as samples: extraction controls (three FLOQSwabs and two Sterivex filter units) and no-template PCR controls. No PCR bands were visible for the controls; they were nevertheless normalized and sequenced as the other samples.

The 16S rRNA library was sequenced on a MiSeq instrument (Illumina), with 300 bp paired end reads (v3 chemistry) and the PhiX control library blended to 10%. Bcl files were processed using RTA v1.18.54 and converted to fastq format using bcl2fastq v.2.17.1.14. Quality of the sequenced data was verified using FastQC v0.11.3.[33] Samples, primers, and barcodes are listed in Tables S3 and S4.

Bioinformatic Analyses. Remnant Illumina adapters were removed using AdapterRemoval v. 2.1.7.[34] Paired sequence reads were merged, demultiplexed, quality filtered, clustered into OTUs, and classified using Moira v. 1.3.0[35] and Mothur v. 1.36.1.[36] Details, settings, and commands are listed in File S1. Samples that had no measurable DNA or did not give clear bands after PCR were excluded from the analyses because results of low DNA samples are prone to being highly impacted by contamination such as from DNA isolation kits.[37] All 23 faucet, two shower hose biofilms (LS14H, LS15H), and one water sample (FS02H) were excluded.Table S5).

Operational Taxonomic Unit (OTU) abundance data were analyzed with the R packages Vegan (v. 2.4−1[38]) and Phyloseq (v. 1.18.1[39]). Rare OTUs (containing <0.005% of reads) were removed prior to diversity analyses[40] and data subsampled without replacement to the smallest sample size (36924 sequences; seed 161018). Nonmetric multidimensional scaling (NMDS) using the Bray−Curtis dissimilarity measure was used to visualize dissimilarities in community composition. Differences were evaluated using analysis of similarities (ANOSIM). Four different alpha-diversity indices were calculated (Observed, Chao1, Shannon, InvSimpson). Kruskal−Wallis Rank Sum Tests were used to evaluate differences. Core microbiomes were defined as OTUs present in all samples with an abundance of at least 0.1% and determined with kOverA OTU filtering from the Genefilter package (v.1.56.0[41]).

Accession Numbers. Demultiplexed fastq files of merged paired-end sequences were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena; study PRJEB22257; samples' accession numbers are listed in Table S5).

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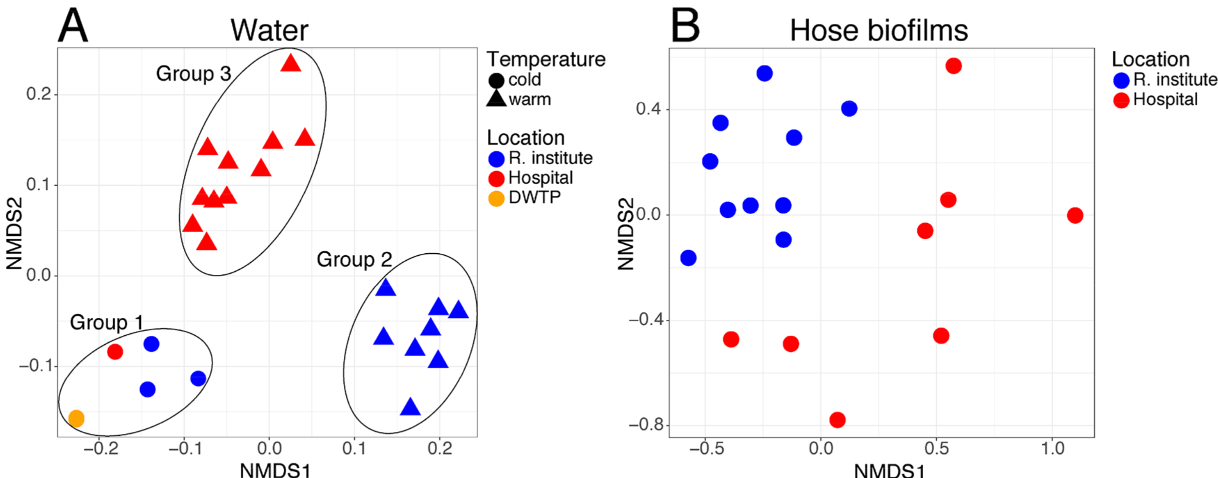

Figure 2. NMDS ordination plots based on Bray−Curtis distances. (A) Water samples. (B) Hose biofilm samples. Note difference in scale between the two plots. See also Figure S1 (NMDS plot including water and hose biofilm samples).

RESULTS AND DISCUSSION

Community Composition of Water and Biofilm Samples. The sequencing approach revealed diverse bacterial communities in both water and shower hose biofilm samples (Figure 1). The faucet biofilm samples had to be excluded from all analyses because no or only very small amounts of DNA were recovered (Table S3), and impacts of kit and laboratory contamination on their community composition[37] could not be ruled out.

Water and hose biofilm communities differed significantly from each other: β-diversity analyses based on Bray−Curtis distances clearly separated the samples based on sample type (biofilm vs water, ANOSIM R 0.988, $p < 0.001$, Figure S1). The effect of CSI treatment was less clear (CSI vs none, biofilm and water samples, ANOSIM R 0.092, $p = 0.034$, Figure S1). Furthermore, water samples contained a higher species richness (Kruskal−Wallis: Chi2 = 30.7, $p < 0.001$) and evenness (Kruskal−Wallis: Chi2 = 27.1, $p < 0.001$) than biofilm samples (Figure S2, Table S7). We defined species richness as the number of observed OTUs in each sample and estimated evenness with the Inverse Simpson Index. The Inverse Simpson Index is a nonparametric diversity index that in essence captures the variance of species abundance distribution. It will rise as the community becomes more even.[42]

Other researchers that investigated microbial communities of drinking water networks have also observed that bulk water and biofilm communities differ significantly from each other.[43−46] Bulk waters have been shown to have a higher species richness and evenness compared to biofilm samples irrespective of the age of the sampled material (under two years[44,46] to over 20 years[43]), the source of the drinking water (groundwater,[45] surface water[43,44]), or the methodology employed to study microbial communities (fingerprinting followed by sequencing[43] or high-throughput amplicon sequencing[44−46]).

Diversity and Taxonomy of Water Samples. β-diversity analyses of the water samples alone revealed three distinct groups (Figure 2A, Table 1): Group 1 contained all cold-water samples, including samples taken at Oset DWTP, water intake samples at the research institute and the hospital, and one shower at the research institute (FS11W). Due to technical difficulties, only cold water was sampled from this shower. Group 2 contained all warm-water samples taken at the research institute, and group 3 all warm-water samples taken at the hospital after CSI. The analyses were based on Bray−Curtis distances, and the evidence was very strong that both temperature (warm vs cold water: ANOSIM R = 0.596, $p < 0.001$) and water treatment (CSI vs no treatment: ANOSIM R = 0.621, $p < 0.001$, Table S6) contributed to this grouping.

The close grouping of all cold-water samples suggests that the community composition in the water discharged from Oset DWTP stayed essentially stable throughout the distribution system and arrived nearly unchanged at the research institute and the hospital over 5 km away. In addition, the community composition changed little within the pipe-system of the research institute as long as the temperature was not changed. In contrast, the community compositions changed significantly within the buildings after the water had passed through the hot-water system at the research institute or the hot-water system and CSI at the hospital.

The three water sample groups also differed in species richness, evenness, and ATP measurements (Figure 3, Table 2). The warm-water samples at the research institute (group 2) had the highest species richness, while no significant richness differences were observed between cold-water (group 1) and warm-water samples at the hospital (group 3). Evenness was significantly higher and less variable in cold-water communities (group 1) than warm-water communities (groups 2 and 3). Furthermore, the ATP results showed that cold water contained the highest living bacterial biomass, while CSI-treated warm water at the hospital contained the lowest.

At the research institute, this suggests an important effect of the hot-water system on the bacterial living biomass and community composition. Others also found that hot- and cold-water communities within the same building are distinct.[46,47] Henne et al.[47] observed that the composition and structure of cold-water communities in temperate regions is highly influenced by seasonal factors such as temperature and precipitation, whereas warm-water communities were more stable. They attributed this stability to the selection and proliferation of thermophilic bacteria in hot-water systems.

At the hospital, ATP and community differences between cold, incoming water and warm, CSI-treated water suggest important effects of the hot-water system in combination with CSI. The effect of these two cannot be differentiated in the present study as only cold-water samples upstream of CSI and

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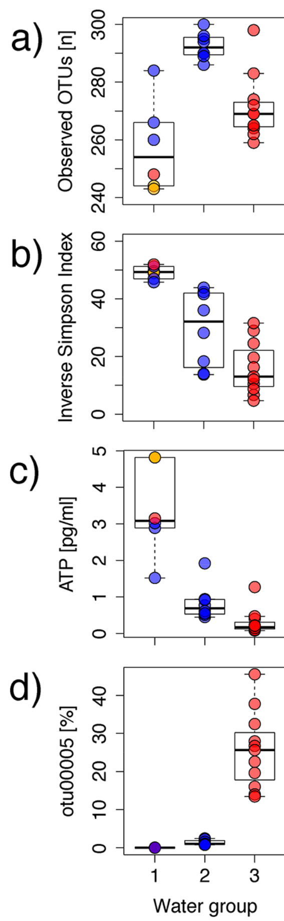

Figure 3. Differences in (a) species richness (observed OTUs), (b) evenness (inverse Simpson index), (c) ATP in water samples, and (d) relative abundance of otu00005 in the three water groups. Statistics in Table 2. Water groups: 1, cold-water samples (without CSI); 2, warm-water samples from the research institute (without CSI); 3, warm-water samples from the hospital (after CSI). See also Diversity and Taxonomy of Water Samples section and Table 1. Blue, sampled at research institute; red, sampled at hospital; orange, sampled at DWTP.

Table 2. Statistics for Differences Observed between the Three Water Groups (Figure 3)

Kruskal–Wallis rank-sum test	Chi2	df	*p*
observed OTUs	14.447	2	<0.001[a]
inverse Simpson I.	16.279	2	<0.001[a]
ATP	18.044	2	<0.001[a]
rel. abundance otu00005	20.871	2	<0.001[a]
pairwise comparisons Wilcox rank sum test	**group1/ group2**	**group1/ group3**	**group2/ group3**
observed OTUs (p adjust bonferroni)	0.0072[b]	0.2905	0.0760
InvSimpson (p adjust bonferroni)	0.0072[b]	0.0033[b]	0.0695
ATP (p adjust bonferroni)	0.0109[c]	0.0032[b]	0.0101[c]
rel. abundance otu00005 (p adjust bonferroni)	0.0072[b]	0.0033[b]	0.0001[a]

[a]$p < 0.001$. [b]$p < 0.01$. [c]$p < 0.05$.

warm-water samples downstream of CSI were taken and processed for microbiome analyses.

ATP and community differences between the warm-water samples at the research institute (group 2) and those at the hospital (group 3) may be attributed to a range of factors (Table 3). While all efforts were made to find two comparable full-scale water distribution systems, one with and one without CSI disinfection, a number of dissimilarities between the two buildings were encountered that are likely to have contributed to the differences in microbial community compositions observed (Table 3).

For example, small differences in warm-water temperature have been shown to have important effects on the microbial composition of drinking water,[48] and hot-water temperatures have been shown to select for thermo-tolerant *Legionella* strains.[8] It is probable that the 5 °C difference in hot-water temperature between the buildings has had an effect on community composition and ATP results. Furthermore, pipe material has an important influence on microbiome composition.[48,49] The pipe materials in the present study are unknown, but we observed consistent differences in the water chemistry parameters of the incoming water at the research institute and the hospital (Table S2). Specifically, zinc values were twice as high and copper values were 25–45 times higher in the incoming water at the research institute compared to the hospital. As both buildings receive water through the same main pipes, these differences indicate that the water pipes connecting the research institute with the main water pipes contain more zinc and copper than the corresponding pipes connecting the hospital. While these differences in water chemistry did not have discernible effects on the community composition of the incoming water samples (all clustered within group 1), it cannot be excluded that differences in pipe materials contributed to the distinct grouping of warm-water samples.

Proteobacteria had the highest relative abundance in all water samples, but the distribution at class level differed between the three groups (Figure 1, Figure S3). The cold-water samples (group 1) contained similar relative amounts of *Alpha-* and *Betaproteobacteria*, while the warm-water samples at the research institute (group 2) were dominated by *Alphaproteobacteria* and the warm-water samples at the hospital (group 3) by *Betaproteobacteria* (Figure S3). Especially one OTU had a very high relative abundance in the bacterial communities of group 3, otu00005, classified as "unclassified *Betaproteobacteria*." On average, this OTU contributed 25% (range 14–45%) of all reads in the warm water samples at the hospital (Figure 3; Tables 2 and S9). In comparison, the same OTU only made up 1.4% of all the reads sequenced from the warm-water samples at the research institute (group 2, range 0–2.5%) and was virtually absent from cold-water samples and biofilms. This indicates that the local conditions at the hospital resulted in a relative enrichment of otu00005. Different proportions of *Proteobacteria* frequently dominate drinking water communities,[1,3,44,50] but such high abundances of a single OTU in drinking water samples has, to our knowledge, not been described.

To characterize otu00005, the most abundant sequence in the OTU (identical to 46% of sequences in otu00005) was used for similarity searches against databases at National Center for Biotechnology Information (NCBI). These searches showed that sequences within otu00005 were similar (>97%) to clones from uncultured bacteria isolated from a diverse range of habitats including the tap water of a Norwegian hospital and Norwegian households, bulk water of the Cincinnati drinking water system, a subsurface thermal spring, dental plaque, and volcanic ash (Table S10). These results indicate that the bacteria represented by otu00005 are likely hydro- and

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Table 3. Differences between the Research Institute and the Hospital That May Have Contributed to Dissimilar Bacterial Communities in Water and Biofilm Samples

	research institute	hospital
in-house disinfection	none	copper−silver ionization (CSI); addition of 200−300 μg/L copper and 30 μg/L silver ions to incoming water
hot-water system	portion of incoming water heated through heat exchange and circulated through warm water pipes	portion of CSI treated water heated and stored in warm-water tanks before circulation in hot-water pipes
hot-water temperature	65 °C	70 °C
filters at water intake	100 μm pore size	100, 20, and 5 μm pore size
water chemistry incoming water	higher zinc and copper values than at the hospital (Tables S1 and S2)	lower copper and zinc values than at the research institute (Tables S1 and S2)
in-house pipe materials	unknown	unknown
faucet materials	brass or brass-like materials, details not known	brass or brass-like materials, details not known
shower-hose materials	flexible plastics, details not known	flexible plastics, details not known
shower-hose disinfection measures	chlorine disinfection once weekly; exchange when necessary	exchange every six months at somatic wards
room details/usage pattern	staff shower rooms located in basement; usage frequency unknown	five showers from patient rooms (somatic wards; 1−2 patients/shower), 3rd and 4th floor; five staff showers, 6th floor; usage frequencies unknown

thermophilic. However, no close similarity was found to 16S rDNA sequences from cultured or genome sequenced bacteria, and thus it is not possible to infer which taxa otu00005 represents or what its ecological function is.

Differences between Faucet and Shower Hose Biofilms. As mentioned above, the faucet biofilm samples were removed from the analyses as no or only very small quantities of DNA were recovered from these samples, especially from samples taken at the hospital (Table S3). Microbiome data based on dilute DNA samples has been shown to be heavily influenced by kit and other laboratory contaminants.[37] In contrast, DNA extraction from hose biofilm samples resulted in variable but substantially higher quantities of DNA (Table S3). Only two of 20 hose biofilm samples were excluded due to low DNA recovery. Both samples were taken at the hospital in the same ward from patient bathrooms (LS14H, LS15H, Tables 1 and S3).

Differences in DNA recovery between faucet and hose biofilms were likely due to differences in substrate material. The shower hoses were made of flexible plastics, whereas the faucets were made of brass or brass-like materials: metal alloys containing zinc and copper. The exact plastic and metallic compositions are not known. Hwang et al.[51] noted that artificial drinking water biofilms grown on brass gave less DNA yield compared to biofilms grown on plastic. Others have shown that substrate material significantly influences the formation potential and diversity of biofilms in drinking water systems.[52] In general, metallic materials appear to have a lower biofilm formation potential than materials based on plastic polymers such as, for example, polyvinyl chloride (PVC) or polyethylene (PE),[53] materials that are frequently used in shower hoses.

Variable amounts of DNA recovered from individual shower hoses may be due to a multitude of factors: First of all, the sampling was not quantitative. While all effort was made to keep the sampling protocol the same for all shower hoses, differences in area swabbed may have occurred. Further, neither shower hose age nor use frequency nor precise material were known and may have varied within and between buildings. These three factors are known to have important effects on the amount and composition of shower hose biofilms.[46,54] Finally, water disinfection in general has been shown to have an effect on total cell concentration in biofilms and biofilm thickness. Hose biofilms exposed to disinfection tend to be thinner and have lower cell concentrations than biofilms not exposed to disinfectants.[46] Thus, less biofilm may be expected in drinking water systems exposed to CSI. Indeed, several CSI system manufacturers have claimed that CSI inhibits or even removes biofilms. This claim may be supported by the observation that we did not recover DNA from nine of 11 faucet biofilm samples taken at the hospital but from all faucet biofilm samples at the research institute (Table S3). However, in contrast, we recovered similar amounts of DNA from shower hose biofilms at the research institute and the hospital. While the amount of DNA recovered is only a rough estimator of biofilm abundance, this indicates that biofilms were present in shower hoses at the hospital despite the CSI system being in use for six years. Thus, the effect of CSI on biofilm abundance is not clear and may be dependent on substrate material. Peer-reviewed studies investigating multispecies biofilms have to date not found an effect of CSI on the amount of biofilm produced.[19,55,56]

Diversity and Taxonomy of Shower Hose Biofilms. Diversity analyses of the shower hose biofilms revealed differences in community composition (ANOSIM $R = 0.2$, $p = 0.022$, Table S6) and species richness (Figure S2, Table S7), between samples recovered from the research institute and the hospital, but the differences were less clear than those observed for the water samples (Figure 2, Figure S2, Table S7). Biofilms recovered from the research institute had a higher observed OTU richness than those recovered from the hospital (Kruskal−Wallis chi2 = 9.691, $p = 0.002$; abstract figure and Figure S2D). Species evenness was similar between the two buildings (Kruskal−Wallis chi2 = 2.85, $p = 0.091$) and much lower when compared to the water samples (Kruskal−Wallis chi2 = 27.239, $p < 0.001$; Figure S2 and abstract figure, Table S7)

All biofilm samples at the research institute and the hospital were dominated by *Proteobacteria*, especially *Alphaproteobacteria* (Figure 3). This taxon often dominates biofilms in drinking water systems.[43,46,54,57] Overall, 292 different OTUs were detected in all shower hose biofilm samples, but only two were present in all samples with an abundance >0.1%. Both these core OTUs were classified as *Alphaproteobacteria*, one as

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Rhizobiales, the other as *Sphingomonadales*. The abundances of these core OTUs varied widely between the samples, but overall, they comprised 28% of all biofilm sequences (Table S8).

Recently, three other studies have characterized the bacterial communities of shower hose biofilms using high-throughput sequencing technology.[46,54,58] Two studies used 16S rDNA amplicon sequencing;[46,54] the third used shotgun metagenomics sequencing in combination with bacterial culturing.[58] Of these, one study used 16S rDNA amplicon sequencing to investigate the effect of shower hose material and biofilm age on community composition in a model system.[54] The authors showed that both age and material had significant impacts on bacterial communities, but that communities became more alike with age.[54] Furthermore, they found that opportunistic pathogens were more common in low-biomass biofilms. The second study investigated a variety of biofilms from shower hoses collected around the world.[46] The results showed that disinfection use in general had important effects on the thickness and diversity of biofilm samples. Disinfection exposed biofilms were thinner and had a lower cell concentration and species richness. Furthermore, similar to the first study, thin biofilms were more likely to host potential opportunistic pathogens.[46] The third study used culturing and shotgun metagenomic sequencing to characterize the biofilm communities of hospital shower hoses.[58] In shotgun metagenomics sequencing, all DNA within a sample is sequenced and not only specific markers, such as in 16S rDNA amplicon sequencing. This study retrieved significantly different communities with both approaches.[58] The culturing approach retrieved a community dominated by *Proteobacteria*, while the metagenomics approach recovered communities dominated by *Mycobacterium*-like taxa. In a different study, nontuberculous *Mycobacteria* were enriched in showerhead biofilms receiving municipal chlorine-treated water but not present in showerhead biofilms receiving chlorine-free water.[59] As many *Mycobacteria* are highly chlorine-resistant,[60] it has been suggested that chlorination may result in *Mycobacteria* enrichment in biofilms.[59] However, considering that opportunistic pathogens including *Actinomycetales* bacteria may be enriched in low-biomass biofilms not exposed to chlorination,[54] it appears that the processes leading to an enrichment of drinking water biofilms with *Mycobacteria* and other potentially pathogenic species are more complex.

Nontuberculous *Mycobacteria* spp. have previously been suggested to be more tolerant to CSI than *Legionella* bacteria,[18] but an enrichment of *Mycobacteria* in biofilms or warm-water samples from the hospital was not detected in the present study. Sequences classified as *Actinomycetales*, thus potentially including *Mycobacteria* and *Mycobacteria*-like taxa, were much less abundant in biofilms compared to water samples (Kruskal–Wallis chi-squared = 21.195, p value < 0.001, Figure S4). Furthermore, no difference in relative abundance between the three water sample groups or between the biofilm groups was detected (Figure S4). Likewise, *P. aeruginosa* has previously been indicated to be more tolerant to CSI treatment than *Legionella* bacteria,[21,27] but *Pseudomonadales* classified sequences were not enriched in the hospital water or biofilm samples (Figure S5). The abundance of *Pseudmonadales* sequences was in general low: <3% relative abundance in all samples, except two hose biofilm samples, one at the research institute (FS11H, 28%) and one at the hospital (staff shower LS20H, 53% relative abundance).

Detection and Diversity of *Legionella*. The CSI system at the hospital was installed in 2009 to combat *Legionella*.[30] Already a few weeks after installation, *Legionella* numbers dropped to zero,[30] and no recolonization has been observed since (R. Almo, Technical department, Lovisenberg Diakonale Hospital, Pers. com.). *Legionella* monitoring at the hospital is based on *Legionella* culture in accordance with ISO 11731-2. The same culture method was used during the present study. In line with the monitoring results, no *Legionella* bacteria were cultured from any of the water or biofilm samples taken at the hospital. However, there were also no *Legionella* bacteria cultured from any of the other water or biofilm samples taken during the present study. Likewise, all ELISA tests were negative. This suggests that active, culturable *Legionella* bacteria were absent or rare in all water and biofilm samples taken at Oset DWTP, at the research institute, and at the hospital.

In contrast to the culturing and ELISA results, limited numbers of sequences classified as *Legionella* were detected in all water samples and some biofilm samples, including those taken after CSI treatment at the hospital (Table S11). However, the relative abundance of *Legionella*-classified sequences per sample was low, ranging from 0 to 2.6% (median 0.3%). In general, *Legionella* sequences were virtually absent from biofilm samples, except for one shower hose biofilm sample at the research institute (FS10H, Table S11). Other researchers have also recovered more *Legionella* from bulk water than from biofilms samples in a diverse range of drinking water systems.[46,61,62]

Overall, six OTUs were classified as *Legionella*; three relatively abundant (Otu00079, Otu00111, Otu00124) and three rare OTUs (Otu00289, Otu00324, Otu00340; Figure 4).

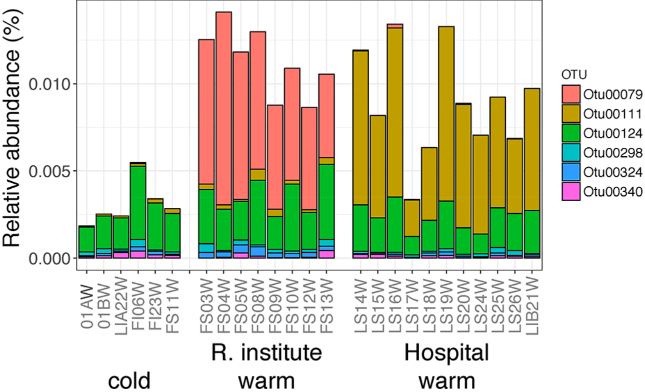

Figure 4. Relative abundance of *Legionella*-classified sequences in all water samples. Samples: 01AW–FS11W, group 1; FS03W–FS13W, group 2; LS14W–LIB21W, group 3. See Diversity and Taxonomy of Water Samples section for discussion of groups.

Interestingly, the three abundant OTUs showed a nonrandom distribution: Otu00124 was present in all water samples with similar proportions. Otu00079 was virtually absent from all cold-water samples and from warm-water samples at the hospital but was most abundant in the warm-water samples at the research institute (Figure 4 and Figure S6). Otu00111 in contrast was most abundant in the warm-water samples at the hospital but was only present as a small fraction in the warm-water samples at the research institute and in all cold-water samples (Figure 4 and Figure S6). These observations suggest that at least a small number of *Legionella* were present in all water samples and that different conditions or locations

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selected for different *Legionella* OTUs. Water temperature is one plausible selective factor, as relatively more *Legionella* sequences were detected in warm-water compared to cold-water samples, but location, CSI treatment, or other unknown factors may also have played important roles. It has for example been shown that property age, shower age, frequency of use, and shower head cleaning have important effects for the abundance of *Legionella* in household showers.[61]

Our finding that a diversity of *Legionella* bacteria are present in drinking water samples are in line with those from other research studies. For example, 16S rDNA fingerprinting methodology[26] showed that a variety of *Legionella* types are present in drinking water, but that the *Legionella* communities change from surface reservoir to tap water. Furthermore, the same authors found that heating caused a shift to thermophilic species and a 3-fold *Legionella* increase. In addition, "heat-and-flush" disinfection using similar temperatures as the hot-water temperatures at the hospital and the research institute has been shown to select for heat-tolerant *Legionella* strains.[8] Furthermore, several disinfection methods and heat may induce a viable but nonculturable (VBNC) state in *Legionella* bacteria.[8] Induction of a VBNC state may be one reason why we (and others[44]) detected *Legionella* through 16S rDNA amplicon sequencing but not through culturing. DNA based PCR and sequencing methodologies cannot discern whether the bacteria detected are active, dead, or in a VBNC state.

Limitations and Further Perspectives. Here, we aimed to characterize the effects of CSI on the bacterial community composition of drinking water and biofilms in a full-scale system. Our results show that the bacterial warm-water communities recovered from buildings with and without CSI are relatively homogeneous within each building but differ significantly between the two buildings. It is tempting to conclude that the differences observed are due to disinfection with CSI treatment.

However, others have shown that building effects (i.e., the accumulated inherent differences between buildings) may explain the majority of variation observed in bacterial communities encountered in drinking water distribution systems.[46] In the present study, we have also encountered a range of factors that may have contributed to differences in community composition in addition to CSI treatment (Table 3). Furthermore, it was not possible to disentangle effects of temperature and CSI in the hospital samples. In future studies, it will therefore be important to include a higher number of buildings as well as cold- and warm-water samples to minimize building effects and to separate the effects of heating and CSI treatment on community composition.

In addition, it will be important to include quantitative measurements for the abundance and viability of bacteria in all samples. Here, we primarily used 16S rDNA amplicon sequencing of DNA extracts, a qualitative approach that includes all DNA present in a sample and does not differentiate between environmental DNA, dead cells, active cells, and those in a VBNC state. However, water transport, changes in temperature, and CSI treatment are likely to have an effect on the number and metabolic activity of cells. These effects were reflected in the dissimilar ATP values measured in the three water groups (Figure 3c). Notably, ATP measurements show only effects on the entire community, not on specific taxa. To be able to investigate the effect of CSI on the abundance and metabolic activity on specific taxa, community analyses based on RNA transcripts, advanced microscopy, and flow-cytometry using live/dead differentiation methods will be useful in the future.

Analyses of specific taxa indicate that different *Legionella* communities were present in cold-water samples, warm-water samples at the research institute, and CSI-treated warm-water samples at the hospital. Furthermore, one unclassified *Betaproteobacteria* OTU was highly enriched in the water samples at the hospital. To characterize the different *Legionella* communities and the enriched OTU in detail, metagenomic shotgun-sequencing may be necessary.

It is also noteworthy that neither *Actinomycetales* nor *Pseudomonadales* appeared to be enriched in the biofilm or water samples at the hospital compared to the other samples. Both nontuberculous *Mycobacteria* spp.[18] and *P. aeruginosa*[21] have previously been indicated to be highly tolerant to CSI treatment. In future studies, it will be interesting to quantify the effect of CSI on the absolute abundance and viability of these potential opportunistic pathogens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b05963.

11 Tables and 6 Figures (PDF)

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All authors contributed to the manuscript and approved the final version.

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Notes

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■ ABBREVIATIONS

ATP adenosine triphosphate

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CSI copper−silver ionization
DWTP drinking water treatment plant
OTU operational taxonomic unit
(q)PCR (quantitative) polymerase chain reaction

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