

1 **Biofilms in Shower Hoses**

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## Abstract

Shower hoses offer an excellent bacterial growth environment in close proximity to a critical end-user exposure route within building plumbing. In a global survey, biofilms from 78 shower hoses from 11 countries were characterized in terms of cell concentration ( $4.1 \times 10^4 - 5.8 \times 10^8$  cells/cm<sup>2</sup>), metals accumulation (including iron, lead, and copper), and microbiome composition (including presence of potential opportunistic pathogens). In countries using disinfectant, biofilms had on average lower cell concentrations and diversity. Metals accumulation (up to 5 µg-Fe/cm<sup>2</sup>, 75 ng-Pb/cm<sup>2</sup>, and 460 ng-Cu/cm<sup>2</sup>) seemed to be partially responsible for discoloration in biofilms, and likely originated from other pipes in the building. While some potential opportunistic pathogen genera (*Legionella*) had positive correlations with biofilm cell concentration, others (*Mycobacterium*, *Pseudomonas*) had surprisingly non-existent or negative correlations with biofilm cell concentrations. In a follow-up study, 15 identical shower hoses were installed for the same time period in the same country, and both stagnant and flowing water samples were collected. Ecological theory of drift and selection helped to explain microbiome composition and diversity differences between biofilm, stagnant and flowing water samples. Shower hose age helped to explain metals accumulation but not biofilm cell concentration, while frequency of use may influence biofilm cell concentration. Given the uncontrolled nature of showers in real homes, more controlled tests are necessary to disentangle individual drivers of biofilm growth in this unique environment. However, given the detection of potential opportunistic pathogens (e.g. *L. pneumophila* in 21/78 shower hoses), shower hose biofilms are clearly a critical element of building plumbing, and a potential target for building plumbing monitoring.

**Keywords:** shower hose; building plumbing; biofilm

## 1. Introduction

Showers have been implicated as a route of infection for opportunistic pathogens (Schoen and Ashbolt, 2011). However, surprisingly little research attention has been paid to these final meters of water distribution, i.e., shower heads (Feazel et al., 2009) and shower hoses (Collins et al., 2017; Proctor et al., 2016; Soto-Giron et al., 2016). Within the building plumbing context, shower hoses are uniquely exposed to a triple threat of exacerbated bacterial growth factors. (1) They are typically exposed to warm water (rather than only cold or hot water), and would be subject to distal end cooling, even if used with properly regulated and controlled hot water recirculation systems (Rhoads et al., 2016). (2) They typically have long stagnation intervals: in a study including more than 700 homes with 2.6 persons/household, showers were used for 1.8 showers/day with 7.8 minutes/shower on average (i.e., stagnant 23.8 hours/day) (DeOreo et al., 2016). (3) The interior hose is made from flexible polymeric materials that leach significant amounts of biodegradable organic carbon; considerably more than hard pipe materials used otherwise in building plumbing (Bucheli-Witschel et al., 2012; Proctor et al., 2016).

Excessive bacterial growth in the last meters before consumer exposure during showers presents a potential health risk. Opportunistic pathogens, including *Legionella pneumophila*, *Mycobacterium avium*, *Pseudomonas aeruginosa*, and *Acanthamoeba polyphaga* can infect consumers' lungs, eyes, or open wounds, causing pneumonia-like symptoms or other infections (Falkinham et al., 2015; Kilvington et al., 2004). The hygienic implications for end-users are real. For example, from 2011-2012, *L. pneumophila* was implicated in 21 outbreaks in the USA alone (Beer et al., 2015), and many were suspected to involve showers. Along the same lines, regular reports in news media show that *Legionella* related disease, or even just *Legionella* detection, is becoming increasingly disruptive and costly, triggering facility shut-downs, remediation measures, and legal actions (Table S1).

Interest in building plumbing opportunistic pathogen management is increasing slowly. For example, regulations in Germany require periodic *L. pneumophila* testing in homes when water boilers

85 exceed 400 L in size (BMJV, 2011), and new Swiss legislation requires testing of shower water in  
86 public buildings (EDI, 2016). However, these monitoring activities are extremely laborious and the  
87 regulations often fall short both in terms of sampling methods (Wang et al., 2017) and preventative  
88 actions. For example, although materials in drinking water household plumbing are typically quality-controlled to some extent (e.g. (CEN, 2013)), the materials used specifically in shower plumbing  
89 are often completely unregulated because shower water is not necessarily considered as 'potable  
90 water' (BVer LMG, 2017). Moreover, while other pipe materials are installed by qualified professionals, shower hoses remain one of few components of water distribution that can be replaced by  
91 the consumers themselves. Consumers' choice in replacement hoses is driven only by functionality  
92 and aesthetics, with ignorance concerning potential health impacts. This is especially concerning  
93 because shower hoses are literally the last meter before water reaches the end-user, and are often  
94 used to more easily bathe patients and the elderly whom are at higher risk for opportunistic patho-  
95 gen infection.

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99 The unique bacterial growth environment and potential relevance to human health necessitate a  
100 better understanding of the biofilms that develop in shower hoses in the final meters of water dis-  
101 tribution. The first goal of this study was a detailed characterization of biofilms in shower hoses col-  
102 lected from around the world, looking at biofilm concentrations of bacteria and metals, microbiome  
103 composition, and the presence of opportunistic pathogens. In addition to this global survey, a fol-  
104 low-up study was conducted to further identify important factors in biofilm formation and to deter-  
105 mine biofilms' relationship to the water phase.

## 106 **2. Materials and Methods**

107

### 108 **2.1. Sample collection**

109 *Global survey:* Shower hoses were collected from 78 showers across 11 countries on 3 continents  
110 (Belgium, Denmark, Germany, Latvia, Portugal, Serbia, South Africa, Spain, the United Kingdom  
111 (UK), and the United States (US)). Volunteers were instructed to detach the shower hose from all  
112 fittings and to gently decant water from the hose. Hoses were sealed with autoclaved ½ inch stop-  
113 pers and stored in a plastic bag for transport to the lab within one week of removal. All volunteers  
114 also completed a brief survey to ascertain age (time since hose installation) and general water  
115 qualities (i.e., chlorination).

116 All samples were processed within one day of arrival to the laboratory. The outer casing  
117 was removed and the flexible plastic hose was cut into sections for analysis. Two 5-cm sections  
118 from either end were stored in Greiner tubes with 0.2 µm filtered mineral water (Evian, France) for  
119 biofilm visualization. A 90-cm section was used for biofilm extraction, and the remainder was dis-  
120 carded. The 90-cm section was filled with 20 mL sterile glass beads (3 mm diameter) and filtered  
121 mineral water. All sections were stored at 4 °C in the dark until analysis.

122 *Follow-up study:* A follow-up study was conducted for 15 showers in Switzerland. All participants  
123 were given the same type of PVC-P shower hose to use for 1.5 years. Hoses and more detailed  
124 information on use patterns were collected as described above. To determine the biofilms' rela-  
125 tionship to the water phase, three 1 L water samples were taken from each home: (1) stagnant -  
126 from the shower hose after overnight stagnation (exact stagnation duration unknown), (2) warm  
127 flowing - from the shower hose after five minutes of flushing with warm water (i.e., showering tem-  
128 perature, 35-45 °C), and (3) cold flowing - from the nearest tap after five minutes of flushing with  
129 cold water (i.e., distribution system water without shower hose influence).

130

### 131 **2.2. Biofilm visualization**

132 Biofilm structure and thickness were characterized with optical coherence tomography (OCT), us-  
133 ing a Spectral Domain OCT Imaging System (930nm, OCT System Ganymede, Thorlabs GmbH,  
134 Dachau, Germany). Scanning electron microscopy (SEM), was performed at the Center for Mi-

135 microscopy and Image Analysis (University of Zurich) on 1 cm<sup>2</sup> pieces that were fixed with 2.5% Glu-  
136 taraldehyde and stored at 4°C in the dark.

137

### 138 **2.3. Biofilm extraction**

139 Biofilm was extracted using a repeated sonication strategy similar to (Proctor et al., 2016). The 90-  
140 cm section with glass beads and filtered mineral water was inverted five times and then sonicated  
141 in a bath (Bandelin Sonorex, Rangendingen, Germany) for five minutes. The water was collected  
142 and replaced with new filtered mineral water. In total, five rounds of sonication and replacement  
143 were completed for each hose. After the final step, beads were removed and discarded, and hoses  
144 were filled with only filtered mineral water for a final rinsing step. The total biofilm suspension was  
145 sonicated with a needle (Sonoplus HD 2200, Bandelin Sonrex, Rangendingen, Germany) for 30  
146 seconds for homogenization and was aliquoted for further analysis.

147

### 148 **2.4. Flow cytometry (FCM)**

149 Staining and FCM analysis was done as described previously (Prest et al., 2013). Briefly, a work-  
150 ing solution of SYBR<sup>®</sup> Green I (SG) (Invitrogen AG, Basel, Switzerland) was prepared by 100x dilu-  
151 tion in anhydrous dimethylsulfoxide (DMSO). Water samples and biofilm suspensions were stained  
152 with SG at 10 µL/mL. Samples were preheated to 35 °C (3 min), then incubated with stain in the  
153 dark for 10 min at 35 °C before measurement. FCM measurements were performed on a BD Accu-  
154 ri C6<sup>®</sup> instrument (BD Accuri cytometers, Belgium). Data analysis was performed using the BD Ac-  
155 curi CFlow<sup>®</sup> software, following the procedure described previously (Prest et al., 2013) to calculate  
156 total cell concentration (TCC). Water TCC was used directly (cells/mL) and biofilm TCC was calcu-  
157 lated using the hose surface area (cells/cm<sup>2</sup>).

158

### 159 **2.5. Elemental Analysis**

160 Homogenized 100 µL aliquots of the biofilm suspensions were acid-digested in 4 mL concentrated  
161 ultrapure HNO<sub>3</sub> (Carl Roth GmbH, Karlsruhe, Germany) and 1 mL ultrapure concentrated hydro-  
162 gen peroxide (Sigma-Aldrich, Buchs, Switzerland) in 15 mL Teflon-tetrafluorometoxil (PTFE-TFM)  
163 tubes in an MLS ultraClave 4 (Milestone Inc., Shelton, USA) at 230°C and 130 bar for 35 minutes.

164 Subsequently, digestates were diluted 10-fold with ultrapure deionised water. Quality control of di-  
165 gestion was performed by co-digesting procedural blanks and certified reference materials SRM  
166 1568b (rice flour) and RTC SRM-008 (sediment), which gave elemental recoveries between 61-  
167 138% (Table S2).

168 Following digestion, elemental concentrations were quantified using a quadrupole dynamic  
169 reaction cell ICP-MS (Agilent 7500cx). For each investigated element, the targeted isotope, reac-  
170 tion mode and detection limit ( $3 \times \sigma$  of >10 blanks) is given in Table S2. Instrumental tuning and  
171 calibration was performed daily and a 1 ppm Sc and 0.1 ppm In and Lu in 1% HNO<sub>3</sub> solution was  
172 used as internal standard. Quality control was conducted by triplicate analysis of selected diges-  
173 tates and by analyzing procedural blanks and aqueous reference standards (Merck X and NIST  
174 1643f).

175

## 176 2.6. DNA extraction

177 The remaining volume of biofilm suspension was filtered on a 0.2 µm polycarbonate Nucleopore®  
178 membrane filter (47 mm diameter, Whatman, Kent, UK). The filter was inserted into a 5 mL tube  
179 and stored at -20 °C before DNA extraction with the Power Water DNA Isolation Kit (MoBio Labor-  
180 atories, Inc., Carlsbad, CA) according to manufacturers' instructions.

181

## 182 2.7. qPCR

183 qPCR was used to quantify specific organisms, including *L. pneumophila* (via macrophage infectiv-  
184 ity potentiator (*mip*) gene), *M. avium*, *Acanthamoeba* spp., and *V. vermiformis* using previously de-  
185 scribed protocols (Kuiper et al., 2006; Nazarian et al., 2008; Rivière et al., 2006; Wang et al., 2012;  
186 Wilton and Cousins, 1992). Reactions were performed using either a ABI7500 system (Thermo  
187 Fischer Scientific) for TaqMan (Bio-Rad) assays (*L. pneumophila* and *Acanthamoeba* spp.) or a  
188 LightCycler 480 for EvaGreen (Bio-Rad) assays (*M. avium* and *V. vermiformis*) (Roche). Reactions  
189 and primers are detailed in Table S3. A 1:10 dilution was used for all samples, and standard  
190 curves using 7 standards on each run, with a limit of quantification (LOQ) of 10 gene cop-  
191 ies/reaction. Only samples above the LOQ in duplicate reactions were considered to be positive for  
192 presence/absence assessment.

193

## 194 **2.8. Amplicon sequencing with Illumina MiSeq**

195 Approximately 1 ng of DNA extract from each sample was subjected to PCR amplification using  
196 modified universal bacterial primers Bakt\_341F and Bakt\_805R (Klindworth et al., 2013), which  
197 target the V3-V5 region of the 16S rRNA gene, and were adapted with a nucleotide tail to facilitate  
198 binding Nextera adapters. Index PCR was performed to add the Nextera XT v2 Index Kit adapters  
199 (Illumina) to the amplicon. PCR reaction conditions are detailed in (Table S4). After each PCR re-  
200 action, products were purified using the Agencort® AMPure® XP system (Beckman Coulter, Inc.,  
201 Brea, CA).

202 Each product was quantified using Qubit 2.0 HS DNA system (Thermo Fisher Scientific).  
203 Samples were normalized to the same concentration before running on the MiSeq platform using  
204 MiSeq Reagent Kit v2 (300-cycles, #MS-102-2002) according to manufacturer's protocol with 10%  
205 PhiX. All sequencing was done at the Genetic Diversity Centre (GDC) of ETH, Zurich. Libraries  
206 were produced separately for the global survey and the follow-up study.

207

## 208 **2.9. Statistical analysis**

209 For correlations in the global survey, non-parametric tests (Spearman, Kruskal-Wallis, as labelled)  
210 were used because data was not normally distributed. Parametric tests (Pearson's correlation)  
211 were used to quantify correlations in the follow-up study.

212 For sequencing data, sequences were merged, trimmed, filtered, and clustered into opera-  
213 tional taxonomical units (OTUs) according to several algorithms (Table S5). Sequences were iden-  
214 tified according to greengenes v.13.5 (DeSantis et al., 2003). In R, phyloseq (McMurdie and  
215 Holmes, 2013) was used for processing. Libraries were rarefied to either 37,629 (global survey) or  
216 82,185 (follow-up) sequences per sample. In this process, two samples from Switzerland (global  
217 survey) were removed from analysis due to low number of reads. Non-metric multidimensional  
218 scaling (NMDS) was used to visualize microbiome similarities using Bray-Curtis dissimilarity. The  
219 adonis and ordiellipse functions from the vegan package (Oksanen et al., 2013) were used to re-  
220 late environmental data to microbiome composition. In the follow-up, the number of core OTUs  
221 was calculated as the OTUs present in all locations of each sample type or overlapping type.

### 222 3. Results

223

224 For each section, results are presented separately for the two experiments – the global survey and  
225 follow-up study. In the global survey, 78 shower hoses with largely unknown histories were collect-  
226 ed from 11 countries. In the follow-up study, 15 identical PVC-P shower hoses were installed in  
227 Swiss homes and collected after 1.5 years of use, together with three types of water samples from  
228 each location.

229

#### 230 3.1. Biofilm concentrations were high but variable

231

##### 232 *Global survey*

233 Biofilm thickness as measured by OCT ranged from non-detectable to 0.40 mm (Figure 1). Thick-  
234 ness varied considerably across different hoses, but also along a single shower hose (two ends  
235 90-100 cm apart) (Figure S1). SEM imaging of a representative biofilm indicates a thick matrix of  
236 cellular, non-cellular organic, and inorganic constituents (Figure 1, Figure S2).

237 The biofilm surface concentration (hereafter, biofilm concentration) was measured with  
238 several methods using the biofilm suspension from the 90-cm section of hose. Biofilm total cell  
239 concentration (biofilm TCC) measured with FCM ranged from  $4.1 \times 10^4$  –  $5.8 \times 10^8$  cells/cm<sup>2</sup> (Figure  
240 2A), with an average of  $4.4 \times 10^7$  cells/cm<sup>2</sup> on each hose. Other measures of biofilm concentration,  
241 including the biofilm concentrations of intact cells and adenosine tri-phosphate, correlated well with  
242 biofilm TCC (Spearman's  $\rho=0.95$  and  $0.87$  respectively,  $p<0.001$ , Figure S3). Biofilm concentra-  
243 tions of 16S rRNA genes (qPCR), total organic carbon (TOC), and optical density (OD<sub>546</sub>) did not  
244 correlate well with biofilm TCC (Spearman's  $\rho=0.52$ ,  $0.57$ , and  $0.65$  respectively,  $p<0.001$ ). Since  
245 TOC includes extracellular polymeric substances (EPS), and OD<sub>546</sub> is influenced by inorganics  
246 (e.g., calcium, and iron), these can serve as proxies for sliminess and color, which are otherwise  
247 hard to quantify (discussed further with Figure S3).

248 Biofilm TCC varied significantly based on country of origin, and this between-country varia-  
249 tion seemed to be related to the use/non-use of disinfectant. When comparing biofilm TCC be-  
250 tween countries that used disinfectant (Belgium, Latvia, Portugal, Serbia, UK, and US, N=39) and

those that did not (Denmark, Germany, South Africa, Spain, and Switzerland N=39), biofilm TCC was significantly higher without disinfectant ( $p < 0.001$ , Kruskal-Wallis, Figure 2A). This dichotomous classification for disinfectant use/non-use oversimplifies the situation. For example, many utilities in South Africa typically use disinfectants, but the samples were taken from homes using untreated borehole water. Also, some systems (e.g., Belgium) were known to use only low concentrations, and other systems (e.g., Latvia) were known for disinfectant loss in distribution. However, switching of these 'border-line' cases (e.g., Belgium, Latvia) did not affect the overall results.

#### *Follow-up study*

In the follow-up study, biofilm TCC ranged between  $0.3 - 2.0 \times 10^8$  cells/cm<sup>2</sup> (average  $7.76 \times 10^7$  cells/cm<sup>2</sup>), which is a narrower range compared to the same 15 locations in the global survey ( $0.04 - 1.2 \times 10^8$  cells/cm<sup>2</sup>, average  $5.36 \times 10^7$  cells/cm<sup>2</sup>). In 9 of 15 locations, biofilm TCC was higher during the follow-up study than in the global survey (Figure 3A). This occurred even though hoses were usually older during the global survey than in the follow-up study (hoses from 12 of 15 follow-up sample locations were estimated to be > 2-8 years old during the global survey, while 3 were known to be < 6 months old).

### **3.2. Frequency of use may affect biofilm concentrations**

Water TCC varied amongst the three types of water samples collected in the follow-up study (Figure 4A), with stagnant water having the highest TCC in 12 of 15 locations. To relate the water phase to biofilm, additional water quality parameters for the cold flowing water and use-patterns were also measured (Table S6). While water quality did not correlate well with biofilm TCC (data not shown), biofilm TCC correlated positively with the frequency of use (Pearson's  $R = 0.72$ ,  $p = 0.004$ , Table S7).

### **3.3. Metals accumulate in biofilms**

280 *Global survey*

281 Color was notably variable across biofilms (e.g., deep red/orange, translucent, white, yellow)  
282 (Figure 5, Figure S4). Presumably, this can be attributed partly to metal deposition. While not de-  
283 tected in all biofilms, biofilm concentrations of iron, lead, and copper were as high as 5  $\mu\text{g-Fe}/\text{cm}^2$   
284 (South Africa), 75  $\text{ng-Pb}/\text{cm}^2$  (Denmark), and 460  $\text{ng-Cu}/\text{cm}^2$  (Latvia). These metals all correlated  
285 to one another (Spearman's  $p>0.75$ ,  $p<0.001$ ), and correlated moderately with optical density of  
286 biofilm suspensions (data not shown, correlations only driven by several high points). Calcium and  
287 magnesium were detected in all biofilms, and correlated separately with one another (Spearman's  
288  $\rho=0.6$ ,  $p<0.001$ ), as might be expected with sorption behavior. There were no strong correlations  
289 between these metals and biofilm TCC (Figure S5).

290

291 *Follow-up study*

292 The concentration of metals tended to be higher during the global survey when hoses were older  
293 than during the follow-up study (Figure 3B,C,D). Concentrations were more similar when hoses  
294 were from the same building and had similar ages (e.g., Hoses 3-6 were from one building and had  
295 similar ages in both experiments Figure 3B,C,D).

296

297

298 **3.4. Microbiome composition reveals a diverse ecological niche in biofilms**

299

300 *Global survey*

301 The number of OTUs observed in each biofilm ranged from 74 to 481. This measure of diversity  
302 correlated significantly but weakly with biofilm TCC (Spearman's  $\rho=0.28$ ,  $p=0.01$ ), and was signifi-  
303 cantly lower amongst biofilms in countries that use disinfectant ( $p=0.01$ , Kruskal-Wallis, Figure 2B).

304 Some similarities could be seen in biofilm microbiome compositions from the same country  
305 (Figure 6A), but country only explains 24% of variation in microbiome composition (Adonis,  
306  $p<0.001$ ). Use of disinfectant was not useful for explaining microbiome composition (4% explained  
307 by disinfectant use, Adonis,  $p<0.001$ , Figure 6B). However, countries consistently using strong dis-  
308 infectant residuals (US, UK, Portugal) cluster at a high positive NMDS1. The most abundant OTUs

309 may drive some of these variations, with the sum of the top ten OTUs accounting for 3 – 41% of  
310 individual microbiomes. This proportion varied by country (Kruskal-Wallis,  $p < 0.001$ ) and was signif-  
311 icantly higher when disinfectant was used (Kruskal-Wallis,  $p < 0.001$ ).

312 Interestingly, biofilm TCC also seemed to be related to microbiome composition. Biofilm  
313 TCC correlated with the relative abundance of several of the ten most abundant genera (Figure  
314 S6). Significant negative correlations were found with *Sphingomonas*, *Pseudomonas*, and *Limno-*  
315 *bacter* (Spearman's  $\rho = -0.56, -0.55, -0.47$ , respectively,  $p < 0.001$ ), while positive correlations were  
316 found with *Dok59*, *Sphingobium*, and *Meiothermus* (Spearman's  $\rho = 0.57$  ( $p < 0.001$ ),  $0.21$  ( $p = 0.17$ ),  
317  $0.21$  ( $p = 0.12$ ), respectively).

318

#### 319 *Follow-up study*

320 Amongst water samples, cold flowing water had the highest number of OTUs, followed by warm  
321 water and stagnant water (Figure 4B). The biofilm had a much lower diversity than all of the water  
322 samples (e.g., between 90 and 98% fewer OTUs than cold water, (Figure 4B)).

323 Microbiome composition also varied between three types of water samples and the biofilm  
324 (Figure 4C). Even at the phylum and class level, each sample type clearly harbored different types  
325 of bacteria (Figure 7). Three taxa (Betaproteobacteria, Alphaproteobacteria, and Bacteroidetes)  
326 that accounted for 91% of biofilm sequences, only accounted for 31% of the cold influent water mi-  
327 crobiome. Seven taxa (OD1, OP3, Deltaproteobacteria, Planctomycetes, Chloroflexi, Chlamydiae,  
328 and Nitrospirae) that accounted for 51% of cold-water microbiomes only accounted for 2% of bio-  
329 film microbiomes. Another four taxa (Acidobacteria, Actinobacteria, Deinococcus-Thermus, and  
330 TM7) were enriched in warm water (16%). Core OTUs were also distinct between the sample  
331 types, with only 6 OTUs shared by all samples (Figure 7). Sample type, however, only explained  
332 18% of microbiome variation (Adonis,  $p < 0.001$ ). Household explained 41% of microbiome variation  
333 (Adonis,  $p < 0.001$ , Figure S7).

334

335

### 336 **3.5. Specific organisms in shower hose biofilms may present risk**

#### 337 *Global survey*

Two opportunistic pathogens, *Mycobacterium avium* and *Legionella pneumophila*, and two amoebae, *Acanthamoeba polyphaga* and *Veramoeba vermiformis*, were detected via qPCR in 11, 21, 4, and 21 hoses respectively. The maximum concentrations on these hoses was  $6.6 \times 10^7$  gene copies (gc)/cm<sup>2</sup>,  $6.6 \times 10^5$  gc/cm<sup>2</sup>,  $2.5 \times 10^4$  gc/cm<sup>2</sup>, and  $2.8 \times 10^5$  gc/cm<sup>2</sup>, respectively. *L. pneumophila* co-occurred with a potential amoebae host (*V. vermiformis*) in six hoses, and it co-occurred with *M. avium* in two hoses.

Several genera that might include opportunistic pathogens were also detected in sequencing data. *Legionella*, *Mycobacterium*, and *Pseudomonas* were detected in 43, 64, and 34 hoses respectively. While 31 of 34 *Pseudomonas* positive hoses were also positive for *Mycobacterium*, only 14 were also positive for *Legionella*. 37 hoses were positive for both *Legionella* and *Mycobacterium*, and 13 hoses were positive for all three genera of interest. Some countries had higher occurrences: *Legionella* was detected in 9 of 10 hoses in Denmark; *Mycobacterium* was detected in all hoses in Belgium, Germany, Latvia, South Africa, Serbia, UK, and US; and *Pseudomonas* was detected in 8 of 9 hoses in UK.

Absolute abundance of these genera was calculated by multiplying the relative abundance with TCC (as done previously (Props et al., 2016)), yielding in some cases substantial maximum concentrations of these genera: *Legionella* ( $7.8 \times 10^5$  cells/cm<sup>2</sup>, Belgium), *Mycobacterium* ( $4.1 \times 10^7$  cells/cm<sup>2</sup>, Belgium), and *Pseudomonas* ( $3.1 \times 10^6$  cells/cm<sup>2</sup>, Switzerland). One hose from the UK had the highest relative abundance of *Mycobacterium* (35%), but because biofilm TCC was low, it only had the 17<sup>th</sup> highest concentration by calculated absolute abundance ( $5.9 \times 10^5$  cells/cm<sup>2</sup>). A hose from Belgium only had 16% *Mycobacterium*, but had 2-orders of magnitude higher absolute abundance ( $2.1 \times 10^7$  cells/cm<sup>2</sup>).

When *Legionella* was detected, biofilm TCC was significantly higher (Kruskal-Wallis,  $p < 0.001$ ) (Figure 8A), indicating that *Legionella* is more likely to be found with a higher biofilm TCC. There was also a significant positive correlation between *Legionella* relative abundance and biofilm TCC (Spearman's  $\rho = 0.36$ ,  $p < 0.001$ ). However, for *Mycobacterium* and *Pseudomonas*, detection was associated with reduced biofilm TCC (Kruskal-Wallis,  $p = 0.06$ , and  $< 0.001$  respectively) (Figure 8D,E). Biofilm TCC was not a predictor for detection for *L. pneumophila*, *M. avium*, or *V.*

366 *vermiformis* (Figure 8B,C,F), but results may have been influenced by the relatively few detects  
367 with qPCR.

368

369 *Follow-up study*

370 Surprisingly, cold influent water was frequently positive for low concentrations of *Legionella* (12/12),  
371 *Mycobacterium* (12/12), and *Pseudomonas* (11/12). Biofilms were more sporadically positive for  
372 these genera (*Legionella* 6/15, *Mycobacterium* 7/15, and *Pseudomonas* 13/15). However, these  
373 three genera had the highest calculated absolute abundances in stagnant water samples ( $6.8 \times 10^3$ ,  
374  $6.9 \times 10^3$  and  $6.2 \times 10^2$  cells/mL respectively for *Legionella*, *Mycobacterium*, and *Pseudomonas*),  
375 and these maximums corresponded with biofilm detection of the genera.

## 376 4. Discussion

377

378

379 The shower hoses used in this study all originated from real homes, and were thus subject to many  
380 unknown and uncontrolled factors contributing to biofilm formation and microbiome composition.  
381 These selective forces include, amongst others, material properties (hose composition and age),  
382 user-specific properties (frequency of use, temperature), biofilm properties (e.g., biofilm cell con-  
383 centration, iron providing oxidizing agents, copper providing toxicity), and selective forces on the  
384 water earlier in distribution (e.g., chlorine, water microbiome composition and abundance). Even  
385 with the more controlled follow-up study, multiple factors simultaneously contribute to observed  
386 results, and it is challenging to disentangle selective forces from one another. However, some  
387 trends and specific interesting examples were observed and are discussed below.

388

389

### 390 4.1 Shower hoses harbor many bacteria.

391 The characteristics common to nearly all shower hoses, including mild-to-warm temperatures  
392 (ranging from room temperature to maximum shower temperatures of c.a. 43 °C), long stagnation,  
393 and nutrient migration from flexible plastic materials (Proctor et al., 2016) likely contribute to high  
394 biofilm TCC. Although variable, biofilm TCC tended to be higher on shower hoses (average  $10^7$   
395 cells/cm<sup>2</sup>) than on hard pipe materials typically used in building plumbing (copper and PEX pipes,  
396  $\sim 5.7 \times 10^5$  cells/cm<sup>2</sup>, (Inkinen et al., 2014)) or plumbing simulators (Pe-Xc,  $\sim 2 \times 10^6$  cells/cm<sup>2</sup> (Proc-  
397 tor et al., 2016)). However, comparison of biofilm data between different studies is influenced by  
398 differences in sampling and analysis methods (Wang et al., 2017). In addition to bacteria, biofilms  
399 also harbor non-bacterial cells (e.g., amoebae) and non-cellular organic and inorganic matter (e.g.,  
400 EPS, metals), such that thick, complex environments emerge (Figure 1).

401

402

### 403 4.2 Disinfectant use during water treatment affects biofilms in buildings.

404 The use of disinfectants during water treatment can lower both the concentration (Prest et al., 2016)  
405 and diversity (Bautista-de los Santos et al., 2016) of bacteria in water. It is unclear, however, how  
406 this would affect biofilms at the distal end of building plumbing systems, where disinfectant residual  
407 is often lost (Lipphaus et al., 2014). In the present study, disinfected drinking water resulted in bio-  
408 films with lower TCC, decreased diversity, and increased relative abundance of the ten most  
409 abundant OTUs (Figure 2, Figure 6). It could thus be argued that either (1) residual disinfectant  
410 acts as a selective force directly on biofilms, or (2) disinfectant acts as an indirect selective force,  
411 reducing the diversity in the source water which in turn reduces the diversity in the seed available  
412 for biofilm colonization. With purely stochastic immigration to the biofilm, diversity in the meta-  
413 community (in this case, the influent water) would be reflected in the biofilm microbiome (Battin et  
414 al., 2007). Since several countries with relatively high disinfectant residuals clustered closer to-  
415 gether (Figure 6B), prolonged exposure to disinfectant may be more selective (e.g., a surviving 're-  
416 sistome' (Jia et al., 2015)).

417

418

#### 419 **4.3 Shower hose material quality is a problematic unknown.**

420 The amount and type of carbon leaching from plastic pipe materials is critical for determining bio-  
421 film concentration and composition (Proctor et al., 2016; Wen et al., 2015). Composition of hoses  
422 in the global survey was clearly variable (Figure 5), but unknown, and therefore no reasonable  
423 conclusions can be inferred. In contrast, identical PVC-P hoses were used in the follow-up study.  
424 This specific material encouraged biofilm proliferation more than other materials (up to  $2 \times 10^8$   
425 cells/cm<sup>2</sup> over 8 months) (Proctor et al., 2016) and this was linked to carbon leaching: under opti-  
426 mal conditions, PVC-P hoses leached large amounts of biodegradable carbon ( $9.7 \mu\text{g-C/cm}^2$  sup-  
427 porting  $4.8 \times 10^7$  cells/cm<sup>2</sup> in 7 days). The generally higher biofilm concentrations during the follow-  
428 up study (Figure 3A) may thus be linked to the common material. However, leaching behavior is  
429 variable, with an initial peak diminishing over time (Bucheli-Witschel et al., 2012). Thus, the com-  
430 mon young age of the materials in the follow-up study can also contribute to the high, narrow range  
431 of biofilm TCC (discussed further below).

432

433

#### 434 **4.4 Are new shower hoses worse than old ones?**

435 Within the same household, accumulation of metals in biofilms seemed to increase with age  
436 (Figure 3B-D), but this was difficult to confirm, since age of hoses during the survey was usually  
437 unknown beyond a minimum (e.g., time of move-in). Furthermore, while passive metal accumula-  
438 tion likely increases with time, primary biofilm development may involve active accumulation (e.g.,  
439 iron as trace nutrient, calcium as biofilm structural support).

440 Biofilm TCC is dependent on availability of nutrients for growth. Carbon leaching diminishes  
441 over time (Bucheli-Witschel et al., 2012), resulting in a nutrient-rich environment in new hoses, and  
442 a nutrient-poor environment in old hoses. Other nutrients necessary for growth (e.g., N and P) are  
443 typically present in oligotrophic concentrations in drinking water, and thus enter the shower hose  
444 based on use frequency over time. Here, in cases where hose age could be estimated (ranging 3  
445 months to 30 years), there was no correlation with biofilm TCC (data not shown). The hose with  
446 the most biofilm ( $5.8 \times 10^8$  cells/cm<sup>2</sup>) was only installed for one year, and TCC was often higher in  
447 the younger biofilms during the follow-up study (Figure 3A). This lack of correlation between age  
448 and biofilm concentration is not unprecedented (Wingender and Flemming, 2004). It is likely that a  
449 growth plateau is reached quickly with shower hose biofilms due to the fast leaching of considera-  
450 ble amounts of carbon (Proctor et al., 2016; Wen et al., 2015).

451

#### 452 **4.5 Metals from the building accumulate at the shower hose.**

453 The biofilm concentrations of metals were similar in hoses from the same buildings (Figure 3B-D,  
454 with hoses 3-6 from the same building). While some (calcium and magnesium) are presumably re-  
455 lated to local influent water quality (e.g., hardness), it is likely that others (lead, copper, iron) at  
456 least partially originate from other pipes and fittings in the building (Gonzalez et al., 2013). While  
457 the latter group can also accumulate from trace concentrations in the water, pipe corrosion is a  
458 well-known problem and information about building plumbing materials is generally not well docu-  
459 mented or known to consumers. Risks from lead in pipes and brass fittings are known, but regula-  
460 tions attempting to limit these lead risks are not applied retroactively to replace building plumbing  
461 components, and building plumbing still contributes significantly to lead in water (Haider et al.,

2002). Metals can also leach from plastic pipes (e.g., lead is used as a stabilizer for certain PVC plastics (Zhang and Lin, 2015)).

**4.6 Specific use patterns affect biofilms.**

Frequency of use has previously been shown to affect tap-specific colonization of opportunistic pathogens (Rudi et al., 2009) and microbiomes in a model hot water distribution system (Ji et al., 2017). Here, the more frequently used hoses in the follow-up study had higher biofilm TCC. With their stagnation patterns, shower hoses could be thought of as batch reactors. Nutrient loads (other than carbon provided by the hose) would then be determined by frequency of use and concentration in water (Table S7). Interestingly, the three hoses with the highest biofilm concentration were used frequently during the workweek, but only rarely on weekends. Thus, nutrient loads were high and stagnation was irregular. Since frequent use both supplies nutrients and potentially disrupts biofilms by changing hydraulics and delivering stress elements (e.g., chlorine, heat), the complexities of irregular use are interesting for future research. Here, in the follow-up study, residual chlorine was not present, but the hose with the lowest biofilm concentration was typically only used for high temperature cleaning of the tub (70 °C), essentially providing a heat-shock to the biofilm.

**4.7 The microbiome of a building plumbing system is explained by drift and selection.**

In the follow-up study, household explained much of the microbiome variation (i.e., water and biofilm samples were similar within a household (Figure S7)). This might be expected as the microbiology of the entire building's plumbing system is inevitably dictated by drift from the cold distribution system water. However, the differences between various water sample types and biofilms were evident across households in terms of TCC, diversity, and microbiome composition (Figure 4, Figure 7). If one considers the path water takes through the home as a microbiome continuum (Proctor and Hammes, 2015), then one can explain these changes through the concepts of drift and selection.

Warm water was less diverse and had lower TCC than cold water. Together with the enrichment of *Deinococcus-Thermus* (Figure 7), which consists almost entirely of extremophiles, this

indicates water heater stress as a strong selective force. Negative relationships between diversity and temperature were previously observed in building plumbing simulators (Ji et al., 2017). However, cold and warm water samples still shared 23 core OTUs. While cold water is the source and metacommunity for warm water, warm water was collected only at moderate temperatures (35-40 °C, i.e., mixing of cold and hot water), and thus there was also direct drift from cold water.

Stagnant water was similar in terms of water TCC, diversity and composition, to its source and metacommunity, the warm water. However, drift from the biofilm phase (i.e., biofilm detachment during stagnation) increased TCC and lowered diversity as the microbiome converged with biofilm microbiomes (Figure 4, Figure 7). Phylogenetic convergence between stagnant water and biofilm samples has been noted previously (Inkinen et al., 2014), and might be tied to the duration of stagnation (Ji et al., 2017).

Biofilms all had low diversity compared to water, and were also different from each other, with only 8 OTUs shared by all biofilms (Figure 7). Organic carbon leaching from the hoses likely provide a positive selective force, and PVC-P is specifically known to select for a low diversity in biofilms compared to other materials (Proctor et al., 2016). Growth in biofilms might be a selective force in and of itself, as reduced diversity in biofilms compared to water has been noted previously in building plumbing (Inkinen et al., 2016) and a chlorinated distribution system (Douterelo et al., 2017), but not a non-chlorinated distribution system (Liu et al., 2014).

#### **4.8 Biofilm composition is driven by multiple factors that co-vary with biofilm TCC.**

Biofilm TCC seemed to drive some components of microbiome composition (Figure S6). While some bacteria may be affected by biofilm thickness itself (e.g., more diverse niche space in thick biofilms with localized gradients (Flemming et al., 2016)), multiple selective forces can affect biofilm TCC and composition simultaneously. For example, *Sphingomonas* had a negative correlation with biofilm TCC. It is resistant to chlorine (Jia et al., 2015), and thus disinfectant may be acting as a selective force that dictates both *Sphingomonas* abundance and biofilm TCC. *Limnobacter* also had a negative correlation with biofilm TCC, but is susceptible to chlorine (Jia et al., 2015). Since it can degrade phenol (Vedler et al., 2013) which leaches from some plastic pipes (Skjevrak et al., 2003), this suggests the positive selective force of plastic pipes when growth conditions are

520 otherwise non-ideal. *Meiothermus* is an interesting example of a genus positively correlating with  
521 biofilm TCC. It is slightly thermophilic, has a reddish color, causes biofouling, and has been isolated  
522 in geothermal springs with iron oxidizing activity (Kolari et al., 2003; Urbietta et al., 2015). Here,  
523 *Meiothermus* accounted for 12% of two notably red biofilms from South Africa (Figure S4B) with  
524 high iron concentrations (0.09 and 5  $\mu\text{g}/\text{cm}^2$ ), suggesting that temperature, iron oxidation, and high  
525 biofilm cell concentrations were all contributing selective factors for the presence of this organism.

526

527

#### 528 **4.9 These biofilms hold some risk.**

529 Toxic metals and potential opportunistic pathogens were found in shower hose biofilms. Metals  
530 exposure is through consumption (drinking), and thus exposure from showers is low, but not obsolete  
531 (e.g., brushing teeth in shower). Additionally, sporadic detection of particulate lead is not uncommon  
532 in building water (Deshommes et al., 2010), and could result from biofilm detachment (i.e.,  
533 dislodged during hose manipulation). However, concentrations of metals tended to be lower than  
534 detected in distributions systems (Lehtola et al., 2004), and thus these biofilms are unlikely to be a  
535 major contributor to risk.

536 All of the reported opportunistic pathogens present a risk specifically for showering, with  
537 routes of infection either through inhalation or wound exposure (Falkinham et al., 2015). It is difficult  
538 to interpret the clinical relevance of the concentrations, since little is understood about (1) how  
539 these biofilms detach into the water phase and (2) what dose is required for infection. Moreover,  
540 for reported genera derived from sequencing data, species and strain level definition was not possible,  
541 and thus these do not necessarily present risks.

542 A common argument for biofilm control is that reducing total biofilm also reduces risk of exposure  
543 to opportunistic pathogens. However, biofilm concentration likely co-varies with or affects  
544 biofilm microbiome composition (as discussed above). *L. pneumophila* is thought to proliferate in  
545 thick biofilms, and recently, a threshold biofilm concentration for *L. pneumophila* growth was proposed  
546 at  $10^6$  cells/ $\text{cm}^2$  (van der Kooij et al., 2017). Most of our biofilm concentrations were above  
547 this threshold, and *L. pneumophila* was even detected below the proposed threshold. Although a  
548 positive relationship between TCC and *L. pneumophila* was not found (Figure 8B), *Legionella* data

549 correlated positively with TCC (Figure 8A), indicating that decreasing biofilm concentration can  
550 protect against this particular risk.

551 Other opportunistic pathogens may have a negative correlation with biofilm TCC (e.g., *My-*  
552 *cobacterium* and *P. aeruginosa* (Meier and Bendinger, 2016; Proctor et al., 2016)), or with other  
553 opportunistic pathogens (e.g., *Legionella* with *P. aeruginosa* (Leoni et al., 2001)). In this study, *My-*  
554 *cobacterium* and *Pseudomonas* correlated negatively with biofilm TCC (Figure 8D,E), and co-  
555 occurred with each other more than with *Legionella*. In a study of shower hose biofilms exposed to  
556 chlorine residual in the US, *Mycobacterium* dominated (Soto-Giron et al., 2016), and thus disin-  
557 fectant resistance may play a role in these correlations. With biofilm management strategies, it is  
558 necessary to weigh the risks of many potential pathogens at the same time. Reducing biofilm con-  
559 centration (i.e. with disinfectant) may reduce risk from some potential pathogens while increasing  
560 risk from others.

561 Stagnant water, which is influenced by detached biofilm, had the highest potential risk of  
562 the three water types tested. However, biofilm was not the only source for these genera, since the  
563 cold water was also frequently positive for potential opportunistic pathogens. In a previous study of  
564 showers in the UK, only 40% of stagnant water samples positive for *Legionella* were also positive  
565 in shower hose biofilm swabs (Collins et al., 2017), further indicating that other sources (e.g., cold  
566 water distribution systems) are likely important in shower exposure.

567

#### 568 **4.10 Shower hose biofilms represent a meaningful sampling point.**

569 Water samples are the easiest type of sample to collect from household plumbing (Wang et al.,  
570 2017). However, < 2% of bacterial biomass of distribution systems is present in the planktonic  
571 phase, with most biomass in biofilms and loosely deposited material (Liu et al., 2014). Our data, in  
572 combination with the little available evidence on real shower environments (Collins et al., 2017;  
573 Feazel et al., 2009; Soto-Giron et al., 2016), suggest that opportunistic pathogens are harbored in  
574 shower-related biofilms. Shower hoses offer one of the most convenient sampling locations for bio-  
575 film monitoring in building plumbing. Most components of building plumbing are not removable and  
576 are difficult to sample (e.g., manual swabbing), thus limiting reproducibility and representativeness.  
577 In contrast, shower hoses can be removed and replaced, offering large sections (350 – 800 cm<sup>2</sup>) of

578 easily accessible biofilms. Such a biofilm-based approach will complement the wealth of  
579 knowledge on building plumbing water, and is necessary for a more complete understanding of  
580 ecology and risk in household plumbing.

581

582 **4.11 Shower hoses can be managed better.**

583 Shower hoses offer a critical area for improvement of building plumbing management strategies.  
584 These hoses typically fall outside potable water material regulations, but clearly microbiology  
585 needs to be considered. As it stands, consumers assume all risks and receive little guidance about  
586 this portion of easily controlled building plumbing. Moreover, shower hoses are not necessary for  
587 all homes, and could be replaced with safer hard pipe material. Hospitals and old-age homes are  
588 generally better informed about limiting risk from shower environments. Programs for quarterly  
589 shower hose and head replacement aimed at reducing risk are available for such facilities (Table  
590 S8). Given the tenuous relationship between age and biofilm TCC in this study, a more aggressive  
591 strategy, like single-use shower hoses (Table S8), may be necessary for high-risk patients.

592

## 593 5. Conclusions

594

595 In a global survey and follow-up study, shower hoses from all over the world were characterized in  
596 detail in terms of biofilm concentrations, metals accumulation, and microbiome composition. While  
597 many factors were uncontrolled, some trends were apparent:

- 598 • When disinfectants were used, cell concentrations and diversity were reduced.
- 599 • Young shower hose age and bad material quality likely contributed to high cell concentra-  
600 tions.
- 601 • Frequent, but irregular use likely contributed to high cell concentrations.
- 602 • The microbiome along the path of water delivery in a building could be explained by drift  
603 and selection.
- 604 • Biofilm microbiome composition was shaped by several factors, some of which co-varied  
605 with cell concentration.
- 606 • Potential opportunistic pathogens were detected, with either a positive (*Legionella*) or nega-  
607 tive (*Mycobacterium*, *Pseudomonas*) correlation with cell concentration.
- 608 • Metals accumulated over time in biofilms, likely originating from other building plumbing  
609 components.

610 While more controlled studies can further explore these trends, it was abundantly clear that shower  
611 hoses offer a perfect biofilm sampling point to complement building water studies, and that shower  
612 hoses should be considered in building plumbing risk management strategies.

613

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627

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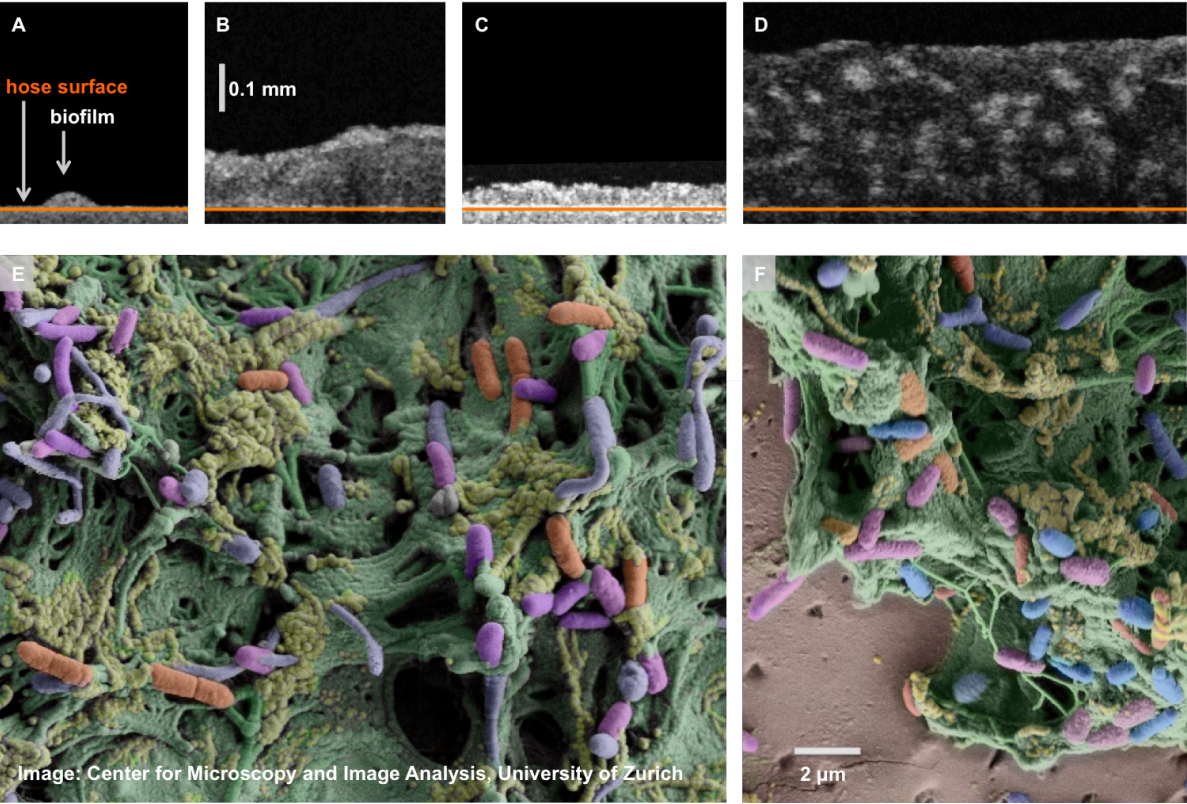
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794 **Figures**

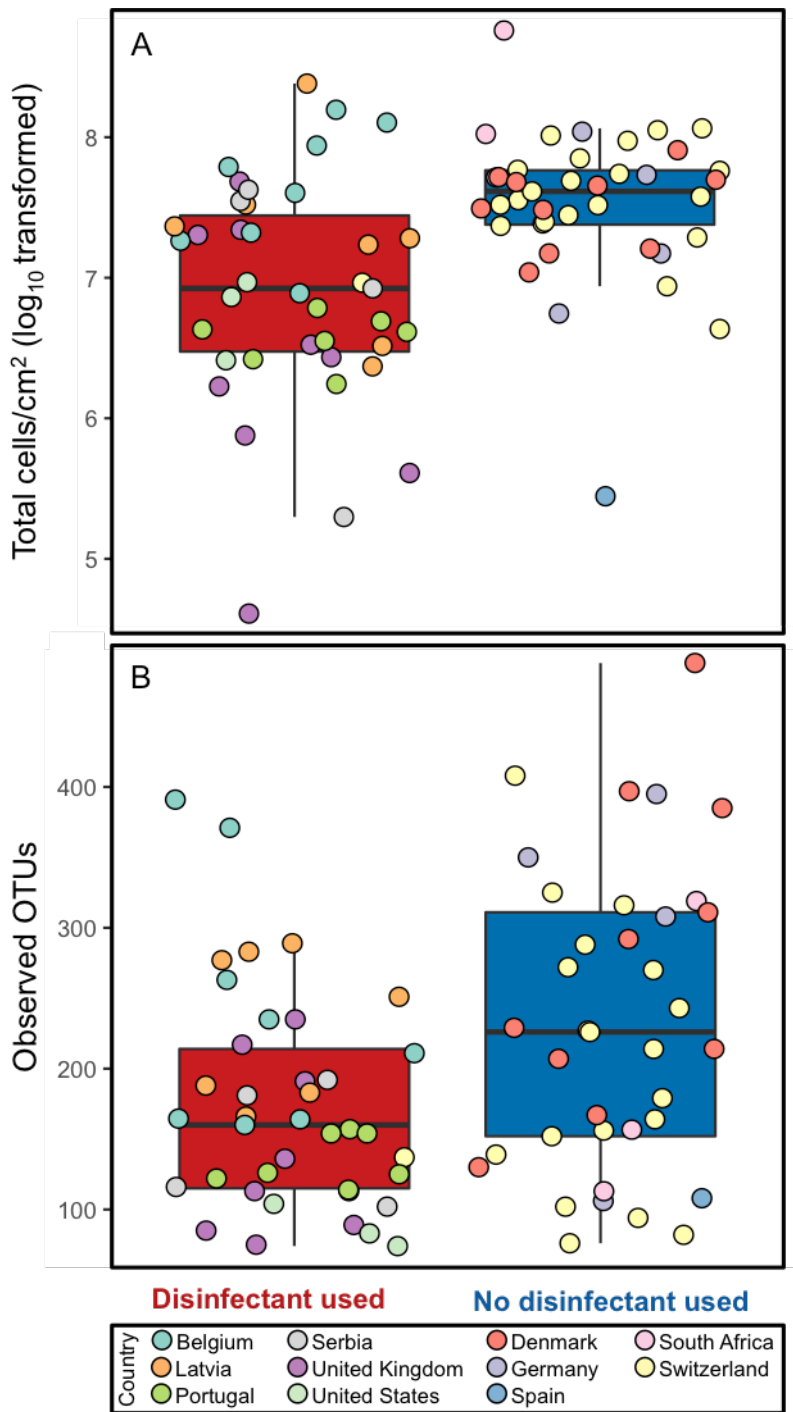
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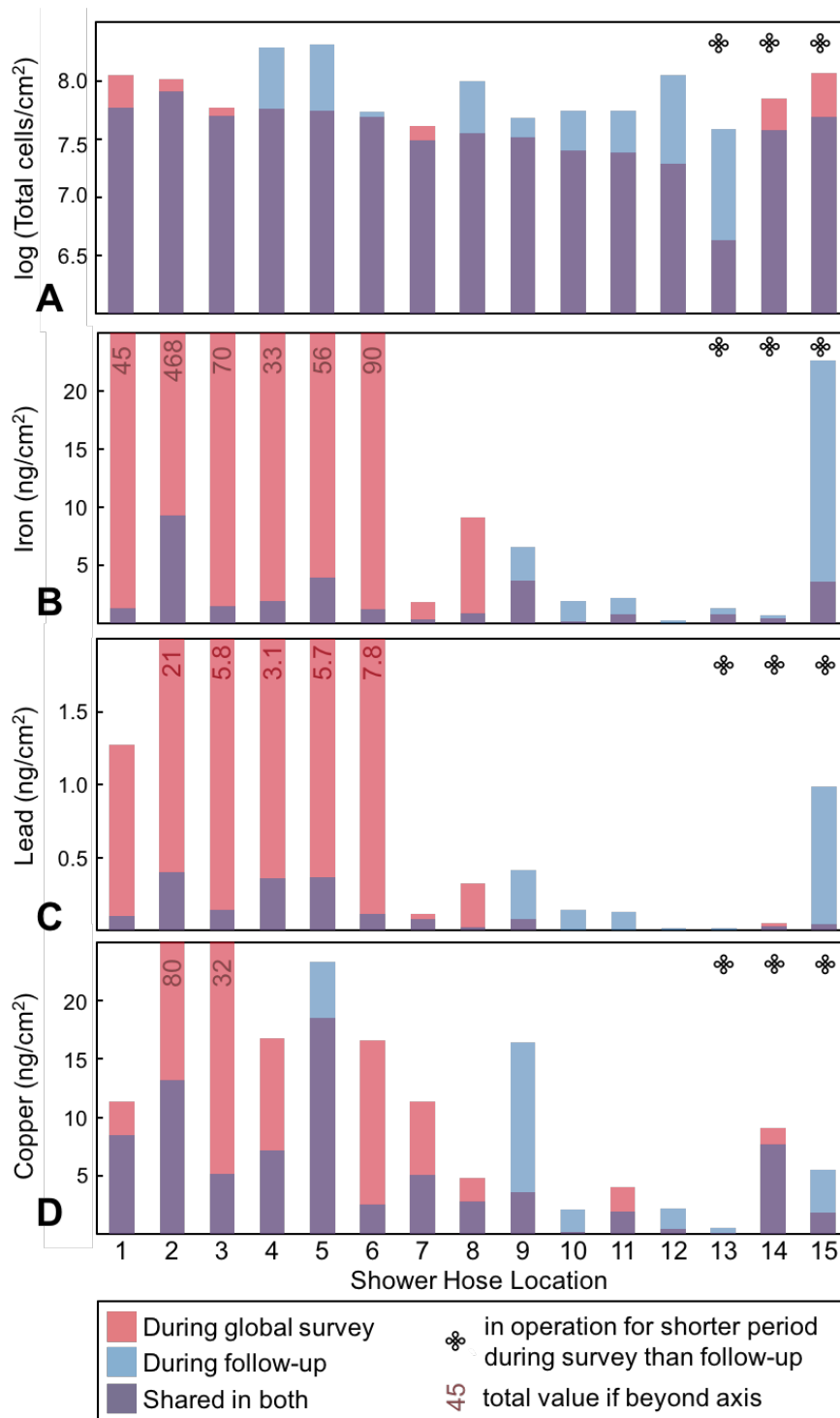
797 Figure 1: Imaging of shower hose biofilms. Top: Optical coherence tomography of biofilms with  
798 various thicknesses from (A) the United States, (B) Switzerland, (C) Denmark, and (D) Germany.  
799 All OCT images are on the same scale. Bottom: Scanning electron microscopy of thick biofilms  
800 from a Swiss shower hose with EPS matrix (green), and cellular (orange, purple, blue) components.  
801 Images (E) and (F) are from the same hose. In (F) hose surface (dusty rose, left bottom) together  
802 with biofilm matrix (right top). SEM images were colored artificially to highlight various biofilm ele-  
803 ments. Original images can be found in Figure S2.

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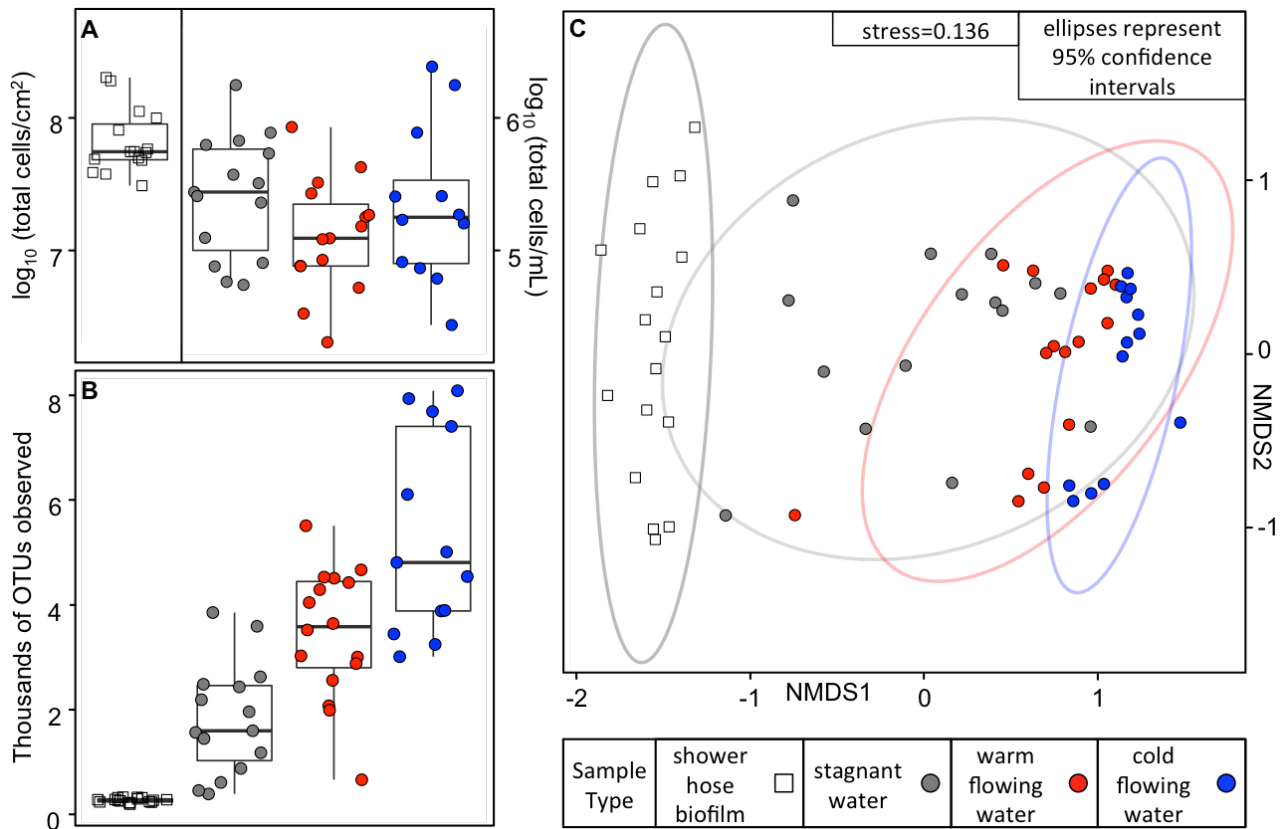
805

806 Figure 2: Biofilm total cell concentration (Biofilm TCC) (A) and number of observed operational  
 807 taxonomical units (OTUs) (B) of shower hoses, grouped by use of disinfectant (red) or lack of disin-  
 808 fectant (blue). Individual sample values are shown as dots, colored by country of sample origin.  
 809 Box and whisker plots represent median and quartile values for each measure.



810

811 Figure 3: Paired comparison of total cell counts (A), iron (B), lead (C), and copper (D) between  
 812 global survey (red) and controlled experiment (blue), with overlapping concentrations in purple.  
 813 Shower hoses are arranged in order of descending cell concentration in global survey, with the ex-  
 814 ception of three hoses on the right. These hoses were known to be in operation <1.5 years prior to  
 815 the global survey, i.e., less than in the controlled experiment, and are indicated with a symbol.  
 816 Concentrations beyond the reasonable axes are labeled with real values due to wide variations.



817

818 Figure 4: (A) Total cell concentration (TCC) (B) Diversity (observed OTUs) and (C) NMDS repre-  
 819 senting Bray-Curtis dissimilarities for biofilm and each group of collected water in the follow-up  
 820 study. For TCC, biofilm TCC is on the left axis and water TCC is on the right axis. Stagnant water  
 821 was collected from the shower hose after a minimum 8 hour stagnation. Warm water was collected  
 822 at “typical shower temperature” after 5 minutes of flow. Cold water was collected from the kitchen  
 823 tap after 5 minutes of flow. For total cells, N=15 except for cold water, where N=12, because sev-  
 824 eral hoses shared nearest cold-water sources. For sequencing data, N also includes triplicate  
 825 measurements for 1 biofilm, and duplicate samples for one warm water and one cold water.

826



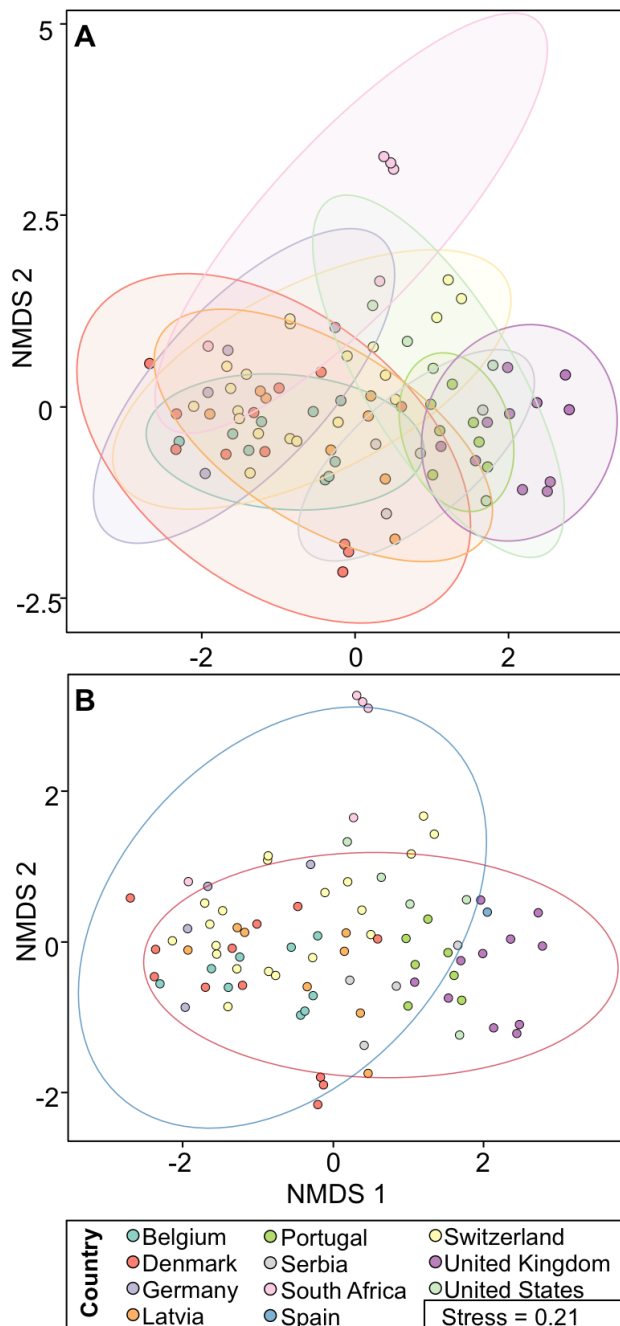
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830

Figure 5: Photos of shower hoses cut open to reveal biofilms. The hoses had diverse diameters and materials, and biofilms on the hoses had different color, coverage, and texture.



831  
 832 Figure 6: Non-metric multi-dimensional scaling (NMDS) representing Bray-Curtis dissimilarities of  
 833 biofilm microbiomes, with each point representing a microbiome measurement of a shower hose,  
 834 colored by country of sample origin. Each panel is the same NMDS superimposed with statistical  
 835 analyses. Colored ellipses representing 95% confidence intervals for (A) country, and (B) use [red]  
 836 or non-use [blue] of disinfectant.

837

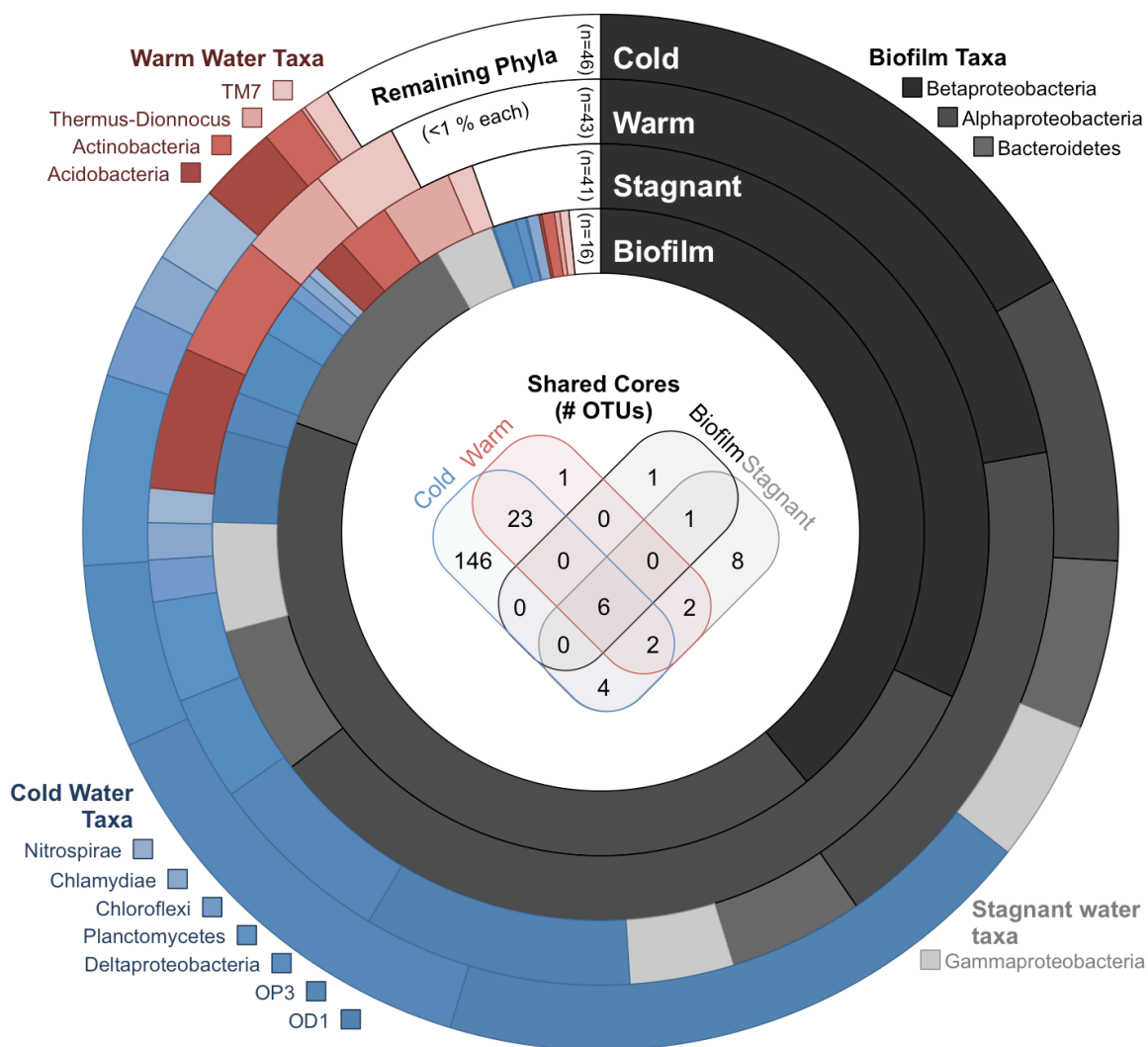


Figure 7: Core OTUs (center) and average relative abundance (outer rings) of Phyla (and Proteobacteria-classes) by sample type (biofilm, stagnant water, warm water, and cold water) in the follow-up study. The number of core OTUs was calculated as the OTUs present in all locations of each type or overlapping type (e.g., 6 OTUs were common to all locations in all sample-types). Each ring represents the average of 15 samples, or for cold water 12 samples due to repeat samples from single households. Phyla are colored by the phase in which they represent the highest proportion - biofilm (black), stagnant (gray), cold (blue), and warm (red). These taxa are also labeled in the outer corners.

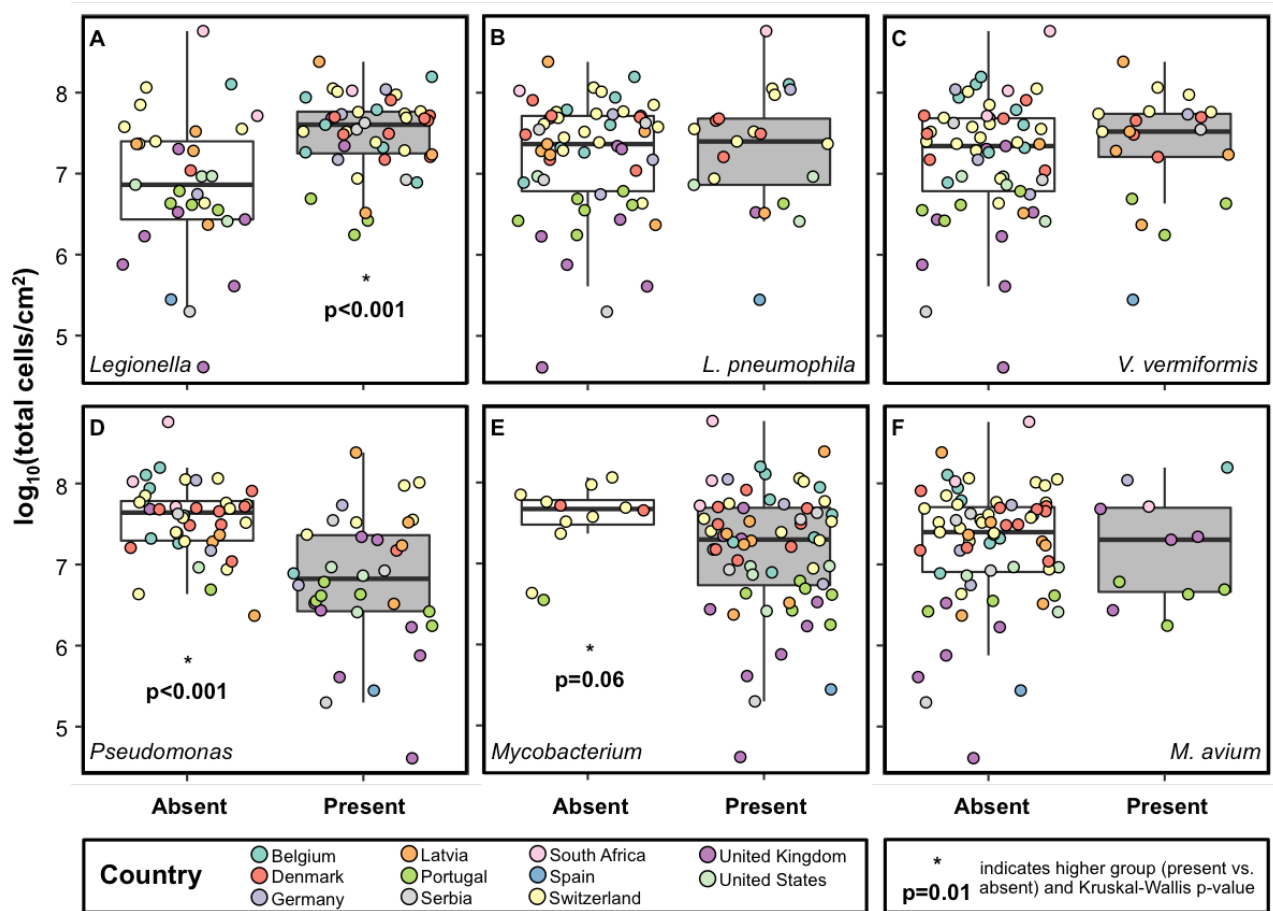


Figure 8: Total cell counts ( $\log_{10}$  transformed) grouped by absence (white, left) or presence (shaded, right) of potential opportunistic pathogens, as measured by detection of genus with amplicon sequencing (A,D,E), or detection of a specific opportunistic pathogen with qPCR (B, C, F). Three opportunistic pathogens containing genera– *Legionella* (A), *Pseudomonas* (D), and *Mycobacterium* (E), two opportunistic pathogens – *L. pneumophila* (B), and *M. avium* (F), and one amoeba known to host *L. pneumophila* – *V. vermiformis* (C) were analyzed. Individual sample values are shown as dots, colored by country of sample origin. Box and whisker plots represent median and quartile values for each measure. (\*) and p-values are displayed under box plots of groups with higher average cell concentration (Kruskal-Wallis).

860     Supplementary information

861

862

863     **Biofilms in Shower Hoses**

864

865     Table S1: News items concerning *Legionella pneumophila*, all published in the first half of 2017.

866

867     Table S2: Elemental Analysis Method Details

868

869     Table S3: qPCR method details

870

871     Table S4: Details for Illumina sequencing

872

873     Table S5: Details for Illumina sequencing data processing

874

875     Figure S1: Optical Coherence Tomography (OCT) images of biofilms from 2 hoses from Belgium  
876     (Hose 1) and Switzerland (Hose 2). All are on an equal scale. A and B are from opposite ends of  
877     the same hose (Hose 1). C, E, and F are from one 5 cm piece on one end of a hose, while D is  
878     from the opposite end of the same hose (Hose 2).

879

880     Figure S2: Original black and white images (zoomed out) of biofilms on shower hoses, correspond-  
881     ing to Figure 1E (top) and 1E (bottom). In Figure 1, only particular sections were shown in order to  
882     focus attention. Color was also added artificially to highlight key biofilm elements.

883

884     Figure S3: Correlation between various measures of biofilm concentration. All scatter-plot points  
885     represent values for individual shower hose samples, colored by country of sample origin. Some  
886     factors (Total cells, intact cells, ATP, and 16S qPCR gc) are transformed as indicated due to the  
887     wide spread of values.

888

889 Table S6: Water quality and use patterns for 15 samples in follow-up study

890

891 Table S7: Correlation values between use patterns and calculated weekly doses and biofilm con-  
892 centration. Calculated weekly loads were derived by multiplying the number of uses per week by  
893 water quality concentrations (nitrogen, phosphorous, magnesium, calcium or total cells in the cold  
894 flowing water, (Table S6)) and the volume inside a shower hose. For all, N=15 for the 15 biofilm  
895 samples. Uses per week alone (e.g., without multiplying by a water quality parameter) had the  
896 strongest correlation with biofilm TCC, while other water quality measurements had only moderate  
897 correlations. That is to say, no single nutrient or load was controlling the system with stronger pre-  
898 dictive power than frequency of alone. It could be either a combination of nutrients/cells or some  
899 other aspect of frequency of use that controls the biofilm TCC.

900

901 Figure S4: Photo from outside of a shower hose, with metal partially removed (left), and the inside  
902 of a shower hose with a particularly red/orange biofilm (right). On the left, biofilm formed yellow  
903 spots, resembling bacterial colonies on agar. On the right, the biofilm had a rough texture and  
904 deep red/brown/orange color.

905

906 Figure S5: Concentrations of metals (lead, iron, copper, calcium, and magnesium) and biofilm total  
907 cell concentration (biofilm TCC), with their correlations. Points represent individual hoses, colored  
908 by country. None of the metals correlated well with biofilm TCC. There were many non-detects for  
909 lead, iron and copper, but when present, there tended to be positive relationships between these  
910 metals – i.e., these metals precipitated together in biofilms. Calcium and magnesium were present  
911 in all biofilms, and had a positive relationship with each other. While the first three metals likely  
912 originate from up-stream pipes in the distribution system, calcium and magnesium likely originate  
913 from hardness in the water.

914

915 Figure S6: Correlational analyses between relative abundance of genera (sum of all OTUs identi-  
916 fied within that genera) and biofilm total cell concentration (TCC) (log10 transformed). Points rep-  
917 resent individual hoses, colored by country. Spearman rank correlations noted for correlations with

918 biofilm TCC, with (\*) indicating  $p < 0.001$ . These are the three most significant positive and negative  
919 correlations from the top 10 most abundant genera, but these do not necessarily represent the  
920 strongest correlations. For example, a significant positive correlation was found between *Legionel-*  
921 *la* and biofilm TCC (Spearman's  $\rho = 0.36$ ,  $p < 0.001$ ). This analysis focused on the top 10 most  
922 abundant genera because more frequent non-detects with less abundant OTUs likely affected re-  
923 sults strongly. Notably, two genera from within the same family (*Sphingomonas* and *Sphingobium*)  
924 were both amongst the top 10 most abundant genera, but had opposite correlations.

925

926 Figure S7: NMDS representing Bray-Curtis dissimilarities between samples in follow-up experi-  
927 ment. Samples consist of 15 biofilms, and matching stagnant water, warm running water, and cold  
928 running water. Ellipses represent 95% confidence intervals for each household. These ellipses are  
929 generally narrow across an NMDS2 range, but extend widely through NMDS1 to capture all sam-  
930 ple types. Household explained 41% of microbiome variation (Adonis). Thus, while there are simi-  
931 larities between sample types (symbol type and color), there are some strong similarities in a  
932 household. This could, for example, be due to drift into the household (cold flowing water) selecting  
933 downstream microbiomes.

934

935 Table S8: Products available for addressing biofilms in shower hoses, including quarterly replace-  
936 ment systems and single-use shower hoses.