Environmental Science & Technology

Article

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Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.7b05963 • Publication Date (Web): 20 Feb 2018

ACS Publications

Environmental Science & Technology is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

TITLE. Microbial community composition of tap water and biofilms treated with or without copper-silver-ionization

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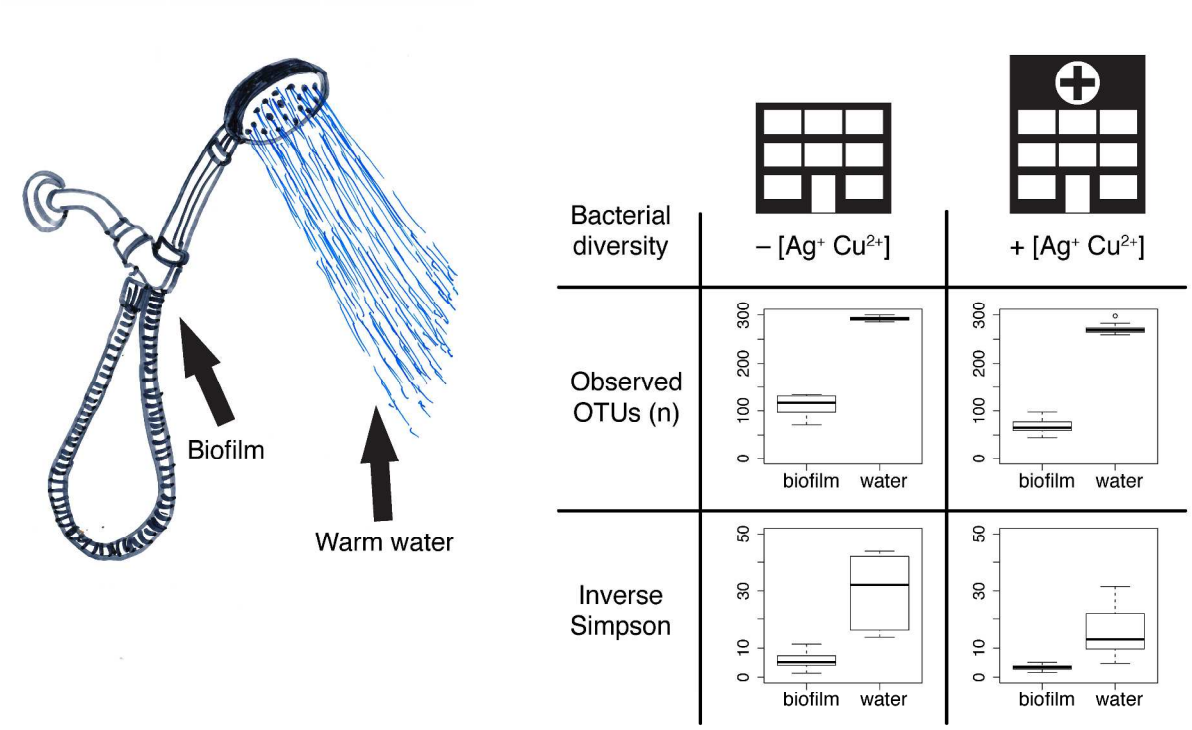
ABSTRACT. Copper-silver-ionization (CSI) is an in-house water disinfection method primarily installed to eradicate *Legionella* bacteria from drinking water distribution systems (DWDS). Its effect on the abundance of culturable *Legionella* and *Legionella* infections has been documented in several studies. However, the effect of CSI on other bacteria in DWDS is largely unknown. To investigate these effects, we characterised drinking water and biofilm communities in a hospital using CSI, in a neighbouring 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, $p < 0.001$).



Abstract figure

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 immuno-compromised 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 is almost 30 % in Europe [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 source 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

re-current incidences [16, 17]. These recurrences are thought to be due to too low levels of 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 sequenced-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 complimentary 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

74 heterotrophic bacteria have been shown to be more tolerant than *Legionella* to CSI in a Finnish
75 hospital [18]. Finally, the effect of CSI on the entire bacterial community has not yet been
76 evaluated.

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

84

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

89 Oset DWTP serves 92% of Oslo's residents [28]. It uses water from Lake Maridalsvannet, a
90 freshwater lake situated north of the city. Routine water treatment at Oset consists of
91 coagulation, sedimentation, filtration, UV irradiation and pH adjustment. In addition, small
92 amounts of sodium hypochlorite, typically 0.1 mg Cl_2 L^{-1}, are added to the treated water even
93 when the UV system works satisfactorily to ensure that the back-up disinfection system is
94 functioning [29].

The research institute and the hospital are neighbouring 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 (approx. 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 ^{o}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, complimentary 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 Table 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

118 throughout the building: five from patient bathrooms (1-2 patients per bathroom) and five from
119 staff changing rooms.

120 The same sampling protocol was used at all sites. First, the water outlets were flushed for 1
121 min and, where possible, the water temperature adjusted to 35°C - 38°C prior to sampling.
122 Temperature adjustment was not possible at Oset DWTP and at the water intakes at the hospital
123 and the research institute; thus only cold-water was sampled at these sites. Firstly, samples for
124 bacterial culturing, ATP and ELISA analyses were taken in separate autoclaved glass bottles: 1x
125 500 ml for ATP, 1 x 1 L for ELISA and 2 x 1 L for *Legionella spp.* culture. The bottles were
126 immediately transported to the laboratory and processed. 0.5 L water was in addition sampled at
127 the hospital for copper and silver analyses. Secondly, for DNA analyses, 10 L of water were
128 filtered through a Sterivex^{TM} 0.22 µm filter unit (Millipore) using sterile silicone tubing and a
129 peristaltic pump (Watson Marlow 120S/DV, 120 rpm, approx. flow 100 ml min^{-1}). The filter was
130 aseptically removed, capped on both sides, transferred to a 50 ml Falcon tube and placed on ice
131 until arrival in the laboratory, where it was frozen at -20 °C and further processed within two
132 weeks. Finally, biofilm samples were taken. For DNA analyses, the showerhead was removed
133 and the inside of the shower hose thoroughly swabbed (FLOQSwab^{TM} with 30 mm breakpoint;
134 Copan Italia). The swab tip was put into a 2 ml Eppendorf tube filled with 1 ml autoclaved and
135 sterile filtered 1x phosphate buffered saline (PBS) solution. Then the shower hose was
136 dismounted and a second biofilm sample taken in the same manner from the faucet to which the
137 hose had been connected. For intake water samples taken directly from faucets, faucet insides
138 were thoroughly swapped as described for shower faucets. The swabs were stored on ice until
139 arrival at the laboratory. Swabs were centrifuged for 30 min (4 °C, 2000 x g), most of the
140 supernatant was carefully removed and the tube containing the swab tip and biofilm pellet was

141 frozen at -20°C until DNA isolation. For bacterial culture, one Copan eSwab (Copan Italia) was
142 used to thoroughly swab the end of the shower hose that had been attached to the faucet. The
143 swab was placed in 1 ml liquid Amies medium, transported to the laboratory on ice and
144 immediately processed.

145 Table 1: Main sample details. For further information, see Supplementary Information.

Room	Water Sample	Hose Biofilm Sample	Sample type	CSI	Filtration	Water temp. □	Temp. category	Water group
Oset DWTP								
Laboratory	01AW	#	treated drinking water	no	no	9.7	cold	1
Laboratory	01BW	#	treated drinking water	no	no	9.7	cold	1
Research Institute								
K637	FI06W	#	water intake	no	yes	9.6	cold	1
K637	FI23W	#	water intake	no	no	8.7	cold	1
U526	*	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

Room	Water Sample	Hose Biofilm Sample	Sample type	CSI	Filtration	Water temp. □	Temp. category	Water group
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	LIA22W	#	water intake	no	no	8.2	cold	1
Technical	LIB21W	#	water intake	yes	yes	37.7	warm	3
430B	LS14W	*	patient shower	yes	yes	36.9	warm	3
439B	LS15W	*	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

Room	Water Sample	Hose Biofilm Sample	Sample type	CSI	Filtration	Water temp. □	Temp. category	Water group
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

146 # No hose biofilm samples taken at DWTP and water intakes *Sample excluded from analyses due to too little DNA or number of sequenced reads.

147

148

149 **Water and biofilm analyses.** For water chemistry analyses, 1 L cold water was sampled in
150 PE- bottles at selected sampling points after flushing for 1 min, stored in opaque cool bags and
151 transported to the laboratories within two hours. Water chemistry analyses were carried out by
152 Oslo's Water and Wastewater authorities (VAV) according to accredited ISO methods.

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

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

158 *Legionella*-specific ELISAs were carried out with the HybriScan®D Legionella kit (Sigma-
159 Aldrich) according to the manufacturer´s instructions and results read on a Multiskan EX
160 (Labsystems) plate reader.

161 Cultivation of *Legionella* from water samples was carried out according to standard methods
162 [31]. In addition, 1 L water was filtered through a second filter. The filter was cut into small pieces,
163 placed in a tube containing 10 ml saline solution (0.9%) and gently shaken for 2 minutes. From
164 this solution 0.1 ml was inoculated on GVPC-agar and BCYE-agar. For cultivation from
165 biofilms, 0.1 ml of the liquid Amies medium was inoculated on GVPC-agar and BCYE-agar, and
166 incubated at 36 ± 1°C for up to 10 days.

167

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 re-analysed 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.

16S PCR, normalization, amplicon pooling and sequencing: The dual-index PCR protocol published by [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 2X Phusion Hot-start II High-Fidelity MasterMix, 2.5 µl forward and 2.5 µl reverse primer (1µM each), 0.75 µl DMSO, 1.75 µl PCR-grade water, and 5 µl template (controls or DNA, max. 4 ng/ µl). The PCRs were run on a Bio-Rad S1000 Thermal Cycler using the following program: 1 x [98°C/30s], 33 x [98°C/15s, 54°C/15s, 72°C/15s], 1 x [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 manufacturer's protocol using 25 µl PCR product and 25 µl 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 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 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.

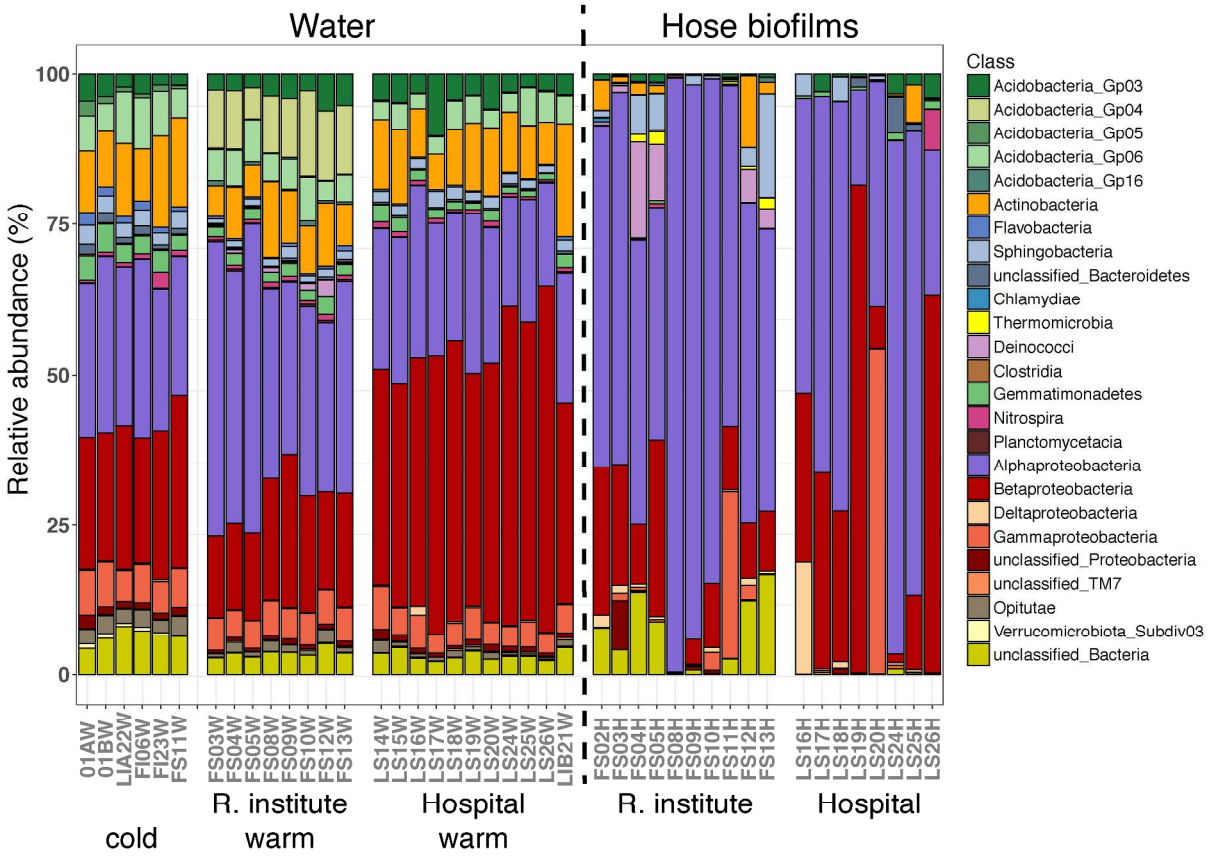
Bioinformatic analyses: Remnant Illumina adapters were removed using AdapterRemoval v. 2.1.7 [34]. Paired sequence reads were merged, demultiplexed, quality filtered, clustered into OTUs and classified using Moira v. 1.3.0 [35] and Mothur v. 1.36.1 [36]. Details, settings and commands are listed in File S1. Samples that had no measureable DNA or did not give clear bands after PCR were excluded from the analyses because results of low DNA samples are prone to be highly impacted by contamination such as from DNA isolation kits [37]. All 23 faucet, 2 shower hose biofilms (LS14H, LS15H) and 1 water sample (FS02H) were excluded (Table S5).

Operational Taxonomic Unit (OTU) abundance data was analysed 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). Non-metric multidimensional scaling (NMDS)

using the Bray-Curtis dissimilarity measure was used to visualise 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).

224 RESULTS and DISCUSSION.



225

226 **Figure 1.** Relative abundance of bacterial Orders in water and hose biofilm samples. Water
227 samples: 01AW-FS11W: Group 1; FS03W-FS13W: Group2; LS14W-LIB21W: Group3. See
228 paragraph "Diversity and taxonomy of water samples" for discussion of water groups and Table
229 1 for all sample details.

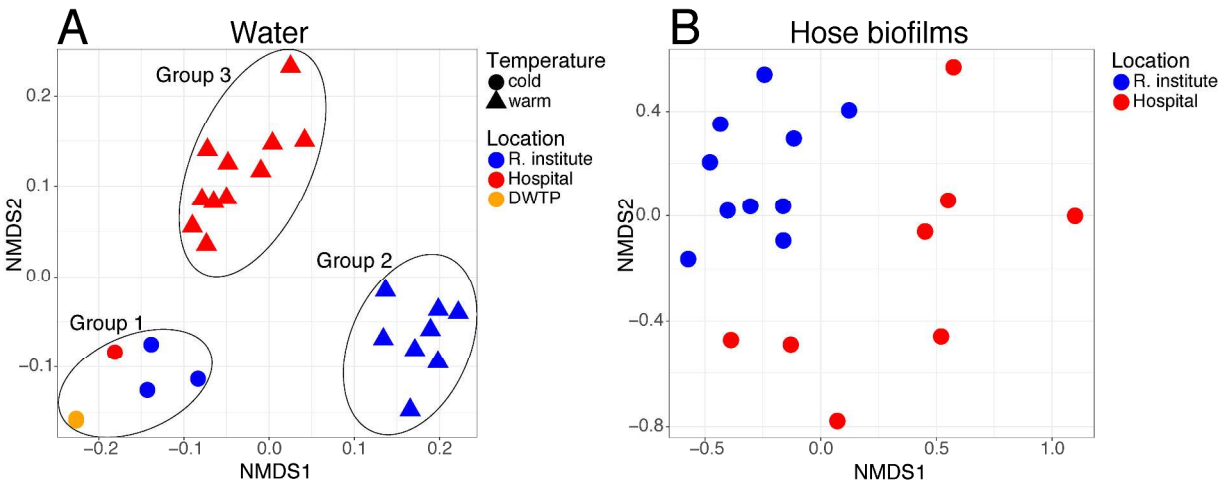
230

231 **Community composition of water and biofilm samples.** The sequencing approach revealed
232 diverse bacterial communities in both water and shower hose biofilm samples (Fig.1). The faucet
233 biofilm samples had to be excluded from all analyses because 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, $p < 0.001$, Fig. S1). The effect of CSI treatment was less clear (CSI vs. none, biofilm and water samples, ANOSIM R 0.092, $p = 0.034$, Fig. 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 (Fig. 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 non-parametric 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 (ground water [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]).



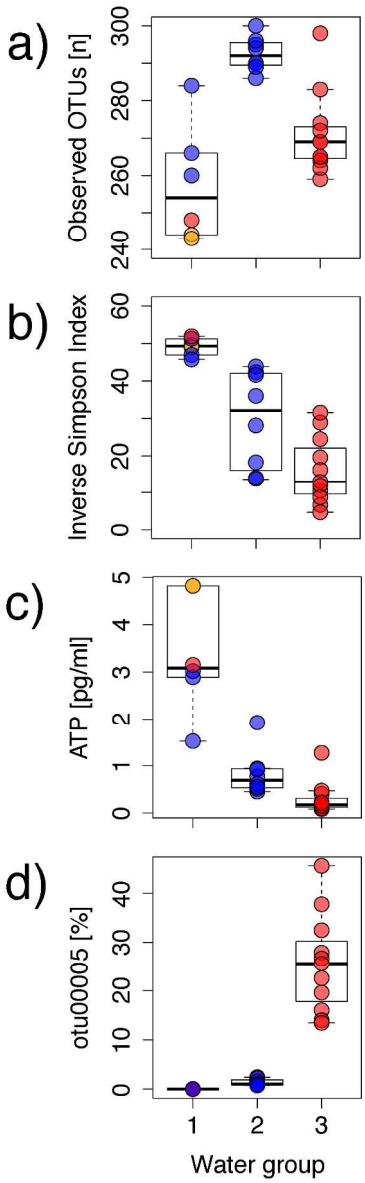
254

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

258

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

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



275

276 **Figure 3.** Differences in a) species richness (Observed OTUs), b) evenness (Inverse Simpson
277 Index), c) ATP in water samples and d) relative abundance of otu00005 in the three water
278 groups. Statistics in Table 2. Water groups: 1 - cold-water samples (without CSI); 2 - warm-
279 water samples from the research institute (without CSI); 3 - warm-water samples from the
280 hospital (after CSI); see also "Diversity and taxonomy of water samples" and Table 1. Blue –
281 sampled at research institute; Red – sampled at hospital; Orange – sampled at DWTP.

282

283 **Table 2.** Statistics for differences observed between the three water groups (Fig. 3). *** p <
284 0.001, ** p < 0.01, * p < 0.05.

Kruskal-Wallis rank-sum test	Chi2	df	p
Observed OTUs	14.447	2	<0.001***
Inverse Simpson I.	16.279	2	<0.001***
ATP	18.044	2	<0.001***
Rel. abundance otu00005	20.871	2	<0.001***
Pairwise comparisons Wilcox rank sum test	**Group1/Group2**	**Group1/Group3**	**Group2/Group3**
Observed OTUs (p adjust bonferroni)	0.0072**	0.2905	0.0760
InvSimpson (p adjust bonferroni)	0.0072**	0.0033**	0.0695
ATP (p adjust bonferroni)	0.0109*	0.0032**	0.0101*
Rel. abundance otu00005 (p adjust bonferroni)	0.0072**	0.0033**	0.0001***

285

286

287 The three water sample groups also differed in species richness, evenness and ATP
288 measurements (Fig. 3, Table 2). The warm-water samples at the research institute (Group 2) had
289 the highest species richness, while no significant richness-differences were observed between
290 cold-water (Group 1) and warm-water samples at the hospital (Group 3). Evenness was
291 significantly higher and less variable in cold-water communities (Group1) than warm-water
292 communities (Group 2 and 3). Furthermore, the ATP results showed that cold-water contained

293 the highest living bacterial biomass, while CSI-treated warm-water at the hospital contained the
294 lowest.

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

302 At the hospital, ATP- and community differences between cold, incoming water and warm,
303 CSI-treated water suggest important effects of the hot-water system in combination with CSI.
304 The effect of these two cannot be differentiated in the present study as only cold-water samples
305 upstream of CSI and warm-water samples downstream of CSI were taken and processed for
306 microbiome analyses.

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

313

314 **Table 3.** Differences between the research institute and the hospital that may have contributed to
315 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 □	70 □
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	Lower copper and zinc values than at the research institute
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 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

316

317 For example, small differences in warm-water temperature have been shown to have important
318 effects on the microbial composition of drinking water [48] and hot-water temperatures have been
319 shown to select for thermo-tolerant Legionella strains [8]. It is probable that this 5□ difference in
320 hot water-temperature between the buildings has had an effect on community composition and
321 ATP results. Furthermore, pipe material has an important influence on microbiome composition
322 [48, 49]. The pipe materials in the present study are unknown, but we observed consistent
323 differences in the water chemistry parameters of the incoming water at the research institute and
324 the hospital (Table S2). Specifically, zinc values were twice as high and copper values were 25-
325 45 times higher in the incoming water at the research institute compared to the hospital. As both

326 buildings receive water through the same main pipes, these differences indicate that the water
327 pipes connecting the research institute with the main water pipes contain more zinc and copper
328 than the corresponding pipes connecting the hospital. While these differences in water chemistry
329 did not have discernible effects on the community composition of the incoming water samples
330 (all clustered within Group 1), it cannot be excluded that differences in pipe materials
331 contributed to the distinct grouping of warm-water samples.

332 *Proteobacteria* had the highest relative abundance in all water samples, but the distribution at
333 class level differed between the three groups (Fig. 1, Fig. S3). The cold-water samples (Group 1)
334 contained similar relative amounts of *Alpha-* and *Betaproteobacteria*, while the warm-water
335 samples at the research institute (Group 2) were dominated by *Alphaproteobacteria* and the
336 warm-water samples at the hospital (Group 3) by *Betaproteobacteria* (Fig. S3). Especially one
337 OTU had a very high relative abundance in the bacterial communities of Group 3, otu00005,
338 classified as "unclassified *Betaproteobacteria*". On average, this OTU contributed 25 % (range
339 14-45 %) of all reads in the warm water samples at the hospital (Fig. 3; Tables 2 and S9). In
340 comparison, the same OTU only made up 1.4 % of all the reads sequenced from the warm-water
341 samples at the research institute (Group 2, range 0-2.5 %), and was virtually absent from cold-
342 water samples and biofilms. This indicates that the local conditions at the hospital resulted in a
343 relative enrichment of otu00005. Different proportions of *Proteobacteria* frequently dominate
344 drinking water communities [1, 3, 44, 50], but such high abundances of a single OTU in drinking
345 water samples has, to our knowledge, not been described.

346 To characterise otu00005, the most abundant sequence in the OTU (corresponding to 46 % of
347 sequences in otu00005) was used for similarity searches against databases at NCBI. These
348 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 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 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 or 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 multi-species biofilms have to date not found an effect of CSI on the amount of biofilm produced [19, 55, 56].

394

395 **Diversity and taxonomy of shower hose biofilms.** Diversity analyses of the shower hose
396 biofilms revealed differences in community composition (ANOSIM R = 0.2, p = 0.02, Table S6)
397 and species richness (Fig. S2, Table S7), between samples recovered from the research institute
398 and the hospital, but the differences were less clear than those observed for the water samples
399 (Fig. 2, Fig. S2, Table S7). Biofilms recovered from the research institute had a higher observed
400 OTU richness than those recovered from the hospital (Kruskal-Wallis chi2 = 9.691, p = 0.002)
401 (abstract figure and Fig. S2D). Species evenness was similar between the two buildings
402 (Kruskal-Wallis chi2 = 2.85, p = 0.091) and much lower when compared to the water samples
403 (Kruskal-Wallis chi2 = 27. 239, p <0.001; Fig S2 and abstract figure, Table S7)

404 All biofilm samples at the research institute and the hospital were dominated by
405 *Proteobacteria*, especially *Alphaproteobacteria* (Fig. 03). This taxon often dominates biofilms in
406 drinking water systems [43, 46, 54, 57]. Overall, 292 different OTUs were detected in all shower hose
407 biofilm samples, but only two were present in all samples with an abundance > 0.1 %. Both these
408 core OTUs were classified as *Alphaproteobacteria*, one as *Rhizobiales*, the other as
409 *Sphingomonadales*. The abundances of these core OTUs varied widely between the samples, but
410 overall, they comprised 28 % of all biofilm sequences (Table S8).

411 Recently, three other studies have characterised the bacterial communities of shower hose
412 biofilms using high-throughput sequencing technology [46, 54, 58]. Two studies used 16S rDNA
413 amplicon sequencing [46, 54], the third used shotgun metagenomics sequencing in combination with
414 bacterial culturing [58]. Of these, one study used 16S rDNA amplicon sequencing to investigate the
415 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, 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 characterise 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 (Order incl. *Mycobacteria*) 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

439 samples from the hospital was not detected in the present study. Sequences classified as
440 *Actinomycetales,* thus potentially including *Mycobacteria* and *Mycobacteria*-like taxa, were
441 much less abundant in biofilm compared to water samples (Kruskal-Wallis chi-squared = 21.195,
442 p-value <0.001, Fig. S4). Furthermore, no difference in relative abundance between the three
443 water sample groups or between the biofilm groups was detected (Fig. S4). Likewise, *P.*
444 *aeruginosa* has previously been indicated to be more tolerant to CSI treatment than *Legionella*
445 bacteria [21, 27], but *Pseudomonadales* classified sequences were not enriched in the hospital water
446 or biofilm samples (Fig. S5). The abundance of *Pseudmonadales* sequences was in general low:
447 < 3% relative abundance in all samples, except two hose biofilm samples, one at the research
448 institute (FS11H, 28 %) and one at the hospital (staff shower LS20H, 53% relative abundance).

449

450 **Detection and diversity of *Legionella.*** The CSI system at the hospital was installed in 2009 to
451 combat *Legionella* [30]. Already a few weeks after installation, *Legionella* numbers dropped to
452 zero [30] and no re-colonisation has been observed since (R. Almo, Technical department,
453 Lovisenberg Diakonale Hospital, pers. com.). *Legionella* monitoring at the hospital is based on
454 *Legionella* culture in accordance with ISO 11731-2. The same culture method was used during
455 the present study. In line with the monitoring results, no *Legionella* bacteria were cultured from
456 any of the water or biofilm samples taken at the hospital. However, there were also no *Legionella*
457 bacteria cultured from any of the other water or biofilm samples taken during the present study.
458 Likewise, all ELISA tests were negative. This suggests that active, culturable *Legionella* bacteria
459 were absent or rare in all water and biofilms samples taken at Oset DWTP, at the research
460 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 0-2.6% (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; Fig. 4). Interestingly, the three abundant OTUs showed a non-random 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 (Fig. 4 and Fig. 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 (Fig. 4 and Fig. S6). These observations suggest that at least a small number of *Legionella* were present in all water samples and that different conditions or locations 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].

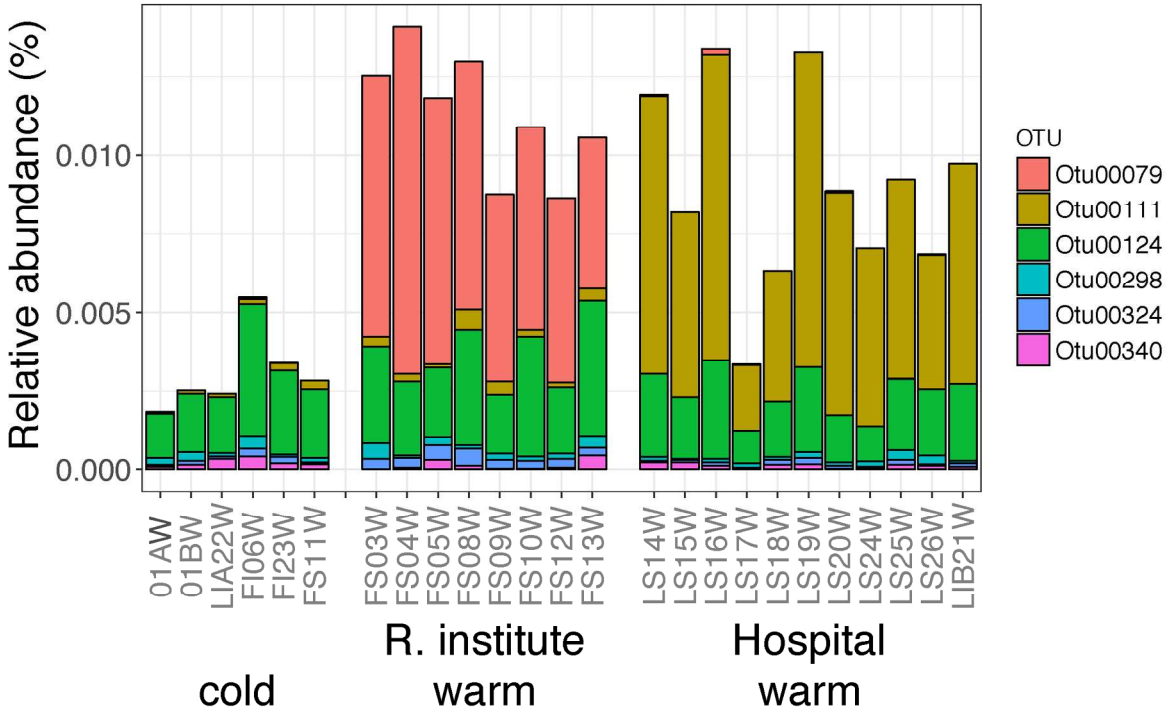


Figure 4. Relative abundance of *Legionella*-classified sequences in all water samples. Samples: 01AW-FS11W - Group 1; FS03W-FS13W - Group 2; LS14W-LIB21W – Group 3. See section "Diversity and taxonomy of water samples" for discussion of groups.

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 three-fold *Legionella* increase. In addition, "heat-and-flush" disinfection using similar temperatures as the hot-water temperatures

496 at the hospital and the research institute has been shown to select for heat-tolerant *Legionella*
497 strains [8]. Furthermore, several disinfection methods and heat may induce a viable but non-
498 culturable (VBNC) state in *Legionella* bacteria [8]. Induction of a VBNC state may be one reason
499 why we (and others [44]) detected *Legionella* through 16S rDNA amplicon sequencing but not
500 through culturing. DNA based PCR and sequencing methodologies cannot discern whether the
501 bacteria detected are active, dead or in a VBNC state.

502

503 **Limitations and further perspectives.** Here we aimed to characterise the effects of CSI on the
504 bacterial community composition of drinking water and biofilms in a full-scale system. Our
505 results show that the bacterial warm-water communities recovered from buildings with and
506 without CSI are relatively homogenous within each building but differ significantly between the
507 two buildings. It is tempting to conclude that the differences observed are due to disinfection
508 with CSI treatment.

509 However, others have shown that building effects (i.e. the accumulated inherent differences
510 between buildings) may explain the majority of variation observed in bacterial communities
511 encountered in drinking water distribution systems [46]. In the present study, we have also
512 encountered a range of factors that may have contributed to differences in community
513 composition in addition to CSI treatment (Table 3). Furthermore, it was not possible to
514 disentangle effects of temperature and CSI in the hospital samples. In future studies, it will
515 therefore be important to include a higher number of buildings as well as cold- and warm-water
516 samples to minimise building effects and to separate the effects of heating and CSI treatment on
517 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 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 (Fig. 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 characterise 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. One file containing 11 Tables and 6 Figures.

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Author Contributions

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

Funding Sources

The Norwegian Institute of Public Health received a grant from the Norwegian Ministry of Health and Care Services to investigate the microbial quality of drinking water in Norway. The sequencing service was provided by the Norwegian Sequencing Centre (www.sequencing.uio.no), a national technology platform hosted by the University of Oslo and Oslo University Hospital, supported by the "Functional Genomics" and "Infrastructure" programs of the Research Council of Norway and the South-Eastern Norway Regional Health Authority.

ACKNOWLEDGMENT

We are thankful to the hospital staff for their commitment and for enabling the sampling campaigns, to Wenche Fonahn, Inger-Anne Kvernrød and Nina Gjølme for their dedicated support and for carrying out the ATP and ELISA analyses as well as *Legionella* culturing and to

the team at VAV for carrying out the physical-chemical water analyses. We thank Fernando Puente-Sánchez for his assistance and advice on running Moira in combination with Mother.

ABBREVIATIONS

ATP adenosine triphosphate; CSI Copper-Silver-Ionization; DWTP drinking water treatment plant; OTU operational taxonomic unit; (q)PCR (quantitative) polymerase chain reaction

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