Microbiology of Household Plumbing
Causes and Consequences

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presented by

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Summary

Drinking water, even when distributed safely, deteriorates in quality after entering the home. The microbiological quality of this building plumbing water is of particular interest because opportunistic pathogens (e.g. *Legionella pneumophila*) are known to proliferate there. Beyond the potential threat to health, microbial growth results in customer complaints about taste, odor, color, turbidity, slime formation, and filter clogging in building plumbing.

While water distributors strive to deliver a product that remains stable in the home, they have minimal influence on the design, maintenance, and operation of building plumbing systems. At the property line, legal responsibility for the water shifts away from the water distributor to the building owner. On top of this, monitoring water stability in the home requires invasive sampling and has potential privacy implications. All of these factors contribute to a poor understanding of the microbiology of water in building plumbing.

Many factors give building plumbing a unique growth environment. Pipe materials are varied and sometimes unregulated. In the building multiple materials are found within inches of each other. With high surface area to volume ratios, and more frequent replacement (e.g. with bathroom remodels), these materials have a considerably higher impact in buildings than in the distribution network. Temperature is high (e.g. hot and mixed water distribution) compared to the distribution network, creating favorable growth conditions. Building distal-ends are also only flowing intermittently (i.e. long stagnation times), allowing for biofilm and water phase interactions. While all of these factors are known to contribute to growth, their impact on bacterial concentration and composition is not completely understood. Moreover, each building is different with respect to design, operation, and location, complicating synthesis of various building plumbing studies.
The research presented here primarily addresses three factors in building plumbing, namely material quality, stagnation, and temperature. These three factors come together at shower hoses, which have (1) flexible polymeric materials that leach large amounts of biodegradable carbon, (2) long stagnation intervals (i.e. once daily showers), and (3) temperatures ranging only from warm to room temperature (i.e. neither hot nor cold). Shower hoses were thus often used as a model system throughout this thesis, for studies of both biofilm development and stagnation dependent water dynamics. As this research was supported by highly quantitative culture-independent methods (i.e., flow cytometry and qPCR), some critical aspects of these methods were also further investigated.

Throughout this work, building plumbing is approached as an ecological system governed by simple ecological principles, primarily that of dispersal (i.e., movement of bacteria) and selection (i.e., favorable growth conditions). This approach allowed for a cohesive understanding of building plumbing behavior in both large data sets from around the world and a series of highly controlled systems. For example, the quality of polymeric material was selective, but the effect could be surpassed by dispersal from the distribution network, and the selective factors ‘upstream’, like chlorination or temperature. During stagnation, spatial dispersal along the continuum of water delivery was insignificant compared to local dispersal from the biofilm to the water phase, which affected both concentration and microbiome composition. Ultimately, controlling bacterial growth in buildings will take advantage of these principles, and will furthermore embrace the inevitable growth in building plumbing pipes and water. Altogether, this research presents valuable steps towards a better understanding and better management of the microbiology within building plumbing.
Microbiology of Household Plumbing – Causes and Consequences

PhD Thesis by Caitlin Proctor

<table>
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<tr>
<th>Biofilm – shaped by</th>
<th>Material</th>
<th>Water</th>
<th>Water during stagnation</th>
<th>Cell count increase</th>
<th>Microbiome shift</th>
<th>The microbiome also shifts</th>
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<tr>
<td></td>
<td>Plastic materials provide food for bacteria</td>
<td>Water intermittently provides nutrients, bacteria, and stressors (e.g. heat)</td>
<td>During stagnation, biofilm detaches, increasing cell concentrations</td>
<td>Before stagnation</td>
<td>After stagnation – biofilm detaches</td>
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From the distribution network to the most distal end, materials, surface area to volume ratio, and typical stagnation times change along a continuum, each microbiome affecting the next.

A microbiological continuum

Whether the time-scale is days or months, biofilm develops over time, typically growing and increasing in diversity, but growth is not typically linear.
Zusammenfassung


üben diese Materialien in Gebäuden einen größeren Einfluss aus als im Verteilnetz. Des Weiteren sind die Temperaturen in Gebäudeinstallationen vergleichsweise hoch (z.B. Warm- und Mischwasserverteilung), was wiederum günstige Wachstumsbedingungen bietet. In den verzweigten Endsträngen von Hausinstallationen fliesst Wasser nur unregelmässig (d.h. lange Stagantionszeiten), was Interaktionen zwischen der Biofilmphase und der Wasserphase erlaubt. Während alle diese Faktoren nachweislich zum Wachstum beitragen, ist ihr Einfluss auf bakterielle Konzentrationen und Gemeinschaftszusammensetzungen noch weitestgehend unbekannt. Des Weiteren ist die Zusammenführung verschiedener Studien schwierig, da jedes Gebäude auf seine Art einzigartig ist, z.B. in Bezug auf Standort, Design und Betrieb.

Die vorliegende Forschungsarbeit bezieht sich hauptsächlich auf drei Faktoren in Gebäudeinstallationen – Materialien, Stagnation und Temperatur. Eine Kombination aus allen dreien findet sich bei Duschschläuchen, welche (1) aus nachgiebigen, polymeren Materialien bestehen, die hohe Mengen an biologisch nutzbarem Kohlenstoff freisetzen, (2) langen Stagnations-Intervallen ausgesetzt sind (d.h., tägliche Dusch-Ereignisse), sowie (3) Temperaturen die sich zwischen warm und Raumbedingung bewegen (d.h., weder heiß noch kalt). Aus diesem Grund wurden Duschschläuche in dieser Forschungsarbeit oft als Modellsystem genutzt; sowohl zur Untersuchung von Biofilmen, als auch von Stagnationsbedingten Wasserdynamiken. Da hierzu höchst quantitative, Kultivierungs-unabhängige Methoden genutzt wurden (z.B. Durchflusszytometrie und qPCR), wurden des Weiteren einige kritischen Aspekte dieser Methoden eingehender untersucht.

In dieser Arbeit werden Gebäudeinstallationen durchgehend als ökologische Systeme gehandhabt, welche von einfachen ökologischen Prinzipien bestimmt werden, insbesondere denen der Ausbreitung (d.h., Bewegung von Bakterien) und Selektion (d.h., begünstigende Wachstumsbedingungen). Diese Herangehensweise erlaubte ein umfassendes Verständnis mikrobieller Dynamiken in Gebäudeinstallationen sowohl auf Grundlage großer, global
# Mikrobiologie in Trinkwasser-installationen von Gebäuden - Ursachen und Auswirkungen

**Doktorarbeit von Caitlin Proctor**

<table>
<thead>
<tr>
<th>Biofilm – beeinflusst durch</th>
<th>Wasser</th>
<th>Material</th>
<th>Wasser bringt periodisch Nährstoffe und Bakterien, aber auch Stressfaktoren (z.B. Hitze)</th>
<th>Wasser während der Stagnation</th>
<th>Änderung im Mikrobiom</th>
<th>Während der Stagnation löst sich Biofilm ab und die Zellkonzentration steigt</th>
<th>Das Mikrobiom verändert sich</th>
<th>Biofilm-Entwicklung erfolgt über die Zeit, ob auf Tages- oder Monate-Skala; nicht typischerweise mit linearem Wachstum, aber zunehmender Artenvielfalt</th>
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<tr>
<td>Material</td>
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<td>Plastik-Materialien als Nahrungsquelle für Bakterien</td>
<td>Vor Stagnation</td>
<td>Anstieg der Zellzahl</td>
<td>Nach Stagnation – Biofilm-Ablösung</td>
<td>Vor Stagnation</td>
<td>JHA- und FHA-Bakterien im Endknoten Wasser</td>
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<td>Wasser bringt periodisch Nährstoffe und Bakterien, aber auch Stressfaktoren (z.B. Hitze)</td>
<td>Nach Stagnation – Biofilm-Ablösung</td>
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<td>Weniger aerobisch (weniger OTU) gleich zeitgleich Biofilm</td>
</tr>
</tbody>
</table>

Materialien, Oberfläche-zu-Volumen-Verhältnisse und Stagnationszeiten ändern sich auf dem Weg vom Verteilernetz bis zum hintersten Distalende, mit der gegenseitigen Beeinflussung der Mikrobiome untereinander.

Biofilm-Entwicklung erfolgt über die Zeit, ob auf Tages- oder Monate-Skala; nicht typischerweise mit linearem Wachstum, aber zunehmender Artenvielfalt.
Summary / Zusammenfassung
Chapter 1

General Introduction
Building plumbing, a unique and complex system

Building plumbing has several factors that contribute to a unique and interesting microbial growth environment, but the work presented here focuses on just three primary factors. Firstly, the pipes in building plumbing are of varied, sometimes unregulated or under-regulated materials. Polymeric materials are of particular interest as they leach organic carbon that can support bacteria growth, especially in biofilms. Since surface area to volume ratios are high in building plumbing (i.e., higher than in the distribution network), these materials and biofilms have a strong impact on water quality.

Secondly, stagnation time is long and uneven. This stagnation leads to biofilm detachment that changes not only the concentration of bacteria in water, but also the composition of the microbiome. Stagnation can thus impact consumers’ exposure, especially in the first minutes of flow.

Lastly, building plumbing has distinct temperature regimes. Water heaters can reach temperatures capable of disinfection, and water in both cold and hot distribution pipes typically reaches room temperature with greater stagnation time. These overall higher temperatures can encourage bacterial growth more than cold distribution network underground pipes, and can select for a different group of bacteria. Moreover, fluctuating temperatures in stagnant distal end pipes likely select for certain bacterial traits.

These three factors (material quality, stagnation, and temperature) have clear implications throughout building plumbing and even in the distribution network (e.g., seasonal variations in temperature, zones with low or no flow). However, they come together most critically at the shower hose, which has ideal bacterial growth temperatures (i.e., room to showering temperature), long stagnation (i.e., once daily showers), and low quality flexible plastic materials (i.e., not approved for drinking water use).
Along with a large global field survey, several test-systems simulating specific aspects of building plumbing are used in this work to test hypotheses about temperature, stagnation, and material quality. Much of this work targets shower hoses as a worst-case scenario, but conclusions can extend beyond this specific environment.

Throughout this work, the boundaries of test-systems are examined carefully. Building plumbing is the final step of a large water delivery system, and thus test-systems must be considered within their wider context. Building plumbing is also considered as an ecological system in this work. The principles of dispersal (movement of bacteria into or out of a microbial community; here, facilitated by bulk-flow of water or biofilm attachment/detachment) and selection (favorable/unfavorable growth conditions) can be used to explain the behavior of bacteria in all systems.

**The tools with which we study building plumbing**

The way in which we study building plumbing systems is, in large part, limited by sampling procedures and methods of analysis. In this work, several new methods are proposed for the observation of building plumbing. For example, shower hose biofilms offer a large area of easily accessible biofilms, improving upon previous methods which involved system disruption or swabbing end points. In-pipe real-time flow cytometry is also introduced in this work.

Throughout this work, both quantitative and qualitative methods were used in concert, since both are necessary to understand the full ecosystem dynamics. To accomplish this, flow cytometric (e.g., cell counts) and molecular (e.g., 16S amplicon sequencing) approaches
were applied side by side. In a final section of the work, these two approaches were critically evaluated both quantitatively (comparing 16S rRNA qPCR and flow cytometric cell counts) and qualitatively (using 16S amplicon sequencing to better understand flow cytometric fingerprints), reinforcing their complementary roles throughout the work.

Thesis Overview

Chapter 2 presents the state of the science for drinking water microbiology. The concept of a microbiome continuum is introduced, wherein each step of drinking water production and distribution affects the microbiome in terms of both numbers and composition as it continues to the household. Ultimately, consumers’ exposure to bacteria in water is the cumulative effect of many engineering decisions and ecological processes.

The challenges to maintaining biological stability along the last meters are introduced. Stagnation in these meters is inevitable, and many design decisions (e.g. pipe material choice) are known to impact the microbiome. The concept of probiotics is also emphasized as an alternative to disinfection based water distribution.

Chapter 3 uses a broad global survey of shower hose biofilms to explore the factors controlling these biofilms. Chlorination proves to be a strong factor, both in terms of biofilm concentration and microbiome composition. With a controlled follow-up study, these ideas are further refined, with an emphasis on use-patterns and dispersal from the network.

In Chapter 4, the importance of material quality in biofilm is tested. Even with a narrow category of materials – flexible synthetic materials – the impact on biofilm concentration and
composition is clear. As shower hoses do not fall into the category of 'potable water pipes', these may skip regulations meant to safeguard against excessive bacterial growth.

Chapter 5 uses a building plumbing simulator to determine the relative contribution of location, temperature, pipe material, and stagnation to the microbiome in both biofilm and water over the course of a year. Ultimately, these factors are presented through the lens of community ecology theory as dispersal and selection processes which assemble a somewhat stable biofilm, that in turn disperses into stagnant water.

In Chapter 6, the observations of water during stagnation across several test systems are used to assemble a conceptual model for the behavior of bacteria during stagnation and flow. Across all test systems, an increase in bacterial concentration during stagnation was accompanied by a shift in the flow cytometric fingerprint and microbiome composition. The chapter features the use of novel real-time flow cytometry measurements taken from directly inside the pipe. Dispersal, through flow of water and biofilm detachment controls the short-term dynamics inside the pipe.

In Chapter 7, flow cytometry, a critical method across all studies presented here, is further explored. In particular, the elusive identities of low nucleic acid (LNA)-content bacteria are revealed through a novel combination of filtration, flow-cytometric, and sequencing techniques. Across several freshwater ecosystems, LNA-content bacteria have a surprising phylogenetic clustering, and the reasons for this are explored.
In Chapter 8, flow cytometry and molecular methods for bacterial enumeration are critically compared. Bacterial enumeration is critical for the studies presented here as well as for all studies of ecological and engineering processes. The correlations, as well as limitations, are then useful for understanding how studies fit into a larger context.

Finally, in Chapter 9, knowledge gained from all studies is synthesized and put into the larger context of drinking water microbiology and the methods with which we study it.
Chapter 2
Drinking water microbiology – from measurement to management
Abstract

New microbial tools enable detailed quantification and characterization of complex drinking water microbiomes. Many opportunities exist from source-to-tap to apply this knowledge towards management of the microbiology. This requires consideration of the microbiome continuum across all phases harboring microbes (biofilms, cells attached to loose deposits and planktonic cells) and across all stages (source, treatment, distribution and premise). Biofilters can be optimized towards specific compound removal and can seed the distribution network with beneficial bacteria. Disinfection aggressively controls the microbiome, but may select for unwanted bacteria. Within premise plumbing, dramatic changes occur with unavoidable stagnation and pipe material influence. To supply safe drinking water sustainably, it is imperative that the field progress from characterization towards management of the drinking water microbiome.
Introduction

The presence of bacteria in drinking water (DW) has been recognized since the earliest microbial studies. Although research initially focused on fecal-associated pathogens, it has broadened considerably in recent years towards including non-fecal opportunistic pathogens (e.g., Legionella, Mycobacteria), process-related microbial problems (e.g., biofouling, biocorrosion), and microbes that are functionally relevant for DW treatment (e.g., nutrient and micropollutant removal) (Benner et al., 2013; Berry et al., 2006; Wang et al., 2013a). The fact is, from source-to-tap, different stages of DW systems offer unique habitats where microbes develop thriving complex communities that in turn influence downstream microbiomes (Figure 1). However, there are still broad knowledge gaps with respect to how these microbiomes are shaped and in turn the consequences for both the DW system and consumers. In order to treat and distribute water sustainably, it is imperative to accept the omnipresence of microbes in DW, understand their behavior, and apply that knowledge to manage them (Read et al., 2011). This review focuses on the microbiology of centralized

![Figure 1: Overview of drinking water systems, highlighting major influences on microbial quantity and composition across the four stages of the microbiome continuum - source, treatment, distribution and household. The figure is theoretical; numerous variations in system configuration occur.](image-url)
Drinking water microbiology – from measurement to management

Treatment and distribution of DW within the context of industrialized countries. It examines potential avenues for microbial management throughout such systems, and highlights critical research areas and opportunities.

Exciting new methods reveal a complex DW microbiome

Next-generation sequencing (NGS) technologies (i.e. 454 pyrosequencing, Illumina, and Ion Torrent) enable high-throughput, high-resolution characterization of the microbiome. This includes amplicon sequencing of the 16S rRNA gene for identifying bacterial community members (Berry et al., 2006; Douterelo et al., 2014; Foster et al., 2012; Holinger et al., 2014; Baron et al., 2014; Prest et al., 2014; Wang et al., 2014b), analysis of RNA for describing the active microbiome (Henne et al., 2012), DNA-based metagenomic analysis for discerning functional capacity (Gomez-Alvarez et al., 2012; Shi et al., 2013), and potential applications for metatranscriptomic analysis of gene expression (Douterelo et al., 2014; Foster et al., 2012). With the exception of straightforward bacterial community analysis, which captures a taxonomic snapshot with resolution ranging from major phyla down to rare taxa, application of these methods in DW research has so far been rather limited.

All community composition studies concur that DW microbiomes are complex, comprising up to 48 phyla and in excess of 4,000 unique operational taxonomical units (OTUs) (Holinger et al., 2014; Wang et al., 2014b; Lin et al., 2014; Pinto et al., 2014; Lautenschlager et al., 2013), with many defined OTUs presently unmatched to known organisms (i.e. “unclassified”). DW microbiomes tend to be dominated by Proteobacteria and share most other phyla, even with considerable differences in geography and treatment processes (Figure 2). In this broad comparison of phyla from similar effluent points of three treatment plants, 15 out of 25 total phyla were shared by at least two samples. With the exception of one candidate phylum (G02), the phyla unique to any one DW system represented 5% or less of sequences from
that specific system (Figure 2). While some of these similarities are potentially driven by the similar low-nutrient profile across all DW systems, it is erroneous to label DW microbiomes as similar based solely on phyla-level data. Substantial differences occur in the distribution of lower classifications and the occurrence of rare taxa (Shade et al., 2014). The relative abundance of Alpha-, Beta-, or Gamma-proteobacteria is inconsistent across different DW microbiomes, and also over time and between stages within one system (Prest et al., 2014; Pinto et al., 2014, 2012). The dynamics of individual and rare OTUs can also differ completely from that of the overall phyla (Pinto et al., 2014; Shade et al., 2014).

This raises the key question of whether a universal DW microbiome can actually be defined. One much-needed step forward would be a meta-study of all existing DW microbiome data, if provision can be made for differences in sampling (i.e. stages, phases, and temporal variation discussed below), biases (e.g. DNA extraction (Hwang et al., 2012a), reagent contamination (Salter et al., 2014), and PCR (Pinto and Raskin, 2012)), and analytical methods (i.e. sequencing

<table>
<thead>
<tr>
<th>Phylum</th>
<th>WTP 1</th>
<th>WTP 2</th>
<th>WTP 3</th>
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<tr>
<td>Proteobacteria</td>
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<td>Chlorobi</td>
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Lowest Relative Abundance Highest

● = reported in all WTP ○ = unique to 1 WTP ▲ = reported in 2 WTP

**Figure 2:** Relative abundance of bacteria at the phylum level from the effluent of three drinking water treatment plants [WTP] as reported: WTP 1 (Pinto et al., 2012), WTP 2 (Prest et al., 2014), and WTP 3 (Lautenschlager et al., 2014). Studies were chosen for illustrative purposes based on use of similar sampling points and methods, and to capture a wide variation in geography and treatment schemes.
platform differences (Liu et al., 2012; Quail et al., 2012). Moreover, there is clear potential for application of more advanced statistical methods to all studies. For example, Pinto and colleagues (Pinto et al., 2014) used such tools to select 66 “key” OTUs from thousands and map the interactions that were responsible for most of the dynamics seen in their DW system, perhaps setting a precedent for a more functional definition of the ‘core’ microbiome.

Research opportunities:

- Establish a comprehensive definition of the ‘core’ microbiome and test for the existence of a general core DW microbiome (versus system-specific ones)
- Implement NGS applications beyond ‘taxonomic snapshots’, with further consideration for metagenome, transcriptome, viruses, and eukaryotic organisms, including free-living amoebae, fungi, and invertebrates (Buse et al., 2013; Delafont et al., 2013) (Figure 3)
- Fundamental studies to identify and annotate unclassified OTUs in DW

Figure 3: Distribution and interactions of the microbiome across various phases (planktonic, biofilm and particle-associated) in a drinking water system. Adapted from Liu et al. and Nescerecka et al. (Liu et al., 21013b, 2014a; Nescerecka et al., 2014).
Chapter 2

Considering stages, phases, and temporal variations

A major challenge with respect to defining a DW microbiome is that it changes dynamically in both absolute abundance and community structure through stages of DW treatment and distribution (Figure 1), with each stage potentially seeding the downstream system (Prest et al., 2014; Lin et al., 2014; Lautenschlager et al., 2013; Pinto et al., 2012; Lautenschlager et al., 2014). In this manner, a microbiome continuum develops, necessitating consideration across all stages for accurate interpretation of the causes of change.

Moreover, the DW microbiome varies considerably over the phases of (i) planktonic cells, (ii) attached biofilm cells, and (iii) bacteria attached to particles or loose deposits (Liu et al., 2013b) (Figure 3). The planktonic phase is arguably most relevant to the consumer, with total cell concentrations typically ranging from $10^3$ - $10^5$ cells/mL (Lautenschlager et al., 2013; Liu et al., 2013a; Nescerecka et al., 2014), but representing less than 2% of bacteria in a distribution network (DN) (Liu et al., 2014a). Biofilms offer an environment protected from oxidative and hydraulic stress, while pipe material can affect biofilms both positively and negatively. Pipe-associated biofilm cell concentrations range from $10^4$ – $10^7$ cells/cm$^2$ (Inkinen et al., 2014; Lehtola et al., 2006), comprise 20-60% of the bacteria in a DW system, and are associated with a range of unwanted reactions (e.g., biocorrosion). Bacteria associated with loose deposits, sediments, and particles represent up to 80% of active biomass, but are often not examined due to sampling difficulties (Liu et al., 2013b, 2014a). This latter phase is highly relevant since it can be mobilized and transported in the network during hydraulic disturbances and has been shown to harbor hygienically relevant microbes, including mycobacteria (Liu et al., 2013b; Douterelo et al., 2013; Thomson et al., 2013). Several authors demonstrated convincingly that the phases differ in both absolute abundance and community composition (Henne et al., 2012; Lin et al., 2014; Liu et al., 2014a; Douterelo et al., 2013; Liu et al., 2014b). It was demonstrated that combining relative abundance measurements (i.e., NGS) with absolute abundance measurements (i.e., flow
Drinking water microbiology – from measurement to management

cytometry (FCM)) and viability assessment (i.e., adenosine tri-phosphate) provides complimentary information for microbiome characterization throughout DW treatment and distribution (Prest et al., 2014; Lautenschlager et al., 2014).

In this regard, cultivation-independent methods that allow accurate quantification of bacterial concentrations (i.e. FCM) have become invaluable as a replacement for cultivation, keeping pace with NGS developments (Douterelo et al., 2014; Prest et al., 2014; Besmer et al., 2014). FCM can also be extremely sensitive to temporal variations in aquatic systems (Besmer et al., 2014). These variations, including a daily pattern in premise plumbing bacterial concentrations (Besmer et al., 2014; Lautenschlager et al., 2010), add yet another dimension to consider when designing sampling/monitoring schemes.

Research opportunities:

• Determine the relevance of each phase to the DW system and the consumer
• Establish consensus of systematic and comparable sampling and analytical methods to include all phases in microbiome descriptions (Liu et al., 2013b, 2013a)
• Develop and apply ecological theories and models to the DW microbiome continuum (Pinto et al., 2014)

Managing microbes from catchment to consumer

A sobering question is how the wealth of microbial data acquired with state-of-the-art methods actually benefits DW management and whether microbial resource management concepts (Read et al., 2011) are applicable to DW systems. The fact is that decisions at all stages of DW treatment and distribution profoundly shape the microbiome. While this provides ample opportunities to purposefully manage DW microbiology, it also highlights the
need for a multi-dimensional approach towards studying these systems and proposing management solutions.

**Bacterial are useful for DW treatment**

A primary goal of DW treatment is the production of safe DW, and thus treatment plants apply multiple hygienic barriers (e.g. UV, membrane filtration, ozonation) (Figure 1). In this respect, quantitative microbial risk assessment (QMRA) is a well-developed concept for managing DW safety, with specific emphasis on source water contaminants (Bichai and Smeeet, 2013). However, treatment plants are not solely anti-microbial environments. Biofiltration (e.g., rapid sand, granular activated carbon, and slow sand filtration) is one of the oldest treatment methods and is operated with encouragement of bacterial growth in biofilms supported on granular material. A basic function of biofiltration is the removal of growth supporting nutrients, essential for producing biologically stable DW (Lautenschlager et al., 2013). This function is common for a wide variety of heterotrophic bacteria as demonstrated in studies of various biofilter communities (Lautenschlager et al., 2014; Wang et al., 2013b; Derlon et al., 2013). More specialized biofiltration functions include the bacterial-mediated removal of unwanted compounds such as ammonium, arsenic, manganese, and iron a variety of micropollutants (Benner et al., 2013; Li et al., 2013; Hedegaard and Albrechtsen, 2014; Gülay et al., 2013; Upadhyaya et al., 2012; Hoyland et al., 2014; Lee et al., 2014). New microbiology tools benefit the understanding and optimization of biofiltration processes by identifying and quantifying major contributors to pollutant removal (Li et al., 2013; Upadhyaya et al., 2012; Hoyland et al., 2014). For example, arsenic removal was linked to the location of active communities within the biofilter and optimized with filter operational parameters (Upadhyaya et al., 2012). In another example, iron oxidation, previously viewed as a purely chemical process, was linked to iron oxidizing bacteria (Gülay et al., 2013). While progress in this field has been made, there is still ample opportunity to further develop bio-remediated removal of micropollutants as an alternative to expensive abiotic processes (Benner et al., 2013).
Biofilters harbor specific communities and consequentially cause significant shifts in the microbiome, accounting for 50-60% change in series, based on β-diversity metrics (Lautenschlager et al., 2014). There is growing evidence that biofilters at the treatment plant may direct the entire DN microbiome (Lin et al., 2014; Lautenschlager et al., 2013; Pinto et al., 2012; Lautenschlager et al., 2014), which could provide operators the opportunity to control and manage the DN microbiome from this easily observed treatment stage.

**Research opportunities:**

- Combine metabolic potential analysis (metagenomics) with optimization of biofilters
- Determine feasibility of bioaugmentation of biofilters with assembled communities (Benner et al., 2013)
- Understand further the role and risks of biofilters in seeding and shaping the DN microbiology (Pinto et al., 2012)

**Disinfection prior to and during distribution**

The final step of DW treatment is critical with respect to the microbiome released into the DN and the tools with which this is studied. Final disinfection eliminates most bacteria and is a key component in risk management (Bichai and Smeets, 2013). Often coupled to final disinfection is the use of residual disinfectants (i.e. chlorine, chloramine) with the purpose of inhibiting microbial growth during distribution. Final and/or residual disinfection has three major implications. (1) Firstly, as it partially or completely destroys the treatment plant microbiome, it effectively eliminates any meaningful seeding of the DN with biofilter communities. (2) Secondly, it means that interpretation of microbiome data is incomplete without viability considerations. Many popular methods (e.g. FCM total cell counts and all DNA-based methods) include effectively dead, and thus potentially irrelevant, cells. In this case, it is imperative to use viability-targeting methodology for quantification and
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characterization (Prest et al., 2014; Henne et al., 2012; Nescerecka et al., 2014; Chiao et al., 2014; Villarreal et al., 2013). Henne and colleagues showed clear differences between RNA and DNA populations (Henne et al., 2012), while the work of Chiao and colleagues clearly demonstrated a completely different DNA-based microbiome after chlorination when applying propidium monoazide to exclude DNA from membrane-compromised cells (Chiao et al., 2014). (3) Finally, some viable bacteria are still detected in systems with disinfection, and thus disinfectants potentially act as a stress-based selective pressure. Uncontrolled growth-related problems arise when disinfectant residual is lost (Nescerecka et al., 2014) and there is growing concern that use of disinfection residuals selects for a microbiome that is resistant to oxidants and increasingly resistant to other stressors such as antibiotics (Shi et al., 2013; Pruden, 2014). There is also evidence that chloramine versus chlorine use selects for different microbiome composition (Holinger et al., 2014; Wang et al., 2014b; Gomez-Alvarez et al., 2012; Wang et al., 2013b; Hwang et al., 2012b; Wang et al., 2014a).

Research opportunities:

- Validate methodologies to assess bacterial activity or viability in conjunction with community analysis of disinfected DW (Chiao et al., 2014; Villarreal et al., 2013)
- Establish importance of extracellular DNA and DNA from dead cells in DW
- Establish relationship between disinfection and selection of multi-resistant microorganisms and determine consequences for the consumer (Shi et al., 2013; Pruden, 2014)

The alternative: distributing a viable microbiome

DN microbiology is essentially dictated by opposing philosophies regarding the use or non-use of disinfectants. The alternative approach to both final disinfection and use of residual disinfectants is the continuous release of a viable microbiome during treatment (Figure 1).
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with control of subsequent regrowth through nutrient limitation (Lautenschlager et al., 2013). Several large European treatment plants use biofiltration as a final treatment step, thus releasing benign communities that remain stable during distribution, with concentrations on the order of $10^4 – 10^5$ cells/mL (Lautenschlager et al., 2010). Wang and colleagues (Wang et al., 2013a) recently proposed the provocative idea to use benign bacteria, either from a designed inoculum or from indigenous bacteria, as “probiotics” in premise plumbing to exclude those that are detrimental to the system or consumers. It can be argued that European systems without disinfectants have unintentionally been employing this strategy with indigenous bacteria at full-scale for decades and are therefore particularly interesting models to study the concept (Box 1).

### Indigenous Communities as Drinking Water Probiotics

**Concept** – Altering system conditions across the entire microbiome continuum to promote establishment of preferred indigenous communities

**Considerations for full-scale implementation** –

1. *Use biofilter seeding* to continuously provide a controllable indigenous inoculum to the distribution network (Pinto et al., 2012; Lautenschlager et al., 2014)

2. *Stop final/residual disinfection* as this is counterproductive to point 1

3. *Apply nutrient limitations* to encourage biostability (Lautenschlager et al., 2010) and reduce risk

4. *Smartly design system* to encourage/discourage desirable/undesirable microorganisms (e.g. hot water configuration (Brazeau and Edwards, 2013), pipe material (Buse et al., 2014a))

5. *Incorporate ‘extreme’ selective antimicrobial measures* targeting specific unwanted bacteria (e.g. bacteriophage or competitor introduction) (Wang et al., 2013a)

6. *Promote consumer acceptance* with proof of concept research (i.e. EU systems) including detailed risk assessment

**Box 1**

### Challenges along the last meters

Within buildings, water effectively stagnates for up to 23 hours per day (Directorate-General for Energy and Transprot - Directorate D - New Energies and Demand Management, 2002). Stagnation allows water temperature to increase to household levels (Lautenschlager et al.,...
and leads to depletion of disinfectant residuals (Nescerecka et al., 2014; Wang et al., 2014a). In addition, stagnation exposes the pipe-biofilm to bio-available nutrients in the water phase and additionally allows exchanges between the suspended cells and pipe biofilm microbiomes. These factors favor microbial growth and considerable changes in both concentration (up to 320% increase) and composition (up to 100% shift) of the DW microbiome have been observed after stagnation (Lautenschlager et al., 2010).

Pipe material is critical to DW microbiology, especially in premise plumbing where pipe diameters are considerably smaller than in the DN, providing a surface-area-to-volume ratio increase. For a typical house, 150 m of 2 cm diameter pipe provides a 94,000 cm$^2$ surface for biofilm development. With DW biofilm concentrations of up to 10$^7$ cells/cm$^2$ (Lehtola et al., 2006), this implies up to 10$^{12}$ DW biofilm bacteria in a single household. It has been demonstrated that pipe diameter (Thomson et al., 2013) and material affect the biofilm microbiome (Wang et al., 2014a; Lin et al., 2013; Zhu et al., 2014; Lu et al., 2014). Metal-based materials (e.g. copper pipes) may be effective at inhibiting short-term biofilm formation compared to synthetic materials (Zhu et al., 2014), but this growth limitation may result in unwanted selection. For example, it was suggested that copper selects for certain opportunistic pathogens (i.e. Legionella pneumophila) and more resistant free-living amoeba (Lu et al., 2014; Buse et al., 2014b). In contrast, polymeric substrates, shown to support extensive bacterial growth (Inkinen et al., 2014; Zhu et al., 2014; Bucheli-Witschel et al., 2012), leach from synthetic pipe material long after installation (Inkinen et al., 2014), although the composition and quantity of these leached polymeric substances differ considerably between different materials (Bucheli-Witschel et al., 2012; Zhang and Liu, 2014). Even small amounts of synthetic material, as added with automatic vs. standard faucets, may induce community shifts (Baron et al., 2014). Pipe material selection presents one of the clearest opportunities to influence the household microbiome, given high surface-area-to-volume ratios.
The hot water distribution system is a distinct part of the premise plumbing system, often with a distinct microbiome (Buse et al., 2013; Inkinen et al., 2014) that presents a different risk to the consumer, including the risk for *Legionella* infection (Wang et al., 2013a; Buse et al., 2014a; Zhang and Liu, 2014; Brazeau and Edwards, 2013). At high boiler temperatures (ca. 60 °C), disinfection may occur, but lower water heater temperatures (i.e. 48 °C), and/or the combination with cold water, and/or stagnation between uses means fluctuating lower temperatures in relevant pipes (Brazeau and Edwards, 2013). Thus, initial high temperatures may provide a selection mechanism followed by more ideal temperatures for regrowth (Brazeau and Edwards, 2013). In one extreme example, biofilms exposed to warm water, as in showerheads, were shown to enrich opportunistic pathogens (Rhoads et al., 2014).

Design of the hot water distribution system, including instantaneous hot water heaters and recirculating lines, may limit this risk (Brazeau and Edwards, 2013).

Designing household solutions is difficult, as each premise varies greatly from the next. Certain prescribed measures of regrowth control may also inadvertently encourage unwanted microbial regrowth and fail without proper upkeep (Baron et al., 2014; Wang et al., 2013b; Rhoads et al., 2014). Thus, legislation with respect to microbiology in premise plumbing is disturbingly limited.

**Research opportunities:**

- Establish effective premise plumbing sampling strategies which balance with practical and privacy issues
- Provide guidance on pipe material selection considering supportive and inhibitory microbiological effects (Wang et al., 2014a; Lin et al., 2013; Zhu et al., 2014; Lu et al., 2014; Buse et al., 2014a; Bucheli-Witschel et al., 2012)
- Demonstrate proposed pathogen mitigation strategies (Lautenschlager et al., 2010; Brazeau and Edwards, 2013; Rhoads et al., 2014) at full-scale
Conclusions

- The DW microbiome develops along a dynamic continuum from source-to-tap wherein engineered strategies have profound microbiological impact.
- Full understanding of the DW microbiome requires a broader and deeper assessment using advanced tools (both quantitative and qualitative) to evaluate stage, phase, and temporal variations.
- The challenge is to evolve from characterization towards understanding, in order to benefit consumer and practitioner acceptance and to drive the knowledge towards sustainable management of the DW microbiome.

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Author Contributions
CP and FH both contributed to conception, literature review, and writing.
References


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*Annotations for selected pieces of high impact.

*1 • The authors used automated in-situ FCM to quantitatively measure the microbiome of several aquatic systems with 15-minute resolution, demonstrating the technology’s potential as a high-resolution quality monitor. Results indicate that temporal fluctuations are a critical consideration for sampling strategies.

*2 • The authors performed disinfection studies with and without removal of dead cells to demonstrate the importance of viability consideration in DW studies. The communities with dead cells removed had a strong community shift, while the total cell communities remained stable.

*3 • The authors clearly established differences in both concentration and composition of the microbiome in the four phases of growth. With just 2% of bacteria in bulk water and up to 80% in loose deposits, the study demonstrates the need to consider all phases for complete DN understanding.

*4 • The authors linked use of upstream materials (Cu, UPVC) to downstream microbiome composition, with copper having potentially negative effects. The study impacts consideration of materials and study design, with the more-complicated system simulating realistic premise plumbing.

*5 • The authors used a large dataset from a real DN to demonstrate spatial dynamics and strong seasonal cycling using interspecific occupancy abundance models. The advance statistical use of NGS data is a critical step towards predictive modeling and management of the DW microbiome.

*6 • The authors used FCM and pyrosequencing in concert to detect community shifts in the DN, with the valuable added measure of absolute abundance. Authors demonstrated that the methods complimented each other for a highly detailed microbiome characterization.

*7 • The authors linked opportunistic pathogen occurrence to both bacterial and eukaryotic OTUs and to controllable elements (disinfectant residual, pipe materials) using a simulated DN. The study moves beyond characterization towards feasible strategies for management of the DW microbiome.
Chapter 3

Biofilms in Shower Hoses

78 shower hoses
11 countries

Biofilms varied in terms of:

- Potential opportunistic pathogen detection
- Cell concentrations
- Microbiome composition
- Metals accumulation

Chapter submitted for publication in a revised format by:

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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 drinking water plumbing. However, the health risks associated with and processes underlying the development of biofilms in shower hoses are poorly studied. In a global survey, biofilms from 78 shower hoses from 11 countries were characterized in terms of cell concentration (4.1 x 10^4 – 5.8 x 10^8 cells/cm²), metal 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. Metal accumulation (up to 5 µg-Fe/cm², 75 ng-Pb/cm², and 460 ng-Cu/cm²) seemed to be partially responsible for discoloration in biofilms, and likely originated from other pipes upstream in the building. While some potential opportunistic pathogen genera (Legionella, detected in 21/78 shower hoses) were positively correlated with biofilm cell concentration, others (Mycobacterium, Pseudomonas) had surprisingly non-existent or negative correlations with biofilm cell concentrations. In a controlled 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 dispersal and selection helped to explain microbiome composition and diversity of different sample types. Shower hose age was related to metal accumulation but not biofilm cell concentration, while frequency of use appeared to influence biofilm cell concentration. This study shows that shower hose biofilms are clearly a critical element of building drinking water plumbing, and a potential target for building drinking water plumbing monitoring.

Keywords: shower hose; building plumbing; biofilm; microbiome; heavy metals
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; Thomson et al., 2013) and shower hoses (Collins et al., 2017; Lienard et al., 2017; Moat et al., 2016; Proctor et al., 2016; Soto-Giron et al., 2016; Whiley et al., 2015). Within the building drinking water 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 are 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 drinking water 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, *Mycobacterium* species were found in shower aerosols of patients with nontuberculous mycobacteria (NTM) pulmonary disease (Thomson et al., 2013). 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 be-
coming increasingly disruptive and costly, triggering facility shut-downs, remediation measures, and legal actions (Table S1).

Interest in building drinking water plumbing opportunistic pathogen management is increasing slowly. For example, regulations in Germany require periodic *L. pneumophila* testing in homes when water boilers exceed 400 L in size (BMJV, 2011), and new Swiss legislation requires testing of shower water in public buildings (EDI, 2016). However, these monitoring activities are extremely laborious and the regulations often fall short both in terms of sampling methods (Wang et al., 2017) and preventative actions. For example, materials in household drinking water plumbing are typically quality-controlled to some extent (e.g. (CEN, 2013)), but the materials used specifically in shower plumbing are often completely unregulated because shower water is not necessarily considered as ‘potable 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 the consumers themselves. Consumers’ choice in replacement hoses is often driven by functionality and aesthetics alone, with ignorance concerning potential health impacts. This is especially concerning because shower hoses are literally the last meter before water reaches the end-user, and are often used to more easily bathe patients and the elderly whom are at higher risk for opportunistic pathogen infection.

The unique bacterial growth environment and potential relevance to human health necessitates a better understanding of the biofilms that develop in shower hoses. The overarching goal of this study was a detailed characterization of biofilms in real-world shower hoses on a global scale. The specific aims were to investigate the importance of engineering and consumer choices on that environment (i.e., disinfectant use, material quality, age of hoses, and specific use patterns), and to explore ecological relationships amongst bacterial communities and the potential risks posed by these biofilms. To achieve these aims, a global survey of shower hoses investigated biofilm concentrations of bacteria and metals, microbiome
composition, and the presence of opportunistic pathogens in 78 shower hoses from around the world. Additionally, a controlled study with shower hose material, hose age, and water type controlled, was conducted to remove some of the variability in the global survey, to identify the key factors in biofilm formation, and to determine biofilms’ relationship to the water phase.
2. Materials and Methods

2.1. Sample collection

Global survey: Shower hoses were collected from 78 showers across 11 countries on 3 continents (Belgium [8], Denmark [10], Germany [4], Latvia [7], Portugal [7], Serbia [4], South Africa [3], Spain [1], Switzerland [21], the United Kingdom (UK) [9], and the United States (US) [4]; Table S2). Volunteers were recruited by word-of-mouth through colleagues, and thus samples were geographically condensed in some countries (i.e., shared workplace). With the exception of 4 hoses from a Swiss office-building locker room, all samples were from domestic settings. Volunteers were instructed to detach the shower hose from all fittings and to gently decant water from the hose. Hoses were sealed with autoclaved ½ inch stoppers and stored in plastic bags for transport to the lab within one week of removal. Shipping time was minimized, but variable by country; hand-delivery (in luggage) and 24-hour shipping were used when possible. All volunteers were also asked to complete a brief survey to ascertain hose age (time since hose installation) and general water quality (i.e., disinfection by chlorination). Water was not collected or analyzed in this portion of the work.

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

Controlled study: A controlled study was conducted for 15 showers in Switzerland where disinfectant is not used. All participants were given the same type of PVC-P shower hose to use for 1.5 years. Hoses and more detailed information on use patterns were collected as
described above. In addition, three 1 L water samples were taken from each home: (1) stagnant - from the shower hose after overnight stagnation (exact stagnation duration unknown), (2) warm flowing - from the shower hose after five minutes of flushing with warm water (i.e., showering temperature, 35-45 °C), and (3) cold flowing - from the nearest tap after five minutes of flushing with cold water (i.e., distribution system water without shower hose influence).

2.2. Biofilm visualization

Biofilm structure and thickness were characterized with optical coherence tomography (OCT), using a Spectral Domain OCT Imaging System (930nm, OCT System Ganymede, Thorlabs GmbH, Dachau, Germany). The long wavelength light allows penetration up to a depth of 2.7 mm (in air, i.e., with a refractive index of 1) with axial and lateral resolutions of 4.4 µm and 15 µm, respectively. For biofilms penetration depths up to 1.7 mm are typically observed due to their higher refractive index. Scanning electron microscopy (SEM), was performed at the Center for Microscopy and Image Analysis (University of Zurich) on 1 cm² pieces that were fixed with 2.5% Glutaraldehyde and stored at 4°C in the dark.

2.3. Biofilm extraction

Biofilm was extracted using a repeated sonication strategy that was optimized and used previously (Proctor et al., 2016). The 90-cm section with glass beads and filtered mineral water was inverted five times and then sonicated in a bath (Bandelin Sonorex, Rangendingen, Germany) for five minutes. The water was collected and replaced with fresh filtered mineral water. The water volume varied with hose diameter. In total, five rounds of sonication and replacement were completed for each hose after which the beads were removed and discarded, and the hoses were filled with only filtered mineral water for a final rinsing step. The total biofilm suspension (volume ranging 84 – 423 mL) was sonicated with a needle (Sonoplus HD 2200, Bandelin Sonrex, Rangendingen, Germany) for 30 seconds for homogenization and was aliquoted for further analyses. A negative control sample for DNA-based anal-
yses was also produced using the same filtered mineral water and homogenization with needle.

2.4. Flow cytometry (FCM)

Staining and FCM analysis was done as described previously (Prest et al., 2013). Briefly, a working solution of SYBR® Green I (SG) (Invitrogen AG, Basel, Switzerland) was prepared by 100x dilution in anhydrous dimethylsulfoxide (DMSO). Water samples and biofilm suspensions were stained with SG at 10 µL/mL. Samples were preheated to 35 °C (3 min), then incubated with stain in the dark for 10 min at 35 °C before measurement. FCM measurements were performed on a BD Accuri C6® instrument (BD Accuri cytometers, Belgium). Data analysis was performed using the BD Accuri CFlow® software, following the procedure described previously (Prest et al., 2013) to calculate total cell concentration (TCC). Water TCC was used directly (cells/mL) and biofilm TCC was calculated using the hose surface area (cells/cm²).

2.5. Elemental Analysis

Homogenized 100 µL aliquots of the biofilm suspensions were acid-digested in 4 mL concentrated ultrapure HNO₃ (Carl Roth GmbH, Karlsruhe, Germany) and 1 mL ultrapure concentrated hydrogen peroxide (Sigma-Aldrich, Buchs, Switzerland) in 15 mL Teflon-tetrafluormetoxil (PTFE-TFM) tubes in an MLS ultraClave 4 (Milestone Inc., Shelton, USA) at 230°C and 130 bar for 35 minutes. Subsequently, digestates were diluted 10-fold with ultrapure deionised water. Quality control of digestion was performed by co-digesting procedural blanks and certified reference materials SRM 1568b (rice flour) and RTC SRM-008 (sediment), which gave elemental recoveries between 61-138% (Table S3).

Following digestion, elemental concentrations were quantified using a quadrupole dynamic reaction cell ICP-MS (Agilent 7500cx). For each investigated element, the targeted isotope, reaction mode and detection limit (3 x σ of >10 blanks) is given in Table S3. Instrumental
tuning and calibration was performed daily and a 1 ppm Sc and 0.1 ppm In and Lu in 1% HNO$_3$ solution was used as internal standard. Quality control was conducted by triplicate analysis of selected digestates and by analyzing procedural blanks and aqueous reference standards (Merck X and NIST 1643f).

2.6. DNA extraction

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

2.7. qPCR

qPCR was used to quantify specific organisms, including *L. pneumophila* (via macrophage infectivity potentiator (*mip*) gene), *M. avium*, *Acanthamoeba* spp., and *V. vermiformis* using previously described protocols (Kuiper et al., 2006; Nazarian et al., 2008; Rivière et al., 2006; Wang et al., 2012; Wilton and Cousins, 1992). Reactions were performed using either an ABI7500 system (Thermo Fischer Scientific) for TaqMan (Bio-Rad) assays (*L. pneumophila* and *Acanthamoeba* spp.) or a LightCycler 480 for EvaGreen (Bio-Rad) assays (*M. avium* and *V. vermiformis*) (Roche). Reactions and primers are detailed in Table S4. A 1:10 dilution was used for all samples, and standard curves using 7 standards on each run, with a limit of quantification (LOQ) of 10 gene copies/reaction. Only samples above the LOQ in duplicate reactions were considered to be positive for presence/absence assessment.

2.8. Amplicon sequencing with Illumina MiSeq

Approximately 1 ng of DNA extract from each sample was subjected to PCR amplification using modified universal bacterial primers Bakt_341F and Bakt_805R (Klindworth et al., 2013), which target the V3-V5 region of the 16S rRNA gene, and were adapted with a nucleotide tail to facilitate binding Nextera adapters. Index PCR was performed to add the Nex-
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tera XT v2 Index Kit adaptors (Illumina) to the amplicon. PCR reaction conditions are detailed in (Table S5). After each PCR reaction, products were purified using the Agencort® AMPure® XP system (Beckman Coulter, Inc., Bera, CA).

Each product was quantified using Qubit 2.0 HS DNA system (Thermo Fisher Scientific). Samples were normalized to the same concentration before running on the MiSeq platform using MiSeq Reagent Kit v2 (300-cycles, #MS-102-2002) according to manufacturer’s protocol with 10% PhiX. All sequencing was done at the Genetic Diversity Centre (GDC) of ETH, Zurich. Libraries were produced separately for the global survey and the controlled study. The negative control was excluded from further analysis due to low DNA yields.

2.9. Statistical analysis

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

For sequencing data, sequences were merged, trimmed, filtered, and clustered into operational taxonomical units (OTUs) according to several algorithms (Table S6). Sequences were identified according to greengenes v.13.5 (DeSantis et al., 2003). In R, phylseq (McMurdie and Holmes, 2013) was used for processing. Libraries were rarefied to either 37,629 (global survey) or 82,185 (controlled) sequences per sample. In this process, two samples from Switzerland (global survey) were removed from analysis due to low number of reads. Non-metric multidimensional scaling (NMDS) was used to visualize microbiome similarities using Bray-Curtis dissimilarity. The adonis and ordiellipse functions from the vegan package (Oksanen et al., 2013) were used to relate environmental data to microbiome composition. In the controlled study, the number of core OTUs was calculated as the OTUs present in all locations of each sample type or overlapping type.
3. Results

For each of the following sections, the results are presented separately for the two experiments – the global survey and the controlled study. In the global survey, 78 shower hoses with largely unknown histories were collected from 11 countries. In the controlled study, 15 identical PVC-P shower hoses were installed in Swiss homes and collected after 1.5 years of use, together with three types of water samples from each location.

3.1. Biofilm and bacterial concentrations were high and variable

Global survey

Biofilm thickness ranged from non-detectable to 0.40 mm (Figure 1) and varied considerably across different hoses and along a single shower hose (OCT, Figure S1). SEM imaging of a representative biofilm indicated a thick matrix of cellular, non-cellular organic, and inorganic constituents (Figure 1, Figure S2).

The biofilm surface concentration (hereafter, biofilm concentration) was measured using the biofilm suspension from the 90-cm section of hose. Biofilm total cell concentration (biofilm TCC) measured with FCM ranged from $4.1 \times 10^4$ – $5.8 \times 10^8$ cells/cm$^2$ (Figure 2A). Other measures of biofilm concentration, including intact cells and adenosine tri-phosphate, correlated well with biofilm TCC (Spearman’s $\rho=0.95$ and 0.87 respectively, $p<0.001$, Figure S3). Biofilm concentrations of 16S rRNA genes (qPCR), total organic carbon (TOC), and optical density ($\text{OD}_{546}$) correlated poorly with biofilm TCC (Spearman’s $\rho=0.52$, 0.57, and 0.65 respectively, $p<0.001$) (discussed further with Figure S3).

Biofilm TCC varied significantly based on country of origin, seemingly related to the use/non-use of disinfectant. When comparing biofilm TCC between 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 does not take disinfectant concentration or type into account. For example, drinking water systems in Belgium were known to use only low disinfectant concentrations, and systems in Latvia were known for disinfectant loss during distribution.

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

**Controlled study**

In the controlled study, biofilm TCC ranged between 0.3 – 2.0 x 10^6 cells/cm² (Figure 4A), which was a narrower range compared to the same 15 locations investigated during the global survey (0.04 – 1.2 x 10^8 cells/cm²). In 9 of 15 locations, biofilm TCC was higher during the controlled study than in the global survey (Figure 3A). This occurred even though hoses...
were usually older during the global survey than in the controlled study (hoses from 12 of 15 controlled sample locations were estimated to be > 2-8 years old during the global survey, while 3 were known to be < 6 months old). The importance of hose age is further discussed in section 4.2.3.

Water TCC varied amongst the three types of water samples collected in the controlled study (Figure 4B), with stagnant water having the highest TCC in 12 of 15 locations. Additional water quality parameters (i.e., aqueous chemistry) for the cold flowing water and use-patterns were also measured and were similarly variable (Table S7). 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 S8).

**Figure 2**: Biofilm total cell concentration (Biofilm TCC) (A) and number of observed operational taxonomical units (OTUs) (B) of shower hoses, grouped by use of disinfectant (red) or lack of disinfectant (blue). 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.
3.2. Metals accumulate in biofilms

Global survey

Color was notably variable across biofilms (e.g., deep red/orange, translucent, white, yellow) (Figure 5, Figure S4), which can presumably be at least partly attributed to metal deposition. (Some color may also be attributed to the proliferation of specific bacteria, as discussed in section 4.3.2). Although not detected in all biofilms, biofilm concentrations of iron, lead, and
copper were as high as 5 µg-Fe/cm² (South Africa), 75 ng-Pb/cm² (Denmark), and 460 ng-Cu/cm² (Latvia). These metals all correlated to one another (Spearman’s ρ>0.75, p<0.001), reflecting the tendency of copper and lead to sorb to amorphous iron minerals in circumneutral aqueous environments. These metals also correlated moderately with optical density of biofilm suspensions (data not shown, correlations strongly affected by outliers). Calcium and magnesium were detected in all biofilms, and correlated separately with one another (Spearman’s ρ=0.6, p<0.001), as might be expected for these chemically-similar alkaline earth metals. There were no strong correlations between metal concentrations and biofilm TCC (Figure S5).

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

**Controlled study**

The concentrations of copper and lead in biofilms tended to be higher in the global survey (where hoses were older) than during the controlled study (Figure 3B,C,D). Concentrations were similar when hoses were from the same building and had similar ages (e.g., Hoses 3-6 were from one building and had similar ages in both experiments Figure 3B,C,D).

**3.3. Microbiome composition reveals a diverse ecological niche**

**Global survey**

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

Some similarities could be seen in biofilm microbiome compositions from the same country (Figure 6A), but the origin of the hose (country) only explained 24% of variation in microbiome composition (Adonis, $p<0.001$). The use of disinfectant was not a useful parameter for explaining microbiome composition (4% explained by disinfectant use, Adonis, $p<0.001$, Figure 6B).
The most abundant OTUs may have driven some of these variations, with the sum of the top ten OTUs accounting for 3 – 41% of individual microbiomes. This proportion was significantly higher when disinfectant was used (Kruskal-Wallis, p<0.001). Biofilm TCC also correlated with the relative abundance of several of the ten most abundant genera (Figure S6). Significant negative correlations were found with *Sphingomonas*, *Pseudomonas*, and *Limnobacter* (Spearman’s $\rho$ = -0.56, -0.55, -0.47, respectively, p<0.001), while positive correlations were found with *Dok59*, *Sphingobium*, and *Meiothermus* (Spearman’s $\rho$ = 0.57 (p<0.001), 0.21 (p=0.17), 0.21 (p=0.12), respectively).

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

Of all investigated water types, cold flowing water had the highest number of OTUs, followed by warm water and stagnant water (Figure 4C). The biofilms had a much lower bacterial diversity than the water samples (e.g., between 90 and 98% fewer OTUs than cold water, (Figure 4C)).

Microbiome composition also varied between three types of water samples and the biofilm (Figure 4D). Even at the phylum and class level, each sample type clearly harbored different types of bacteria (Figure 7). Three taxa (Betaproteobacteria, Alphaproteobacteria, and Bacteroidetes) that accounted for 91% of biofilm sequences, only accounted for 31% of the cold influent water microbiome. Seven taxa (OD1, OP3, Deltaprotobacteria, Planctomycetes, Chloroflexi, Chlamydiae, and Nitrospirae) that accounted for 51% of cold-water microbiomes only accounted for 2% of biofilm microbiomes. Another four taxa ( Acidobacteria, Actinobacteria, Deinococcus-Thermus, and TM7) were clearly enriched in warm water (16%). Core OTUs were also distinct between the sample types, with only 6 OTUs shared by all samples (Figure 7). Sample type (i.e., three water types, biofilm), however, only explained 18% of microbiome variation (Adonis, p<0.001). Household (i.e., building of sample origin) explained 41% of microbiome variation (Adonis, p<0.001, Figure S7).

3.4. Specific organisms in shower hose biofilms may present risk

Global survey

Two opportunistic pathogens, Mycobacterium avium and Legionella pneumophila, and two amoebae, Acanthamoeba ploypagha 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)/cm², $6.6 \times 10^5$ gc/cm², $2.5 \times 10^4$ gc/cm², and $2.8 \times 10^5$ gc/cm², 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.
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.

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.
Chapter 3

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 x 10^5 cells/cm², Belgium), *Mycobacterium* (4.1 x 10^7 cells/cm², Belgium), and *Pseudomonas* (3.1 x 10^6 cells/cm², Switzerland). This calculation of absolute abundance changes perception of the data; e.g. the hose with the highest relative abundance of *Mycobacterium* (35%) only had the 17th highest concentration by calculated absolute abundance (5.9 x 10^5 cells/cm²) because biofilm TCC was low.

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 the relative abundance of *Legionella* and biofilm TCC (Spearman’s p=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 of *L. pneumophila*, *M. avium*, or *V. vermiformis* (Figure 8B,C,F), which may have been caused by the relatively few detects of these species with qPCR.

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Surprisingly, cold influent water was consistently positive for low concentrations of *Legionella* (12/12), *Mycobacterium* (12/12), and *Pseudomonas* (11/12). Biofilms were more sporadically positive for these genera (*Legionella* 6/15, *Mycobacterium* 7/15, and *Pseudomonas* 13/15). Of all investigated water types, the highest calculated absolute abundances of these three genera were found in the stagnant water samples (6.8 x 10^3, 6.9 x 10^3 and 6.2 x 10^2 cells/mL respectively for *Legionella*, *Mycobacterium*, and *Pseudomonas*), and these maximums corresponded with detection of the genera in the respective biofilms.
Biofilms in Shower Hoses

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).

Controlled study
4. Discussion

Several interesting trends could be disentangled from the data despite the uncontrolled and unknown factors that are inevitable with samples from real homes. Although all investigated shower hoses have similarities (e.g., flexible materials, installed after mixing valves), each shower hose presented a unique environment (4.1), which harbored many bacteria (4.1.1), and inorganic mineral deposits (4.1.2). These environments were heavily influenced by engineering and consumer choices (4.2) such as disinfectant use (4.2.1), hose material (4.2.2), age of hoses (4.2.3), and specific use patterns (4.2.4). Ecological relationships between microbiomes were evident (4.3), especially concerning the dispersal and selection of bacteria within buildings (4.3.1), and composition that co-correlated with biofilm TCC (4.3.2). While some risk could be attributed to shower hose biofilms (4.4.1), we present several options for monitoring (4.4.2) and managing (4.4.3) these risks.

4.1 Shower hoses are a unique environment

4.1.1. Shower hoses harbor many bacteria. The physiochemical conditions common to nearly all shower hoses, including mild-to-warm temperatures (ranging from room temperature to maximum shower temperatures of c.a. 43 °C), long stagnation, and nutrient migration from flexible plastic materials (Proctor et al., 2016) may promote high biofilm TCC. Although a literature comparison of biofilm data is complicated by differences in sampling and analysis methods (Wang et al., 2017), our study indicates that biofilm TCC tends to be higher on shower hoses (average $10^7$ cells/cm$^2$) than on hard pipe materials typically used in building drinking water plumbing (copper and PEX pipes, $\sim 5.7 \times 10^5$ cells/cm$^2$, (Inkinen et al., 2014)) or drinking water plumbing simulators (Pe-Xc, $\sim 2 \times 10^6$ cells/cm$^2$ (Proctor et al., 2016)). In addition to bacteria, the studied shower hose biofilms also harbored non-bacterial cells, likely including fungi (Moat et al., 2016) and amoebae (Figure 8, (Lienard et al., 2017)). Visualizations of the shower hose biofilms indicate a thick, complex matrix (Figure 1), with non-
cellular organic and inorganic matter (e.g., EPS, mineralized metal precipitates) that likely affects bacterial proliferation.

4.1.2. Metal accumulation originates from upstream pipes in the building. The biofilm concentrations of metals varied considerably, but were similar in hoses from the same buildings (Figure 3B-D, with hoses 3-6 from the same building). While elements like calcium and magnesium are presumably related to local influent water quality (e.g., hardness), metals such as lead, copper, and iron may also originate from upstream pipes and fittings in the building (Gonzalez et al., 2013). Pipe corrosion is a well-known problem and building drinking water plumbing materials are generally not well documented or known to consumers. For instance, risks from lead in pipes and brass fittings are known, but regulations are not applied retroactively to replace building drinking water plumbing components, and building drinking water plumbing still contributes significantly to lead in water (Haider et al., 2002). In addition, certain metals can also leach from plastic pipes (e.g., lead is used as a stabilizer for certain PVC plastics (Zhang and Lin, 2015)).

4.2 Engineering and consumer choices influence this environment

4.2.1 Disinfectant use during water treatment affects biofilms in buildings. The use of disinfectants during drinking water treatment can lower both the concentration (Prest et al., 2016) and diversity (Bautista-de los Santos et al., 2016) of bacteria in drinking water. It is unclear, however, how this would affect biofilms at the distal end of building drinking water plumbing systems, where the disinfectant residual is often lost (Lipphaus et al., 2014). In the present study, disinfected drinking water resulted in biofilms with lower TCC, decreased diversity, and an increased relative abundance of the ten most abundant OTUs (Figure 2, Figure 6). It could thus be argued that either (1) residual disinfectant acts as a selective force directly on biofilms, or (2) disinfectant acts as an indirect selective force, reducing the diversity in the source water, which in turn reduces the diversity in the seed available for biofilm
colonization. With purely stochastic immigration to the biofilm, diversity in the metacommunity (in this case, the influent water) would be reflected in the biofilm microbiome (Battin et al., 2007). Since several countries with relatively high disinfectant residuals clustered closer together (Figure 6B), prolonged exposure to disinfectant may be more selective (e.g., a surviving ‘resistome’ (Jia et al., 2015)). Lack of stronger correlation to biofilm composition may be due to the fact that exact type and concentration of disinfectant were not known in this study.

4.2.2 Shower hose material quality is a problematic unknown. The amount and type of carbon leaching from plastic pipe materials is critical for determining biofilm concentration and composition (Proctor et al., 2016; Wen et al., 2015). Composition of hoses in the global survey was clearly variable (Figure 5), but was not determined in the global survey, in part due to difficulties quantifying compounds that have already leached out of the pipe (e.g., plasticizers (Heim and Dietrich, 2007; Skjevrak et al., 2003)). In contrast, identical PVC-P hoses were used in the controlled study. This specific material encouraged biofilm growth more than other materials (up to $2 \times 10^8$ cells/cm$^2$ over 8 months) (Proctor et al., 2016) and this was linked to carbon leaching: under optimal conditions, PVC-P hoses leached large amounts of biodegradable carbon (9.7 µg-C/cm$^2$ supporting $4.8 \times 10^7$ cells/cm$^2$ in 7 days). The generally higher biofilm concentrations during the controlled study (Figure 3A) may thus be linked to the common material. However, leaching behavior is variable, with an initial peak diminishing over time (Bucheli-Witschel et al., 2012). Thus, the common young age of the materials in the controlled study can also contribute to the high, narrow range of biofilm TCC (discussed further below).

4.2.3 Are new shower hoses worse than old ones? Within the same household, accumulation of metals in biofilms seemed to increase with age (Figure 3B-D), but this was difficult to confirm, since age of hoses during the survey was usually unknown beyond a minimum (e.g., time of move-in). Furthermore, while passive metal accumulation through metal precip-
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Iteration increases with time, primary biofilm development may involve active accumulation (e.g., iron as trace nutrient, calcium as biofilm structural support).

Biofilm TCC is dependent on availability of nutrients for growth. Carbon leaching diminishes over time (Bucheli-Witschel et al., 2012), resulting in a nutrient-rich environment in new hoses, and a nutrient-poor environment in old hoses. Other nutrients necessary for growth (e.g., N and P) are typically present in oligotrophic concentrations in drinking water, and thus enter the shower hose based on use frequency over time. Here, in cases where hose age could be estimated (ranging 3 months to 30 years), there was no correlation between hose age and biofilm TCC (data not shown). The hose with the most biofilm ($5.8 \times 10^8$ cells/cm$^2$) was only installed for one year, and TCC was often higher in the younger biofilms during the controlled study (Figure 3A). A lack of correlation between age and biofilm concentration has been observed before (Wingender and Flemming, 2004) and may be explained by a growth plateau that is reached quickly after fast exhaustion of easily available and leachable carbon (Proctor et al., 2016; Wen et al., 2015).

4.2.4 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). In this study, the more frequently used hoses in the controlled study had higher biofilm TCC. With their stagnation patterns, shower hoses could be thought of as batch reactors. Nutrient loads (other than carbon, which is provided by the hose itself) are determined by frequency of use and their concentration in water (Table S8). Interestingly, the three hoses with the highest biofilm concentration were used frequently during the workweek, but only rarely on weekends. Thus, the nutrient loads in these hoses were high, but 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 should be the theme of future research. Here, in the controlled 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.

4.3 Simple ecological relationships evident in microbiomes

4.3.1 The microbiome of a building drinking water plumbing system is explained by dispersal and selection. In the controlled 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 drinking water plumbing system is inevitably dictated by dispersal from the cold distribution system water. However, clear 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 dispersal 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. While the DNA-based sequencing methods cannot distinguish between live and dead bacteria, the percentage of intact cells in warm water remained high (average above 50%), indicating that live bacteria contributed substantially to this result (Table S7). 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 dispersal from cold water.
Biofilms in Shower Hoses

Stagnant water was similar in terms of water TCC, diversity and composition, to its source and metacommunity, the warm water. However, dispersal 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 drinking water 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).

4.3.2 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 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 otherwise non-ideal. *Meiothermus* is an interesting example of a genus positively correlating with biofilm TCC. It is slightly thermo-
philic, has a reddish color, causes biofouling, and has been isolated in geothermal springs with iron oxidizing activity (Kolari et al., 2003; Urbieta et al., 2015). Here, *Meiothermus* accounted for 12% of two notably red biofilms from South Africa (Figure S4B) with high iron concentrations (0.09 and 5 µg/cm²), suggesting that temperature, iron oxidation, and high biofilm cell concentrations were all contributing selective factors for the presence of this organism.

### 4.4 Quantifying and managing risk from shower hose biofilms

#### 4.4.1 Shower hose biofilms hold risks.**

Toxic metals and potential opportunistic pathogens were found in shower hose biofilms. Metal exposure is through consumption (drinking), and thus exposure from showers would be low, but not obsolete (e.g., brushing teeth in shower). Additionally, particulate lead is sporadically detected in building drinking water (Deshommes et al., 2010), and could result from biofilm detachment (i.e., dislodged during hose manipulation). However, the metal biofilm concentrations in the shower hoses (e.g., 75 ng-Pb/cm²) are probably lower than detected in distributions systems (Lehtola et al., 2004), and thus the metal concentrations in the biofilms are unlikely to be a major contributor to risk.

All of the reported opportunistic pathogens present a risk specifically for showering, with routes of infection either through inhalation or wound exposure (Falkingham et al., 2015). The presence of opportunistic pathogens in these biofilms agrees with their identification in previous local surveys (Collins et al., 2017; Moat et al., 2016; Soto-Giron et al., 2016). However, it is difficult to interpret the clinical relevance of the concentrations, since little is understood about (1) how these biofilms detach into the water phase and (2) what dose is required for infection. Moreover, for reported genera derived from sequencing data, species and strain level definition was not possible, and thus these do not necessarily present risks.
Biofilms in Shower Hoses

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

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

Stagnant water, which is influenced by detached biofilm, had the highest potential risk of the three water types tested. However, biofilm was not the only source for these genera, since the cold water was also frequently positive for potential opportunistic pathogens. In a previous study of showers in the UK, only 40% of stagnant water samples positive for Legionella were also positive in shower hose biofilm swabs (Collins et al., 2017), further indicating that other sources (e.g., cold water distribution systems) may be equally important for shower exposure.
4.4.2 Shower hose biofilms represent a meaningful sampling point. Water samples are the easiest type of sample to collect from household drinking water plumbing systems (Wang et al., 2017). However, < 2% of bacterial biomass of distribution systems is present in the planktonic phase, with most biomass in biofilms and loosely deposited material (Liu et al., 2014). Our data, in combination with the available evidence on real shower environments (Collins et al., 2017; Feazel et al., 2009; Soto-Giron et al., 2016), suggest that opportunistic pathogens are harbored in shower-related biofilms. Shower hoses offer one of the most convenient sampling locations for biofilm monitoring in building drinking water plumbing (i.e., excluding wastewater plumbing). Most components of building drinking water plumbing are not removable and are difficult to sample (e.g., manual swabbing), thus limiting reproducibility and representativeness. In contrast, shower hoses can be removed and replaced, offering large sections (350 – 800 cm²) of easily accessible biofilms. An aged biofilm may also be more representative of the building drinking water plumbing system than a grab water sample (i.e., accumulation of metals over time, variable disinfection concentrations and temperature in water). Such a biofilm-based approach will complement the wealth of knowledge on building drinking water plumbing water, and is necessary for a more complete understanding of the ecology and risks of household drinking water plumbing.

4.4.3 Shower hoses can be managed better. Shower hoses offer a critical area for improvement of building drinking water plumbing management strategies. These hoses typically fall outside potable water material regulations, but clearly microbiology needs to be considered. As it stands, consumers assume all risks and receive little guidance about this portion of easily controlled building drinking water plumbing. Moreover, shower hoses are not necessary for all homes, and could be replaced with safer hard pipe material. Hospitals and old-age homes are generally better informed about limiting risk from shower environments. Programs for quarterly shower hose and head replacement aimed at reducing risk are available for such facilities (Table S9). Given the tenuous relationship between age and biofilm TCC in this study, a more radical strategy, like single-use shower hoses (Table S9), may be
necessary for high-risk patients. Less aggressive strategies, such as allowing shower hoses to dry between uses, are not likely sufficient to prevent colonization by potential opportunistic pathogens (Whiley et al., 2015). In any case, an effective management of building drinking water plumbing should be backed up by effective monitoring (4.4.2 above).
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5. Conclusions

In a global survey and controlled study, shower hoses were characterized in detail in terms of biofilm concentrations, metal accumulation, and microbiome composition. While many factors were uncontrolled, some trends were apparent:

- When disinfectants were used, cell concentrations and diversity were reduced.
- Young shower hose age and bad material quality likely contributed to high cell concentrations.
- Frequent, but irregular use likely contributed to high cell concentrations.
- The microbiome along the path of water delivery in a building could be explained by dispersal and selection.
- Biofilm microbiome composition was shaped by several factors, some of which co-varied with cell concentration.
- Potential opportunistic pathogens were detected, with either a positive (*Legionella*) or negative (*Mycobacterium, Pseudomonas*) correlation with cell concentration.
- Metals accumulated over time in biofilms, likely originating from other building drinking water plumbing components.

While more controlled studies should further explore these trends, it is clear that shower hoses offer a perfect biofilm sampling point to complement building water studies, and that shower hoses should be considered in building drinking water plumbing risk management strategies.
Acknowledgements

The authors acknowledge financial support from MERMAID, a Marie Sklodowska-Curie Initial Training Network, under grant no. 607492, and the Swiss National Science Foundation (SNF grant number 31003A_163366/1). Jurg Sigrist and Lisa Neu contributed to lab-work. Standards for opportunistic pathogen qPCR reactions were supplied by the lab of Amy Pruden (Virginia Tech), with special thanks to Emily Garner for coordination. Karin Beck and Helmut Bürgmann assisted with 16S qPCR data acquisition and evaluation. Sequencing data analyzed in this paper were generated in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich, with support and protocols from Aria Minder and Silvia Kobel, and with raw sequencing data analysis performed by Jean-Claude Walser. Lastly, the authors thank all sample volunteers, especially local coordinators, including Marta Vignola, Ameet Pinto, Marta Kinnunen, Svetlana Perovic, Stefanie Imminger, Xiaofei Wang, Alina Nescerecka, and Sam Van Nevel.

Author Contributions

CP led experimental design, data acquisition, data interpretation, and writing. MR and BV contributed to data acquisition and interpretation. FH contributed to experiment design, data interpretation, and writing.
Chapter 3

References


Biofilms in Shower Hoses


EDI, 2016. The Swiss Federal Department of Home Affairs. Regulation of the EDI about drinking water and water in publicly accessible baths and showers. (TBDV) 807.022.11.


Chapter 3


doi:10.1038/ismej.2017.14


doi:10.1007/s10295-003-0047-z


Liu, G., Bakker, G.L., Li, S., Vreeburg, J.H., Verberk, J.Q., Medema, G.J., Liu, W.T., Van Dijk, J.C.,
Biofilms in Shower Hoses


75
abundances. ISME J. doi:10.1038/ismej.2016.117


Biofilms in Shower Hoses


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

<table>
<thead>
<tr>
<th>Article title</th>
<th>Description</th>
<th>Link</th>
<th>Screenshot Page (After Table S9)</th>
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<td>Parisian Macao battling Legionnaire’s Disease outbreak (April 25, 2017)</td>
<td>A casino in China may have been involved in 3 cases of Legionnaire’s disease for elderly visit. Before test results are in, the casino is doing precautionary measures like closing the swimming pools, jacuzzis, and fountains. The article speculated about possible drops in tourism to the casino.</td>
<td><a href="https://calvinayre.com/2017/04/25/casino/parisian-macao-legionnaires-disease-outbreak/">https://calvinayre.com/2017/04/25/casino/parisian-macao-legionnaires-disease-outbreak/</a></td>
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<td>Walton pool and gym reopen almost three months after legionella bug outbreak (February 24, 2017)</td>
<td>A gym/athletic center in the UK involved in a late 2016 Legionella outbreak, with tests of shower water coming back positive for the bacteria, was forced to close for three months in order to modify the water system and eliminate <em>Legionella</em>. The period was hard to estimate and reopening was frequently pushed back.</td>
<td><a href="http://www.clactonandfrintongazette.co.uk/news/15115947.Pool_and_gymreopen_almost_three_months_after_legionella_bug_outbreak/">http://www.clactonandfrintongazette.co.uk/news/15115947.Pool_and_gymreopen_almost_three_months_after_legionella_bug_outbreak/</a></td>
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Table S2: Additional information about shower hoses used in global survey.

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<th>Country (# of samples)</th>
<th>Location (Number of samples)</th>
<th>Chlorine use</th>
<th>Notes on water</th>
<th>Estimated minimum age (years)</th>
<th># people using shower</th>
<th>Household type</th>
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* Locker room in an office building. Use-patterns are variable, but as these showers were also used in the controlled study, more information about use patterns is available in Table S6.
Table S3: Elemental Analysis Method Details

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<th>Element</th>
<th>Target isotope (m/z)</th>
<th>Reaction mode (1=no gas, 2=H, 3=He)</th>
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Table S4: qPCR method details

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<th>Sequence</th>
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<th>Reaction timing</th>
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<td>denaturation</td>
<td>(Wang, 2012)</td>
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<td>120 95 °C</td>
<td>(Nazari an, 2008)</td>
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<td>2</td>
<td>30 72 °C</td>
<td></td>
</tr>
<tr>
<td><strong>Mycobacterium avium – 16S rRNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYCGEN-F (5µM)</td>
<td>AGA GTT TGA TCC TGG CTC AG</td>
<td>0.8</td>
<td>denaturation</td>
<td>(Wang, 2012)</td>
</tr>
<tr>
<td>MYCAV-R (5µM)</td>
<td>ACC AGA AGA CAT GCG TCT TG</td>
<td>0.8</td>
<td>120 95 °C</td>
<td>(Wilton, 1992)</td>
</tr>
<tr>
<td>water</td>
<td></td>
<td>2.4</td>
<td>40 cycles</td>
<td></td>
</tr>
<tr>
<td>Evagreen</td>
<td>EvaGreen Supermix (Bio-Rad)</td>
<td>5</td>
<td>8 98 °C</td>
<td></td>
</tr>
<tr>
<td>Template</td>
<td></td>
<td>1</td>
<td>30 68 °C</td>
<td></td>
</tr>
</tbody>
</table>
**Table S5: Details for Illumina sequencing**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicon PC Primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakt_341F</td>
<td>CCTACGGGNGGCGWGCAG</td>
<td>Klindworth, 2013</td>
</tr>
<tr>
<td>(S-D-Bact-0341-b-S-17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakt_805R</td>
<td>GACTACHVGGGTATCTAATCC</td>
<td></td>
</tr>
<tr>
<td>(S-D-Bact-0785-a-A-21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nextera adapter tail before forward</td>
<td>TCG-TCG-GCA-GCG-TCA-GAT-GTGTAT-AAG-AGA-CAG-CA</td>
<td></td>
</tr>
<tr>
<td>Nextera adapter tail before reverse</td>
<td>GTC-TCG-TGG-GCT-CGG-AGA-TGTGTA-TAA-GAG-ACA-GAG</td>
<td></td>
</tr>
<tr>
<td>PCR Details</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td>Holding Cycling Reps Cycling Kti/Mix and Reaction Chemistry</td>
<td>Template/Notes</td>
</tr>
<tr>
<td>Amplicon PCR</td>
<td>95 °C 95 °C 0:30 19 X 54 °C 0:30 5:00 72 °C 0:30 1U KAPA 2G robust HotStart Polymerase (KAPA Biosystems, Boston, USA), 1 x reaction buffer B, and 0.4 µM of each primer in a final volume of 25 µL. Sensoquest Labcycler Basic used.</td>
<td>Two sets of frame-shifted primer sets were used on each replicate extraction per sample: Sets 0 and 2 for replicate A and sets 1 and 3 for replicate B 2 µL DNA template (0.8-50 ng)</td>
</tr>
<tr>
<td>Index PCR</td>
<td>95 °C 95 °C 0:30 10 X 55 °C 0:30 3:00 72 °C 0:30 1 X KAPA HiFi HotStart Ready Mix and 5 µl of each of the respective Nextera index primers in a total reaction volume of 50 µl</td>
<td>Pooled amplicon PCR product</td>
</tr>
<tr>
<td>Additional Steps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purification of Amplicon PCR product</td>
<td>Agencort AMPure beads XP system (Beckman Coulter)</td>
<td>Supplier’s protocol</td>
</tr>
<tr>
<td>Purification of Index PCR product</td>
<td>Agilent Bioanalyzer</td>
<td>Supplier’s protocol</td>
</tr>
<tr>
<td>Quality Control of Index PCR product</td>
<td>KAPA library quantification kit</td>
<td>Supplier’s protocol</td>
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</table>
Table S6: Details for Illumina sequencing data processing

<table>
<thead>
<tr>
<th>Step</th>
<th>Algorithm/Version</th>
<th>Parameters</th>
<th>Citation</th>
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</thead>
<tbody>
<tr>
<td><strong>Quality Control</strong></td>
<td>FastQC v.0.10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Merge Reads</strong></td>
<td>FLASH v1.2.9</td>
<td>minimum overlap: 40</td>
<td>(Magoc and Salzberg, 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>maximum overlap: 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max mismatch density: 0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Trim adaptor sequences and sort frame shifts</strong></td>
<td>Cutadapt v1.4</td>
<td>error rate: 0</td>
<td>(Martin, 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Quality Filtering</strong></td>
<td>PRINSEQ-lite v0.20.4</td>
<td>size range: 450-550 bp</td>
<td>(Schmieder and Edwards, 2011).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>minimum mean quality score: 25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>no ambiguous nucleotides</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC range: 20-80</td>
<td></td>
</tr>
<tr>
<td><strong>OTU clustering</strong></td>
<td>usearch v7.0.1090</td>
<td>identity cutoff: 97%</td>
<td>(Edgar, 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>abundance sorting: 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chimera filtering</td>
<td></td>
</tr>
</tbody>
</table>
Figure S1: Optical Coherence Tomography (OCT) images of biofilms from 2 hoses from Belgium (Hose 1) and Switzerland (Hose 2). All are on an equal scale. A and B are from opposite ends of the same hose (Hose 1). C, E, and F are from one 5 cm piece on one end of a hose, while D is from the opposite end of the same hose (Hose 2).
Figure S2: Original black and white images (zoomed out) of biofilms on shower hoses, corresponding to Figure 1E (top) and 1F (bottom). In Figure 1, only particular sections were shown in order to focus attention. Color was also added artificially to highlight key biofilm elements.
Figure S3: Correlation between various measures of biofilm concentration. All scatter-plot points represent values for individual shower hose samples, colored by country of sample origin. Some factors (Total cells, intact cells, ATP, and 16S qPCR gc) are transformed as indicated due to the wide spread of values.

Further discussion about biofilm concentration measures:
- TCC, ICC, and ATP had strong correlations with one another. These all target primarily bacteria. ICC and ATP both target living biomass.
- qPCR did not correlate well with TCC in this study. This could be because the efficiency of extra processing steps (e.g., DNA extraction) was affected unequally by inorganic deposits that varied unequally across biofilms (e.g., iron).
- TOC was likely influenced by both cells and extracellular polymeric substance (EPS) production. It may have captured some aspects of 'sliminess', which is otherwise difficult to quantify.
- OD did not correlate well with TCC. While cells likely contributed to OD, other factors contributed to the color, and thus the OD of the suspended biofilms. For example,
red color can be caused by inorganic (iron deposition) and organic (*Meiothermus* bacteria). Hardness deposition (calcium, magnesium) likely contributed with opaque white particles. OD did correlate with several metals (Spearman’s ρ for lead:0.68, iron:0.67, copper:0.86, calcium:0.40, and manganese:0.51).

**Methods.**

**Intact cells (ICC) analysis for biofilm suspensions**
Measurement of ICC was the same as for TCC, except for the stain used. Propidium iodide (PI; 30 mM) was mixed with the SYBR® Green I working solution to a final PI concentration of 0.3 mM. 200 µL was stained with SGPI at 10 µL/mL. Samples were preheated to 35 °C (3 min), then incubated with stain in the dark for 10 min at 35 °C before measurement. Flow cytometric measurements were performed, as described previously, using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium). Data analysis was performed using the BD Accuri CFlow® software, following the procedure described previously (Prest et al., 2013) to calculate ICC.

**Adenosine tri-phosphate (ATP) analysis for biofilm suspensions**
Total ATP was determined using the BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turnier Biosystems, Sunnyvale, CA, USA) as described elsewhere (Hammes et al., 2010). A biofilm suspension sample (100 µL) and the ATP reagent (100 µL) were warmed to 38 °C simultaneously in separate sterile Eppendorf tubes. The sample and the reagent were combined and then the luminescence was measured after 20 second reaction time at 38 °C. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known ATP standard (Promega). ATP was measured in triplicate, and the relative standard deviation among technical replicates was below 4%.

**16S qPCR**
Quantification of the 16S gene were completed as previously described (Proctor et al., 2016). Briefly, the primers Bact349F/Bact806R and probe Bac516F (Takai and Horikoshi, 2000) and were performed using LightCycler 480 Probes Master hot start reaction mix (Roche). Either 100-fold or 1000-fold sample dilutions were used. qPCR reactions were performed on a LightCycler 480-II (Roche) and analyzed using the LightCycler 480 ver. 1.5.1 software (Roche).

**Total organic carbon (TOC)**
TOC concentration was determined by thermal oxidation to CO₂ and infrared detection with the non-purgeable organic carbon method according to EN 1484 (TOC-VCPH, Shimadzu, Kyoto, Japan). All samples were diluted tenfold with a purified water with TOC < 5 µg/mL organic carbon.

**Optical Density**
Optical density of biofilm suspensions was measured on an Uvikon 930 spectrophotometer (Kontron Instruments, Germany).
Table S7: Water quality and use patterns for 15 samples in the controlled study

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of People</th>
<th>Uses per Week</th>
<th>Notes</th>
<th>Household use patterns</th>
<th>Nitrogen (mg/L)</th>
<th>Phosphorous (µg/L)</th>
<th>Magnesium (mg/L)</th>
<th>Calcium (mg/L)</th>
<th>Total Cells (cells/mL)</th>
<th>% Intact cells</th>
<th>% Intact Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>14</td>
<td></td>
<td></td>
<td>4.7</td>
<td>7.8</td>
<td>2.5</td>
<td>5.00</td>
<td>2.44E+06</td>
<td>46</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td>0.9</td>
<td>7.3</td>
<td>7.5</td>
<td>49.2</td>
<td>2.55E+05</td>
<td>40</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>locker room</td>
<td>8</td>
<td>*same room as 6</td>
<td>often stagnant on weekend</td>
<td>2.56</td>
<td>6.7</td>
<td>11.5</td>
<td>71.0</td>
<td>7.72E+05</td>
<td>42</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>locker room</td>
<td>20</td>
<td>*same room as 5</td>
<td>often stagnant on weekend</td>
<td>3.14</td>
<td>4.9</td>
<td>12.4</td>
<td>76.1</td>
<td>1.77E+06</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>locker room</td>
<td>30</td>
<td>*same room as 4</td>
<td>often stagnant on weekend</td>
<td>3.14</td>
<td>4.9</td>
<td>12.4</td>
<td>76.1</td>
<td>1.77E+06</td>
<td>34</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>locker room</td>
<td>9</td>
<td>*same room as 3</td>
<td>often stagnant on weekend</td>
<td>2.56</td>
<td>6.7</td>
<td>11.5</td>
<td>71.0</td>
<td>7.72E+05</td>
<td>38</td>
<td>58</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>2</td>
<td>**same household as 14</td>
<td></td>
<td>1.0</td>
<td>6.8</td>
<td>9.6</td>
<td>56.5</td>
<td>1.85E+05</td>
<td>55</td>
<td>52</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>15</td>
<td></td>
<td></td>
<td>3.68</td>
<td>3.0</td>
<td>1.3</td>
<td>7.19</td>
<td>2.70E+04</td>
<td>70</td>
<td>73</td>
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<tr>
<td>9</td>
<td>5</td>
<td>16</td>
<td></td>
<td></td>
<td>1.0</td>
<td>3.0</td>
<td>8.0</td>
<td>50.7</td>
<td>1.59E+05</td>
<td>63</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
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<td>21</td>
<td></td>
<td></td>
<td>0.92</td>
<td>3.9</td>
<td>7.9</td>
<td>51.0</td>
<td>6.07E+04</td>
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<td>76</td>
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<tr>
<td>11</td>
<td>2</td>
<td>14</td>
<td></td>
<td></td>
<td>6.0</td>
<td>3.0</td>
<td>9.6</td>
<td>32.4</td>
<td>2.53E+05</td>
<td>57</td>
<td>75</td>
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<tr>
<td>12</td>
<td>2</td>
<td>17.5</td>
<td>often stagnant on weekend</td>
<td></td>
<td>1.11</td>
<td>6.9</td>
<td>8.5</td>
<td>53.7</td>
<td>7.27E+04</td>
<td>63</td>
<td>77</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>9.5</td>
<td>Typically only used for high temperature cleaning</td>
<td></td>
<td>2.30</td>
<td>7.2</td>
<td>13.1</td>
<td>113.9</td>
<td>8.10E+04</td>
<td>72</td>
<td>77</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>14</td>
<td>**same household as 7</td>
<td></td>
<td>1.0</td>
<td>6.8</td>
<td>9.6</td>
<td>56.5</td>
<td>1.85E+05</td>
<td>47</td>
<td>62</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>11</td>
<td></td>
<td></td>
<td>0.84</td>
<td>9.4</td>
<td>6.6</td>
<td>47.6</td>
<td>1.69E+05</td>
<td>54</td>
<td>51</td>
</tr>
</tbody>
</table>

* Hoses 3 – 6 were from two locker rooms (men’s, women’s) in the same building.
** Hoses 7 and 14 were from two bathrooms in the same apartment.
† Hot water samples as described in methods.
Water Quality Methods
Water quality parameters were quantified by the AuA laboratory of Eawag, Switzerland. Briefly, nitrogen was measured via chemiluminescence utilizing Shimadzu TOC-L CSH. Phosphorus (Total-P) was measured after chemical digestion with potassium peroxide in the autoclave (Truttnauer/Systec 2540 EL) at 121°C followed by the spectrophotometric determination of ortho-phosphate after the reaction to phosphorus-molybdenum-blue-complex. Total-P was measured on a Spektrophotometer Varian Cary 50 Bio. Magnesium and calcium were measured using Ion chromatography (Column: Metrohm C6 – 250/4.0) with a Metrohm 930 Compact Flex. Intact cells were measured as described with Figure S3. The percentage is determined by dividing the intact cell concentration by the total cell concentration.

Table S8: Correlation values between use patterns and calculated weekly doses and biofilm concentration. Calculated weekly loads were derived by multiplying the number of uses per week by water quality concentrations (nitrogen, phosphorous, magnesium, calcium or total cells in the cold flowing water, (Table S7)) and the volume inside a shower hose. For all, N=15 for the 15 biofilm samples. Uses per week alone (e.g., without multiplying by a water quality parameter) had the strongest correlation with biofilm TCC, while other water quality measurements had only moderate correlations. That is to say, no single nutrient or load was controlling the system with stronger predictive power than frequency of alone. It could be either a combination of nutrients/cells or some other aspect of frequency of use that controls the biofilm TCC.

<table>
<thead>
<tr>
<th>Calculated weekly load</th>
<th>Correlation with biofilm total cell concentration [log_{10}(cells/cm^2)]</th>
<th>(Pearson's test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R</td>
</tr>
<tr>
<td><strong>Uses</strong></td>
<td>use/week</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>Total nitrogen</strong></td>
<td>mg/week</td>
<td>0.61</td>
</tr>
<tr>
<td><strong>Total phosphorous</strong></td>
<td>µg/week</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>Magnesium</strong></td>
<td>mg/week</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Calcium</strong></td>
<td>mg/week</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>Total cells</strong></td>
<td>log_{10}(cells)/week</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Figure S4: Photo from outside of a shower hose, with metal partially removed (left), and the inside of a shower hose with a particularly red/orange biofilm (right). On the left, biofilm formed yellow spots, resembling bacterial colonies on agar. On the right, the biofilm had a rough texture and deep red/brown/orange color.
Figure S5: Concentrations of metals (lead, iron, copper, calcium, and magnesium) and biofilm total cell concentration (biofilm TCC), with their correlations. Points represent individual hoses, colored by country. None of the metals correlated well with biofilm TCC. There were many non-detects for lead, iron and copper, but when present, there tended to be positive relationships between these metals – i.e., these metals precipitated together in biofilms. Calcium and magnesium were present in all biofilms, and had a positive relationship with each other. While the first three metals likely originate from up-stream pipes in the distribution system, calcium and magnesium likely originate from hardness in the water.
Chapter 3 – Supplementary Information

Figure S6: Correlational analyses between relative abundance of genera (sum of all OTUs identified within that genera) and biofilm total cell concentration (TCC) (log10 transformed). Points represent individual hoses, colored by country. Spearman rank correlations noted for correlations with biofilm TCC, with (*) indicating p<0.001. These are the three most significant positive and negative correlations from the top 10 most abundant genera, but these do not necessarily represent the strongest correlations. For example, a significant positive correlation was found between *Legionella* and biofilm TCC (Spearman’s ρ=0.36, p<0.001). This analysis focused on the top 10 most abundant genera because more frequent non-detects with less abundant OTUs likely affected results strongly. Notably, two genera from within the same family (*Sphingomonas* and *Sphingobium*) were both amongst the top 10 most abundant genera, but had opposite correlations.
Figure S7: NMDS representing Bray-Curtis dissimilarities between samples in the controlled study. Samples consist of 15 biofilms, and matching stagnant water, warm running water, and cold running water. Ellipses represent 95% confidence intervals for each household. These ellipses are generally narrow across an NMDS2 range, but extend widely through NMDS1 to capture all sample types. Household explained 41% of microbiome variation (Adonis). Thus, while there are similarities between sample types (symbol type and color), there are some strong similarities in a household. This could, for example, be due to drift into the household (cold flowing water) selecting downstream microbiomes.
Table S9: Products available for addressing biofilms in shower hoses, including quarterly replacement systems and single-use shower hoses.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Description</th>
<th>Link</th>
<th>Screenshot Page (After Table S9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quarterly Replacement</td>
<td>TSafe® Replacement Hand and Wall Showers</td>
<td>Shower hoses and heads made with antibacterial coatings. With four different colors, they can be replaced quarterly, or taken off for cleaning.</td>
<td><a href="https://www.grahamassetmanagement.co.uk/DataBaseDocs/nav_6024781__replacement_shower_brochure_oct_2016.pdf">https://www.grahamassetmanagement.co.uk/DataBaseDocs/nav_6024781__replacement_shower_brochure_oct_2016.pdf</a></td>
</tr>
<tr>
<td></td>
<td>Dupal L8® Shower</td>
<td>The company replaces antimicrobial shower heads and hoses quarterly, and collects them for recycling. The system uses four different colors to keep track of replacement.</td>
<td><a href="https://www.antibacterialshower.co.uk/">https://www.antibacterialshower.co.uk/</a></td>
</tr>
<tr>
<td></td>
<td>Challis Ag+® Antimicrobial shower hoses</td>
<td>Made with anti-microbial plastics, and four different colors to keep track of replacement. The company delivers new hoses and collects old ones for recycling.</td>
<td><a href="http://www.alchallis.com/environment/environmental_policy.php">http://www.alchallis.com/environment/environmental_policy.php</a></td>
</tr>
<tr>
<td>Single-use</td>
<td>Steri-Spray Steri-Cleanse Showering Attachment</td>
<td>Flexible single-use hose which easily connects to Steri-Spray shower systems. Designed to be used once to limit patient-to-patient contamination, it also ensures that biofilm does not grow.</td>
<td><a href="http://www.steri-spray.com/shower-attachment/">http://www.steri-spray.com/shower-attachment/</a></td>
</tr>
</tbody>
</table>

References

Web screenshots (referred to in Table S1 and Table S9).
Biofilms in Shower Hoses


Key Features & Benefits

• Reduced maintenance and cleaning costs
• Unique removable faceplate so shower can be cleaned between
• Coloured locking pawl matches the colour on the faceplate lock ring
• Locking rings & GRP washer
• Reduced risk of biofilm formation
• No dependence on water pressure
• Easy to install
• WRAS approved
• UK manufactured

Hand Shower and T-Safe®

The T-Safe® Replacement Hose Kit

• 3/4" or 1" male thread
• TWIG® and GRP
• No dependence on water pressure
• No interference with shower function
• Easy to install
• Manufactured in the UK
• WRAS approved
• UK manufactured

Replacement Hand and Wall Showers

• 3/4" or 1" male thread
• TWIG® and GRP
• No dependence on water pressure
• No interference with shower function
• Easy to install
• Manufactured in the UK
• WRAS approved
• UK manufactured

Legionnaires’ disease diagnosed in Fresno nursing home patient

The Apache Summit Mall, one of the largest gathering places in Fresno, has been shut down after Legionnaires’ disease was diagnosed in a patient who stayed there. The patient, who was staying at the hotel for a convention, was diagnosed with the disease, which is caused by a type of bacteria that can be found in warm water. The hotel has been closed for cleaning and disinfection, and the mall has been temporarily shut down as well.

The Legionnaires’ disease outbreak is the most serious in the United States in recent years. The disease is caused by a type of bacteria that can be found in warm water, and it can be spread through the air when the water is heated. The bacteria can cause pneumonia and other severe respiratory illnesses, and it can be fatal in some cases.

The hotel has been closed for cleaning and disinfection, and the mall has been temporarily shut down as well. The hotel and mall will be cleaned and disinfected for at least 24 hours before they can reopen.

The Fresno County Department of Public Health has been working with the hotel and mall to ensure that the cleaning and disinfection process is thorough. The department has also been working with the hotel and mall to ensure that the cleaning and disinfection process is thorough.

The Apache Summit Mall is located in the heart of downtown Fresno and is home to a number of popular restaurants and stores. The mall is one of the busiest in the United States, and it is a popular destination for shoppers, diners, and tourists.

The hotel and mall are not the only places that have been affected by the Legionnaires’ disease outbreak. Several other hotels and public places in the United States have been affected by the disease in recent years.

The Legionnaires’ disease outbreak is the most serious in the United States in recent years. The disease is caused by a type of bacteria that can be found in warm water, and it can be spread through the air when the water is heated. The bacteria can cause pneumonia and other severe respiratory illnesses, and it can be fatal in some cases.

The hotel and mall will be cleaned and disinfected for at least 24 hours before they can reopen. The department has also been working with the hotel and mall to ensure that the cleaning and disinfection process is thorough.

The Fresno County Department of Public Health has been working with the hotel and mall to ensure that the cleaning and disinfection process is thorough. The department has also been working with the hotel and mall to ensure that the cleaning and disinfection process is thorough.
Chapter 3 – Supplementary Information

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11 of 16
Biofilms in Shower Hoses

Benefits To The Hospital Trust

- challis showerheads can significantly reduce bacteria growth on showerheads, helping prevent biofilm growth and scale formation.
- challis showerheads have a fixed spray face which is easy to clean, making any changes to your current cleaning regime. But with them, you can save up to 50% on water, energy & carbon reduction.
- new showerheads will save money, as they are WRAS approved.
- maintenance staff install new showerheads/hoses.
- before the start of the second quarter new showerheads delivered to each ward.
- we supply the appropriate number of showerheads/hoses to the hospital.
- each water head is bagged ready for collection.

Infection Control Benefits

- challis showerheads are not associated with legionella bacteria to grow on them. Challis showerheads have a fixed spray face which is easy to clean, making any changes to your current cleaning regime.
- because of this, challis showerheads can significantly reduce bacteria growth on showerheads, helping prevent biofilm growth and scale formation.
- challis showerheads have a fixed spray face which is easy to clean, making any changes to your current cleaning regime.
- with them, you can save up to 50% on water, energy & carbon reduction.
- maintenance staff install new showerheads/hoses.
- before the start of the second quarter new showerheads delivered to each ward.
- we supply the appropriate number of showerheads/hoses to the hospital.
- each water head is bagged ready for collection.

Water, Energy & Carbon Reduction

- we are the only commercial manufacturer to have OPEX accreditation, so we can reduce your operational costs of 12 months of savings of the ROI.
- challis showerheads have a fixed spray face which is easy to clean, making any changes to your current cleaning regime.
- with them, you can save up to 50% on water, energy & carbon reduction.
- maintenance staff install new showerheads/hoses.
- before the start of the second quarter new showerheads delivered to each ward.
- we supply the appropriate number of showerheads/hoses to the hospital.
- each water head is bagged ready for collection.

Environmentally friendly - showerheads recycled instead of incinerated.

Less need to store and handle cleaning chemicals.

Fast ROI - showerheads can save up to 50% on water, energy & carbon reduction.

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Maintenance staff install new showerheads/hoses.

We supply the appropriate number of showerheads/hoses to the hospital.

Each water head is bagged ready for collection.

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Chapter 4

Biofilms in shower hoses – choice of pipe material influences bacterial growth and communities

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Caitlin R. Proctor, Marja Gächter, Stefan Kötzsch, Franziska Rölli, Romina Sigrist, Jean-Claude Walser, Frederik Hammes
Abstract

Flexible polymeric pipe materials are commonly used as shower hoses or connections to faucets in the last meters of building plumbing, but these tend to leach high concentrations of carbon that encourage bacterial growth. Here we compared the microbiological impact of six such materials, with both a short-term material comparison test and a daily shower simulator operating for eight months. Materials ranked differently in the migration potential and biomass formation potential assays of the comparison test, but overall these results correlated ($R^2 > 0.77$) with long-term biofilm development in the shower simulator. The biofilm concentration after eight months ranged from $2 \times 10^6$ cells/cm$^2$ on the control material (PE-Xc) to $2 \times 10^8$ cells/cm$^2$ on a typical shower hose (PVC-P). However, differences between four of six materials was much less pronounced after eight months than in early months. Communities characterized with 16S rRNA amplicon sequencing clustered with both material and time ($R^2 = 0.31$; $R^2 = 0.25$), and correlated strongly with biofilm concentration ($R^2 > 0.74$). A universal core consisting of 7 genera accounted for 44% of all sequences, and accounted for more of young and high-biomass biofilms. Genera containing opportunistic pathogens were more common in low-biomass pipes. We conclude that choice of materials is not only critical for determining biofilm concentration, but also community composition. Our results show that a seemingly small choice in plumbing material in the final meters of distribution can make a considerable difference in the building plumbing ‘exposome’.

Keywords: shower; drinking water; biofilm; pipe material; plastics
1. Introduction

Flexible materials are commonly used in building plumbing due to their versatility. The final connections to various faucets and appliances (e.g., showerhead or extendable kitchen faucet) are often flexible hoses made from synthetic polymers, rubber, or silicone. Many of these materials leach biodegradable organic carbon in concentrations that essentially nullify carbon reduction efforts in drinking water treatment (Bucheli-Witschel et al., 2012; LeChevallier et al., 1996; van der Kooij, 1992), resulting in biologically unstable water with increased planktonic and biofilm concentrations (Rittmann and Snoeyink, 1984). Flexible materials are not properly considered under water quality regulations, despite the fact that there is clear evidence that flexible materials result in dramatically more biofilm formation than hard plastics, stainless steel, or copper pipes (Bucheli-Witschel et al., 2012; Zhu et al., 2014). Moreover, these final connections are coming under increased scrutiny for biofilm development due to concerns over the establishment and proliferation of opportunistic pathogens (Feazel et al., 2009), and the potential exposure of consumers (Ashbolt et al., 2010). Thus, it is essential that the quality of these final connection materials in the last meter of drinking water distribution be critically assessed for their contribution to biological growth.

The importance of material choice with respect to microbiology in drinking water applications is typically considered only on broad scales, e.g., where metals are compared with plastics and/or cement (Wang et al., 2014; Lin et al., 2013; Zhu et al., 2014; Lu et al., 2014; Ji et al., 2015). These studies demonstrated large-scale differences in nutrient leaching behaviors that contribute to considerable differences in biofilm concentrations and composition. However, within the finer scales of one category such as flexible plastics, there can still be considerable differences in material quality that need to be explored and understood thoroughly (Wen et al., 2015). As government regulations slowly begin to consider the biological impact of pipe materials (DVGW, 2007), industry together with researchers have
developed standardized short-term tests that are useful in developing new materials (Bucheli-Witschel et al., 2012; Wen et al., 2015). While these tests are useful for material evaluation and comparison, it is unknown how well they represent the materials’ behavior in practical use. Materials tend to change their quality with age as leaching diminishes and biofilm establishes (Inkinen et al., 2014; Quevauviller et al., 1991). In addition, realistic use of materials in buildings involves temperature and stagnation selection factors that are not necessarily reproduced in short-term tests or in reactors designed to model distribution systems. Many of these selective factors are favorable for microbial growth and likely dictate development of a specific community that could even encourage opportunistic pathogen proliferation (Feazel et al., 2009).

In the present study, biofilm development was investigated in six plastic pipe materials, commercially advertised for use as potable water or shower hoses. We used a slue of methods to quantify and characterize the microbial communities that developed during (1) material comparison tests and (2) an 8-month daily shower simulation. The novelty of this work is the specific focus on flexible material and the comparison between data from material tests and actual biofilm growth during use. Our results show that a seemingly small choice in plumbing material in the final meters of distribution can make a considerable difference in the building plumbing ‘exposome’.
2. Materials and Methods

2.1 Materials tested

Six commercially available pipe materials with widely varying qualities were chosen for comparison (Table 1). Material A was a hard plastic pipe specifically approved for drinking water use in Europe, and was used as a control against the five flexible pipes. Material B was a flexible pipe, also approved for drinking water, typically used to connect taps (e.g., kitchen faucets) to the main water supply of a house. Material C was a silicone shower hose that meets German drinking water material standards, and Material D was a shower hose with a silver-ion antibacterial coating. Materials E and F were typical shower hoses with a 4-fold price difference. Where possible, information on the material type and legislative certifications is provided in Table 1.

Table 1: Overview of the six materials chosen for comparison

<table>
<thead>
<tr>
<th>Type</th>
<th>Category</th>
<th>Material</th>
<th>Additional details</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Hard</td>
<td>PE-Xc</td>
<td>Approved by DVGW &amp; SVGW *</td>
</tr>
<tr>
<td>B</td>
<td>Flexible</td>
<td>PE-X **</td>
<td>Approved by DVGW &amp; SVGW *</td>
</tr>
<tr>
<td>C</td>
<td>Flexible</td>
<td>Silicone</td>
<td>Approved by DVGW *</td>
</tr>
<tr>
<td>D</td>
<td>Flexible</td>
<td>Unknown</td>
<td>Coated with patented inorganic silver-ion coating</td>
</tr>
<tr>
<td>E</td>
<td>Flexible</td>
<td>PVC-P</td>
<td>With ash addition; expensive hose</td>
</tr>
<tr>
<td>F</td>
<td>Flexible</td>
<td>PVC-P</td>
<td>Cheap hose compared to F+E</td>
</tr>
</tbody>
</table>

* Approval by DVGW (German Technical and Scientific Association for Gas and Water) and SVGW (Swiss Technical and Scientific Association for Gas and Water) are identical and require passing the KTW guideline for migration\textsuperscript{16} and the W270 guideline for microbial enhancement\textsuperscript{13}. Due to purchase in Switzerland or Germany, only these certifications are listed. The material may concurrently pass guidelines and standards from other countries (e.g. BPP [NL], BS 6920 [UK]).

\textsuperscript{16} Cross-linking method unknown.
2.2 BioMig material test package

The BioMig test package (Wen et al., 2015), which was designed to quantify the organic carbon migration potential and biological impact of materials in contact with drinking water, was performed on all six materials. Briefly, the test package consists of two assays, namely a migration potential assay and a biomass formation potential (BFP) assay. The migration potential assay involves seven consecutive 24 hour incubation cycles at 60 °C and measures the total organic carbon (TOC) and the growth potential of bacteria on the TOC that leached from materials on days 1, 3, and 7. Growth potential of the leachate was measured in an assay with an inoculated drinking water microbial community and incubation for 6 days at 30 °C with shaking at 120 rpm (Prest et al., 2016). The BFP assay quantifies both biofilm and planktonic growth with flow cytometry after 14 days of incubation of the test material together with a drinking water microbial community at 30 °C with shaking. Both assays use a surface-to-volume ratio of 1 cm⁻¹, and bottled mineral water (Evian, France) serves as test water and inoculum. In growth potential assays, a minimal medium mix with phosphate (10 mg/L), nitrogen (12 mg/L), and iron (0.3 mg/L) is added to ensure that carbon is the only limiting nutrient.

2.3 Realistic building plumbing simulator (BPS)

A realistic building plumbing simulator (BPS) was constructed to simulate conditions of discontinuous warm water flow that drinking water and shower hoses might experience in a typical system (e.g. daily shower use) (Figure 1). Each pipe material (length 1.5 m, diameter 7 – 11 mm) was connected in triplicate to a building’s hot water connection. The system was connected to an ordinary hot water tap in a laboratory building. A magnetic valve controlled flow, which was split between test materials using X-splitters and 8 mm food-industry quality hoses. The 18 test material hoses were connected in triplicate, with ball valves to control flow in individual hoses.
hot water system where the maximum temperature was 42 °C, and which was flushed once daily for 15 minutes at a flow rate of 1 L/min with the use of a magnetic valve on a timer. While this flow rate was considerably lower than in a real shower, it allowed use of the water pressure directly from the same tap. Between flushing cycles, the BPS was exposed to ambient room temperature (18 °C – 23 °C). All connections between the tap and the BPS were made with a similar length of polyurethane flexible pipe suited for use in the food industry and associated X-splitters (Festo, Esslingen am Neckar, Germany). Ball valves (Festo, Esslingen am Neckar, Germany) placed at the beginning of the test lines were used to control flow and water pressure to be approximately equal between all 18 lines. Water used for flushing was non-chlorinated drinking water (Dübendorf, Switzerland). The water is characterized in detail in Supplementary Table S1.

2.4 Biofilm sampling and detachment

After one, two and eight months of operation, biofilm samples were collected by cutting 30 cm from the distal end of each pipe. The 30 cm length was chosen in order to achieve enough total biofilm suspension volume for analysis and sufficiently capture the heterogeneity of the biofilms, while considering the limitation of total length of pipe. Of the 30 cm cut at each sampling event, 28 cm was used for biofilm removal and quantification via repeated sonication. Each end of each pipe was plugged using a stopper specific to the pipe material. The pipe segment was filled with sterile glass beads (3 mm diameter) and 0.2 µm filtered bottled mineral water (Evian, France). After this stabilization step, samples were stored at 4 °C for a maximum of 3 days before detachment and analysis. For biofilm detachment, the pipe segment with stoppers and glass beads was sonicated by submersion in a sonication bath (Bandelin Sonorex, Rangendingen, Germany) for 5 minutes, and the biofilm suspension (without the glass beads) was removed and collected. This sonication procedure was repeated 5 times with freshly filtered bottled mineral water added each time. After the fifth sonication, glass beads were discarded and the pipes were filled with filtered bottled mineral water and inverted 30 times. This final rinse water was added to the biofilm
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suspension. The entire biofilm suspension (32 – 150 mL) was sonicated with a Bandelin Sonorex device (Sonopuls HD 2200, Bandelin Sonorex, Rangendingen, Germany) with needle at 40% intensity with 50% power for 30 seconds. In the eighth month, a biofilm extraction blank consisting of the filtered water with needle sonication was processed alongside biofilm samples. The biofilm suspension was then subjected to quantification by the means described below. Blanks were not included for each material, and thus initial colonization on the materials may contribute to inter- and intra- hose variation.

2.5 Fluorescent staining and flow cytometry of water samples and biofilm suspensions

Staining and flow cytometric analysis was done as described previously (Hammes et al., 2008; Berney et al., 2008; Prest et al., 2013). In short, for a working solution, SYBR® Green I (SG) (Invitrogen AG, Basel, Switzerland) was diluted 100x in anhydrous dimethylsulfoxide (DMSO). For measurement of intact cells, propidium iodide (PI; 30 mM) was mixed with the SYBR® Green I working solution to a final PI concentration of 0.3 mM. These working solutions were stored at -20 °C until use. From every water sample, 500 µL was stained with SG or SGPI at 10 µL/mL. From every biofilm suspension, 200 µL was stained with SG or SGPI at 10 µL/mL. Samples were preheated to 35 °C (3 min), then incubated with stain in the dark for 10 min at 35 °C before measurement. Flow cytometric measurements were performed, as described previously (Prest et al., 2014), using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium) equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm. The flow cytometer is equipped with volumetric counting hardware, calibrated to measure the number of particles in 50 µL of a 500 µL sample; or 100 µL of a 200 µL sample. Measurements were performed at pre-set flow rate of 66 µL/min. A threshold value of 800 a.u. was applied on the green fluorescence channel (FL1). Data analysis was performed using the BD Accuri CFlow® software, following the procedure described previously (Prest et al., 2013). Briefly, bacterial signal was selected using electronic gating on density plots of green fluorescence (FL1; 533 nm), and red fluorescence.
(FL3; > 670 nm). Total cell concentration (TCC) was calculated with results from SG stain, and intact cell concentration (ICC) was calculated with results from SGPI stain.

2.6 Adenosine tri-phosphate (ATP) analysis for biofilm suspensions

Total ATP was determined using the BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA, USA) as described elsewhere (Hammes et al., 2010). A biofilm suspension sample (100 µL) and the ATP reagent (100 µL) were warmed to 38 °C simultaneously in separate sterile Eppendorf tubes. The sample and the reagent were combined and then the luminescence was measured after 20 second reaction time at 38 °C. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known ATP standard (Promega). ATP was measured in triplicate for all samples, and the relative standard deviation among technical replicates was always below 4%.

2.7 Total organic carbon (TOC)

TOC concentration was determined by thermal oxidation to CO₂ and infrared detection with the non-purgeable organic carbon method according to EN 1484 (TOC-VCPH, Shimadzu, Kyoto, Japan). All samples were diluted tenfold with a purified water with TOC < 5 µg/mL organic carbon.

2.8 DNA extraction

After analysis, the remaining volume of biofilm suspension or water sample was filtered using sterile techniques on a 0.22 µm polycarbonate Nucleopore® membrane filter (47 mm diameter, Whatman, Kent, UK). The filter was inserted into a 5 mL tube and stored at -20 °C before DNA extraction with the Power Water DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to manufacturers’ instructions. The sample processing strategy of filtering the biofilm suspension, which was treated as a liquid sample throughout, leant itself to use of the Power Water DNA Isolation Kit.
2.9 16S qPCR

qPCR reactions were performed on a LightCycler 480-II (Roche) and analyzed using the LightCycler 480 ver. 1.5.1 software (Roche). Quantification of bacterial 16S rRNA genes was carried out using the primers Bact349F/Bact806R and probe Bac516F (Takai and Horikoshi, 2000) (Supplementary Information, Table S2). Reaction conditions are detailed in Supplementary Table S3, and were performed using LightCycler 480 Probes Master hot start reaction mix (Roche), each primer at a concentration of 0.9 µmol/L, and the probe at 0.3 µmol/L. Results were evaluated using the point-fit method for absolute quantification.

Total reaction volumes were 10 µl with a sample volume of 2 µL. DNA extracts with DNA concentrations ranging from 0.1 to 106 µg/mL were diluted 10- and 100-fold in AE buffer (Qiagen) and measured in triplicate. 100-fold dilution results were used, except where results were below the limit of quantification and 10-fold dilution results were used.

Quantification standards were obtained from a suitable PCR product cloned into pGEM T-Easy vector (Promega), purified with a Qiaprep Spin Miniprep Kit (Qiagen), quantified using the Qubit System (Thermo Fisher Scientific) and diluted in 10-fold serial dilutions in AE buffer to concentrations from 500 million to 50 copies per reaction. Standards were measured in quadruplicate. High linearity ($R^2 = 0.998$) in standards was observed. There were no signs of inhibition with a standard spiked into a sample (data not shown).

Limit of detection was determined by the quantification of the biofilm extraction blank that consisted of filtered water processed alongside biofilm suspensions. DNA was extracted from this blank and it was diluted as other samples. Limit of quantification was defined as the most dilute standard detected with standard deviation of Cp (crossing point) < 0.5.

2.10 Amplicon sequencing with Illumina MiSeq

Approximately 1 ng of DNA extract from each sample was subjected to PCR amplification using modified universal bacterial primers Bakt_341F and Bakt_805R(Klindworth et al.,
which target the V3-V5 region of the 16S rRNA gene, and were adapted with a tail to facilitate binding Nextera adapters (Supplementary Information, Table S2). Index PCR was performed to add the Nextera XT v2 Index Kit adaptors (Illumina) to the amplicon. PCR reaction conditions are detailed in Supplementary Table S3. After each PCR reaction, products were purified using the Agencort® AMPure® XP system (Beckman Coulter, Inc., Bera, CA).

Each product was quantified using the Kapa Library Quantification Kit performed on 7500 Fast Real-Time PCR System (Applied Biosystem). Using these concentrations, samples were normalized before running on the MiSeq platform using MiSeq Reagent Kit v2 (300-cycles, #MS-102-2002) according to manufacturer’s protocol. Due to low quality in the first run, a second run, with 50% PhiX instead of 5%, was run to improve quality. All sequencing was done at the Genetic Diversity Centre (GDC) of ETH, Zurich.

2.11 Statistics
To compare quantification of biofilm from the realistic BPS, all measurements of detached biofilm suspensions were normalized to the pipe surface area from which the biofilm was removed. These biofilm surface-normalized concentrations are referred to as biofilm concentrations throughout the text. In the case of TCC, ATP and 16S gene copies, concentrations were then log transformed. All subsequent statistical tests were performed in R (v 3.2.3) (R Development Core Team, 2014) with an alpha of 0.05. A Shapiro-Wilk test was performed and all measures except log (TCC) were found to be not normally distributed, and thus non-parametric methods were used to compare means with these measures. T-tests and Tukey’s HSD were used for TCC, and non-parametric Kruskal-Wallis (de Mendiburu) tests were used for all other measures. Spearman’s rank correlation coefficients were used to compare biofilm concentration metrics.
2.12 Sequencing statistics
The libraries from the two runs were subject to stringent quality control, and these steps, trimming methods, and merging steps are detailed in Supplementary Table S4. The two runs were not significantly different (p=1.0, Adonis) and were thus merged for subsequent analysis in order to increase the total number of reads. The UPARSE workflow was used to cluster the reads into OTUs and create count tables (Supplementary Table S4). A total of 5.6 million (95%) amplicons (e.g. merged and quality filtered reads) from 57 samples were mapped to 828 OTUs. USEarch-UTAX classifier was used to assign taxonomy for the OTUs based on the green-genes database version 13/5 (McDonald et al., 2012). All samples were subsequently scaled to the minimum depth of 14,880 sequences per sample.

In R, Phylloseq (McMurdie and Holmes, 2013) (v 1.14.0) was used for community analysis. Non-metric multi-dimensional (NMDS) scaling using Bray-Curtis dissimilarity was performed for visualization of results. From the vegan package, Adonis (Non-parametric permutation, MANOVA) tests were used to fit experimental factors and the Envfit function was used to fit vectors for numerical variables to the NMDS ordination (Oksanen et al., 2013).

2.13 Measuring realistic stagnation scenarios
In order to determine what stagnation scenario is “realistic” for the building plumbing elements tested in this study, 7 households recorded their water usage during one week. Focus was placed on the kitchen faucet and the shower hose, two locations where flexible hosing is almost always used in Swiss households. Households varied from 2-5 persons/household. Participants were asked to mark the half-hour period in which the tap was opened. For maximum stagnation time, it was also assumed that any marked use could be at the beginning or the end of the half hour period. To calculate total stagnation time, 2 minutes of use per kitchen faucet opening, and 15 minutes of use per shower were used.
3. Results and Discussion

Six different water pipe materials (Table 1) were compared in short-term material tests and a long-term building plumbing simulator (Figure 1). Migration and growth potential assays showed clear material differences (Figures 2 and 3) and largely predicted growth in the BPS operated over 8 months (Figures 4 and 6). Bacterial communities were influenced by both material and time (Figure 7). Core communities (Figure 8) and community membership reflected the differences between materials and the dynamic changes in biofilms over time.

3.1. Short-term material tests show differences in migration and growth behavior between materials

The six materials reacted differently in a comprehensive test package (BioMig (Wen et al., 2015)) that measures migration potential at 60 °C and biomass formation potential (BFP) at 30 °C. Figure 2 shows results from the migration potential assay, with migrated TOC (Figure 2a) and growth potential of the leachate (Figure 2b) from 3 of the 7 migrations (days 1, 3, and 7) stacked to visualize total migration potential. Materials A-C, approved for use in drinking water (Table 1), leached less TOC (0.4 – 4.2 µg/cm²) than Materials D-F (Figure 2a), and had relatively less biological growth supported by the leachate (4.5 – 5.1 x 10⁶ cells/cm²) (Figure 2b). In contrast, Materials E and F leached higher amounts of carbon (9.7 – 10.4 µg/cm²) and the leachate supported substantially more growth (3.8 – 4.8 x 10⁷ cells/cm²). An interesting outlier to this trend was Material D, which leached by far the highest total amount of TOC (54.9 µg/cm²), and supported the least growth (2.7 x 10⁶ cells/cm²). This variant behavior is directly attributed to the inorganic silver-ion coating with antimicrobial properties, which seems to inhibit growth during the first migration (with 38.47 µg/cm² organic carbon leaching, but no detectable growth on this leachate). This effect diminished in later migrations, indicating that much of the coating was removed with the first migration. The efficiency of conversion from leached carbon to cell growth also varied considerably. This proportion of assimilable organic
Biofilms in Shower Hoses – choice of pipe material influenced bacterial growth and communities

carbon (AOC) can be calculated using the expectation that 1 µg of carbon produces $10^7$ cells (Wen et al., 2015). Material C has notably efficient conversion, leaching nearly 100% AOC compared to Materials A, B, E, and F ranging 10% – 65%, and Material D, with antimicrobial inhibition, at 0.5% assimilable carbon (Supplementary Figure S1). With this wide range, it is evident that using TOC leaching from pipes as a sole proxy for biological growth potential is completely erroneous. Many existing materials tests quantify TOC leaching from pipes, but their focus is rather on sensory aspects or potential disinfectant byproduct formation (Heim and Dietrich, 2007; Brocca et al., 2002; Skjevrak et al., 2003; Ryssel et al., 2015), and thus they strive to include this non-utilizable portion, as the complex structures that bacteria cannot break down may contribute to taste and odor compounds.

Figure 2: Migration potential tests for six tested materials showing (A) the total organic carbon (TOC) and (B) growth potential of the migrated carbon. Data are stacked values from three of seven sequential migrations (Mig1, Mig3, and Mig7), with each migration conducted at 60 °C and lasting 24 hours. Error bars are adjusted standard deviations from duplicate migration tests, with triplicate growth potential tests from each duplicate.

The results from the BFP assay are shown in Figure 3, with growth after 14 days in the planktonic and biofilm phases stacked. Similar to the results of the migration potential assay, there is a clear division between Materials A-C and Materials D-F, with Materials A-C
supporting less growth (total BFP of 1.7 – 6.7 x 10^7 cells/cm^2) than Materials D-F (total BFP = 2.9 – 8.3 x 10^8 cells/cm^2). The ranking amongst Materials A-C varies between the BFP and migration potential assays, but the differences between these materials are not extreme in either assay (12% difference in growth supported by leachate and 10% difference in total BFP). The high growth supported by Material D further suggests that any inhibitory effect of the patented inorganic silver-ion coating was short-lived. It also may be localized, as 74% of the growth occurred in the planktonic phase, away from the inhibitory surface. Differences between the migration test and the BFP test can also be driven by the difference in temperatures (60 °C in migration compared to 30 °C in BFP), which could influence both the rate of leaching and the specific compounds released. Results from similarly classified materials ranged from quite different to extremely similar to previous results with the same test-package (Wen et al., 2015), indicating that variation in production quality and further production details (e.g. percent flexibilizer added) can be critical to performance of the materials.

3.2. Realistic use conditions differ from material tests

A survey of 7 households revealed that the maximum stagnation time for an individual flexible pipe typically occurred overnight in shower hoses. The maximum stagnation time observed in one week in a shower hose ranged between 13.5 hours (5 person household) and 49.5 hours (2 person household). The average maximum stagnation time was 28.1
hours for showers, and only 17 hours for kitchen faucets. Interestingly, while the maximum time differed considerably by location in the household, the estimated total stagnation time was similar (23.8 hours per day for kitchen faucets and 23.5 hours per day for showers). Material tests (section 3.1 above) use fixed conditions (time/temperature) over a short duration. For practical reasons, these conditions are not consistent with the actual long-term use of the products, but are rather designed to capture the maximum potential impact of the material in a reasonable amount of time. To measure realistic use, the BPS was run with shower temperature water (42 °C) for a short time (15 minutes), with stagnation during 23.75 hours at ambient room temperature, and a total duration of 8 months. According to this survey, a stagnation time of 23.75 hours reflects shower usage in a household of 1-2 persons.

3.3. Biofilm development in the realistic BPS is influenced by material and time

Over the course of 8 months, biofilm growth observed with a variety of methods (TCC, qPCR for 16S, TOC, and ATP) in a BPS using discontinuous water flow similar to that of a typical daily shower, was highly variable and dependent upon both material and time (Figure 4).

The materials with approval for drinking water applications (A-C) had significantly lower biofilm concentration than Materials D-F across all four measures of biofilm (Figure 4) (p < 0.001, t-test; p < 0.001, Kruskal-Wallis Test). This is driven by Materials A and F, the extremes in each category. When comparing individual materials pair-wise, the only significant differences involved A (monthly averages ranging 7.9 x 10^4 – 6.3 x 10^6 cells/cm²) or F (monthly averages 2.5 x 10^7 – 2.0 x 10^8 cells/cm²). A was significantly lower than all materials except B, and Material F was significantly higher than all materials except D (p < 0.05, Tukey HSD). The similarity between Materials B-E becomes especially obvious in month 8, when average biofilm concentrations ranged only from 1.6 – 3.2 x 10^7 cells/cm². This diminished effect of material with time may be attributed to diminished nutrient leaching.
Figure 4: Biofilm formation on the six tested hose materials quantified with four methods after 1, 2, and 8 months of operation in a realistic building plumbing simulator (BPS). (A) Total cell concentration (TCC), (B) 16S rRNA gene copies (16S) measured by qPCR, (C) total organic carbon (TOC), and (D) adenosine tri-phosphate (ATP) were measured on biofilm suspensions and normalized to the available surface area. Error bars are from biological triplicates of each material type. Technical triplicates for each measurement (for ATP and 16S) are not included in standard deviation calculation. BF Blank refers to a biofilm extraction blank processed with other samples during the month 8 analysis.
While an increase in biofilm concentration is clear (Figure 4), it is difficult to draw conclusions about when or if biofilms entered steady state using only three time points. Biofilm concentrations in month 8 were significantly higher than other months using TCC (material averages ranging $6.3 \times 10^6 - 2.0 \times 10^8$ cells/cm$^2$ compared to months 1 and 2 ranging $7.9 \times 10^4 - 3.2 \times 10^7$ cells/cm$^2$) ($p < 0.03$, Tukey HSD). However, using the other measurements of biofilm concentration, month 8 was not always significantly different than earlier months ($p > 0.05$, Kruskal multiple comparisons). While other studies suggest that steady state for drinking water biofilms is reached in two to three months or less (Lehtola et al., 2004, 2002), it is likely that more time was required in this scenario, with discontinuous flow and inconsistent temperatures. With the high amounts of carbon leaching from the pipes, growth is limited based on the additional influx of nitrogen, phosphorous and other nutrients from the water during flushes. Biofilm concentrations in this study were also higher than those in distribution systems with continuous flow (Liu et al., 2013; Lehtola et al., 2006) and systems with chlorine (Wang et al., 2012b), and had the most similarity (via qPCR) to biofilms on PEX within a building (Inkinen et al., 2014). Due to destructive sampling, a higher temporal resolution could not be achieved in the current study.

Biofilm concentration data from qPCR, TCC, and ATP correlated strongly with each other (Figure 5) ($p > 0.85$, Spearman Rank Correlation). ATP had the strongest correlation to intact cells (ICC) ($p = 0.92$, Spearman Rank Correlation), showing that these two measures of viability and activity are complementary for biofilms, as previously demonstrated for drinking water samples (Siebel et al., 2008). TOC had weaker correlations ($p$ ranging 0.52 to 0.65, Spearman Rank Correlation) with all other measures of biofilm concentration. This is not surprising, as TOC includes organic matter other than cells, such as extracellular polymeric substance (EPS).
Figure 5: Correlations between biofilm concentration measures. For each scatterplot, n = 54, with measurements from 3 time-points on 3 triplicates of 6 material types. Spearman-rank correlation values are superimposed on plots with potential values ranging 0 (no correlation) to 1.00 (perfect correlation). Values less than 0.7 in blue, > 0.7 and < 0.9 in green, > 0.9 in red. Values for the Spearman-rank correlation are identical whether or not values are log-transformed.

3.4. BioMig data predicts material behavior in realistic BPS

The BioMig test package largely predicted the behavior of the six test materials in the realistic BPS for an extended time period. This test package produces several metrics of material performance, but the most comparable metric to biofilm formation in the BPS
(Figure 4A) is the biofilm biomass formed in the BFP test (Figure 3). A log-log correlation between the two TCC measurements by month (Figure 6) showed that the BioMig metric predicted the behavior of the month 2 measurements best, \( R^2 = 0.83 \) (linear regression), compared to month 1 \( R^2 = 0.78 \), and month 8 \( R^2 = 0.77 \). A 1:1 ratio is not achieved in any of the tests, with biomass quantification typically about 1 log lower in the realistic BPS than the BFP test. While the absolute numbers are not the same, both the rankings and degree of difference are reasonably well predicted with the BioMig test package, even when both hard and flexible pipes are considered together.

Monthly variations in correlation patterns are likely related to differences in the growth conditions of the BioMig test and the BPS. In the BioMig test, inorganic nutrients and oxygen are supplied in excess for the 14-day test period, whereas in the BPS, inorganics are supplied only intermittently (water replaced at each shower event) over 8 months. On top of this, growth potential tests of the water used in the BPS showed that it was primarily carbon-limited, but that inorganic nutrient limitations (nitrogen and phosphorous) quickly hindered growth, even with AOC concentrations as low as 1 mg/L (Supplementary Information, Table S5). This theory of multiple nutrient limitations is supported with the data from materials A-C in early months, which has the highest linearity. Here, the inorganics supplied by intermittent water flow were...
likely sufficient to match the low amount of carbon leaching from materials. There is a clear need to investigate the growth of complex bacterial communities under conditions of continuously diminishing organic carbon supply and intermittent supply of inorganic nutrients in more detail.

3.5. Bacterial community structure driven by both material and time

The bacterial communities of all hoses consisted of 19 bacterial phyla from which individual phyla were present above 1% of any given sample. This is comparable to the phyla-level diversity found in other studies (e.g. 9 phyla (Liu et al., 2014) and 14 phyla (Wang et al., 2014)). Five dominant phyla (Proteobacteria, Bacteroidetes, Planctomycetes, Actinobacteria, and Acidobacteria) accounted for 94% of all sequences (range 67% – 99.9% per sample). The most dominant phylum was Proteobacteria (78.8% of all reads; range 23% – 99.9% per sample), most of which were Alpha-Proteobacteria (range 3% – 25% per sample) which often dominates drinking water biofilms (Inkinen et al., 2014; Henne et al., 2012; Liu et al., 2014; Douterelo et al., 2015).

Bacterial communities clustered according to both material and month, with material forming tight clusters within the month that they were measured (Figure 7). Both month and material were significant factors for explaining the differences in community structure (p < 0.001, Adonis) with material explaining slightly more variation than month ($R^2 = 0.31$ and 0.25 respectively, Adonis). The different materials leach different amounts (Figure 2) and different types (Bucheli-Witschel et al., 2012) of organic carbon, which can in turn select and promote growth of different bacteria from the same source (water phase). Such differences have been previously demonstrated with obvious differences in pipe material (e.g. metal, cement, and plastic (Wang et al., 2014)), but not for such a narrow functional group of pipe materials. Neither bacterial concentrations (Figure 4) nor bacterial community structure (Figure 7) stabilized within the experimental time frame. With continued growth and diminishing carbon
leaching from pipe materials, nutrient and oxygen gradients develop and change dynamically, and thus the bacterial communities also shift. Seasonality in the drinking water (Pinto et al., 2014) may also be reflected in the biofilm phase due to daily seeding. Further studies will investigate these dynamics with higher temporal resolution than was possible here due to destructive sampling methods.

**Figure 7**: NMDS calculated with Bray-Curtis similarity between samples from six different hoses during eight months in a building plumbing simulator. Color is by material and symbol shape is by month. Dotted lines and arrows link biological triplicate samples for two materials to highlight the clustering through time. Three technical replicates were applied to one Material F (month 8) DNA extract, and these are linked with a gray triangle. BLK is a blank of the biofilm extraction procedure.
All four measures of biofilm concentration correlated with NMDS analysis ($p < 0.001$, EnvFit), with a range of correlations in a similar direction ($R^2$ range 0.37 – 0.88) (Supplementary Information, Figure S2). Interestingly, when biofilm concentrations measured by TCC were similar in month 8 for Materials B-E ($1.6 – 3.2 \times 10^7$), communities also clustered closely (Figure 7). This co-variance further indicates that the nutritional additions of the pipe material which influenced biofilm concentrations (Figure 3) also drove the community structure. It was recently proposed that such knowledge could be used to manage bacteria under the framework of drinking water ‘probiotics’ (Wang et al., 2013; Proctor and Hammes, 2015). This concept suggests that engineers should encourage the establishment of benign bacterial communities instead of aiming to kill or otherwise minimize all bacteria (e.g. disinfection, anti-bacterial pipes). If materials can be designed to leach carbon that selects for a desirable community structure in the long-term, then shower hoses may offer a very practical avenue for application of these concepts. This is further discussed below with respect to genera that include opportunistic pathogens.

### 3.6. Core communities reflect dynamic changes in biofilms

In order to see which taxa were the most important for community clustering, core communities were calculated by looking at genera that were present in 100% of samples. Figure 8 shows the Universal Core, Transient Core and Material Cores for each set of biological replicates. The Universal Core consisted of genera common to all biofilms (54 samples). The Material Cores consisted of genera common to a particular material throughout time (9 samples). The Transient Cores were common to all samples within a sampling period (18 samples). Genera could belong to both the Material Core and the Transient Core.
Seven genera were common to all biofilm samples (Universal Core), and these genera accounted for 44% of all sequences from normalized samples (range 2% – 98% per sample).

In a similar analysis of drinking water samples from 5 networks in various locations across the US, 78% of all sequences belonged to a universal or network-specific core, even with much more varied pipe materials (Ji et al., 2015). Despite diversity generally being higher in drinking water communities than biofilms (Henne et al., 2012; Liu et al., 2014), material-specific niches develop within biofilms (Liu et al., 2014) which result in more localized biofilm community variation compared to bulk water phase. In all materials, the core community decreased over time, concurrent with diversity increasing in most, but not all materials (Supplementary Information, Figure S3).

The trends for these core communities could be separated by whether materials encouraged low-biomass (A-C) or high-biomass (D-F) development (Figure 4). Materials D-F generally had larger Universal Cores than Materials A-C, and had a higher proportion of Transient Core than Material Core. Materials A-C had smaller Universal Cores, and a higher proportion of Material Core than Transient

**Figure 8:** Core communities by combined relative abundance for six materials at three time points (months 1, 2, and 8) in a building plumbing simulator. The Universal Core (black) represents genera common to all samples, while the Transient Core (grey) was common to all samples during a given month. Material Cores (material-specific color) were present in all samples of a material, and genera shared by the Transient Core and the Material Core are hatched with gray and material-specific color.
Core. The high amounts of material-specific organic carbon leaching Materials D-F likely favored the growth of a few fast-growing bacteria. These few fast-growers, once established, are not likely to be completely displaced (high Universal Core). However, together with rapid biofilm development the available niches change drastically as carbon leaching diminishes and nutrient and oxygen gradients develop, and thus, new bacteria are able to outcompete (higher Transient Core, lower Material Core). With limited organic carbon from the material (A-C), there is little growth to select for universally suited bacteria (low Universal Core). While the bacteria that first attach randomly are not necessarily displaced by competition, these do not necessarily match the bacteria suited for growth in all biofilms (higher Material Core). These biofilms are less subject to changing niches (lower Transient Core).

3.7. Membership in communities is dependent on materials

The seven genera common to all samples were Caulobacter, Bradyrhizobium, Sphingomonas, Methyloversatilis, Legionella, and Phenylbacterium, and unclassified genera within Comamonadaceae. Bradyrhizobium and Comamonadaceae have previously been associated with regrowth in pure water (Proctor et al., 2015), and might be expected as background. Comamonadaceae contributed up to 24% of a single biofilm sample, and thus should be considered an important family for biofilm formation in drinking water. The family Comamonadaceae has also been associated with biological instability in the same region (Lautenschlager et al., 2010), and may have been so prevalent in that study due to detachment from biofilms.

The most prevalent of the core genera, Caulobacter, was most abundant during month 1 and generally decreased with time. Members of Caulobacter are frequently studied for swarm cell growth phase and advantages with both attachment and continuous dispersal within biofilms (Hughes et al., 2012; Bodenmiller et al., 2004; Berne et al., 2010). However, to our knowledge, Caulobacter is not frequently reported as a key member of drinking water.
Biofilms in Shower Hoses – choice of pipe material influenced bacterial growth and communities

biofilms. Other taxa were more prevalent with increasing time. Syntrophobacterales, a strictly anaerobic and sometimes sulfate reducing order, previously associated with EPS in anaerobic membrane bioreactors (Yu et al., 2012), is most abundant in month 8. As biofilms thicken, oxygen and nutrient gradients are created (Liu et al., 2014), and foster these more diverse metabolisms.

Other prevalent taxa had behavior specific to materials, further emphasizing the importance of material choice. The order Sphingomonadales was prevalent across all samples, but was lowest with Material F. *Sphingomonas* have previously been identified in distribution system biofilms (Douterelo et al., 2015; Liu et al., 2014) and in shower curtain biofilms (Kelley et al., 2004). Other orders, Actinomycetales and Solirubrobacterales, were mostly prevalent in low-biomass supporting materials (A-C).

In contrast, some orders tended to appear in high-biomass supporting materials. Sapropirales was most prevalent with Materials D, E and F in later months. *Sediminibacterium*, an iron-oxidizing genera within this order, accounted for most of this, and has been identified in several other drinking water systems (Wang et al., 2012a; Kahlisch et al., 2012). An orange color, perhaps related to iron deposition from earlier plumbing in the system, was most notable on Material D, due to the white colored material that was easily stained (Table 1). On Material F, *Bdellovibrio* was particularly prevalent. As this genus of bacteria are parasites on other bacteria (Welsh et al., 2015), it likely thrived in the high-biomass environment.

While sequencing resolution did not allow for identification of specific opportunistic pathogens, several genera that include opportunistic pathogens were observed. One of the 7 Universal Core genera was *Legionella*, with a maximum of 9% in a sample (month 2 on Material D). The maximum warm water temperature was 42 °C both in this BPS and throughout the building, and this temperature range has been associated with *Legionella*...
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colonization (Bédard et al., 2015). It is likely that *Legionella* was present in the building’s plumbing and inoculated the test system, enabling establishment there. *Pseudomonas* was most common in Materials A and B during month 2, making up 72% and 24% of these samples respectively. *Mycobacterium* and *Nocardia* were mostly associated with Materials C and A, with 16% of the month 2 samples on Material C identified as *Mycobacterium*. Interestingly, Materials A-C, which generally supported a lower total biomass, had higher relative abundance attributed to these four genera (*Legionella, Pseudomonas, Nocardia, and Mycobacterium*) than Materials D-F (Supplementary Information, Figure S4A). Even when considering absolute abundance, Materials A-C consistently had higher values for *Mycobacterium* and *Nocardia* than Materials D-F (Supplementary Information, Figure S4B). Opportunistic pathogens have been reported in shower plumbing previously (Feazel et al., 2009), yet the patterns of their prevalence seem to be complex. This study suggests that plastics supporting low-biomass preferentially select for certain opportunistic pathogen containing genera. While this study was not specifically a pathogen study, the data warrants further investigation into this phenomenon. It may also support the probiotics concepts proposed by Wang et al. (Wang et al., 2013), in that a material with strong leaching behavior that supports a higher biomass can better protect against certain opportunistic pathogens.

3.8. Implications for regulations

Standard commercially available shower hoses (Materials E and F) were made from the same material (PVC-P). Differences between these materials observed in all tests are attributed to unknown production details (e.g. percentage and type of flexibilizer added). However, in all tests excepting the first month of the BPS, these two materials were ranked in the top three biomass producing materials (Figures 2, 3, 4). Interestingly, these hoses are not specifically approved for use in drinking water applications (Wen et al., 2015), yet can be used in shower hoses, as a shower is not legally considered as a drinking water source. This regulation makes little sense, as the most frequently cited biological health concern for
growth in drinking water is opportunistic pathogens, which infect via inhalation, as in showers (Falkinham et al., 2015). At the same time, the microbial communities that materials select for are not considered in material certification processes at all. Certain opportunistic pathogens may have actually preferred the certified materials (Supplementary Information, Figure S4). This study is a clear indication that the testing of the microbiological impact of materials in contact with drinking water should be expanded considerably and should be extended towards all pipe and plumbing materials to which the consumer is ultimately exposed.
4. Conclusions

This study demonstrates the importance of attention to detail when planning a building plumbing system. In shower hoses, stagnation times can easily reach 28 hours in real households, and temperatures vary only between 20 °C and 42 °C – conditions which are optimal for bacterial growth. Functionally similar materials intended for use as shower hoses or final connections, all plastic and most flexible, had distinct microbiological reactions along several threads:

- In short term material comparison tests, materials with approval for use in drinking water applications released less organic carbon and supported less growth than those that were not regulated.

- Growth in a realistic BPS with discontinuous warm water flow reflected that of a comprehensive material-testing package (BioMig) designed to assess migration behavior and biological impact of materials in water.

- Bacterial community development was dependent on both material and month in a realistic BPS, with tight clusters by material shifting in a similar direction over time.

- Even with the same influent water, only 44% of sequences were from genera shared across all samples, with a higher proportion shared in young and high-biomass biofilms, demonstrating that the materials and the biofilms developing on them offer significant space for diversification.

- The community structure was related to biofilm concentration. After 8 months, four of six materials supported similar biofilm concentrations and had similar communities, drawing into question the long-term efficacy of some advanced material designs.

- The occurrence of certain potential opportunistic pathogens may be negatively correlated with biofilm concentration.
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Author Contributions

CP led experimental design, data acquisition, data interpretation, and writing. MG contributed to data acquisition and interpretation. SK, FR, and RS assisted with data acquisition. J-CW contributed to sequencing data processing. FH contributed to experiment design, data interpretation, and writing.
References


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Biofilms in Shower Hoses – choice of pipe material influenced bacterial growth and communities

abundance modeling to predict bacterial community dynamics in the drinking water microbiome.


Takai K, Horikoshi K. (2000). Rapid detection and quantification of members of the archaeal


Supplementary Information

Table S1: Characteristics of Dübendorf tap water. Two types of samples were taken: cold running water to represent the distribution system and hot stagnant water to represent the water that entered the building plumbing simulator (BPS). The first may represent the water entering the system at the end of the 10 minute flushing, while the second represents the water entering at the beginning. Values were averaged for three samples taken on separate days.

<table>
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<tr>
<th></th>
<th>TOC (mg/L)</th>
<th>DOC (mg/L)</th>
<th>Total N (mg-N/L)</th>
<th>Total P (µg/L)</th>
<th>Ca(^{2+}) (mg/L)</th>
<th>Mg(^{2+}) (mg/L)</th>
<th>pH</th>
<th>TCC (cells/mL)</th>
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Abbreviations as follows: TOC – total organic carbon; DOC – dissolved organic carbon; Total N – total Nitrogen; Total P – total Phosphorous; TIC – total inorganic carbon; Ca\(^{2+}\) – calcium; Mg\(^{2+}\) – Magnesium; TCC – total cell concentration.

Table S2: Primers and Probes used for 16S qPCR and 16S Amplicon PCR. Several similar but clearly distinct reactions were used over the course of this study.

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<th>Name / Alternative</th>
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<td>Bakt_805R (S-D-Bact-0785-a-A-21) // 785 R</td>
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**Table S3**: PCR and qPCR Reaction Conditions. Several similar but clearly distinct reactions were used over the course of this study.

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**Table S4: Quality Control Steps. Multiple criteria were used to control Illumina sequencing data.**

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Figure S1: Assimilable Organic Carbon (AOC). The percentage of assimilable carbon in the leachate of 6 pipe materials over three migrations (Day 1, Day 3, and Day 7), using the total of three migrations. This is calculated by comparing the total amount of growth on the leachate to the total amount of organic carbon leached, with the assumption that 1 µg of carbon produces $10^7$ cells. Growth supported by the leachate is first converted to equivalent carbon by dividing by $10^7$, then equivalent carbon is divided by the total organic carbon (TOC) in the leachate. Data label added for Material D, with an extremely low value. This relationship between TOC and AOC (Figures 2 A and B) is interesting in that it shows that the type of carbon leaching from each substance varies considerably.

Table S5: Nutrient Amendment Study. Warm unchlorinated Dübendorf water was incubated for 72 hours in sterile glass vials with nutrient amendments. The increase in cell concentrations compared to a control with no nutrient additions is reported. This study follows the approach of Prest et al (Prest et al., 2016). This shows that the nutrient limitations of the Dübendorf water are complex.

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Nutrients added</th>
<th>Log increase over unamended water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphorous (in excess)</td>
<td>Nitrogen (in excess)</td>
</tr>
<tr>
<td>I</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
**Figure S2**: NMDS with five explanatory variables fitted using EnvFit. (1) Month. Measures of biofilm surface concentration: (2) total cells, (3) 16S rRNA gene copies by qPCR, (4) adenosine tri-phosphate, (5) total organic carbon. All vectors are scaled to their $R^2$ correlation values, and $p < 0.001$ for all measures. This complements Figure 7.
Figure S3: Inverse Simpson Index. The indicator of richness and evenness is higher when communities are more diverse. Samples are grouped by material (color) and month in which the samples were taken. For each point n = 3, and error bars represent standard deviation. Lines connect samples of the same material through time. Samples tend to get more diverse with time.
Biofilms in Shower Hoses – choice of pipe material influenced bacterial growth and communities

Figure S4: Genera that may contain opportunistic pathogens – (A) relative and (B) absolute abundances. Throughout, each color represents one of four genera that contain opportunistic pathogens (Pseudomonas, Mycobacterium, Legionella, and Nocardia). In (A), relative abundances are reported according to month (1, 2, 8) within each material (A-F), with averages reported for each.
group. An inset in the upper right corner shows higher detail (range 0-5%). All groups with a relative abundance > 5% are labeled with percentage values. In (B), absolute abundances are reported according to material (A-F) within each month (1, 2, 8), with separate graphs for each genus. Absolute abundances are calculated as below using the relative abundance from [S4A] and the absolute abundance from figure [4B]. These values were subsequently log transformed. A similar approach was used by Prest et al. to combine flow cytometry and community abundance data (Prest et al., 2014).

Formula and example calculation:

\[
\text{[Relative abundance (\%)]} \times \text{[quantification by 16S qPCR]} = \text{calculated absolute abundance}
\]

\[
\text{for example:}
\]

\[
[72.62 \%] \times [1.1 \times 10^7 \text{ gene copies/cm}^2] = 7.96 \times 10^6 \text{ calculated gene copies/cm}^2
\]

\[
\log_{10}(7.96 \times 10^6) = 6.9 \log \text{ (calculated gene copies/cm}^2)
\]

The genera were chosen due to the possible presence of organisms that cause opportunistic pathogen infections (often with pneumonia symptoms). One representative is given for each below:

- *Pseudomonas* – *Pseudomonas aeruginosa*
- *Mycobacterium* – *Mycobacterium avium* complex
- *Legionella* – *Legionella pneumophila*
- *Nocardia* – *Nocardia asteroides*

**References**


Biofilms in Shower Hoses – choice of pipe material influenced bacterial growth and communities
Chapter 5

Community assembly through dispersal and selection governs the drinking water microbiome in building plumbing
Community assembly through dispersal and selection governs the drinking water microbiome

Abstract

In the final meters of building plumbing, bacterial growth and the microbiome composition can be affected by many factors. In this study, we explored how 1) pipe material quality (using five polymeric pipes), 2) temperature (hot and cold water distribution), 3) location (two identical test-systems with different distribution networks), and 4) time (stagnation time and operational time) affect total cell concentrations (TCC) and microbiome composition in both biofilms and stagnant water. The results of this large-scale study (i.e., 420 sequencing and 1,320 quantitative data-points) could be explained using the theory of community assembly through selection and dispersal. Material was a strong selective factor for bacterial abundances. Materials leaching high amounts of assimilable organic carbon produced higher TCC (p<0.05) and lower richness than materials leaching less carbon. However, dispersal (movement of bacteria into communities) had a stronger effect on the overall microbiome composition. Temperature was similar in the hot and cold water systems during extended stagnation, but the hot and cold systems still had distinct behavior (i.e., reduced richness in hot water and biofilm microbiomes). Thus, selection in water heaters affected dispersal into the test-system. Location also dictated dispersal, with stronger effects in the hot water system, likely due to differences in water heater configurations. With extended stagnation water-TCCs increased, and water microbiomes became more similar to biofilms. There was also a strong correlation between water-TCC and biofilm-TCC ($R^2 = 0.61$ at one location), indicative of dispersal from biofilm into water. Although biofilms reached stability relatively quickly (e.g., up to 50% of maximum biofilm concentration during one year of operation measured after only 7 days), richness increased over the operational year, indicative of dispersal from water to biofilm. The framework used in this study can be applied to all building plumbing systems to better understand the ecological behavior. Moreover, it was clear that selecting good pipe material and water heater configuration is critical for the microbiome in building plumbing.

Keywords: building plumbing, polymeric materials, stagnation, water heater
1. Introduction

The final meters of drinking water distribution through building plumbing are at high risk for excessive bacterial growth due to variable and generally higher temperatures, high surface-area-to-volume ratios, and long stagnation times (Proctor and Hammes, 2015). During stagnation, temperatures at distal ends of building plumbing typically reach room temperature (i.e., typically warmer than distribution network temperature), irrelevant of the delivery temperature (Rhoads et al., 2015). Use patterns vary considerably between buildings (DeOreo et al., 2016), and the long stagnation times are known to contribute to increased cell concentrations (Lautenschlager et al., 2010; Lipphaus et al., 2014). Surface-area-to-volume ratios are typically much higher than in distribution networks. With larger areas available for biofilm development and pipe leachate diluted by a smaller volume, this potentially makes biofilm and pipe choice more important for water quality. Many different pipe materials are available for building plumbing, and the choice often falls to the building owner, architect, or plumber.

Pipe materials can greatly affect microbiology, for example by offering growth inhibition (copper), or growth support (polymeric pipes leaching bioavailable carbon). While building plumbing studies have elucidated the differences in microbiological impacts between clearly different materials (e.g., stainless steel, copper, versus plastics (Buse et al., 2014; Inkinen et al., 2014; Lehtola et al., 2004; Moritz et al., 2010), few have studied the differences within a single category (e.g., flexible synthetic pipes (Proctor et al., 2016)). That said, it is readily recognized that functionally and aesthetically similar materials (e.g., hard polymeric pipes) can still vary considerably (Wen et al., 2015). Moreover, materials producers regularly try to improve the leaching behavior of pipe materials. However, as most laboratory material tests are short-term (Wen et al., 2015; DVGW, 2007), the long-term impact of materials in real systems is not yet understood. Since carbon leaching from polymeric pipes diminishes over time (Bucheli-Witschel et al., 2012), material is likely critical for initial establishment of biofilms, but the long-term impacts may be negligible.
It is also well known that water quality can vary from location to location. This in turn affects microbiome composition. While much of this is attributed to macro-changes in water treatment practices and source water (Holinger et al., 2014), water chemistry (e.g., pH), and small treatment differences can also impact microbiome composition (Ji et al., 2015). The impact of source water can be difficult to test, because it confounds with building specific variations in field surveys (e.g., use-patterns), and because budget often limits the implementation of controlled pilot-scale test systems to one water distribution network.

Many building plumbing studies focus on the behavior of opportunistic pathogens (e.g., *Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa*), which can cause disease amongst vulnerable populations and known to proliferate in building plumbing (Falkinham et al., 2015). However, control of all of these opportunistic pathogens is difficult, especially because of differing optimal growth conditions between opportunistic pathogens (Proctor et al., 2016; Leoni et al., 2001; Meier and Bendinger, 2016) (Chapter 3). It is thus necessary to understand the ecology of the entire system in order to target these opportunistic pathogens (Wang et al., 2013; Proctor and Hammes, 2015). For example, a better understanding of initial biofilm colonization and invasion could inform a probiotic approach to drinking water where ‘good microorganisms’ are used during a building’s commission to prevent pathogen invasion (Wang et al., 2013; Proctor and Hammes, 2015).

In this study, we explored the microbiological aspects of both biofilm and stagnant water in a plumbing test system that featured five polymeric pipe materials and two water temperatures (simulating hot and cold water distribution systems) installed in two locations in Switzerland for an operational period of one-year. The reproducible test systems allowed for systematic comparison of controlling factors for both abundance and composition of bacterial microbiomes. The ecological processes of dispersal and selection were useful for explaining changes over operational time and extended stagnation time, as well as the differences between two locations and two temperatures, and amongst five pipe materials.
2. Materials and Methods

2.1. Test-system design and operation

Two identical test systems were built in two locations in Switzerland (Locations 1, 2). The water quality between the two locations was similar in many respects (e.g., similar pH, high hardness), but had several major differences (the source for Location 1 water was up to 20% lake water along with ground and spring water, while Location 2 was entirely from groundwater and springs) (Table S1). Both waters were limited by carbon (assimilable organic carbon tests showed carbon limitation, data not shown). Location 2 had 1.6 times as much phosphorous, and 9.8 times as much sulfate as Location 1. These locations were chosen based on space availability to construct test-systems.

Each test system (Figure 1) consisted of 30 test pipe loops of 9 m each, together with connecting influent and effluent pipe. Half (15) were designed to deliver 53 °C water to the pipe loops (hot) (temperature regulated with a flow-mixer), while the other 15 were connected to the cold water supply (cold).

Each set of 15 pipe loops consisted of 5 materials (A-E, Table 1) in triplicate. Each pipe loop was equipped with a water sampling point with a metal outlet. Further details about the mechanics of the pipe system can be found with Figure S1, and photos can be found with Figure S2.

Figure 1: Schematic of test system. Black arrows represent flow direction. Black triangles represent the location of the water sampling points (identical location on all pipes). Each thin colored line represents a test pipe-loop of materials A-E (Table 1), which initially measured 9 m long. Influent (blue-cold, red-hot) and effluent lines (gray) consisted of identical pipe material (PE-Xc). See Figure S1 for more details about connections. Pipe lengths and diameters are not drawn to scale.
Community assembly through dispersal and selection governs the drinking water microbiome

Each loop was flushed with either hot (53 ± 2 °C) or cold water four times daily for 30 s at 15 L/min. Between flushing events water was allowed 2, 4, 8, or 10 hours of stagnation, with 10 hours of stagnation occurring overnight before sampling. The flushing schedule was modified three times to allow for sampling after extended stagnation as noted (Table 2).

Temperature measured in influent lines preceding test-pipes ranged from 22.3 – 61.9 °C in the hot system in both locations. (Water temperature during flush was regulated with a flow-mixer which only occasionally failed the design goal of 53 ± 2 °C). Cold water varied from 10.8 – 27.2 °C with some evidence of seasonality (i.e., slightly lower temperatures in the winter). Water temperatures in pipes followed a similar pattern throughout the day at each location (Figure S3). During water delivery, temperatures would increase (hot) or decrease (cold), but returned to a similar room temperature during extended stagnation. The room temperature between the two locations varied slightly, with

### Table 1: Materials used in test system.

<table>
<thead>
<tr>
<th>Code</th>
<th>Material</th>
<th>Inner diameter (mm)</th>
<th>Pipe Distributor</th>
<th>TOC migrated (μg-C/cm²)¶</th>
<th>Low-leaching or High-leaching ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PE-Xa</td>
<td>15</td>
<td>1</td>
<td>0.3</td>
<td>Low</td>
</tr>
<tr>
<td>B</td>
<td>PE-Xb</td>
<td>15</td>
<td>2</td>
<td>1.12</td>
<td>High</td>
</tr>
<tr>
<td>C</td>
<td>PE-Xc</td>
<td>14.4</td>
<td>1</td>
<td>0.64</td>
<td>Low</td>
</tr>
<tr>
<td>D</td>
<td>PE-RT</td>
<td>16</td>
<td>2</td>
<td>1.00</td>
<td>High</td>
</tr>
<tr>
<td>E</td>
<td>PE-Xb ‡</td>
<td>16</td>
<td>1*</td>
<td>13.25</td>
<td>High</td>
</tr>
</tbody>
</table>

‡ not certified for drinking water use – only for use with hot water in hydronic heating systems.

* produced at a different location from other pipes from company 1.

‡ Migration tests and designation explained with Figure S4.

### Table 2: Sampling schedule for each location. Samples were taken at both locations after approximately equal days of operation. The only exception was sampling 5 (Day 56). At Location 2, electronically controlled flushings were interrupted due to power loss, resulting in an extended stagnation time of 5 days. Stagnation time was intentionally extended for sampling events 7, 10 and 11.

<table>
<thead>
<tr>
<th>Sampling Event</th>
<th>Location 1</th>
<th></th>
<th>Location 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Operating (days)</td>
<td>Stagnation time</td>
<td>Operating (days)</td>
<td>Stagnation (days)</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>10 hours</td>
<td>7</td>
<td>10 hours</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>10 hours</td>
<td>14</td>
<td>10 hours</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>10 hours</td>
<td>28</td>
<td>10 hours</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>10 hours</td>
<td>42</td>
<td>10 hours</td>
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<tr>
<td>5</td>
<td>56</td>
<td>10 hours</td>
<td>56</td>
<td>5 days</td>
</tr>
<tr>
<td>6</td>
<td>91</td>
<td>10 hours</td>
<td>91</td>
<td>10 hours</td>
</tr>
<tr>
<td>7</td>
<td>119</td>
<td>2 days</td>
<td>119</td>
<td>2 days</td>
</tr>
<tr>
<td>8</td>
<td>161</td>
<td>10 hours</td>
<td>161</td>
<td>10 hours</td>
</tr>
<tr>
<td>9</td>
<td>252</td>
<td>10 hours</td>
<td>259</td>
<td>10 hours</td>
</tr>
<tr>
<td>10</td>
<td>286</td>
<td>34 days*</td>
<td>286</td>
<td>27 days*</td>
</tr>
<tr>
<td>11</td>
<td>364</td>
<td>78 days**</td>
<td>373</td>
<td>87 days**</td>
</tr>
</tbody>
</table>

* Referred to as 1 month in the text
** Referred to as 2.5 months in the text
Location 1 in a more protected basement laboratory and Location 2 in a less protected warehouse. At Location 1, the 3,000 L water heater was located 50 m away from the test system, and only 1,500 L was used per day in the building. At Location 2, the 150 L water heater was dedicated for the test system, was located only 4 m from the test system, and was almost completely emptied during each water flush.

2.2. Sampling

Samples were taken according to the schedule (Table 2). Samples were always taken in the morning after the 10-hour overnight stagnation unless otherwise noted. Water samples were taken by flaming the metal outlet, opening the outlet, wasting the first 100 mL, and collecting 500 mL - 1 L of water from the pipes into muffled (550 °C for 2 hours) Schott bottles with sterilized caps. Water volumes were adjusted according to calculated volume inside pipes, which shortened over time. Caps were sterilized with 60 minutes in a 60 °C sodium persulfate bath (100 g/L) and rinsing with ultrapure water, as previously described (Wen et al., 2015). Influent cold water samples were taken by flushing the nearest tap outside the test system until temperature stabilized and taking duplicate 1-L samples.

After water samples were taken, biofilm samples were taken by emptying water from the section and cutting away 30-cm of pipe using sterilized pipe cutters. The pipe-sections were filled with filtered (0.15 µm pore-size Geberit Hygiene Filter, Geberit, Switzerland) cell-free mineral water (Evian, France) with sterilized plastic stoppers to hold water in the pipes. Stoppers were sterilized as described above. After sampling, new fittings were used to seal the system again with the now shorter pipe loop. Negative samples using new (i.e., never in test-system) sterilized (as above) 30-cm sections of pipes A, B, and E were used during each sampling event and treated alongside test pipe-sections.

Water and biofilm samples were transported in coolers to the laboratory and processed on the same day (with the exception of biofilm filtration, which was completed within 72 hours).
2.3. Sample Processing

Water: Upon arrival to the laboratory, water samples were shaken and aliquoted for total organic carbon (TOC) analysis (c.a. 20 mL) and cell concentration with flow cytometry (FCM) (c.a. 1 mL). The remainder of water was used for filtration and DNA extraction.

Biofilm: Biofilm was extracted by submerging the water-filled pipe sections completely in a sonication bath (Bandelin Sonorex, Rangendingen, Germany) and sonicating for three 5-minute rounds, rotating the pipes 120° in between sonication rounds. Biofilm suspensions were additionally sonicated with needle (Sonoplus HD 2200, Bandelin Sonrex, Rangendingen, Germany) for 30 seconds for homogenization. Aliquots (c.a. 1 mL) were taken for biofilm suspension analysis with FCM, and the remainder was filtered after a maximum of 24 hours storage at 4 °C.

2.4. TOC analysis

TOC concentration was determined by thermal oxidation to CO₂ and infrared detection with the non-purgeable organic carbon method according to EN 1484 (TOC-VCPH, Shimadzu, Kyoto, Japan). TOC concentrations were converted to mg TOC/cm² using surface-area-to-volume ratios in each pipe.

2.5. FCM analysis

Staining and flow cytometric analysis was done as described previously (Prest et al., 2013). Briefly, a working solution of SYBR® Green I (SG) (Invitrogen AG, Basel, Switzerland) was prepared by 100x dilution in anhydrous dimethylsulfoxide (DMSO) for measuring total cell concentration (TCC). For measurement of intact cell concentration (ICC), propidium iodide (PI; 30 mM) (Invitrogen AG, Basel, Switzerland) was mixed with the SYBR® Green I working solution to a final PI concentration of 0.3 mM. Water samples and biofilm suspensions were stained with SG or SGPI at 10 µL/mL. Samples were stained and incubated at 37 °C in the
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dark for 13 min before measurement. Flow cytometric measurements were performed on a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium). Data analysis was performed using the BD Accuri CFlow® software. Fixed standard gates were applied to separate bacteria from background signals and low-nucleic acid (LNA)-content bacteria from (HNA)-content bacteria (Prest et al., 2013), with slight adjustments as necessary. Water-TCC and biofilm-TCC were calculated using surface-area-to-volume ratios or surface area of biofilm extracted. Percentage of intact cells (%IC) was calculated by dividing water-ICC by water-TCC. Percentage of HNA content-bacteria (%HNA) was calculated by dividing the concentration of HNA-content bacteria by water-TCC.

2.6. DNA extraction

For both water and biofilm suspension samples, the remaining volume after other analyses was shaken and filtered onto a 0.2 μm polycarbonate Nucleopore® membrane filter (47 mm diameter, Whatman, Kent, UK), and exact volume was recorded. Typically, triplicate samples were combined onto one filter by subsequently filtering the volumes, but for 20 data-points, triplicate pipes were separated for analysis (Table S2). When filtering was slow for some water samples (i.e., high total cell concentration), the total filtered volume was reduced (i.e., filtered volume ranged from 815 – 3080 mL).

Filters were inserted into a sterile 5 mL tube and stored at -20 °C before DNA extraction with the Power Water DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to manufacturers’ instructions.

2.7. Amplicon sequencing with Illumina MiSeq

1 ng of DNA extract from each sample was subjected to PCR amplification using modified universal bacterial primers Bakt_341F and Bakt_805R (Klindworth et al., 2013), which target the V3-V5 region of the 16S rRNA gene. Primers were adapted with a nucleotide tail to facilitate binding Nextera adapters. For 19 samples, PCR replicates were included. Index PCR
was performed to add the Nextera XT v2 Index Kit adaptors (Illumina) to the amplicon. PCR reaction conditions are detailed in Table S3. After each PCR reaction, products were purified using the Agencort® AMPure® XP system (Beckman Coulter, Inc., Bera, CA).

Each product was quantified using Qubit 2.0 HS DNA system (Thermo Fisher Scientific). Samples were normalized to the same concentration before running on the MiSeq platform using MiSeq Reagent Kit v2 (300-cycles, #MS-102-2002) according to manufacturer’s protocol with 10% PhiX. All sequencing was done at the Genetic Diversity Centre (GDC) of ETH, Zurich. Two libraries were created due to the high number of samples, and were subsequently combined for analyses.

2.8. Statistical Analysis
Paired t-tests were used to compare averages of biological triplicates for matching groups. T-tests were used when paired analysis was not possible. Unless otherwise noted, all time points, except where there was an accidental extended stagnation, were used for these tests.

For sequencing data, sequences were merged, trimmed, filtered, and clustered into operational taxonomical units (OTUs) according to several algorithms (details in Table S4). Sequences were identified according to greengenes v.13.5 (DeSantis et al., 2003). In R, phyloseq (McMurdie and Holmes, 2013) was used for processing. Libraries were rarefied to 27,180 sequences per sample. Non-metric multidimensional scaling (NMDS) was used to visualize microbiome similarities using Bray-Curtis dissimilarity. The adonis and ordiellipse functions from the vegan package (Oksanen et al., 2013) were used to relate environmental data to microbiome composition.
3. Results

This study utilized two identical test systems with five different polymeric pipe materials installed in two geographically separate locations, simulating both hot and cold water distribution in buildings (Figure 1). With a long operational period (364/373 days), it was possible to monitor growth dynamics after commissioning of a new system. Normal operation captured aspects of varying stagnation time (daily flushes after 2, 4, 8, and 10 hours, with sampling after the 10 hour stagnation) and temperatures (Figure S3) that likely affect microbiological growth conditions. Additionally, extended stagnation events (2 days – 2.5 months) captured behavior under extreme yet plausible building stagnation events (i.e., weekends, summer holidays). Both locations primarily used non-chlorinated groundwater, but with key differences in minor sources, water chemistry (Table S1), microbiome, and building plumbing outside the test system. The five pipe materials were chosen to represent a range of hard polymeric pipe materials typically used in building plumbing. Laboratory material tests revealed that the pipes essentially divided into two classes – low-leaching (A, C) and high-leaching pipe materials (B, D, E) (Table 1, Figure S4).

Below we present how the test systems responded in terms of total organic carbon (TOC) in the water phase [3.1], total cell concentration (TCC) in the water [3.2] and biofilm [3.3] phases, overall microbiome diversity and structure [3.4], and concentration of specific organisms [3.5] to the controlling factors of time (operational and stagnation), location, temperature, and material.

3.1. TOC leaching

TOC, as measured in the test system, includes all forms of organic carbon (i.e., both biodegradable and non-biodegradable leachate from pipes, and other organic matter like cells). TOC was most strongly affected by operational time, stagnation time, and materials.
In most pipes, TOC was high initially (i.e., first 7-14 days after system 'commissioning') and then dropped to remain steady (Figure S5), indicative of a diminished leaching capacity with operational time, a phenomenon frequently observed in materials tests (Bucheli-Witschel et al., 2012). The exception to this trend is the increased TOC during extended stagnation events (Days 119, 286, 373/364, and Day 56 for Location 2). Extended stagnation allowed for both more leaching from pipes and biomass increase (i.e., growth/biofilm detachment). Extended stagnation times had the highest impact on TOC accumulation early in operation. For example, after only 5 days of stagnation at Day 56, Location 2 water had higher TOC concentrations than after 1-month of stagnation at Day 386 (p=0.04, paired t-test). However, within a similar operational stage, extending stagnation did increase TOC. From Day 286 with 1-month stagnation to Day 364/373 with 2.5-months stagnation, there was a significant TOC increase (p<0.001 for both locations, paired t-tests). Due to these differences, some subsequent analyses were conducted separately for “early-stage” (Days 0 – 42), “steady-stage” (Day 56+), and for “extended stagnation” (Days 119, 286, 373/364, and Day 56 for Location 2).

Influent TOC was not statistically different between the two sites (means for Location 1 = 0.59, and Location 2 = 0.63 mg/L, t-test, p=0.085), but location did impact TOC in test system water. Location 1 samples had a higher TOC (p=0.03, paired t-test). When looking only at the early-stage when carbon leaching was strongest, these differences were only apparent in the hot water system (p=0.044, paired t-test, further discussed with Figure S5).

There was not a significant difference in TOC between hot and cold water systems (p>0.05, paired t-test). While increased carbon leaching was expected with higher temperatures (Figure S6), test system temperatures were variable, and cold and hot systems both reached room temperature during stagnation (Figure S3).
There was a clear statistical difference between the high- and low-leaching materials (i.e., early stage alone, \( p<0.001 \), t-test, mean high = 0.29, mean low = 0.25 \( \mu g-C/cm^2 \)). With extended stagnation, these results became more pronounced (\( p<0.001 \), t-test, mean high = 0.37, mean low = 0.26 \( \mu g-C/cm^2 \)).

### 3.2. Water-TCC

Water-TCC generally increased during stagnation in the test system (shortest sampled stagnation: 10 hours) compared to the influent water (fully flushed). Within the test system, water-TCC responded to material, but not to location or temperature (Figure 2).

Water-TCC did not have strong temporal trends over operational time, but had a strong response to extended stagnation time. As with TOC, water-TCC was more strongly affected by extended stagnation early in operation. Water-TCC was significantly higher after the 5-day stagnation event early in operation (Day 56, Location 2) than after the 1-month stagnation later in operation (\( p = 0.02 \), paired t-test). However, within the same operational stage, extending stagnation increased water-TCC. A 2-
Community assembly through dispersal and selection governs the drinking water microbiome
day stagnation (Day 119) produced significantly higher water-TCC than the previous and fol-
lowing sampling events (Day 91 and 161, both with 10-hour stagnation) for all materials at
both locations (p< 0.001, paired t-tests) (average increase of 1.8 × 10^5 at Location 1, and 3.2
x 10^5 at Location 2). Later in operation, the 2.5-months stagnation (Day 364/373) produced
higher water-TCC than the 1-month stagnation (Day 286), but the increase was only signifi-
cant at Location 1 (p=0.03, paired t-test).

Location was not a significant factor for water-TCC, percentages of intact cells (%IC), or per-
cectages of high-nucleic acid (HNA)-content bacteria (%HNA). Temperature also was not a
significant factor for water-TCC (Location 1: p=0.79, paired t-test; Location 2:p=0.20, paired
t-test) (Figure 2). However, both %IC and %HNA were higher in hot water samples than cold
water samples, suggesting changes in viability (%IC) and microbiome composition (%HNA)
(%IC mean for hot = 74%, cold = 71%, p<0.001, t-test; %HNA: mean for hot = 80%, cold =
60%, p<0.001, paired t-test) (Figure S7, Figure S8).

The water-TCC for high-leaching materials was higher than for low-leaching materials
(p<0.05, t-test) (Figure S9). Both %IC and %HNA were higher amongst high leaching mate-
rials as well (%IC: mean high = 77%, low = 70%, p<0.001, t-test; %HNA mean high = 73%,
mean low = 65%, p<0.001, t-test) (Figure S7, Figure S8).

3.3. Biofilm-TCC
Biofilm-TCC was affected by material, and showed little response to location, temperature, or
operational time (Figure 2, Figure S5, Figure S9).

Biofilm growth patterns varied considerably based on material. Low-leaching materials had
small and unsteady increases in biofilm-TCC during the early-stage, and high-leaching mate-
rials had nearly stable biofilm-TCC immediately (Figure S5). For example, for the cold bio-
films at Location 1, Material A started at 2% of the maximum biofilm-TCC (1.6 x 10^4 cells/cm^2,
Day 7; maximum: $8.1 \times 10^5$ cells/cm$^2$, Day 56), while Material E started at 50% of the maximum biofilm-TCC ($1.6 \times 10^6$ cells/cm$^2$, Day 7; maximum: $3.2 \times 10^6$ cells/cm$^2$, Day 364). This suggests fast growth for high-leaching materials. However, for both of these sample groups, the minimum biofilm-TCC occurred at Day 14 ($7.4 \times 10^3$ cells/cm$^2$ for Material A; $1.6 \times 10^5$ cells/cm$^2$ for Material E). Biofilm heterogeneity (i.e., introduced by loop geometry) may contribute to unsteady increases.

Location and temperature did not strongly affect biofilm-TCC (Figure 2), but material did. High-leaching materials tended to have higher biofilm-TCC than low-leaching materials (Figure 2, Figure S9), but this was not consistent at all sampling events (Figure S5).

In the early stage, water-TCC correlated well with biofilm-TCC (for log$_{10}$ transformed values of water-TCC and biofilm-TCC, $R^2 = 0.61$ and 0.47 for Locations 1 and 2, p<0.001) (Figure 3, Figure S10). This correlation was largely driven by the difference in behaviors between high-leaching pipes and low-leaching pipes (Figure 3). This correlation was not strong outside the early stage (i.e., when including the steady-state samples with equal stagnation time, $R^2 = 0.36$ and 0.28 for Locations 1 and 2). With extended stagnation time, the ratio of water-TCC to biofilm-TCC increased dramatically (Figure S11), interfering with correlations between the two.

**Figure 3:** Total cell concentrations, log transformed, in biofilm (x-axis) and water (y-axis) samples from the same pipe during the early stage of operation (Days 7, 14, 28, and 42). Low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) are separated to highlight differences in materials. Water cell concentrations are expressed in cells/cm$^2$ to account for differences in surface area to volume ratio between pipes.
3.4. Microbiome analysis

3.4.1 Reproducibility

PCR replicates (19 duplicate groups) and biological replicates (20 triplicate groups) showed reproducibility in the study. Richness, as measured by number of observed OTUs, was fairly similar across PCR replicates with less than 9.2% variation in water samples, and less than 26% variation in biofilm samples. The same measure was also fairly stable amongst biological replicates with an average of 12.4% variation for water replicate groups, and 19.6% for biofilm replicate groups. The composition of the communities was consistent amongst biological replicates as well (e.g., in Figure S12, a water biological replicate group is indicated for sampling event 2). Negative biofilm controls were included, but due to low DNA yields (i.e., resulting from high-quality sampling), these were only occasionally included in analysis, and patterns were not discernable for these samples.

3.4.2 Richness

Water samples always had a higher richness (number of observed OTUs) than biofilm samples (p<0.001, paired t-test) (Figure 4). Richness indicates the diversity within a sample. With a lower number of observed OTUs, only a few types of bacteria are present, while with a higher number of OTUs, a more diverse group (i.e., including rare OTUs) of bacteria are present. For water samples, richness decreased when water-TCC increased (Figure S13). Both stagnation time and material likely contributed to this result. Richness decreased with extended stagnation time (Figure S14), and with high-leaching materials (Figure S15), both of which had higher water-TCC. Richness in biofilms did not correlate with biofilm-TCC.

In biofilms, richness tended to increase with time, especially in cold samples. Water richness remained more stable, but there was a slight increase over operational time amongst cold high-leaching material samples (Figure S16). Location was important for influent water samples, with higher richness at Location 1 than Location 2 (p < 0.001, t-test). This in turn,
seemed to affect the test-system water, with higher richness at Location 1 than Location 2 (p<0.001, t-tests) (Figure 4), but location was not important for biofilm richness. Temperature strongly affected richness, with more observed OTUs in hot samples (p<0.001, t-tests) (Figure 4).

3.4.3 Microbiome composition and structure

Influent water microbiomes were relatively stable over time, but differed between the two locations (Figure 5). The difference between locations accounted for 47% of variation amongst influent samples, while time only accounted for 26% of this variation (adonis).

Test system microbiomes were similar across temperature, location, and phase (water vs. biofilm), with somewhat clear clusters for each of these sub-groups by NMDS (Figure 5). Temperature was the most important of these factors, accounting for 12% of variation (adonis, excluding influent and negative controls, p<0.001). Thus, further analysis was done after dividing samples by temperature.
Location was critical for microbiome composition amongst hot samples, with clustering clearly dividing samples along these lines, but was less important amongst cold water microbiomes (Figure 6). Operational time accounted for more variation in microbiome composition than material, and both of these factors accounted for more variation than phase (adonis in cold and hot systems, respectively: $R^2 = 0.18$ and 0.21 for sampling event, 0.16 and 0.16 for material, and 0.04 and 0.04 for phase) (Figure S12, Figure S17). This suggests that overall changes in environment (over time and between materials) affect both biofilm and water together. Early stage samples seemed to have the greatest dissimilarity (i.e., large spread in Figure S12 for Day 7 vs. tighter clustering for later sampling events).

**Figure 5:** Non-metric multidimensional scaling (NMDS) representing bray-curtis dissimilarities between samples, with colors representing temperature (red for hot, blue for cold, black for influent and negative control samples), and shape representing the phase (squares for biofilm, triangles for water) and location (empty for location 1, filled for location 2). Ellipses represent 95% confidence intervals of clustering by location, phase, and temperature. Labels were added to ellipses to enhance understanding.
Figure 6: NMDS representing bray-curtis dissimilarities between samples. Samples are divided as to whether they originate from the cold system (top) or the hot system (bottom). Colors represent the time the sample was taken (pinks for early stage, green for late stage), while shape represents the phase (squares for biofilm, triangles for water) and location (empty for location 1, filled for location 2).
To further explore the composition of the microbiome, two representative sampling events from the stable stage were taken for further analysis (Day259/252 with normal 10-hour stagnation and Day 286 with 1-month stagnation) (Figure S18). When looking at the microbiome composition at these two sample points, differences between temperatures were obvious even at the phylum level. OD1, TM7, Planctomyceyes, Deltaproteobacteria, and Gammaproteobacteria were clearly favored in the cold system, while Themi, Chlorobi, and Betaproteobacteria were clearly favored in the hot system. When stagnation was increased from 10 hours to 1 month, the proportion of OTUs shared between phases increased from 9% to 35% of water OTUs in the hot system, and from 17% to 47% of water OTUs in the cold system. While this was mostly due to the decrease in water OTUs, it is interesting that the OTUs that persisted during extended stagnation were shared with the biofilm phase. Finally, while the cold and hot biofilm microbiome compositions remained somewhat stable, there was a clear shift in the water samples with the extended stagnation. Bacteroidetes was enriched with increased stagnation in both hot and cold water. Chlorobi, and Betaproteobacteria were enriched in the hot water, while OD1 was enriched in the cold water. Thermi declined in the hot water, from 33% of 10-hour stagnated water samples to just 3% of 1-month stagnated water samples.

3.5. Specific organisms

The sum of the top ten most abundant OTUs, several genera containing potential opportunistic pathogen, and OTUs linked to specific metabolisms were tracked using the microbiome composition data. The sum of the top ten most abundant genera was higher amongst hot water samples than either influent or cold water samples. This sum was also higher with high-leaching materials. Interestingly, this mirrored %HNA behavior.

Three genera that might contain potential opportunistic pathogens were detected (Legionella, Mycobacterium, and Pseudomonas) in many sequencing samples (310, 311, and 263 out of 420 samples, respectively). The absolute abundance of these genera was calculated by mul-
tiplying the relative abundance with the TCC. For *Legionella* and *Mycobacterium*, these calculated concentrations were affected by location and temperature, while for *Pseudomonas*, temperature and location seemed to make no difference (Figure 7). Non-detects likely contributed to the lack of trends for *Pseudomonas*. At Location 1, both *Legionella* and *Mycobacterium* concentrations tended to be higher amongst cold water than hot water (p<0.01 for both genera in both biofilm and water). Additionally, samples with high-leaching materials tended to have higher concentrations of both of these genera. At Location 2, concentrations of both *Legionella* and *Mycobacterium* tended to be higher in hot samples, but differences were not always significant. Similar trends were found for both calculated absolute abundances and relative abundances (data not shown).

**Figure 7:** Calculated absolute concentrations of three genera (*Legionella*, *Mycobacterium*, *Pseudomonas*) for water (top) and biofilm (bottom) samples. Non-detects are not shown, but the number is indicated. Boxplots for influent (black), cold (blue), and hot (red) samples mark the median and quartile measurements. Empty boxplots are for Location 1, and shaded ones for Location 2. The color of individual points indicates the material for particular samples, with low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) separated.
The Nitrospirae phylum was more abundant in water samples than biofilm samples, and most abundant in influent samples (Figure S19). Several OTUs were identified as having metabolisms related to sulfate, (e.g., Sulfuricurvum, Geobacter, Thermodesulfovibrionaceae). The sum of the three most abundant of these OTUs had the highest abundance in Location 2 influent samples (Figure S19). Sulfate concentration was also highest at Location 2 (Table S1).

4. Discussion

Bacteria in the biofilm and stagnant water of the test systems differed in terms of TCC, microbiome composition, and presence of specific bacteria. Many of these differences could be attributed to the phase (biofilm vs. water), temperature (hot vs. cold systems), location (Location 1 vs. 2), and material (i.e., high-leaching vs. low-leaching). When one considers the delivery of water as a continuum of microbiomes, each affecting the next (Proctor and Hammes, 2015), all of these trends fall into a complete picture of an ecological system governed by simple community assembly principles, especially those of dispersal and selection (Vellend, 2010). As the study simulated the conditions in a home, there are also practical implications for the engineering of hot and cold water distributions systems.

4.1. Selection

Selection, to put it simply, is the process by which a species is favored for survival and/or growth, that is, one species has a higher fitness value than another (Vellend, 2010; Kinnunen et al., 2016). This favoring process can be either through discouraging competing bacteria from growing while an unaffected group survives (here, referred to as negative selection), or through encouraging certain bacteria to grow by offering favorable conditions (e.g., additional nutrients) for which a specific organism is suited (here, referred to as positive...
The three most prominent selective forces in this study were temperature, organic carbon from polymeric pipes, and protection in biofilm.

### 4.1.1 Temperature

High temperature was a strong negative selective force in this study, reducing richness and strongly influencing microbiome composition. The proportion of the most common bacteria, %IC, and %HNA cells were also increased with high temperatures. Together, these factors suggest that selection by high temperature restricted the growth of some bacteria, while others survived and even thrived. Previously, increased temperature, along a range of hot water temperatures (Ji et al., 2017), and compared to cold water distribution systems (Henne et al., 2013) has been associated with lowered richness.

This selective effect might be readily expected in a system with consistently high temperatures, where few bacteria can survive high temperatures (i.e., constant selection). However, not all portions of hot water distribution systems undergo consistently high temperature. Rather heat is lost during distribution (even with recirculation), and distal ends cool to room temperature (Bédard et al., 2015; Rhoads et al., 2015) (i.e., temporally-variable selection). In this system, both hot and cold water, reached room temperature during stagnation (Figure S3), following models created for domestic buildings (Zlatanović et al., 2017b). Thus, both the cold and hot water system distal ends have similar temperatures during the majority of extended stagnation in a building.

Temperature indeed has different selective effects whether applied consistently or transiently. The biofilm in recirculating lines (i.e., always receiving high temperature water) and distal ends (i.e., cooling down to room temperature) of hot water distribution systems often have distinct microbiomes (Inkinen et al., 2016; Ji et al., 2017). Hot water distal end biofilms even showed more similarity to cold water distal end biofilms than to the recirculating line biofilms in a building (Inkinen et al., 2016), reflecting their similar temperatures during extended stag-
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nation time. Moreover, selection and heat inactivation are dependent not only on the temperature achieved, but also the time for which it is achieved. For example, *Legionella*, while inactivated (5-log reduction) within 5 minutes at 60 °C (Cervero-Aragó *et al.*, 2015), it can also be recovered after temporarily entering the viable but non-cultivable stage (i.e., recovered after temperatures cool) (Dusserre *et al.*, 2008; Allegra *et al.*, 2011).

Within the hot water system, biofilms and water also have distinct exposures to high temperature. Distal end biofilms are repeatedly exposed to moderately high temperatures during water flushing, but temperatures quickly drop. Water is exposed to high temperatures in the water heater only once, but for a prolonged period. It is not surprising then that bacteria of the Thermi phylum, known for heat-tolerance (Griffiths and Gupta, 2007), were enriched in hot water (Figure S18). This phylum was not particularly enriched in hot biofilms or in water stagnant for extended periods, but biofilms and 1-month stagnant water experienced high temperatures for only a short period compared to the time at room temperature.

While temperature selection was as uniformly applied as possible, differences between two locations still emerged, for example with the behavior of *Legionella* and *Mycobacterium*. This highlights the importance of dispersal (discussed further below). While water entering the test-system was the same temperature, the water heaters at the two locations were designed differently, and thus the ‘upstream selection’ or the history of the water was different. At Location 1, water had a residence time of c.a. 2 days, while the water heater at location 2 was nearly emptied during each use (i.e., residence time in the water heater before entering the pipes for the tested 10-hour stagnation was only 8 hours). This could explain why *Legionella* and *Mycobacterium* were effectively controlled by temperature at Location 1, while temperature made no difference for these organisms at Location 2.

Practically, temperature selection has an interesting implication for system design. Water heaters are typically designed to have short residence times (i.e., to save energy wasted on
oversized tanks), but this may limit the effectiveness of high temperatures used to inactivate potential opportunistic pathogens. Often hot water pipes are equipped with insulation to mitigate heat losses, both for energy savings and maintenance of adequate temperatures. However, cooling is inevitable with long stagnation, and insulation only slows cooling to prolong the period of optimal growth for certain opportunistic pathogens (Bédard et al., 2015). Convective mixing (i.e., where upward facing pipes from recirculating lines promote temperature-based water turnover), too, can alter the temperature that bacteria are exposed to and prolong the temperature selection favorable for opportunistic pathogens (Rhoads et al., 2016). In this study, insulation and recirculating lines were not used, and thus water was able to quickly cool, perhaps limiting (but not eliminating) potential opportunistic pathogen growth.

4.1.2 Carbon leaching from pipes

In this study, pipe materials provided a positive selective force. Organic carbon leaching from polymeric pipes provides food for bacteria, encouraging bacteria that are efficient at utilizing those specific substrates over bacteria that utilize other substrates. This organic carbon leaching has been shown to influence both the total amount of bacteria (Wen et al., 2015; Bucheli-Witschel et al., 2012) and microbiome composition (Proctor et al., 2016) that form on polymeric pipes. Moreover, the amount of carbon leaching from new pipes likely eclipses the carbon available in the distribution network (Bucheli-Witschel et al., 2012), even with the possibility of seasonal variations in biodegradable TOC in the distribution network (Zlatanović et al., 2017a).

In this study, there was already a clear difference between the influent microbiome and that of stagnant water inside pipes, with a drop in diversity during stagnation that was consistent across pipe materials and time. While many factors contributed to this (e.g., temperature inside buildings vs. outside as a selective force or dispersal from biofilm), nutrient availability was likely a strong selective factor. In the distribution network, water was carbon limited (Table S1), and the microbiomes were extremely diverse. Other alternative nutrients (i.e., nitro-
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gen cycling) can become a dominant positive selective force in the distribution network (Prest et al., 2016). In this study, with high sulfate concentrations at Location 2, there was also strong evidence for sulfate related metabolisms, and at both locations, Nitrospirae, a phylum active in nitrogen cycling, was more abundant in the influent water than stagnant system water (Figure S19). After extended stagnation in new polymeric pipes, heterotrophs likely out-compete oligotrophic autotrophs that proliferate in distribution networks.

Differences between pipe materials are well known to induce changes in the microbiome. However, studies typically compare a wider range of materials, for example pitting the toxicity of copper against the carbon provided by PEX pipes (Buse et al., 2014; Inkinen et al., 2014; Lehtola et al., 2004; Moritz et al., 2010), or examining a wide range of low-quality materials (Proctor et al., 2016). Here, most of the pipes were of relatively good quality, leaching much less organic carbon than flexible synthetic materials (Wen et al., 2015; Proctor et al., 2016).

The amount of organic carbon leaching from the pipes still differed (i.e., high-leaching vs. low-leaching). The impact on TCC was clear – i.e., with more carbon, more bacteria were present. These effects were strongest in the early stage. For example, an accidental stagnation on Day 56 at Location 2 resulted in considerable water-TCC increase, while later extended stagnation events of greater length did not produce such spikes. The microbiome also shifted between high-leaching and low-leaching materials, with clear impacts on richness and the proportions of HNA cells, most abundant OTUs, and IC. Increased carbon availability with high leaching pipes resulted in less diverse communities – likely dominated by a few fast growing bacteria capable of efficient and specific TOC consumption.

These results challenge both a neutral and niche model of biofilm development. One would expect an increase in diversity with increased biomass under neutral theory, and an increase in diversity with increased substrate availability under niche theory (Pholchan et al., 2013).
However, the character of the carbon was not assessed in this study. Another study comparing neutral and niche models noted differing yields depending on whether bacteria were using simple or complex carbons (Pholchan et al., 2013), which could also affect these results.

Finally, the selective effects of pipe materials likely diminish over time, together with decreased carbon leaching. In this study, there was evidence that factors related to dispersal (discussed below) had an increasing importance with time, with cold biofilms increasing in richness over time. Thus, pipe materials may only directly select the initial microbiome, but these effects are long lasting (i.e., initial biofilm is not easily displaced).

### 4.1.3 Biofilm versus water

Biofilms and water had undeniable differences in terms of richness regardless of other factors. However, there were still similarities between the two phases based on temperature and location (i.e., other selective forces and dispersal). The reduced diversity in biofilm has been noted in other studies of drinking water distribution systems (Inkinen et al., 2016; Henne et al., 2012; Douterelo et al., 2017), but the phenomenon is not consistent (Liu et al., 2014), nor are the reasons entirely clear. Several concurrent selective forces are likely at play to differentiate the two microbiomes.

The concept of limited diffusion of nutrients into a biofilm is not new (Stewart, 2012), but most biofilm models consider constant flow, which creates a convective barrier to diffusion. Models also typically consider flow of nutrients from the water to the biofilm base (e.g., one-way). On the polymeric pipes of building systems, biofilms undergo primarily stagnant conditions, such that diffusion is not hindered. Additionally, a counter-diffusion occurs, with carbon originating at the biofilm base. Thus, one must consider counter-diffusion models wherein biofilms are often more productive (Kinh et al., 2017). Counter-diffusion would mean that bacteria forming biofilms on pipes have first access to carbon leaching, especially early in biofilm development when carbon leaching is highest. Thus, there is a spatial element to se-
lection by carbon leaching that affects biofilms more strongly than the water phase. Bacteria in water only have access to this leached carbon after it diffuses through a biofilm, which likely consumes most of it. However, other nutrients that are delivered by the water (i.e., nitrogen, phosphorous) are only available to biofilm bacteria after the water phase utilizes it. Consistent with this, *Nitrospirae*, which utilizes nitrogen, was more dominant in the bulk water phase than in biofilms.

Bacteria function was not analyzed explicitly in this study. Another similar study found that functionality was largely similar across biofilm and water phase, even when microbiomes were taxonomically distinct (Ji et al., 2017). However, it has been suggested that only some drinking water bacteria are suited for biofilm growth, and that even bacteria suited for both undergo strong phenotypic change when switching between phases (Liu et al., 2016). For example, bacteria with flagella would be more abundant in the water phase, while functionally similar bacteria that produce EPS would be more abundant in the biofilm phase, whether this difference is phenotypic (i.e., switching) or genotypic (i.e., different bacteria). While not based solely on functionality, this still represents a form of selection.

Biofilm is also known to produce extra polymeric substances (EPS), which can protect bacteria from negative selective forces in the bulk water phase (i.e., high temperatures) (Flemming et al., 2016). Additional structural elements provide an ecosystem different from the water phase. Biofilm structure, EPS production, and inorganics were not monitored in this study.

While biofilm and water differences were the primary driver of microbiome composition previously (Ji et al., 2017), this was not the case in our study. Temperature and location (especially for hot samples) were very important, and with extended stagnation, the dissimilarity between water and biofilm decreased. This is indicative of the overriding importance of dispersal in water distribution systems.
4.2. Dispersal

Dispersal refers to the physical movement of bacteria into or out of a microbial community (immigration and emigration events) (Vellend, 2010; Kinnunen et al., 2016). While dispersal is complicated, with a plethora of spatially linked microbiomes (Battin et al., 2007), in the case of drinking water delivery into the home, a primary mode of dispersal is the bulk flow of water delivery with the creation of a microbial continuum (Proctor and Hammes, 2015).

There are also local dispersal events with movement of bacteria between water and biofilm phases. It is critical to define the spatial boundaries for the community in consideration to understand movement across the boundary.

4.2.1. Dispersal from the network

The cold influent water from the network controls the microbiome of the entire building plumbing system. Influent water had the highest diversity in this study, consistent with previous studies (Chapter 3) (Ji et al., 2017). After transport into the building, selective forces decreased diversity (i.e., high temperatures), but there is not another obvious source for diversity to regularly increase under ideal conditions. Of course, in real systems, bacteria can be introduced with maintenance or back-siphonage from a tap (Walker et al., 2000). In this study, 15,121 OTUs of the total 17,960 OTUs in the system were detected in influent water. The difference (2839 OTUs) could have been 1) transient distribution network bacteria that were not detected with grab influent samples, 2) present from pipe system construction, or 3) too rare to detect in influent water. Rare distribution network species proliferating in building plumbing is a common phenomenon. The most obvious case is potential opportunistic pathogens like Legionella pneumophila that are not detected in distribution network water, but are detected in water heating tanks (Wadowsky et al., 1982).

Thus, selective forces acting upstream in the local network (e.g., high sulfate concentrations), will ‘trickle down’ into the building plumbing. For example, in this study it was clear that the high species richness in Location 1 influent water accounted for the increased richness with-
in the test system. High sulfate concentrations at Location 2 increased sulfate metabolisms which persisted into the test system.

4.2.2. Dispersal from the water heater

Dispersal via bulk-flow also played a key role in the temperature-based differences at each site. While dispersal into the entire building is determined by the influent water samples, selective forces in the water heater additionally control the hot water distribution system microbiome. While distal pipes do not reach a high temperatures for prolonged period, the microbiome transported into the hot water distribution system experiences a longer period of high temperature selection, as discussed above. Both the hot biofilm and hot stagnant water thus have a restricted available microbiome for further selection within the pipe.

The hot water microbiomes differed by location. While selection by temperature remained similar within the test system (delivery at 53 ± 2 °C), the dispersal seed was different at each location, due to the difference in upstream selection (i.e., residence time in water heater, discussed above). The influent communities were also different at each location, and may have responded differently to the selective forces in the water heater. Upstream selection reduced the microbiome available for dispersal into distal ends in terms of richness at both sites. This relatively low richness likely translated to stronger divergence between the two sites.

The cold water microbiomes, on the other hand, were more similar between sites than hot water microbiomes. At both locations, cold stagnant water originated from a diverse influent microbiome, and thus the optimal bacteria for carbon and stagnation conditions were likely present and able to assemble similarly in biofilms at both sites. That is, while the hot water limits the similar downstream selective factors from assembling a similar microbiome, the cold water does not.
4.2.3. Dispersal between biofilm and water phases

The influent water supplies the initial microbiome for both biofilm and stagnated water. However, dispersal between stagnated water and biofilm occurs in both directions (attachment/detachment). The increased diversity in biofilm over time in cold samples likely results from random attachment of bacteria from the water phase. Each water delivery acts as a new source for dispersal into biofilm, and with greater time, selection from carbon from pipes decreases (i.e., diminished carbon leaching). Even though bacterial concentrations were somewhat stable over time, random attachment could fit a neutral assembly model.

With extended stagnation, biofilm detachment likely overtakes growth in the planktonic phase. After one month of stagnation, water shared more OTUs with biofilm, although this was mostly due to a decrease in water richness. Other studies have had similar observations, with decreased richness and evenness with extended stagnation time (Lautenschlager et al., 2010) and more shared OTUs with greater stagnation time (Ji et al., 2017). Moreover, proportions of phyla were not consistent with extended stagnation, as might occur with growth in the water phase. It is important to note that even normal stagnation time for this system was 10 hours, which likely allowed for both biofilm detachment and attachment to the biofilm.

4.3. Practical implications for building plumbing system design

Building plumbing systems were not initially designed to consider microbiological growth in water. Thus, studies like this may help to inform better designs for the future.

Temperature was the most important factor in this system, with hot and cold water systems clearly exhibiting different behavior. Survival of Legionella pneumophila is often studied in relation to temperature, as it is expected to proliferate in hot water distribution systems, but die with proper temperature control. However, multiple studies demonstrate that real hot water distribution systems do not maintain high temperatures, and that intermediate tempera-
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tures with distal cooling can exacerbate *L. pneumophila* growth issues (Rhoads et al., 2015; Bédard et al., 2015). In this study, *Legionella* and *Mycobacterium* had increased abundance amongst cold water pipes at Location 1, but not at Location 2. While temperatures experienced in the pipes were similar at each location, differences in water heater design affected dispersal into the system. It is important to note that only genera were considered in this study, and these do not necessarily represent opportunistic pathogens. This study does, however, indicate that cold water systems are at risk for *Legionella* and *Mycobacterium* proliferation. Both cold and hot pipes experienced room temperature for the majority of stagnation time providing similar growth conditions. At Location 1, the hot distribution system was effective at limiting *Legionella* proliferation, but it was due to control upstream rather than constant selective control at the distal end.

The materials in this study provided a new carbon source that was more substantial than organic carbon available in the distribution network. Overall the quality of materials was quite good, with four out of five pipes approved for drinking water use. Differences were not as extreme as amongst metals and polymeric pipes, nor as between a range of flexible synthetic materials. However, there were still differences attributable to carbon-leaching behavior, indicating that pipe quality is an important parameter for water quality in homes.
5. Conclusions

The ecological theory of community assembly through dispersal and selection was useful for explaining the microbiological behavior in this test system simulation building plumbing.

- Water and biofilms had some similarity because they were subject to the same upstream dispersal, but the two microbiomes were distinct due to multiple selection factors within each.

- The two locations were similar, but differences in influent microbiome (i.e., more diverse microbiome at Location 1), and chemical composition (i.e., increased sulfate at Location 2) affected the test-system by dictating dispersal.

- Hot and cold distribution systems were distinct in terms of microbiome composition, but not in terms of TCC and TOC leaching from pipes. While upstream selection changed the dispersed microbiome, selection was not continuous, as water in both systems reached room temperature during stagnation.

- Materials provided a positive selection factor, increasing organic carbon available for growth. While differences between pipe materials were not extreme, high-leaching pipes had higher TCC and lower diversity than low-leaching pipes.

- Although biofilms reached numerical stability relatively quickly, both biofilm and water microbiomes changed over time, for example with increasing diversity in cold biofilms, likely due to continual dispersal from the water phase and decreasing importance of carbon leaching from pipes.

- Stagnation time greatly impacted the microbiome and TCC in water. With extended stagnation time, biofilm detachment became important.

Ultimately, the framework applied in this study can be applied to any building plumbing water distribution system. In this study, even with two very similar water profiles, building factors changed specific outcomes. However, the same ecological principles guided behavior at both locations, and generalizations about temperature and material could be made.
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Author Contributions

CP led data acquisition, interpretation, and writing and contributed to planning. FR, RS, and SK contributed to experimental design, data acquisition, and interpretation. FH contributed to experiment design, data interpretation, and writing.
Chapter 5

References


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Supplementary information

Table S1: General influent water quality at each location, measured with standard methods. At location 1, water is sourced from groundwater (c.a. 75%), treated lake water (15-20%) and spring water (5%) with ratios varying throughout the year. It is part of a large typical municipal drinking water distribution system. Aside from the lake water, water is only treated with UV disinfection prior to distribution. At Location 2, water is sourced from groundwater (majority) and spring water. Typically water is only treated with UV disinfection prior to distribution. The distribution network is primarily industrial users, and due to associated atypical use patterns, water is sometimes chlorinated, but frequency data was not available (i.e., used temporarily with problems).

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<td>Ammonium µg N/L</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Nitrite µg N/L</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Nitrate mg N/L</td>
<td>2.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Total Nitrogen mg N/L</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>o-P µg /L</td>
<td>12.1</td>
<td>21.8</td>
</tr>
<tr>
<td>T-P µg /L</td>
<td>14.2</td>
<td>22.1</td>
</tr>
<tr>
<td>Chloride mg/L</td>
<td>12.2</td>
<td>18.3</td>
</tr>
<tr>
<td>Sulfate mg/L</td>
<td>8</td>
<td>75</td>
</tr>
<tr>
<td>Dissolved Organic Carbon mg/L</td>
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<td>0.7</td>
</tr>
<tr>
<td>Total Organic Carbon mg/L</td>
<td>0.8</td>
<td>0.7</td>
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</tbody>
</table>
Figure S1: Detail schematic of each triplicate pipe-loop

Figure S2: Photos of test system at Location 1.
**Figure S3:** Temperature in hot (red) and cold (blue) water pipes, and room temperature (gray) during a typical day. Temperature was measured every five minutes with sensors located in influent pipes that preceded test-pipes, but came after the mixer for the hot water system. The day was chosen at random, but represents typical behavior. The peaks occasionally exceeded the goal of 53 ± 2 °C, for a maximum of 61.9 °C at both locations.

**Table S2:** Samples used for DNA extraction and 16S amplicon sequencing. Additionally, 19 PCR replicates (e.g., separate PCR on the same DNA extracts) were used for randomly selected samples. Four critical samples were lost due to low DNA or sequence yield, namely, in location 2, the cold water sample from Day 259, and in Location 1, from the cold, both the water and biofilm from Day 28. Additionally, from location 2, one biological replicate from the cold water on Day 42 was lost.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Materials with Triplicate pipes separated</td>
<td>C only</td>
<td>B only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influent water Locations</td>
<td>1, 2</td>
<td>1, 2</td>
<td>1, 2</td>
<td>1, 2</td>
<td>1, 2</td>
<td>1, 2</td>
<td>1, 2</td>
<td>1, 2</td>
<td>1, 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **Biofilm**                   |    |    |    |    |    |    |    |    |    |    |    |
| Materials with Triplicate pipes separated | none | B only |    |    |    |    |    | E |    |    |    |
| Negative control Locations*   | 1, 2 | 2   | 1, 2 | 2  | 2  | 1, 2 |    |    |    |    |    |

*(some left out due to low DNA yield)
### Table S3: Details for Illumina sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Bakt_341F</td>
<td>CCTACGGGNGGCWGAG</td>
<td>Klindworth, 2013</td>
</tr>
<tr>
<td>(S-D-Bact-0341-b-S-17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakt_805R</td>
<td>GACTACHVGGGTATCTAATCC</td>
<td></td>
</tr>
<tr>
<td>(S-D-Bact-0785-a-A-21)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Amplicon PC Primers**

- Nextera adapter tail before forward: TCG-GCA-GCG-TCA-GAT-GTGTA-AAG-AGA-CAG-GA
- Nextera adapter tail before reverse: GTC-TGG-GCT-CGC-AGA-TGTGA-TAA-GAG-ACA-GAG

## PCR Details

<table>
<thead>
<tr>
<th>Assay</th>
<th>Holding</th>
<th>Cycling Reps</th>
<th>Cycling</th>
<th>Kti/Mix and Reaction Chemistry</th>
<th>Template/Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplicon PCR</strong></td>
<td>95 °C</td>
<td>19 X</td>
<td>54 °C 0:30</td>
<td>1U KAPA 2G robust HotStart Polymerase (KAPA Biosystems, Boston, USA), 1 x reaction buffer B, and 0.4 µM of each primer in a final volume of 25 µL.</td>
<td>2 µL DNA template (0.8-50 ng) Two sets of frame-shifted primer sets were used on each replicate extraction per sample: Sets 0 and 2 for replicate A and sets 1 and 3 for replicate B</td>
</tr>
<tr>
<td></td>
<td>5:00</td>
<td>72 °C 0:30</td>
<td></td>
<td></td>
<td>Sensoquest Labcycler Basic used.</td>
</tr>
<tr>
<td><strong>Index PCR</strong></td>
<td>95 °C</td>
<td>10 X</td>
<td>55 °C 0:30</td>
<td>1 X KAPA HiFi HotStart Ready Mix and 5 µl of each of the respective Nextera index primers in a total reaction volume of 50 µl</td>
<td>Pooled amplicon PCR product</td>
</tr>
<tr>
<td></td>
<td>3:00</td>
<td>72 °C 0:30</td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

## Additional Steps

<table>
<thead>
<tr>
<th>Step</th>
<th>System</th>
<th>Protocol</th>
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</thead>
<tbody>
<tr>
<td>Purification of Amplicon PCR product</td>
<td>Agencort AMPure beads XP system (Beckman Coulter)</td>
<td>Supplier’s protocol</td>
</tr>
<tr>
<td>Purification of Index PCR product</td>
<td></td>
<td>Supplier’s protocol</td>
</tr>
<tr>
<td>Quality Control of Index PCR product</td>
<td>Agilent Bioanalyzer</td>
<td>Supplier’s protocol</td>
</tr>
<tr>
<td>Quantification of Index PCR product</td>
<td>KAPA library quantification kit</td>
<td>Supplier’s protocol</td>
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</table>
Table S4: Details for Illumina sequencing data processing

<table>
<thead>
<tr>
<th>Step</th>
<th>Algorithm/Version</th>
<th>Parameters</th>
<th>Citation</th>
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<tbody>
<tr>
<td>Quality Control</td>
<td>FastQC v.0.10.1</td>
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<td></td>
</tr>
<tr>
<td>Merge Reads</td>
<td>FLASH v1.2.9</td>
<td>minimum overlap: 40</td>
<td>(Magoc and Salzberg, 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>maximum overlap: 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max mismatch density: 0.2</td>
<td></td>
</tr>
<tr>
<td>Trim adaptor sequences and sort frame shifts</td>
<td>Cutadapt v1.4</td>
<td>error rate: 0</td>
<td>(Martin, 2011)</td>
</tr>
<tr>
<td>Quality Filtering</td>
<td>PRINSEQ-lite v0.20.4</td>
<td>size range: 450-550 bp</td>
<td>(Schmieder and Edwards, 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>minimum mean quality score: 25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>no ambiguous nucleotides</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC range: 20-80</td>
<td></td>
</tr>
<tr>
<td>OTU clustering</td>
<td>usearch v7.0.1090</td>
<td>identity cutoff: 97%</td>
<td>(Edgar, 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>abundance sorting: 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chimera filtering</td>
<td></td>
</tr>
</tbody>
</table>
Figure S4: Migratory behavior of materials in test system (A-E, Table 1). Total organic carbon (TOC) was measured on days 1, 3, and 7, with migration at 60 °C. Concentration (mg-C/L) was converted using the tests’ surface-area-to-volume ratio (1:1) for a final concentration in relationship to surface area (µg-C/cm²). Assimilable organic carbon (AOC) was measured by incubating migratory water with additional nutrients and cells, and measuring subsequent growth (10^6 cells/cm²). For both tests, material E measured much higher than other materials, and is thus on the secondary right-axis. The migration test was done according to the BioMig protocol, a standardized materials test (Wen et al., 2015).

Materials A-D are approved for potable water use, while material E is not. As potable drinking water pipe materials must pass stringent migration guidelines, the difference between these two classes is not surprising. Still, amongst materials approved for potable use, materials A and C had less migration than materials B and D. Based on this analysis, materials A and C were classified as “low-leaching materials”, while materials B, D, and E were classified as “high-leaching materials”. This dichotomous classification simplifies complicated material behaviors, but is nonetheless useful for describing the experiment.
Community assembly through dispersal and selection governs the drinking water microbiome

**Figure S5**: Total organic carbon (TOC, top row), and total cell counts for Water (middle row) and biofilm (bottom row) through time. All points are averages for three triplicate pipes of the same material (color).
Further Discussion on Figure S5:

Difference in TOC leaching between sites – The TOC leaching was higher at Location 1, especially for the hot water system. This may result from slight differences in hot water system operation between the two buildings. For example, Location 2 was in a warehouse that was less protected from seasonal temperatures than Location 1. The difference in ambient temperature may account for some difference in leaching behavior.

It is also very important to note that the TOC was not measured in the influent hot water (i.e., water freshly delivered from the water heater). Water heaters are known to consume TOC, but it is unknown if the different water heaters consumed a different amount of TOC. Moreover, the mixing valve that was programmed to deliver water to the test system at 53 ± 2 °C may have used different ratios of hot (with reduced TOC) and cold (with more TOC) water, impacting comparisons between the locations.

Figure S6: Migration under sterile conditions at 30 °C (blue) and 60 °C (red). Migration was much higher for Material E, so this is displayed on an alternative axis (right) with the same units. Concentration (mg-C/L) was converted using the tests’ surface-area-to-volume ratio (1:1) for a final concentration in relationship to surface area (µg-C/cm²).
Community assembly through dispersal and selection governs the drinking water microbiome

Figure S7: Percentage of intact cells (IC) in stagnant water samples, when it was measured (not measured for Day 28 or Day 56). Boxplots for cold (blue), and hot (red) samples mark the median and quartile measurements, with empty boxplots for Location 1, and shaded ones for Location 2. The color of individual points indicates the material for particular samples, with low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) separated. Several outliers were cut from the data set because (3 points at location 2), theoretically, the intact cell ratio should not exceed 1.

Figure S8: Percent of high-nucleic acid (HNA) cells in the water phase. Boxplots for influent (black), cold (blue), and hot (red) samples mark the median and quartile measurements. Empty boxplots are for Location 1, and shaded ones are for Location 2. The color of individual points indicates the material for particular samples, with low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) separated.
**Figure S9:** TCC in water (top) and biofilm (bottom) phase, broken out by material category (low-leaching, high-leaching), location, and temperature. Boxplots for influent (black), cold (blue), and hot (red) samples mark the median and quartile measurements. Empty boxplots are for Location 1, and shaded ones are for Location 2. The color of individual points indicates the material for particular samples, with low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) separated.
Community assembly through dispersal and selection governs the drinking water microbiome

Figure S10: Total cell concentrations, log transformed, in biofilm (x-axis) and water (y-axis) samples from the same pipe during the early stage of operation (Days 7, 14, 28, and 42). Shading indicates location (Empty for Location 1, filled for location 2). Water cell concentrations are expressed in cells/cm² to account for differences in surface area to volume ratio between pipes. Linear correlation lines are shown.

Figure S11: Total cell concentrations, log transformed, in biofilm (x-axis) and water (y-axis) samples from the same pipe during the all stages of operation. Symbol type indicates stagnation length (Gray circles for Normal overnight stagnation, orange diamonds for extended stagnation (5 days at Day 56 [Location 2 only], 2 days at Day 119, 27/34 days at Day 286, and 87/78 days at Day 373/364). Water cell concentrations are expressed in cells/cm² to account for differences in surface-area-to-volume ratio between pipes. Correlations were not strong, and the Water: Biofilm ratio increased with increased stagnation (i.e., shift up on graph).
Figure S12: NMDS representing bray-curtis dissimilarities between samples. Samples are divided as to whether they originate from the cold system (top) or the hot system (bottom). Colors represent the time the sample was taken (pinks for early stage, green for late stage), while shape represents the phase (squares for biofilm, triangles for water) and location (empty for location 1, filled for location 2). Ellipses represent 95% confidence intervals of clustering by time point. For hot samples, ellipses were calculated to account for both time and location since locations clustered separately.
Community assembly through dispersal and selection governs the drinking water microbiome

Figure S14: Number of observed OTUs in water phase, broken out by whether stagnation time was normal, overnight (top), or extended (5 days at Location 2 on Day 56, 2 days at Day 119, 27/34 days at Day 286; top). Boxplots for influent (black), cold (blue), and hot (red) samples mark the median and quartile measurements. Empty boxplots are for Location 1, and shaded ones are for Location 2. The color of individual points indicates the material for particular samples, with low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) separated.

Figure S13: Comparison of richness (observed OTUs) and total cell count for water (top) and biofilm (bottom) samples. Samples are colored according to their temperature/sample type. In water samples, trend lines are added for visualization of trends.
Figure S15: Number of observed OTUs in water (top) and biofilm (bottom) phase, broken out by material category (low-leaching, high-leaching), location, and temperature. Boxplots for influent (black), cold (blue), and hot (red) samples mark the median and quartile measurements. Empty boxplots are for Location 1, and shaded ones are for Location 2. The color of individual points indicates the material for particular samples, with low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) separated.
Figure S16: Total number of observed OTUs in each sample for water (top row) and biofilm (bottom row) through time. All points are from Illumina sequencing data for combined samples, except for Material E at time point 10, where biological replicates were analyzed separately and data was analyzed afterwards. Where PCR replicates were done, averages were taken. Several data points are missing due to low DNA yields - see Table S2.
Figure S17: NMDS representing bray-curtis dissimilarities between samples. Colors represent the material for the sample, whether from a high-leaching material (B, D, E) or a low-leaching sample (A, C). Samples are divided as to whether they originate from the cold system (top) or the hot system (bottom). Shape represents the phase (squares for biofilm, triangles for water) and location (empty for location 1, filled for location 2).
Figure S18: Relative abundances of phyla (colors) for water (inner circle) and biofilm (outer circle) for sampling event 9 (top), which had normal stagnation, and sampling event 10 (bottom) which had 1-month of stagnation. Differences between hot (left) and cold (right) communities can be seen. The number of OTUs for each phase and that are shared between phases is indicated. Certain phyla are enriched or diminished with extended stagnation, while biofilms remain more stable.
Figure S19: Relative abundances (ratio) of OTUs in the Nitrospirae phylum (left) and with sulfate-related metabolisms (right), in water (top) and biofilm (bottom) phase. Boxplots for influent (black), cold (blue), and hot (red) samples mark the median and quartile measurements. Empty boxplots are for Location 1, and shaded ones are for Location 2. The color of individual points indicates the material for particular samples, with low-leaching materials (A or C, purple) and high-leaching materials (B, D, or E, green) separated.
Community assembly through dispersal and selection governs the drinking water microbiome
Chapter 6

Biofilm detachment during stagnation and flow in building plumbing

To be submitted for publication in revised format by:

Caitlin R. Proctor, Lisa Neu, Dominik Peter, Jürg Sigrist, Marja Gächter, Michele Prevost, Frederik Hammes
Abstract

The water in building plumbing is exposed to extended stagnation times, which can degrade high water quality entering from the distribution network. While many studies observed a bacterial washout with flushing, the exact cause of this washout is not yet understood. In this work, we apply flow cytometry (FCM) and 16S amplicon sequencing to both field samples and several building plumbing simulators in order to investigate the behavior of bacteria in the pipe during extended stagnation and subsequent flushing. Across several test-systems, the highest bacterial loads in water consistently occurred with the first flush, with up to 14% of all bacteria in the water of a 10-minute shower exiting the pipe in the first 20 seconds. Using novel real-time FCM combined with direct in-pipe sampling, we were able to differentiate between biofilm detachment occurring during stagnation (i.e., accounting for up to 56% of the total cell increase from network water in the first 100 mL flush) from biofilm detachment occurring with physical force during flow (i.e., accounting for 44% of the increase). This cell concentration increase during stagnation occurs much quicker than is possible for growth in the water phase alone, with up to a 51-fold increase in only one hour of stagnation. Microbiomes of this first flush also showed high similarities to biofilms, with decreased richness compared to fully flushed water. However, not all organisms followed the same trends during stagnation. *Legionella pneumophila* concentrations during stagnation did not follow total cell concentrations, potentially affected by temporal variability in the building itself. All of these observations were used to construct a simple conceptual model for the behavior of biofilms and water in pipes during stagnation and flow. Dispersal is identified as the primary driving force for short-term changes in the water phase, with dispersal both from the network (via bulk-flow), and between biofilm and water (i.e., attachment/detachment). Such a conceptual model can help inform building water sampling strategies to better target and quantify exposure risks.

**Keywords:** biofilm detachment; building plumbing; stagnation; real-time flow cytometry
1. Introduction

By the time drinking water reaches consumers’ homes, it can be several days old (Prest et al., 2016), and the water age can increase substantially in the building due to stagnation at individual taps (DeOreo et al., 2016). Increased water age (i.e., hydraulic residence time) in the distribution network has been implicated in loss of chlorine residual, bacterial growth, and even gastrointestinal disease (Prest et al., 2016; Tinker et al., 2009). Similarly, extended stagnation in buildings has been linked to loss of chlorine residual and increases in bacterial concentrations (Lipphaus et al., 2014; Lautenschlager et al., 2010; Rhoads et al., 2016; Zhang et al., 2015). The change in water quality occurs quickly during stagnation and can substantially impact the amount of bacteria to which consumers are exposed (i.e., doubling bacteria concentrations overnight (Lautenschlager et al., 2010)). Thus, the idea of biological stability (i.e., growth, biofilm detachment, and pipe breakages affecting bacterial concentrations (Prest et al., 2016)) is arguably more important for water in buildings than in distribution networks. While the well-studied distribution networks typically have monitoring and low temperatures to maintain and control biological stability, homes are not monitored, stagnate most of the time, and have bacteria friendly temperatures, together contributing to an uncontrolled loss of biological stability. Moreover, opportunistic pathogens (e.g., Legionella pneumophila) are known to proliferate in homes, but not in distribution networks.

Studying biological stability can differ substantially between distribution networks and building plumbing. Growth conditions are inherently different, as building plumbing has higher surface-area-to-volume ratios due to small diameter pipes and it has near constant stagnation. While stagnation in distribution network occurs, it is avoided by design, while it is unavoidable in building plumbing. The high surface-area-to-volume ratios increase the potential impact biofilms have on water quality. Stagnation has been proven to affect bacteria in water, with the first liter of water from a stagnant pipe having (1) more bacteria than flushed water, and (2) a different microbiome (Lautenschlager et al., 2010; Zhang et al.,
Biofilm detachment during stagnation and flow in building plumbing

2015). Both of these effects are typically attributed to growth of network water bacteria (i.e., in the planktonic phase). However, 98% of biomass in drinking water systems is in the biofilm phase or loose deposits (Liu et al., 2014), and recent studies have shown that the stagnated water microbiome is similar to the biofilm microbiome (Chapter 3, Chapter 5, (Ji et al., 2017)). Thus, it is not unreasonable to assume that biofilm detachment plays a strong role in the bacterial increase during stagnation.

Biofilm detachment can occur as erosion of single cells or the sloughing of biofilm aggregates, and can be passive (driven by external factors) or active (biological processes within the biofilm) (Rittmann et al., 2003; Kaplan, 2010; Morgenroth, 2003). In the context of drinking water, erosion and sloughing driven by physical force is most commonly studied, with a focus on flow rates (Douterelo et al., 2013; Lehtola et al., 2006; Manuel et al., 2007; Stewart, 2012). While water flow creates zones of slow-flow around biofilms that retard diffusion and encourage tall biofilm clusters (Stewart, 2012), stagnant water would not create such an inhibitory layer. Additionally, in building plumbing, biofilm detachment by physical force (i.e., by shear force (Picireanu et al., 2001)) would be limited to a short period (i.e., 7.8 minutes/day for a shower hose biofilm (DeOreo et al., 2016)). The mixture of flow and stagnation in building plumbing essentially creates a semi-batch reactor: new nutrients and biomass are introduced during flow and interact with biofilm (i.e., fixed biomass) during stagnation. These conditions would fundamentally change the nature of biofilm detachment compared to constant flow systems, but there are preciously few published studies concerning biofilm detachment without flow in drinking water plumbing systems (Manuel et al., 2007; Hunt et al., 2004).

In this study, four different test-systems were used to investigate the behavior of bacteria during stagnation in building plumbing pipes, to determine how and why biological stability is lost in the final meters of water distribution. Most studies of building plumbing water are limited to measurements of end-of-pipe samples, which cannot differentiate between the
phenomena of (a) biofilm detachment with flow (b) biofilm detachment during stagnation and (c) growth in the water phase. Here, novel real-time flow cytometry (FCM) methods were used to enumerate bacteria directly inside the pipe during stagnation and flow with millisecond resolution to help differentiate these phenomena. Alongside highly quantitative FCM, 16S amplicon sequencing and *Legionella pneumophila* quantification were used to assess the microbiome shift during stagnation. Results were then synthesized into a conceptual model describing the short-term behavior of bacteria inside building plumbing pipes.
2. Materials and Methods

2.1. Experimental setups

Test-system A: Four real showers from the gym of an office building in Dübendorf, Switzerland were analyzed (Showers 1-4). First, water grab samples for manual FCM measurements were collected using a large glass funnel and clean plastic tube to funnel water into 50 mL tubes Granier tubes. Using flow rates (varying from 1.7 – 2.6 L/minute) and cumulative volume, flow time was calculated. Samples were later collected for sequencing, in a similar manner as above, by collecting four water samples in muffled Schott bottles with autoclaved caps. The first sample was the first 500 mL flushed (1), and the second sample was the next 1 L of water (500 – 1,500 mL) (2). After 6 minutes of flow, two “fully-flushed” samples were taken with the shower hose attached (3) and without the shower hose (4). One week after these water samples were collected, biofilms were removed, extracted, and characterized as described previously (Chapter 3). Exact ages of shower hoses were not known, but were likely older than five years.

Test-system B: Shower events were simulated with three shower hoses that had been used regularly for 1 or more years (Hoses 1-3). Hoses 1 and 3 were each collected from two person households in Zürich, Switzerland, and have unknown ages, likely older than five years. Shower hose 2 was collected from the gym shower of a laboratory and office building in Dübendorf, Switzerland, and had been installed for 1 year and 2 months. Hoses were flushed for 5 minutes with warm (maximum temperature 42 °C), non-chlorinated tap water with a flow rate of approximately 6 L/min after varying stagnation times. Hoses were kept filled with water during stagnation, simulating typical stagnation conditions in the home. During a shower event, eight outflow samples were taken for manual FCM measurements (the first flush samples: 0-100 ml, 100-200 ml, 200-300 ml, and after flushing for 1, 2, 3, 4 and 5 min). Additionally, the first flush sample (0-100 ml) was sonicated (30 seconds, 40 % power, 50% intensity) to separate clumps of cells. Additional real-time FCM measurements...
were made directly in the pipe (described below). After water samples were collected, biofilms were removed and extracted as described previously (Chapter 3).

**Test-system C:** As described in (Proctor *et al.*, 2016), hoses made from six flexible synthetic materials (Materials A-F) were installed in triplicate in a shower simulator, typically flushed once per day with warm water (maximum temperature 42 °C) at 1 L/minute per hose. The flushing schedule was temporarily altered to achieve stagnation times of 1 hour to 8 days (192 hours), during the second and third month of operation. Water samples were collected for manual FCM measurements by closing off the hose and gently decanting water from the pipe (i.e., the tap was not opened and network water was not mixed in). Additionally, real-time FCM measurements were taken directly in the pipe during normal flushing events (after 24 hours stagnation), after seven months of operation.

**Test-system D:** Nine replicates (Hose A-H) of one shower hose (identical to Material F from Proctor *et al.*, 2016) were installed in a fixed horizontal installation designed to minimize flow disruptions and heterogeneity (i.e. disturbed as little as possible). Typically, warm water (maximum temperature 42 °C) was flushed twice per day for 15 minutes at a low flow rate of 0.35 L/minute in each hose. First flush samples were taken by collecting the first 130 mL (filling completely a 100 mL Schott bottle). After ten minutes of flow, fully flushed samples were taken. For additional experiments, typical flushing schedules were altered to achieve stagnation times of 1 hour to 24 hours. Samples were analyzed with manual FCM measurements and with Legiolert (below). All experiments took place 1 year – 1 year and 3 months after installation of the test-system.

All four test-systems described here used non-chlorinated water from Dübendorf, Switzerland, and were installed in the same building complex. Further characterization of the water is available elsewhere (Proctor *et al.*, 2016; Lautenschlager *et al.*, 2010). It should be
noted that in test-system B, biofilms were not developed in the same place (i.e., transplanted hoses), but the water used for biofilm development was also non-chlorinated.

2.2. Manual FCM measurements

When necessary, samples were diluted 1:10 with filtered (0.22 µm Millex-GP, Millipore), bottled mineral water (Evian, France). Samples were stained according to standardized protocol (SLMB, 2012) by incubation for 10 minutes at 37°C with SYBR® Green I (Life Technologies, Eugene OR, USA; final concentration 1:10,000) in the dark. Cell counts were determined using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium) with a flow rate of 66 µl/min. Fixed standard gates were applied to separate bacteria from background signals to calculate total cell concentrations (TCC), as well as to separate low nucleic acid (LNA)-content bacteria from high nucleic acid (HNA)-content bacteria to calculate percentage of LNA cells (Prest et al., 2013).

2.3. Real-time FCM and in-pipe measurements

For real-time FCM measurements, a newly developed automated staining and incubation module was used to continuously extract, stain, and incubate water samples (Besmer et al., 2017). The device uses a peristaltic pump to draw stagnant and flowing water from directly inside pipes via a needle with a flow rate of 0.3 ml/min. Sample was stained in a mixing chamber with stain (SYBR Green I; diluted 1:20,000 in TRIS-buffer) continuously flowing at 0.3 mL/min for a combined flow rate of 0.6mL/min. From the mixing chamber, the stained sample flows into an incubation loop (0.75 mm PEAK tubing, 35 °C) for 10 minutes of flow time. The sample then flows into a sampling port compatible with a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium). Data was collected continuously using the “unlimited run” function as previously described (Arnoldini et al., 2013; Besmer et al., 2017) at a flow rate of 14 µL/min. Fixed gating was used as described above during analysis.
2.4. Legiolert

Samples were analyzed for *L. pneumophila* using the Legiolert system (IDEXX, Germany) according to manufacturers’ protocol. Briefly, the method detects a bacterial enzyme present when *L. pneumophila* grows in 100 mL in specifically designed Quanti-Trays. Detection is based on color and turbidity seen after 7 days, allowing an estimation of the most probable number (MPN). The new method has been critically evaluated and has reasonable comparisons to plating, often with greater recovery (Sartory *et al.*, 2017).

2.5 DNA extraction and community analysis

Water samples from test-system A, ranging from 500 – 1100 mL, were filtered onto 0.2 µm polycarbonate Nucleopore® membrane filter (47 mm diameter, Whatman, Kent, UK). Filters were inserted into a 5 mL tube and stored at -20 °C before DNA extraction with the Power Water DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to manufacturers’ instructions. Biofilm samples for test-system A were extracted and analyzed according to protocols in Chapter 3.

Amplicon sequencing was done as described in previously (Proctor *et al.*, 2016). Briefly, 1 ng of DNA extract was amplified with modified primers Bakt_341F and Bakt_805R (Klindworth *et al.*, 2013) targeting the V4-V5 region of the 16S rRNA gene. Index PCR was performed to add Nextera XT v2 Index Kit adaptors (Illumina) to the amplicon. Each PCR product was purified using the Agencort® AMPure® XP system (Beckman Coulter, Inc., Bera, CA). Product concentrations were normalized using Qubit 2.0 HS DNA system (Thermo Fisher Scientific). A pooled library was analyzed on the MiSeq platform using MiSeq Reagent Kit v2 (300-cycles, #MS-102-2002) according to manufacturer’s protocol with 10% PhiX. All sequencing was done at the Genetic Diversity Centre (GDC) of ETH, Zurich. Sequences were merged, trimmed, filtered, and clustered into operational taxonomical units (OTUs) according to several algorithms (Chapter 3). Sequences were identified according to greengenes v.13.5 (DeSantis *et al.*, 2003). In R, phyloseq (McMurdie and Holmes, 2013)
and vegan (Oksanen et al., 2013) packages were used for processing. Libraries were rarefied to 20,788 sequences per sample. Non-metric multidimensional scaling (NMDS) was used to visualize microbiome similarities using Bray-Curtis dissimilarity.
3. Results and Discussion

This study synthesizes data from four distinct test-systems that captured various aspects of water stagnation and flow in building plumbing. The test-systems ranged from realistic (real showers in test-system A, real shower hoses transplanted to a controlled tap in test-system B), to highly controlled with lower flow rates (six flexible hoses with different materials installed in triplicate in test-system C, and nine shower hoses of the same material in test-system D). All were installed in the same laboratory building, fed with non-chlorinated drinking water. Despite differences between test-systems and experimental conditions, several conclusions could be drawn about bacterial loads [3.1], the nature of biofilm detachment [3.2], and in-pipe behavior during stagnation [3.3]. Moreover, community changes with stagnation were explored [3.4]. Results were finally synthesized into a conceptual model [3.5].

3.1. Bacterial loads are highest in the first flush

Bacterial loads, as measured by total cell concentration (TCC) with flow cytometry (FCM), were highest in the first minute of flow and decreased rapidly thereafter (Figure 1, Figure 2, Figure 3). This finding was consistent whether using the outflow of showers with showerheads (test-system A), shower hoses without showerheads (test-system B), or even when measuring directly inside the pipe of simulated showers (test-system C).

In one example of a real shower (Shower 2, test-system A), the shower user would have been exposed to a cumulative total of $1.9 \times 10^9$ cells over the course of a 10 minute shower, but 22% of this exposure occurred already in the first minute (10% of the full shower time), and 14% of this exposure occurred in the first 20 seconds (3.3% of the full shower time). The drop in bacterial loads occurred quickly. Consumers would have been exposed to an average of $1.7 \times 10^8$ cells in the first 20 seconds of flow (averaging the four real showers of test-system A), but this dropped 71% to only $5.1 \times 10^7$ cells in the next 20 seconds.
**Figure 1:** Total cell concentrations of water from real showers during the first 1.5 minutes of flow (showers 1-4, test-system A). Water was collected in sequential 50 mL aliquots. Flow rate was slightly different between showers (ranging 1.7 – 2.6 L/min). For a volume-based analysis, please see Figure S1.

**Figure 2:** Total cell concentrations and percent of low-nucleic acid (LNA)-content bacteria of water from a laboratory shower simulator with three different shower hoses (test-system B). All samples are taken during a five-minute flushing event after 24 hours of stagnation. The first three samples are sequential 100 mL samples during the first flush, and subsequent samples are taken after 1, 2, 3, 4, and 5 minutes of flow. Lines and error bars represent average and standard deviation for triplicate flushing events taken from each hose (hose 1-3). Insets show detail for the first 6 seconds of flow.
Figure 3: Total cell concentrations of water measured directly inside the pipe using real-time flow cytometry, from a low-speed laboratory shower simulator (test-system C). Lines and error bars represent average and standard deviation from repeat flushing events from two hoses that were made of either material F (pink, with triplicate measurements) or material C (purple, with duplicate measurements). Samples were measured continuously before, during (in blue) and after a fifteen minute flushing event, after 24 hours of stagnation. For more information on the simulator and biofilm, please refer to published work (Proctor et al., 2016).

Such a washout curve of bacteria from drinking water pipes is not unprecedented after stagnation (Prest et al., 2013; Lautenschlager et al., 2010; Zhang et al., 2015). Other water parameters (e.g., temperature, chlorine residual) also show such a washout effect because the water quality changes during stagnation in the building. During stagnation, chlorine residual is consumed and temperatures increase (e.g., with building insulation) ((Rhoads et al., 2016; Zhang et al., 2015), Chapter 5). Hot water distribution systems, green buildings, and large buildings can have an even longer period to reach ‘fresh’ distribution network water because these have a larger stagnant volume to flush and lower water use (Hawes et
al., 2017; Rhoads et al., 2016). However, while the changes controlling these physical (temperature) and biochemical (chlorine) parameters are understood, the reasons for biological changes are not yet entirely clear.

3.2. Biofilm detachment occurs during both flow and stagnation

Real-world drinking water studies are inherently limited to the use of end-of-pipe water measurements, which cannot differentiate between bacterial (a) detachment by physical force (i.e., with shear force of water flow (Picioreanu et al., 2001)), (b) detachment due to processes within the biofilm (i.e., erosion during stagnation, active biofilm dispersal (Rittmann et al., 2003; Hunt et al., 2004)), and (c) growth in the water phase (i.e., not detachment). While some studies use these end-of-pipe measurements to determine growth and detachment rates via mass balance (Manuel et al., 2007), doing so relies heavily on assumptions (e.g., biofilm growth is equal to detachment rates, growth is steady in both biofilm and water phases). For building plumbing, a constant growth rate is an unreasonable assumption given the semi-batch reactor nature of stagnant pipes. In this section, we used a novel technique to measure water directly in the pipe during stagnation and flow. By doing this, we could differentiate between (a) detachment with physical force during flow and (b) detachment due to processes that occur during stagnation. Discussion of the third option, (c) growth in the water phase, is presented in the next section (3.3).

With these in-pipe measurements, high TCC (i.e., 4 - 15 times higher than after 15 minutes flow (Figure 3)) was observed before any flow started. When flow began, a steady drop in TCC was observed. This is an important finding that indicates that high amounts of biofilm detachment occurred during stagnation, rather than when flow began. If the high TCC in the first volume of water exiting the pipe (i.e., first flush) were dominated by detachment during flow (i.e., driven by shear force), an initial ‘bump’ would have occurred only when flow started. Several real-time FCM studies, including some with higher flow rates and with
different stagnation times had a similar trend (Figure S3, Figure S4). The possible mechanisms for this portion of biofilm detachment are further discussed in section 3.5.

While the in-pipe measurements did not suggest that biofilm detachment during flow contributed significantly to the increase in TCC, sloughing of aggregates and erosion of single cells due to shear force could not be completely disregarded in these test-systems. FCM, measuring essentially single cells, would not correctly measure large aggregates of detached biofilm without an additional clump disruption step. Moreover, in-pipe measurements described above were taken with a needle carefully positioned in the center-line of flow, and thus may not have captured all dynamics across the cross-section of pipe.

In another experiment (test-system B, Figure 4), in-pipe measurements were compared to the first flush (here, the first 100 mL of water exiting the pipe) during the same shower simulations. The in-pipe measurement taken before flow started (i.e., increase during stagnation) accounted for an average of 56% of the total first flush increase in TCC (with the increase calculated as the difference between first 100 mL and a sample taken after 5 minutes of flow). Thus, less than half of the increase in TCC (i.e., erosion, single-cells detaching) could be attributed to biofilm detachment with physical force.
Biofilm detachment during stagnation and flow in building plumbing

Shear forces likely contributed continuously to TCC increases in the water during flow. Even with long flow times, TCC was not stable, indicative of occasional sloughing or inconsistent erosion (Figure 1, Figure 2, Figure 3). The minimum TCC was also different between the test-systems, although the same building was used in all test-systems (i.e., same influent). This was most noticeable within test-system C (Figure 3), where the two pipe materials had different TCC after 15 minutes of flow. These two pipe materials were in the same test-system, but had different hose diameters (0.8 and 0.7 cm for materials C and F, respectively) and different biofilm bacterial concentrations (1.4 x 10^7 and 2.2 x 10^6 cells/cm² for materials C and F, respectively) (Proctor et al., 2016). Both biofilm amount and the surface-area-to-volume ratio could have affected the relative impact of biofilm detachment during flow.

Throughout these test-systems, flow-rates varied considerably. This affected the rate of bacterial washout, but it is not entirely clear how. Flow-rate affects both the speed with which distal-end stagnant water leaves the system (i.e., washout of the biofilm detached during stagnation) and biofilm detachment rates during flow (Picioreanu et al., 2001; Lehtola et al., 2006). As an example, the simulator with a low flow rate (1 L/minute, test-system C) had a very slow bacterial washout curve (i.e. 4-5 minutes of flow for TCC to drop 75-85%). In test-system B, with 6 L/minute flow, it only took 2-3 seconds of flow for TCC to drop an average of 75-85% percent. Volume relationships alone cannot explain this discrepancy (i.e., it only takes 15-20 seconds for test-system C to reach the same volume washout as 2-3 seconds in test-system B; see volume axes in Figure S1, Figure 2, Figure 3). While the method for testing (i.e., end-of-pipe vs. in-pipe measurements) also likely accounts for this particular discrepancy, the same effect was also seen with identical methods (Figure S3, Figure S4).

It is thus clear that flow rate affected washout in other ways. It is possible that with a high flow-rate, the water was more efficiently pushed out (i.e., all streamlines pushed at once rather than slowly mixed into the center streamline), or that biofilm was more quickly detached with higher shear forces. The difference in shear force is difficult to calculate. Test-
systems A-C all had turbulent flow as determined by Reynold’s number (attributable largely to small pipe diameters). However, due to the small pipe diameters, short lengths, limited head loss, and difficulty estimating pipe roughness, the exact shear force in each system is an unknown. Further tests could more accurately measure these, or could use stepped flow-rates in a single system to more accurately determine the effect of shear under building plumbing conditions. Such tests could also reveal differences in biofilm strength between different operational systems.

3.3 Bacterial concentrations increase quickly with stagnation time

Increasing stagnation time resulted in increased water TCC across all test-systems: (test-system C, Figure 5; test-system D, Figure 6; test-system B, Figure S2). Such an increase with stagnation time was reported previously (Lautenschlager et al., 2010). While previously attributed to growth of water phase bacteria (Lautenschlager et al., 2010; Zhang et al., 2015), several lines of evidence supported that this was primarily due to biofilm detachment instead, including (1) the high rate of increase during stagnation, (2) similar rates regardless of age of pipe, and (3) a strong relationship between maximum TCC and biofilm concentration.

We argue here that the rate of increase, which ranged from 0.04 hr\(^{-1}\) – 0.09 hr\(^{-1}\) in various pipes during the first 24 hours of stagnation (Figure 5), far outpaced any growth that was possible in the water. When the warm water from this building was incubated with stagnation in glass bottles, little to no growth was seen (Figure 5, control). Even considering that the minimum TCC (i.e., starting point) was variable due to previous biofilm detachment with flow, TCC multiplied 34-fold for material C and 51-fold for material F after only one hour of stagnation (comparing the 1 hour stagnation point of Figure 5 to the minimum TCC during flow in Figure 3). In the next hour of stagnation, TCC continued to increase 1.2-1.5 fold in these materials. The doubling time for bacteria in drinking water is usually reported in the range of days, not minutes (Boe-Hansen et al., 2002; Prest et al., 2016).
Biofilm detachment during stagnation and flow in building plumbing

Figure 5: Total cell concentrations of stagnant water in flexible hoses made of 6 materials (Materials A-F) in a low-speed laboratory shower simulator (test-system C), and in a glass control bottle (gray), after increasing amounts of stagnation time. Water samples were taken by closing off hoses and gently decanting water (i.e., no water flow from network). For test-system samples, lines and error bars represent average and standard deviation from triplicate hoses of each material. For the control study, the same tap water was stagnated in 40 mL glass bottles with similar surface-area-to-volume ratios. Biofilm concentrations at the time of the study for each material are as follows: A – 3.4 x 10⁵ cells/cm²; B – 1.8 x 10⁶ cells/cm²; C – 5.1 x 10⁶ cells/cm²; D – 1.1 x 10⁷ cells/cm²; E – 1.5 x 10⁷ cells/cm²; F – 2.9 x 10⁷ cells/cm² (Proctor et al., 2016).

The increase in TCC during stagnation was similar whether using newly installed pipes (Figure 5, approximately 2 months in test-system) or aged pipes (Figure 6, more than 1 year in test-system). The network water used in this study had little carbon available for growth (0.37 μg/L of assimilable organic carbon), which would theoretically support only 3.7 x 10⁵ cells/mL growth (Vital et al., 2010; Hammes and Egli, 2005). Thus, the test-systems’ synthetic pipes, which leach carbon, are likely the primary carbon growth substrate (Proctor et al., 2016). If the increase during stagnation were driven by growth in the water phase alone, a higher rate of increase would be expected in new pipes, when more carbon is available for growth (Proctor et al., 2016; Wen et al., 2015). Even so, approximately 1,000...
μg/L of assimilable organic carbon would be necessary for the water phase cells to grow from $10^5$ cells/mL to $10^7$ cells/mL, which is far more than any pipe can provide in a short time, even under optimal leaching conditions (Proctor et al., 2016; Wen et al., 2015).

Finally, the effect during stagnation was highly variable between the six materials in test-system C (Figure 5). Although the same influent water went into all pipes, maximum TCC and the rate of initial increase were closely related to biofilm amounts (i.e., more biofilm, higher TCC). Altogether, this suggests that biofilm detachment, rather than growth, is driving the dynamics of TCC increase in the water during stagnation.

**Figure 6:** Total cell concentrations (lower left), percent of low-nucleic acid (LNA)-content bacteria (upper left), and *L. pneumophila* concentrations of water from a low-flow laboratory shower simulator with eight hoses (A-H) from the same material (test-system D) taken after increasing amounts of stagnation time. All samples taken were the first flush (130 mL) flowing from each hose after the specified stagnation time. For *L. pneumophila*, the upper limit for the test (Legiolert, IDEXX) is indicated with a dotted line. For flow cytometry measurements (left), flowing water was taken at the same time of measurement as a reference (i.e., this water was not stagnated). For samples taken after 17.5 hours of stagnation, the average and standard deviation of triplicate measurements, taken on different days, are shown, representing the same data as the stagnant water in Figure S10.
The prevalence of biofilm detachment, both during stagnation and with flow, suggests that multiple flushes and extended stagnation would eventually deplete the biofilm of bacteria. On the contrary, only a minute percentage of the biofilm is actually detached during stagnation. For example, in test-system B, the increases in TCC over 24 hours for in-pipe measurements accounted for only 0.06 – 0.66\% of biofilm (all water TCC converted to cells/cm² for comparison to biofilm concentrations in Table S1). Even the first flush (i.e., also including some biofilm detached with flow) only accounted for an additional 0.11-0.44\% of the biofilm. The results were similar in real showers after overnight stagnation (increases for the first 50 mL of flush accounted for 0.24 – 0.87\% of biofilm, test-system A). Biofilm detachment during stagnation also slowed down after 24 hours (Figure 5, Figure S2), suggesting that equilibrium was reached with time, preventing biofilm detachment that would far outpace replenishment. Replenishment occurs via attachment from the water phase and, more importantly, via growth in the biofilm phase (i.e., utilizing carbon from pipes, occurring during stagnation).

### 3.4 The community shifts during stagnation

Increases in TCC during stagnation were accompanied by a fast shift in the bacterial FCM fingerprint. While flushed water (i.e., network water) had a high percentage of LNA-content bacteria (% LNA) (Figure 2), stagnated water had a low % LNA, and this remained fairly stable over increasing stagnation time (Figure 6, Figure S5). This type of FCM fingerprint change likely indicates a microbiome composition shift (Chapter 7, (Prest et al., 2014; Props et al., 2016)). Since bacterial microbiome compositions are typically quite different between biofilms and network water (Chapter 3, 5), the increase in HNA-content bacteria may be further indicative of biofilm detachment. However, since growth is also dominated by HNA-content bacteria (Prest et al., 2013), further investigations into the microbiome were necessary to determine the cause for this shift.
The composition of the microbiome was analyzed in test-system A for shower hose biofilms and several water samples (referred to here as: first flush - the first 500 mL exiting the pipe; second flush - the liter following first flush; fully flushed with/without hose - taken after 6 minutes before and after detaching the shower hose). The microbiome was clearly different between water and biofilm samples (Figure 7), as has been previously reported (Chapter 3, Chapter 5). There was far lower richness amongst biofilm samples than water samples, and increasing diversity with flushing time (Figure S6).

When tracking the core microbiome composition (i.e., OTUs shared between sample groups, Figure S7, Figure S8), the similarities between biofilm and stagnant water are clear. While an average of 82% of the biofilm OTUs were detected in the first flush, this quickly dropped (second flush = 69%, fully flushed with hose = 55%, fully flushed without hose = 47% shared). A shift in community during stagnation (as measured with the first flush) has been observed previously (Chapter 3, 5) (Lautenschlager et al., 2010; Ji et al., 2017; Zhang et al., 2015), but this is often attributed to selective growth in the water phase. However, especially when combining absolute abundance data (Figure S8), the contribution of local biofilm bacteria to this stagnant first flush is clear.

Biofilm detachment can also be investigated by tracking specific organisms. In test-system D, *Legionella pneumophila*, an opportunistic pathogen known to grow in building plumbing, was measured in first flush samples (here, first 100 mL exiting the pipe) after varied stagnation time. Interestingly, the earlier findings with respect to biofilm detachment and stagnation time did not hold as strongly for *L. pneumophila* in this test-system. Specifically, *L. pneumophila* initially increased with stagnation, indicating detachment from the biofilm, but after extended stagnation, it decreased in concentration (Figure 6).
Several explanations can account for this *L. pneumophila* behavior. The method used for *L. pneumophila* detection is growth based. Thus, the decrease measured may only indicate a drop in cultivability after biofilm detachment, rather than a decrease in concentration. *L. pneumophila* growth conditions are complex, usually requiring certain temperatures and either amoebae or biofilm hosts for replication (Taylor *et al*., 2009; Thomas and Ashbolt, 2011; Bédard *et al*., 2015). Thus, a loss in cultivability or death during stagnation would not be surprising, especially since the bulk water cools during extended stagnation (Chapter 5). Furthermore, culture methods do not tend to correlate with other bacterial quantification methods in drinking water (Van Nevel *et al*., 2017).

When interpreting the results across several experiments, it is critical to remember that the building water is not entirely stable during flushing (e.g., biofilm erosion and sloughing during flushing, inconsistent washout in Figure 1). Furthermore, the cold water in this building has previously been shown to vary with time (i.e., related to use-patterns) (Besmer *et al*., 2014).
Since the last water flushing into a pipe becomes the stagnated water, any temporal variability in the microbiome at this point would give a different ‘starting point’ before stagnation, changing the relative importance of biofilm detachment on the final community composition. This may be particularly true for \textit{L. pneumophila}. Sometimes \textit{L. pneumophila} concentrations were higher in flushed samples than in stagnant (17.5 hours) samples (Figure S10), and \textit{L. pneumophila} showed temporal instability in hot water of the building despite relative TCC stability (Figure S9, Figure S12). The temporal instability of \textit{L. pneumophila} could be linked to the operation of the water heater (further discussed with Figure S9, Figure S11), and the results indicate presence of \textit{L. pneumophila} in the rest of the building plumbing (i.e., not just the distal-end biofilm). If growing elsewhere in the building (e.g., another stagnant distal end biofilm), this bacterium could have been introduced to the sampled pipes either during flow (e.g., turbulent eddies at junctures) or during stagnation (e.g., active swimming through stagnant water).

### 3.5. A conceptual model for short-term biofilm detachment dynamics

All of the results above fit into a simple conceptual model for water microbiomes during stagnation and flow at distal ends in building plumbing and in the absence of chlorine (Figure 8). Most of these short-term dynamics are driven by biofilm detachment (and attachment) and the bulk flow of water. Together these processes can be thought of as “dispersal”, or the movement of bacteria into or out of a microbial community (i.e., immigration and emigration events for the water phase community located at the distal ends of pipes) (Vellend, 2010; Kinnunen \textit{et al.}, 2016; Battin \textit{et al.}, 2007). Selection, or the difference in fitness between bacteria favoring some bacteria over others (Vellend, 2010), is also at play, but is probably more active on longer time-scales (Chapter 5).

The water that initially stagnates (Figure 8A) originates via dispersal facilitated by bulk flow from the distribution network (i.e., fully flushed water). It also may include some cells dispersed from biofilms elsewhere in the building and distribution network (i.e., detached
with shear force during flow). This network water has low TCC (Figure 3) and high richness or diversity, especially compared to the biofilm (Figure S6).

During stagnation, the biofilm cells disperse into the surrounding water (biofilm detachment) (Figure 8B), increasing the water TCC (Figure 5, Figure 6), and driving a community shift in the water phase (Figure 7, Figure S6, Figure S7). While some dispersal occurs in the opposite direction (bacteria attaching to biofilm), this process is clearly dominated by dispersal out of the biofilm, resulting in a minimal loss for the biofilm (i.e., <1%), but a significant increase for the water phase (i.e., 1500%+). Even with increased similarity between water and biofilms due to this dispersal, selection ensures that the microbiomes remain somewhat distinct, as biofilm (sessile growth, protected from environmental stress) and water (suspended, low nutrients) have different conditions for continued survival.

The mechanism for dispersal out of the biofilm during stagnation was not entirely clear, and requires more study. However, continuous dispersal from biofilm has been observed during stagnation after flow previously (Hunt et al., 2004). In that case, it was hypothesized as a response to starvation. In the case of shower hose biofilms, fresh water would bring an influx of the limiting nutrients (i.e., nitrogen phosphorous). Both high and low nutrient conditions have been proposed as regulating dispersal from the biofilm (Kaplan, 2010). Other possibilities include cell-division-mediated dispersal (i.e., due to fast growth of biofilm with influx of nutrients), swarming/seeding dispersal, quorum sensing, and peptide signals (Kaplan, 2010). Regardless of the method of biofilm detachment during stagnation, it is likely that the process is selective as well. Only the top of the biofilm is directly available for surface detachment processes like cell-division-mediated dispersal (Kaplan, 2010; Morgenroth, 2003). Other methods of active dispersal (e.g., seeding/swarming dispersal) may act pervasively throughout the biofilm, but have only been specifically observed in a few species (Kaplan, 2010).
Figure 8: Conceptual diagram of water and biofilm microbiome interactions during stagnation and flow in a pipe with established biofilm (i.e. short term dynamics).

A. At the beginning of stagnation, water bacteria are primarily comprised of network bacteria [bacteria with white outline]. The water TCC is low (see Figure 3), but the microbiome has high richness or diversity (i.e., many colors) (see Figure S6). Biofilm bacteria (bacteria with black outline) are present in much higher concentrations, but have relatively little diversity (i.e. fewer colors) (see Figure S6).

B. With increasing stagnation time, dispersal between the biofilm and water is dominated by dispersal out of the biofilm. This results in increased TCC in the water (see Figure 5, Figure 6), and a community shift towards lower diversity with more OTUs shared with the biofilm (see Figure 7, Figure S6, Figure S7). The biofilm, however remains essentially stable, as <1% detaches.

C. During flow, water TCC rapidly decreases as the previously detached biofilm cells are pushed out of the pipe (see Figure 1, Figure 2, Figure 3). Some biofilm aggregates are also sloughed with flow (see Figure 4), but an insignificant portion of the biofilm is lost. With sufficient flushing, the water microbiome shifts back with dispersal from the network, becoming more diverse (see Figure 7, Figure S6, Figure S7).

Regardless of the means of dispersal from the biofilm, the relative impact (i.e., percent increase in TCC) is likely dependent both on the amount of biofilm (i.e., more cells in biofilm available to disperse, Figure 5), and upon the surface-area-to-volume ratio (i.e., more available biofilm surface area to affect a smaller volume). The impact of biofilm detachment would be less obvious in a pipe with less biofilm or with a lower surface-area-to-volume ratio (e.g., larger diameter). Thus, while dispersal from the biofilm may occur similarly further back in the building plumbing system, because larger pipes are used and stagnation is less long (i.e., flow based on use at multiple distal ends), the impact on TCC is less severe.
During flow, this stagnated water is pushed out (Figure 8B), together with biofilm cells and aggregates detached with shear forces (Figure 4), becoming the “first flush” that consumers are exposed to. Fresh water from the distribution network is transported into the building pipes during flow, quickly decreasing TCC (Figure 1, Figure 2, Figure 3), and shifting the microbiome (Figure 7, Figure S6, Figure S7). This dispersal from the network might be affected by temporal variability upstream (e.g., variable temperature in water heater providing uneven selection processes upstream). As stagnation begins (Figure 8A), biofilm cells again disperse into the water (Figure 8B).

Here, only short time-scale changes in the local microbiome were considered. On this time-scale, the biofilm community remains essentially stable. The net change in biofilm bacterial concentration is <1% during each water exchange while the net change in water TCC is >100%. On a longer time-scale, shifts in selection factors (e.g., diminishing nutrient sources) would likely cause changes in the biofilm microbiome (Chapter 4, 5). Moreover, dispersal can also occur along spatial continuums (Battin et al., 2007) in addition to the temporal continuum discussed here.
5. Conclusions

- Despite differences in flow rate, surface-area-to-volume ratio, and biofilm age, the results from all of the test-systems support several clear conclusions.
  - Bacterial exposure is highest from the first flush from distal ends in building plumbing [3.1].
  - Biofilm detachment during stagnation and flow contribute to the high TCC in the first flush [3.2]
  - Bacterial concentrations increase continuously during stagnation due to biofilm detachment [3.3], but <1% of biofilm detaches.
  - Biofilm detachment results in a community shift in the water phase, but does not fully explain the behavior/dynamics of all organisms [3.4].
- The concept of dispersal (via biofilm detachment and bulk flow of water) is critical for understanding the short-term dynamics discussed here.
- These dynamics can also inform a more controlled sampling strategy for building plumbing studies and water quality monitoring.
  - It was clear that the exact stagnation time affected water TCC, and this should thus be kept equal for proper comparison between samples.
  - Since L. pneumophila concentrations decreased with long stagnation time, a short stagnation (i.e., 4-8 hours) may be ideal for detecting this organism.
  - Distal end biofilms had a high impact on the microbiome of the first flush, where total cell concentrations were highest, and thus sampling biofilms could be informative of risk.
  - Due to dynamics in the entire building, absence of an organism in biofilm and first flush does not guarantee that it isn’t present in the building. Temporal dynamics need to be considered in sampling campaigns (sampling once per day does not always capture relevant dynamics), and the entire building needs to be considered when designing for biological stability.
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Author Contributions

CP led experimental design, data acquisition, data interpretation, and writing. LN, DP, JS, and MG contributed to data acquisition. MP contributed to data acquisition and interpretation. FH contributed to experiment design, data interpretation, and writing.
References


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Supplementary information

Figure S1: Total cell concentrations of water from real showers during the first 2.5 liters of flow (showers 1-4, test-system A). Water was collected in sequential 50 mL aliquots. Flow rate was slightly different between showers (ranging 1.7 – 2.6 L/min). For a time-based analysis, please see Figure 1. By volume or by time, the trend is remarkably similar, with total cell concentrations reaching a baseline after approximately 0.5 L of flow in all four showers.

Figure S2: Total cell concentrations water from a shower hose installed in a high-speed shower simulator (Hose 1, test-system B). Water samples are the first flush (i.e. first 100 mL flowing out of pipe) (dark, top) and after five minutes of flow (light, bottom) after increasing amounts of stagnation time (ranging from 1 hour to 48 hours). For 24 hours, the average and standard deviations are shown for triplicate flushing events (see Figure 2). While the first flush samples increase in concentration rapidly with greater stagnation time, the samples taken after five minutes flow show remarkable reproducibility.
Figure S3: Real-time flow cytometry raw-data for one replicate measurement from a hose with Material F in test-system C (Figure 3). Flow cytometry was used to measure continuously from inside the pipe for the last five minutes of a 24-hour stagnation (left), during 15 minutes of flow (“flow time”) and during 5 minutes of subsequent stagnation (right). Here, the left axis is green fluorescence and each dot represents one particle. With so many particles, dots are represented rather as density with darker black representing more particles. Thus, on the left, there are more particles than on the right. This range of green fluorescence represents bacterial cells, with particles sitting higher up thought to have more fluorescence and essentially be larger.

Figure S4: Real-time flow cytometry raw-data for replicate measurements from shower hose A (test-system B) under 1 hour (top) or 48 hours (bottom) of stagnation time. Flow cytometry was used to measure continuously from inside the pipe for the last minutes of a the indicated stagnation time (1 hour or 48 hours) (left), during 5 minutes of flow (between green bars) and during subsequent minutes of subsequent stagnation (right). Here, the left axis is green fluorescence and each dot represents one particle. With so many particles, dots are represented rather as density with darker black representing more particles. Thus, on the left, there are more particles than on the right for the 48-hour stagnation time. As this range of fluorescence represents bacteria, total cell concentrations (TCC, cells/µL, right axis) are plotted over density with a red line. Points are achieved by counting dots within a specified time bin. With short stagnation, the drop when flow started is difficult to capture. However, for 48 hours stagnation, the drop during flow is evident.
Figure S5: Additional flow cytometric data to complement total cell concentrations in Figure 5. The percent of low nucleic-acid (LNA)-content bacteria is from SYBR green (SG) measurements, while the percent of intact cells represents the proportion of bacteria remaining within the gate with SYBR green propidium iodide (SG-PI) staining (SGPI cells/SG cells). Samples were stagnant water in hoses made of 6 materials (Materials A-F) in a low-speed laboratory shower simulator (test-system C) and in a glass control bottle (gray), after increasing amounts of stagnation time. These water samples were taken by closing off hoses and gently decanting water from hoses (i.e. no water flow). For test-system samples, lines and error bars represent average and standard deviation from triplicate hoses of each material. For the control study, the same tap water was stagnated in 40 mL glass bottles with similar surface-area-to-volume ratios.

Intact Cells

The measurement of intact cells was done identically to the measurement of total cells except for the stain used. Propidium iodide (PI; 30 mM) was mixed with the SYBR® Green I working solution to a final PI concentration of 0.3 mM. 200 µL was stained with SGPI at 10 µL/mL. The percentage of intact cells (% Intact cells) was calculated by dividing intact cell concentration by total cell concentration.
Figure S6: The richness (here, as measured by number of observed OTUs in each sample) in samples taken from four real showers (showers 1-4, test-system A). For each shower, five samples were measured: shower hose biofilm, two samples representing the first flush (0-500 mL and 500-1500 mL), and two samples from fully flushed (after 6 minutes of flow) water, either with or without the shower hose in tact.
Figure S7: Core microbiomes in samples taken from four real showers (showers 1-4, test-system A). For each shower, five samples were measured: shower hose biofilm, two samples representing the first flush (0-500 mL and 500-1500 mL), and two samples from fully flushed (after 6 minutes of flow) water, either with or without the shower hose in tact. Core microbiomes are calculated by adding the relative abundance for OTUs falling into each of several ‘core’ categories, including the building core, those OTUs shared by all 20 samples (black), the biofilm core, shared by all four biofilm samples (orange), the water core, shared by all 16 water samples (purple), and shower specific cores, shared by all 5 samples within a shower (green). Shower cores and building/water cores could overlap, and are represented by dual shading. Many OTUs did not fall into any of these categories (other, white). Shower 1, in which the 500-1500 mL sample looks most similar to the 0-500 mL sample, was measured first amongst showers, and thus may have captured building stagnation which was flushed before sampling other showers.
Figure S8: Calculated absolute abundance of core microbiomes in samples taken from four real showers (showers 1-4, test-system A). For each shower, five samples were measured (four shown here): shower hose biofilm (not pictured), two samples representing the first flush (0-500 mL and 500-1500 mL), and two samples from fully flushed (after 6 minutes of flow) water, either with or without the shower hose in tact. Core microbiomes (shown by relative abundance in Figure S7) are calculated by adding the relative abundance for OTUs falling into each of several ‘core’ categories, including the building core, those OTUs shared by all 20 samples (black), the biofilm core, shared by all four biofilm samples (orange), the water core, shared by all 16 water samples (purple), and shower specific cores, shared by all 5 samples within a shower (green). Shower cores and building/water cores could overlap, and are represented by dual shading. Many OTUs did not fall into any of these categories (other, white). Absolute abundances were calculated by multiplying the core relative abundance described above with the total cell concentration (TCC) of each sample.
Figure S9: Total cell concentrations (top) and *L. pneumophila* in a hot water distribution system measured during one continuous day (red triangles with line), and on other days of the week (including weekends) at 9:00 am in a low-speed continuously flowing tap of a building (test-system E). Temperature in the continuously flowing water was also measured over several days (Figure S11) and a typical day is shown here. Temperature was likely related to operation of the water heater, with the one strong peak representing a once-daily heating to 60 °C, and subsequent peaks corresponding to hot water use in the building.

**Test-system E:** To measure the microbiological background of the building (relevant to all systems above), one hot water tap and one cold water tap were opened for continuous flow over the course of a 24 hour period. On other days, additional samples were taken after a minimum of 10 minutes of flow at each tap. Flow rate was low (0.2 L/minute), with the intention of getting the 'background' of the hot water distribution system without greatly disrupting normal flow schedules. Grab samples were periodically taken and analyzed with manual FCM measurements and with Legiolert. Additionally, temperature was measured with 2-minute resolution using 176T4 Datalogger with a Thermoelement Typ K temperature sensor (Testo, Lenzkirch, Germany).
**Figure S10:** Concentrations of adenosine tri-phosphate (ATP, top left), total cells (bottom left) and *Legionella pneumophila* (right) in paired first flush (stripes) and fully flushed (black outline) samples from 9 replicate shower hoses in a low-speed shower simulator (test-system D). Samples were taken by collecting the first 130 mL flowing through the system (stagnant first flush), waiting for 10 minutes of flow, and taking another 130 mL sample. Each color represents a separate triplicate measurement (i.e., different days), while the same color indicates a paired measurement (i.e., on the same day). On the left, measures of total bacteria were somewhat stable between hoses and between replicate measurements. Additionally, a clear trend, where fully-flushed samples have lower bacterial activity and concentrations than stagnant samples emerges. However, for *L. pneumophila*, there is heterogeneity between hoses and between samplings on the same day. Surprisingly, the fully flushed samples sometimes have higher concentrations than their paired stagnant samples (especially for the third event, pink).

**ATP Methods**

Total ATP was determined using the BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA, USA) as described elsewhere (Hammes et al., 2010). A biofilm suspension sample (100 µL) and the ATP reagent (100 µL) were warmed to 38 °C simultaneously in separate sterile Eppendorf tubes. The sample and the reagent were combined and then the luminescence was measured after 20 second reaction time at 38 °C. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known ATP standard (Promega). ATP was measured in triplicate, and the relative standard deviation among technical replicates was below 4%.
**Figure S11:** Temperature measurements from a continuously flowing hot tap (with very low flow rate) in an office and laboratory building (test-system E). On the left, each day has a different color. Occasional low spikes during the hours of 9-16 (pink, blue, red lines) are due to manual interruptions in measurement. For all days measured, water cooled substantially overnight, but stayed within a range during the day. On the right, a typical week-day is separated out (black, top left), and two weekend days (gray, bottom right) are separated out for clarity. The first high spike of each week day seemed to happen at similar times, but the number and timing of intermediate spikes was not the same. On weekends, water temperature remained low overnight for a longer period (i.e. a delayed first spike instead of 6:30, and a quicker 'cool-down', starting around 16:00 instead of 18-20:00).
Figure S12: *L. pneumophila* (top) and total cells (bottom) in hot (left, red) and cold (right, blue) as it related to temperature in low-flow continuously flowing of a building (test-system E). Additionally, the correlation between *L. pneumophila* and total cells is shown on the far-right. Hot water typically had high concentrations, except for one sample taken at 53 °C, with non-detect. Cold water typically had non-detects or very low concentrations of *L. pneumophila* (note different scale). Data for the hot water distribution system is also represented in Figure S9.

Table S1: Biofilm total cell concentrations in the three shower hoses used for test-system B. Biofilm was extracted and measured according to the protocols outlined in Chapter 3. For Hose 3, only 70 cm was evaluated because holes in the hose (due to wear and tear) prevented robust evaluation of a longer section.

<table>
<thead>
<tr>
<th>Shower hose</th>
<th>Length evaluated</th>
<th>Total cell concentration (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90 cm</td>
<td>2.5 x 10⁸</td>
</tr>
<tr>
<td>2</td>
<td>90 cm</td>
<td>1.6 x 10⁸</td>
</tr>
<tr>
<td>3</td>
<td>70 cm</td>
<td>3.5 x 10⁷</td>
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</tbody>
</table>
Chapter 7

Phylogenetic clustering of small low nucleic acid-content bacteria across diverse freshwater ecosystems

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Chapter 7

Abstract

Here we used flow cytometry (FCM) and filtration paired with amplicon sequencing to determine the abundance and composition of small low nucleic acid (LNA)-content bacteria in a variety of freshwater ecosystems. We found that FCM clusters associated with LNA-content bacteria were ubiquitous across several ecosystems, varying from 50 to 90% of aquatic bacteria. Using filter-size separation, we separated small LNA-content bacteria (passing 0.4 µm filter) from large bacteria (captured on 0.4 µm filter) and characterized communities with 16S amplicon sequencing. Small and large bacteria each represented different sub-communities within the ecosystems’ community. Moreover, we were able to identify individual operational taxonomical units (OTUs) that appeared exclusively with small bacteria (434 OTUs) or exclusively with large bacteria (441 OTUs). Surprisingly, these exclusive OTUs clustered at the phylum level, with many OTUs appearing exclusively with small bacteria identified as candidate phyla (i.e. lacking cultured representatives) and symbionts. We propose that LNA-content bacteria observed with FCM encompass several previously characterized categories of bacteria (ultramicrobacteria, ultra-small bacteria, candidate phyla radiation) that share many traits including small size and metabolic dependencies on other microorganisms.

Keywords: low nucleic acid (LNA)-content bacteria; filterable bacteria; freshwater; flow cytometry; small cells; candidate phyla radiation (CPR)
1. Introduction

Bacteria constitute the smallest forms of independent life, and considerable effort has been made to theoretically calculate, locate, and characterize the smallest bacterial representatives (Luef et al., 2015; Duda et al., 2012). Different terminologies, potentially overlapping and not always clearly defined, are used in this field including ultramicrobacteria (UMB), ultra-small bacteria (USB), ultramicrocells, nanoarcahea, and nanoplankton. Focusing within bacteria, UMB are defined as bacteria less than 0.1 µm$^3$ in size (Duda et al., 2012). This upper limit is 1 order of magnitude smaller than a typical Escherichia coli cell (1 µm$^3$), and 9 orders of magnitude smaller than the largest known bacterium, Thiomargarita namibiensis, (2.2 x 10$^8$ µm$^3$ (Schulz et al., 1999)). Similarly, USB have been studied as having small genomes and have been isolated following 0.2 µm filtration (Luef et al., 2015). The terms ultramicrocells and nanoplankton (marine ecology) are similarly defined by filterability (Duda et al., 2012). The exact upper boundary of these groups of “small bacteria”, while defined (Duda et al., 2012), remains somewhat arbitrary. Filtration with small pore size (i.e. 0.2 µm filterable) is often used for the isolation or enrichment of these groups (Miteva and Brenchley, 2005; Luef et al., 2015; MACDonell and Hood, 1982; Hahn et al., 2003), but many bacteria may well exist on the fringe of this border (Duda et al., 2012).

Since particle-size and nucleic acid-content can be quickly assessed using flow cytometry (FCM), it may be a useful tool for identifying small cells. FCM has been used extensively in natural aquatic environments including wastewater (Harry et al., 2016; Günther et al., 2016), drinking water (Van Nevel et al., 2017), process water (Props et al., 2016), seawater (Marie et al., 2014), and lake water (Neuenschwander et al., 2015). Using this method, a bimodal distribution of cells is frequently observed, with two dominant cell clusters separated by fluorescence intensity and/or light scatter signals after staining of nucleic acids. These two groups are commonly referred to as high (HNA) and low (LNA) nucleic acid-content bacteria.
(Lebaron et al., 2002; Wang et al., 2009; Gasol et al., 1999; Bouvier et al., 2007), based on an inferred correlation between observed fluorescence intensity and cellular DNA/RNA content, the target for the fluorescent dyes. Initial studies suggested that LNA-content bacteria represented the dead or inactive fraction of microbial communities (Lebaron et al., 2002), but subsequent research contradicted this by showing their growth (Wang et al., 2009) and substrate uptake (Bouvier et al., 2007). Moreover, using cell sorting, Bouvier et al. (2007) identified LNA-content bacteria that specifically had small genome sizes, and Vila-Costa et al. (2012) characterized distinct phylogenetic communities of LNA-content bacteria in marine waters. In a finding particularly relevant to the present study, Wang et al. (2009) demonstrated that 0.45 µm membrane filtration essentially separated HNA- and LNA-content bacteria, thus establishing a link between LNA-content bacteria and the size and filterability of bacterial cells.

Filtration for size-separation is therefore useful for studying LNA-content bacteria as well as UMB and USB. Such techniques were previously used for the isolation of bacteria in the candidate phyla (i.e. candidate phyla radiation (CPR) lacking culturable representatives but likely representing a distinct clade) (Brown et al., 2015), and have recovered proposed symbiont bacteria and oligotrophic bacteria (Nelson and Stegen, 2015; Hood and MacDonell, 1987). All of these groups are proposed to resist traditional culturing for various reasons, including obligate oligotrophy and dependencies on substrates supplied by other species in nature and not typically supplied in culture media (i.e., auxotrophy). To overcome difficulty with culturing these groups of organisms, some approaches use filtration to remove large competitors and isolate small bacteria (Hahn et al., 2004; Luef et al., 2015). Caution should be taken using filtration to isolate small bacteria since large bacteria with one small dimension (e.g. long with small diameter) can also pass through filters (Wang et al., 2008).
In this study, we approach this concept of small bacteria using FCM and filtration paired with amplicon sequencing. We hypothesize that the cluster of LNA-content bacteria observed with FCM are physically small and thus easily separated with 0.4 µm filtration, are ubiquitous across and even dominate some aquatic environments, and that these LNA-content bacteria are phylogenetically distinct from large HNA-content bacteria. We test these hypotheses both within and across several freshwater ecosystems including lake water, river water, wastewater effluent, groundwater and non-chlorinated tap water. Comparing traits of our small bacteria to other bacteria isolated with filtration, including the broadly defined groups of UMB, USB, CPR, and symbiont bacteria, we propose that LNA-content bacteria can encompass all of these categories that in fact share many traits, (including small size and metabolic dependencies on other microorganisms).
2. Materials and Methods

2.1. Sampling

A total of 47 samples were taken from 22 different sampling sites in Switzerland in five categories of aquatic ecosystems, i.e. groundwater, river water, lake water, (non-chlorinated) tap water, and wastewater (secondary effluent) (Figure 1A, Table S1). One river and one lake sampling site were sampled a total of 12-15 times each to assess temporal dynamics. Samples were taken in muffled (560 °C for 3 h) glass bottles. Volumes per sampling site ranged from 500 to 25'000 ml depending on the expected concentration of bacteria in the respective ecosystem (Table S2). Samples were transported and stored at 4 °C and processed within 24 h.

2.2. Filtration

Filtration volumes were adjusted between 100 and 5'000 ml per filter based on FCM total cell concentration (TCC) measurements to approximately equalize number of cells captured (Table S2). Three types of filters were captured in duplicate for each sample (Figure 1B). The first filter captured the entire community with direct filtration onto 0.2 µm membrane filters ("all bacteria") (Nuclepore® track-etched polycarbonate membranes, 47 mm, Whatman, UK) using sterilized filtration units (Nalgene™, Thermo Fisher Scientific, USA) mounted on sterilized glass bottles. Separately, a two-step filtration was performed to obtain size-based groups. Another water sample was first filtered onto 0.4 µm membrane filters (large bacteria) (Nuclepore™ track-etched polycarbonate membranes, 47 mm, Whatman, UK), and the resulting filtrate was subsequently filtered again on 0.2 µm filters (small bacteria). Filters from the paired filtration step (Large, small) and direct filtration (All) were then stored at -80 °C until DNA extraction.
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Figure 1: Sample collection, treatment, and statistical analysis. A) 47 samples were collected from 22 sampling sites classified in 5 ecosystems. B) Each sample was processed in duplicate, and for each duplicate, 3 different groups were collected: “All bacteria”, which was filtered directly onto a 0.2 μm filter, “Large bacteria” (red), which was filtered directly onto a 0.4 μm filter, and “Small bacteria” (blue), which was the filtrate from the 0.4 μm filter captured on a 0.2 μm filter. The table represents the comparative groups used for OTU (operational taxonomic unit) classification in C), namely the large and small bacteria for filter pairs A and B. C) Each OTU from community sequencing data was classified into 5 categories based on its appearance in the large and small bacteria group of a filter pair or sample. For all categories, it was permissible that an OTU appeared in both the large and small bacteria groups of a filter pair. Unclassifiable was a catch-all for OTUs not meeting the criteria of the other categories, and eliminated OTUs did not meet abundance cutoffs.
2.3. **Flow cytometry**

The TCC of all water samples was determined with FCM before and after 0.4 µm filtration in triplicate. FCM sample preparation and measurements were based on the standard method 333.1 accredited in Switzerland (SLMB, 2012). In short, 200 µl of the water samples were pre-warmed (3 minutes, 37 °C) and then stained with 2 µl of fluorescent stain (SYBR Green I, Life Technologies, Eugene OR, USA; final concentration 1:10'000). After 10 minutes of incubation at 37 °C in the dark, 50 µl were measured on a BD Accuri C6 flow cytometer (BD Accuri, San Jose CA, USA) at a flow rate of 66 µl min⁻¹ with a lower threshold on the green fluorescence (FL1-H) channel at 1’000. Fixed standard gates were applied to separate bacteria from background signals and LNA from HNA bacteria (Prest *et al.*, 2013) (Figure 2). A 10-fold dilution with 0.1 µm filtered Evian water was performed before measurement for samples expected to have high cell numbers.

2.4. **DNA extraction**

Microbial DNA was extracted from preserved filters by enzymatic digestion and cetyltrimethyl ammoniumbromide (CTAB) extraction following a published protocol with minor adaptations (Llirós *et al.*, 2008). In short, enzymatic cell lysis was performed on filters by subsequent incubations with Lysozyme, Proteinase K, and RNase A (Proteinase K volume was increased to 10 µl, and RNase to 5 µl). Cells were lysed with a CTAB buffer, and unwanted materials were extracted with chloroform isoamyl alcohol (we used 49:1 instead of 24:1 v:v ratio). DNA was precipitated with ethanol and DNA redissolved in TE buffer. Sample replicates were extracted separately. DNA concentrations in the extracts ranged from 0.8 to 50 ng µl⁻¹.

2.5. **Amplicon sequencing with Illumina MiSeq**

16S rRNA amplicon sequencing was performed as described previously (Proctor *et al.*, 2016). Bacterial primers 341F and 785R (Klindworth *et al.*, 2013) were used, adapted with a
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tail incorporating frame-shifts that were used to separate replicates during PCR amplification. Products were purified with the Agencort AMPure beads XP system (Beckman Coulter, Inc., Bera, CA) and Nextera index primers were added with Index PCR. Index PCR product was purified, quality controlled and quantified by qPCR. Details for all steps are in Table S3. Equal amounts (4 nM) of PCR product were then pooled for sequencing on the Illumina MiSeq platform following standard protocols for the MiSeq Reagent Kit v3 600 cycles (MS-102-3003).

Multiple algorithms were used for sequence quality control and merging, trimming, and filtering reads, as well as OTU clustering (Table S4), using preferred elements of established pipelines. FastQC v0.11.2 (Magoc and Salzberg, 2011) was used for quality control. FLASH v1.2.9 was used for merging reads with a minimum overlap of 14, maximum overlap of 250, and max mismatch density of 0.25. Cutadapt v1.5 (Martin, 2011) was used with an error rate of 0 at full-length to trim adaptor sequences and sort frame shifts. Quality filtering was done with PRINSEQ-lite v0.20.4 (Schmieder and Edwards, 2011) with a size range of 390-440 bp, mean quality score of 25, maximum of 1 ambiguous nucleotide, GC range of 30-70, and low complexity filter dust/25. Finally, OTU clustering was done with usearch v7.0.1090 (Edgar, 2010) with identity cutoff of 97%, abundance sorting with minimum size of 2 and chimera filtering applied.

Sequences were classified taxonomically according to greengenes v.13.5 (DeSantis et al., 2003) using usearch v10.0.240 linux 64 and sintax (classifier). In R, phyloseq (McMurdie and Holmes, 2013) was used for processing. Sequences identified as Archaea and Chloroplasts were removed from the dataset. All samples considered had more than 4'000 reads in each of the six related samples (3 groups and 2 replicates). Raw sequence data is available under ascension number PRJEB23669.
2.6. Exclusivity analysis

For exclusivity analysis (Figure 1C), rare OTUs that did not reach at least 20 reads in at least two samples were excluded from the dataset (i.e. 43,616 OTUs reduced to 5,029 OTUs for consideration). Only large and small bacteria were considered for this analysis (not the “all bacteria” group). Relative abundances of OTUs per filter were calculated from the total number of reads, and OTUs with abundances < 0.25% per filter were ignored on that filter.

Each OTU was categorized into one of five categories: “exclusively small”, “exclusively large”, “non-exclusive”, “unclassifiable”, or “eliminated” (Figure 1C). For these definitions we considered occurrence of OTUs on (1) corresponding filter pairs, i.e. a pair of a 0.2 µm filter and 0.4 µm filters used to process the same sample replicate and (2) filter replicates, i.e. two filters of the same pore size (either 0.2 µm or 0.4 µm) that both received the same sample and thus should in theory contain the same number and composition of bacteria (technical replicates, Figure 1B,C). Exclusivity was determined by whether an OTU was present in only one group (Large or small bacteria) on both technical duplicate filters from at least one sample. If this criteria was met, it was tolerated that in other samples the OTU (1) was only present in that same group on only one of two technical replicates (e.g. low abundance preventing reproducibility), (2) was present in both groups of a filter pair (e.g. matrix effects trapping small bacteria on 0.4 µm filter, dimensions near border of filterability), or (3) was not present at all. OTUs that were present only in the small bacteria group of a filter pair in one sample and only in the large bacteria group of a filter pair in another sample were classified as non-exclusive. These non-exclusive OTUs could be further divided as to whether this (1) occurred in two separate samples with duplicate filters matching, or (2) occurred without duplicate filters matching. Eliminated OTUs were all those not meeting any of the abundance criteria (all filters having low abundance (less than 0.25%)). Unclassifiable OTUs were either (1) in too low abundance (nearly all filters having less than 0.25%) or (2) too
often co-occurring to be considered exclusive. Exclusivity analysis was performed in excel using exported OTU tables (Supplementary Information – Exclusivity Analysis).

A phylogenetic tree constructed with greengenes v13.9 was plotted to reflect these classifications using the plot_tree function in ggplot2 (Wickham, 2009). The phylogenetic tree was constructed using OTU sequence alignment created with PyNAST, and with gaps removed. Phylogenetic trees were then constructed using FastTree using gamma 20 likelihood for boot-strap values. For ease of interpretation, many OTUs not meeting certain exclusivity benchmarks were removed from trees.

2.7. Community analysis

For non-metric multidimensional scaling (NMDS) community analyses, a different OTU filtration was used. Reads from duplicate filters were merged and all OTUs that did not reach three or more reads in three or more samples were removed (i.e., 43,616 OTUs reduced to 16,254 OTUs). The dataset was then rarefied to the minimum number of reads in the merged dataset (9'781 reads, representing 16,049 OTUs). In phyloseq, NMDS was performed for visualization of community similarities using Bray-Curtis dissimilarity. In R, from the vegan package (Oksanen et al., 2013), adonis analysis was performed to quantify relative importance of each factor for community composition.

2.8. Scanning electron microscopy (SEM)

Water samples from a small artificial experimental pond system (ecosystem not included in any other analyses) that had a naturally high proportion of LNA bacteria (90%), were filtered directly onto a 0.2 µm filter. Samples were fixed with 2.5% gluteraldehyde solution. Final preparation and imaging was done by the Center for Microscopy and Image Analysis (University of Zurich).
3. Results and Discussion

In this study, we demonstrate that so-called LNA-content bacteria are ubiquitous across several freshwater ecosystems (3.1) and that they are small in size and thus separable by filtration with 0.4 µm filters (3.2). Using amplicon sequencing, we demonstrate that size-filtration accounts for some deviation in community composition (3.3), and that this could be attributed to some exclusive OTUs (3.4), which had a particular phylogenetic make-up (3.5).

3.1. LNA-content bacteria are ubiquitous

Forty-seven samples from 22 sampling sites in five different natural and engineered aquatic ecosystems (Figure 1A) contained distinct clusters in FCM data identified as LNA-content bacteria (Figure 2, Figure S1 for quantification, Figure S2 for additional examples). We defined LNA-content bacteria as the bacterial cluster(s) with green fluorescence intensity below a defined instrument-specific threshold in the FCM density plots, and HNA-content bacteria as the cluster(s) above that threshold (Figure 2). To ensure comparability, all samples were analyzed with the exact same protocol and the same FCM gate was used for all samples to select for HNA- and LNA-content bacteria (Prest et al., 2013). The FCM detection of LNA-content bacteria was robust even with different staining protocols, variables, instrumentation, and operators (Figure S3, Figure S4). Additionally, similar LNA-content bacteria clusters have been observed when only considering intact cell counts (Ramseier et al., 2011), indicating that these ubiquitous cells are likely alive.

River water and tap water samples showed distinct LNA- and HNA-content bacteria clusters with similar relative abundance (around 50%, Figure 2), although river water samples had approximately ten times higher absolute abundance (Figure S1). Groundwater and wastewater effluent samples were both dominated by LNA-content bacteria (up to 90%,
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems

Figure 2), although wastewater effluent samples had nearly 100 times higher absolute abundance than groundwater (Figure S1). The data concurs with previous studies describing LNA-content bacteria in diverse ecosystems including river water (Wang et al., 2009), and seawater (Bouvier et al., 2007; Lebaron et al., 2002). Despite relative consistency within ecosystems, the underlying factors contributing to differences in LNA-content bacteria relative abundance in different ecosystems remain elusive. For example, it goes without argument that groundwater and wastewater effluent samples have vastly different environmental conditions, yet both ecosystems show similar dominance of LNA-content bacteria.

Interestingly, lake water samples did not show clear separation between LNA- and HNA-content bacterial clusters (Figure 2E, Figure S2 for additional examples). In general, the lake water samples showed more FCM clusters than the other ecosystem samples, and the cluster within the LNA gate had particularly high median fluorescence relative to the other samples (Figure 2H). The lake water data challenges the perspective of a simplistic separation between only two major groups (i.e. LNA- and HNA-content bacteria). In fact, several previous studies have observed multiple FCM clusters in complex microbial communities (e.g., Figure S4 with DAPI stained samples and more sophisticated optical instrumentation) (Koch et al., 2013; Nishimura et al., 2005; Günther et al., 2012, 2016). Still, most freshwater environments had a nearly bimodal distribution of fluorescence intensity.
Figure 2: Typical flow cytometric density and histogram plots from the five investigated natural and engineered freshwater ecosystems (groundwater A & C, river water B & D, lake water E & H, tap water F & I, wastewater G & J) stained with SYBR Green I. Dotted black lines indicate electronic gates separating bacteria from background. Blue and red gates/dotted lines indicate electronic gates separating LNA and HNA content bacteria. FL1-A = green fluorescence intensity; FL3-A = red fluorescence intensity.
3.2. Filtration separates small LNA- and large HNA-content bacteria

Filtration selects for bacteria with a sufficiently small diameter to pass through the filter pores. Filtration (0.4 µm) retained the majority of HNA-content bacteria from samples but allowed passage of LNA-content bacteria, substantially increasing the relative abundance of LNA-content bacteria in the filtrate (Figure S5). Subsequent filtration of the 0.4 µm filtrate on 0.2 µm filters thus enabled the separate collection of communities dominated by HNA-content bacteria (0.4 µm filter) and LNA-content bacteria (0.2 µm filter) respectively (Figure 1B). For simplicity we will from here on refer to these as large bacteria and small bacteria. This filtration approach was used previously for the enrichment of particularly small LNA-content bacteria (Wang et al., 2009), and similar sequential size-separating filtration techniques have been used to study differences between attached and free-living biomass (Mohit et al., 2014; Ganesh et al., 2014) and to enrich for small bacteria of interest (Neuenschwander et al., 2017).

Figure 3 shows SEM images of a pond sample rich in small LNA-content bacteria on a 0.2 µm filter (i.e., all cells colored in Figure 3 can be considered “small”). These bacteria had an average diameter of 0.18 µm, an average length of 0.57 µm, and an average volume of 0.016 µm$^3$ (n=12); they would thus easily pass a 0.4 µm filter. This follows the small cell sizes for LNA-content bacteria (0.05 µm$^3$) and 0.2 µm filterable bacteria (0.009 µm$^3$) previously shown (Wang et al., 2009; Luef et al., 2015) and fits within the theoretical limits of minimum cell sizes for bacteria (Luef et al., 2015). In addition to this microscopy evidence, there was also a strong correlation between qPCR and FCM cell counts throughout ecosystems (including both archaea and bacterial primers, Spearman’s $\rho=0.72$, $p<0.001$, Figure S6). Archaea contributed an average of 16 % to the directly filtered (all) 16S rRNA gene copies, indicating that archaea (i.e., nanoarchaea (Ludington et al., 2017)) may be of
interest in future studies. These results indicate that LNA-content cells as measured by FCM are in fact bacteria and archaea, rather than an FCM artifact or non-bacterial particles such as viruses, free DNA, or auto-fluorescent particles. The qPCR data further suggests that small bacteria may have slightly fewer 16S rRNA gene copies per cell than large bacteria (Figure S6). Low rRNA operon copy number has been linked with oligotrophic bacteria like S. alaskensis (Roller et al., 2016). S. alaskensis was also identified as passing 0.2-µm filtration in ocean water (Nakai et al., 2011), and is often studied as a UMB.

Figure 3: Scanning electron microscopy (SEM) image of bacteria from a stagnant pond sample rich in LNA content bacteria (> 90%), filtered onto a 0.2 µm pore-size filter. Filter pores are visible as black holes, bacteria are highlighted in blue/purple shades and extracellular filaments are highlighted in green. Colors were added artificially, and the original image can be found in Figure S13.
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems

Thus, our data links low fluorescence after nucleic acid staining (Figure 2), low FCM scatter (Figure S4), filterability (Figure S5), small cell size (Figure 3), and low DNA content (Figure S6) to LNA-content cells. These links are supported by literature, where low fluorescence is linked to low DNA content (Veldhuis et al., 1997), and low scatter is linked to small cell size (Wang et al., 2009; Lebaron et al., 2002; Shapiro, 2003; Gasol et al., 1999). Filterability further confirmed the small cell size (at least diameter) (Gasol and Moran, 1999; Wang et al., 2009; Luef et al., 2015), while small cell sizes have also been linked to small genome sizes (Duda et al., 2012) and low DNA content. Since many of the physical similarities were initially proposed to be linked to temporary physiological state (e.g. starvation (Morita, 1997)), further characterization was required to determine if these distinct physical characteristics linked to a phylogenetically distinct community.

3.3. Bacterial community differences are driven by environmental conditions and filtration

Bacterial communities captured on all filter sizes (Figure 1B) were characterized with 16S amplicon sequencing to determine the differences in community composition attributable to size and other factors. Ecosystem (e.g., lake water vs. river water) was the most important factor for community composition (Figure 4), accounting for 46% of all community variations (Adonis, p<0.001). The five freshwater ecosystems were chosen to be diverse, so this outcome was expected. While not quantified in this study, multiple factors including nutrient conditions, hydraulics, and temperature vary dramatically different between these five ecosystems. Notably, two similar and linked ecosystems, tap water and its primary source in this study, groundwater, clustered close to each other.

It was clear that clustering of bacterial communities by size (i.e. large/small) only occurred within individual ecosystems. The community composition of the combined community ("all
bacteria”) consistently clustered in between the small and large bacterial groups that contributed to it. However, the community composition of the small and large groups separated considerably from each other. When only considering these two groups (i.e. leaving “all” out of analysis), size and its interactions with ecosystem accounted for 27% of community variations (9.3% and 18%, respectively, Adonis, p<0.001). Looking at each individual ecosystem (i.e. Figure 5, focusing on river water), the separation of communities by size is even more apparent. Size (small vs. large) accounts for 24 % of community variation within one ecosystem, and is a significant factor (p<0.001) in all ecosystems with more than four samples (Adonis, Table 1; Table S5 for analysis including “all bacteria” samples). However, within any ecosystem, the sampling site (e.g., River A vs. River B) was a significant factor, often accounting for more variation than size. Sampling site is important amongst rivers for similar reasons to why ecosystem is important when looking at all samples – different environmental conditions select for the total community. Thus, large and small bacteria have distinct community compositions, but they are not completely distinct subsets of the total community.

It has been suggested that HNA and LNA taxonomy is dependent on location and time (freshwater springs, (Martinez-Garcia et al., 2012)), and that percentage of LNA-content bacteria as measured by FCM varies seasonally (rivers, (Liu et al., 2016)). As a test for temporal stability within a sampling site, samples taken over four months (June - September) from River Site A were further analyzed (Site A, Figure 5). Both community composition by size (as measured with 16S amplicon sequencing) (Figure S7) and the percentage of LNA-content bacteria (as measured with FCM) (Figure S8) remained relatively stable in this site. This may indicate that samples were representative in terms of their size groups.

While there was a clear separation between the two size groups, the small and large bacteria were not completely independent, indicating species overlap and a common
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems
dependence on environmental conditions. In some previous studies, separation through cell
sorting failed to see a clear separation of LNA- and HNA-content bacteria communities
(Martinez-Garcia et al., 2012; Longnecker et al., 2005), which may be due to OTU overlap
between sizes. While our filtration approach to separate small and large bacteria has
imperfections (i.e. filter cross contamination (Figure S9)), we were able to characterize a
wide array of ecosystems at a great depth, identifying approximately $10^8$ cells for each
sample. Alternative methods, like cell sorting, are limited and time-intensive for capturing
rare organisms in low-biomass environments (e.g. tap water, ground water). The depth of
our sequencing data allowed us to further investigate the causes for community differences
and overlap as well as discrepancies with previous studies. In the next section, an analysis
of individual OTUs was used to determine which bacteria were truly phylogenetically distinct
and exclusive by size.

**Figure 4:** Non-metric multidimensional scaling (NMDS) of bacterial communities (characterized with
16S amplicon sequencing) calculated with Bray-Curtis dissimilarity between samples from five
different ecosystems (marked by color: Groundwater, Wastewater, River water, Lake water, and Tap
water), with three different size groups by shape. 'All bacteria' is the total community, directly filtered
onto a 0.2 µm filter. Large bacteria is the HNA-dominated community collected on a 0.4 µm filter, and
Small bacteria is the LNA-dominated community in the 0.4 µm filtrate, collected on a 0.2 µm filter. In
NMDS plots, points that are closer together represent bacterial communities more similar to each
other than those further away. A low stress indicates a robust diagram.
Figure 5: Non-metric multidimensional scaling (NMDS) of bacterial communities (characterized with 16S amplicon sequencing) calculated with Bray-Curtis dissimilarity between samples from four different rivers (Site A-D), with three different groups by filter pore size. Color is by sampling site, and shape is by size group. ‘All bacteria’ is the total community, directly filtered onto a 0.2 µm filter. Large bacteria is the HNA-dominated community collected on a 0.4 µm filter, and Small bacteria is the LNA-dominated community in the 0.4 µm filtrate, collected on a 0.2 µm filter. In NMDS plots, points that are closer together represent bacterial communities more similar to each other than those further away. A low stress indicates a robust diagram.

Table 1: Relative importance of various factors in bacterial communities within each ecosystem, calculated by Adonis. For each explanatory variable (Sampling site, size, and interactions between these two factors), the percent of variation explained by the variable and the p-value for statistical significance of the factor are expressed. Sampling site refers to the specific location for each sample (i.e. River A vs. River B). For this analysis, only large and small bacteria groups were included for size (“All bacteria” was excluded from analysis). Large bacteria is the HNA-dominated community collected on a 0.4 µm filter, and Small bacteria is the LNA-dominated community in the 0.4 µm filtrate, collected on a 0.2 µm filter.

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<td>Tap water</td>
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3.4 Individual OTUs are exclusive to each size across five diverse ecosystems

The forthcoming analysis is based on the classification of all OTUs into five categories namely (1) exclusively small, (2) exclusively large, (3) non-exclusive, (4) eliminated, and (5) unclassifiable (Figure 1C). Of the 5,029 OTUs that passed the first abundance cut-off (> 20 reads in > 2 samples), 434 OTUs were classified as exclusive to the small bacteria and 441 OTUs were classified as exclusive to the large bacteria (Figure 6). These OTUs occurred exclusively in one size group (small or large), and appeared on both technical duplicate filters from at least one sample. The relative abundance of these two categories reflected expected trends, with exclusively small OTUs more abundant in the small bacteria community and exclusively large OTUs more abundant in the large bacteria community (Figure 7), and these size-exclusive OTUs contributed to a substantial portion of the community on each filter.

The chosen exclusivity levels were lenient enough to allow presence on both sized filters in some samples, and thus sometimes exclusively small OTUs appeared with the large bacteria community and vice versa. Applying a higher level of exclusivity, wherein co-occurrence in both size filters was never allowed (i.e. not allowing for the cross-contamination described above) or including rare abundances (<0.25%) resulted in far fewer OTUs for analysis. However, we accepted the contamination risk and potential bias, given the likelihood of cross-contamination on filters (Figure S9) and the specificity desired when comparing across ecosystems.

Another 38 OTUs, which we called non-exclusive, were classified as small in some filter pairs and large in other filter pairs. For 12 of these 38 OTUs, this occurred in duplicate for both small and large fractions (i.e., dark green Figure 7). This could be due to differences between ecosystem or sampling site conditions (i.e. the same OTU has different
It could also indicate small species which are exclusively intracellular symbionts in some samples (appearing large), while exclusively free-living in other samples (appearing as small). These non-exclusive OTUs could be quite abundant, especially in lake and river samples (Figure 7).

**Figure 6:** Phylogenetic tree colored by OTU occurrences in each size constructed with 1,224 of > 40,000 OTUs found in water samples. Circle area represents the number of samples (both technical duplicates) in which an OTU was consistently exclusively appearing with either large bacteria (0.4 µm filter, red) or small bacteria (0.2 µm filtered after 0.4 µm filter, blue), with this number ranging from 1 to 16 samples. OTUs which were at times exclusive to both sizes in a filter pair (non-exclusive) are marked in green. Extraneous OTU branches that never met these criteria (unclassifiable) were removed from the figure. Several phyla and a class of interest are labeled. For more detailed phylogenetic identification, see Figure S10a.
Relative abundances of OTUs classified with the described exclusivity criteria (exclusively small [blue], exclusively large [red], non-exclusive [green], and unclassifiable. eliminated and rare OTUs[white/gray]) in each size group of each ecosystem. For each ecosystem (Groundwater, Wastewater, River water, Lake water, and Tap water), the total relative abundance for all filters in a particular size (small, large bacteria) is shown. Large bacteria is the HNA-dominated community collected on a 0.4 μm filter, and Small bacteria is the LNA-dominated community in the 0.4 μm filtrate, collected on a 0.2 μm filter. Non-exclusive OTUs are further divided into whether they occur in duplicate (I) or not (II). OTUs not meeting initial cutoffs are marked as “rare”, and not meeting secondary cutoffs are marked as “eliminated”. Overlap (e.g. exclusively large OTUs in the small bacteria community) is due to leniency that OTUs may occur on both filters (0.2 and 0.4 μm filters) of a filter-pair, so long as it does not appear exclusively on the opposite filter anywhere (e.g. non-exclusive OTU).

The remainder of OTUs were either eliminated due to low relative abundance (eliminated – 3,805 OTUs), or could not be classified in any of the above categories (unclassifiable - 264 OTUs). Most of these unclassifiable OTUs had consistent, but not complete low abundance (i.e., 262 OTUs had <0.25% on 87% or more of filters considered). Another 2 OTUs were often in high abundance, but were always co-occurring (i.e., appeared in >0.25% in both large and small filters consistently). These 2 OTUs were taxonomically identified as
Pelagibacterales and ACK-M1 of Actinomycetes. These remaining groups, together with the OTUs failing to meet the first abundance cutoff (38,587 OTUs) represented a large portion of the community (Figure 7), and may represent a bias in our analysis methods. For example, the large percentage of non-analyzable OTUs in groundwater may owe to the high diversity (making relative abundances for each OTU lower) and low number of samples (only 3 distinct samples).

This data aligns with previous arguments that LNA-content bacteria are viable unique microorganisms (Bouvier et al., 2007; Wang et al., 2009) and refutes the notion that small LNA-content bacteria are simply dead/inactive cells (Lebaron et al., 2002; Servais et al., 2003). It seems unlikely for an entire species (OTU) to be consistently dead/inactive across many samples and ecosystems with vastly different nutrient conditions. However, since some OTUs were not clearly or consistently classified exclusively as small or large OTUs, this may follow the theory of Bouvier et al. (2007) that while some bacteria are “intrinsic to each fraction” (small or large OTUs), others can “exchange between fractions” (non-exclusive OTUs).

3.5 Small and large OTUs cluster on phylum level

When looking at the phylogenetic classification of exclusively small and large OTUs, a remarkably clear pattern emerged (Figure 6, Figure S10, Figure S11, Figure S12). The OTUs classified as exclusive to each size were frequently grouped at a high taxonomic level (i.e., phylum). This provides further evidence that a bacterium’s size (filterability), and thus its classification as a LNA- or HNA-content bacterium, is part of a fundamental and evolutionarily well-preserved trait, rather than linked to its temporary physiological state. Moreover, this separation of the two bacteria classes occurred even when considering five diverse ecosystems. Correlation between some phenotypic traits and phylogenetic
relationships has been suggested in bacteria previously (Martiny et al., 2015), and thus this strong relationship between phylogeny and log-scale differences in size is not entirely surprising.

It should be noted that the high level clustering between sizes, while remarkable, was not entirely consistent (e.g., phyla distributions not exclusive between large and small fractions (Figure S11)). Small OTUs could be found within phyla dominated by large OTUs and vice versa (e.g., Bacteroidetes, Deltaproteobacteria, Figure S11). Especially when considering the phyla-level relative abundance of OTUs falling into different size-exclusive categories (Figure S12), it is clear that 1) much of several phyla could not be easily divided into the two size-based groups (i.e., low abundance, non-exclusive, eliminated, and unclassifiable OTUs), 2) that some phyla have considerable variability in sizes (e.g., Proteobacteria, Verrucomicrobia), and that phyla were not exclusively found in either one fraction or another. Previous studies linking cell size to phylogeny have also noted variability in size within a phylum (Stepanauskas et al., 2017). As many OTUs were discarded from our analysis (i.e., low abundance), even more size variation is also possible within each phylum. Nonetheless, more information about the phyla that were dominated by one size or another provides some deeper insight into why this size-based phylogenetic clustering occurs.

### 3.5.1 - Phyla associated with small OTUs

Many of the 434 OTU associated with small bacteria were attributed to the so-called “candidate phyla radiation” (CPR), which do not yet have cultivated representatives (i.e. Parcubacteria (OD1), Gracilibacteria (GN02), Saccharibacteria (TM7), Dependentiaceae (TM6), and Omnitrophica (OP3)). Many CPR bacteria have been associated with small genomes and have similarities with symbiotic bacteria (Brown et al., 2015; Kantor et al., 2013). Reduced genomes would be consistent with observations of UMB (Duda et al., 2012) and with low fluorescence after staining of nucleic acids. Furthermore, several taxa were
associated with symbiotic or predatory relationships with other microorganisms. Symbionts are associated with genome reduction which may reduce their ability to live independently (McCutcheon and Moran, 2011). Although shown to be growing with an innovative metagenomic approach (Brown et al., 2016), growth rates among CPR are slow, which may further contribute to difficulties with isolating and culturing these small bacteria. Like these groups, LNA-content bacteria are also difficult to culture (Wang et al., 2009). Altogether, this may suggest that many observed exclusively small OTUs (i.e., bacteria passing a 0.4 µm filter, LNA-content bacteria) lack sufficient genomes to produce all necessary cellular building blocks, and rather depend on metabolites from other cells. Rapid FCM observation of LNA-content bacteria may offer an easy method to quantify these otherwise difficult to study bacteria.

Many exclusively small OTUs fell into the proposed Patescibacteria superpylum. Parcubacteria (OD1) has previously been associated with a small size (ultra-small bacteria passing 0.2 µm filter) (Luef et al., 2015; Miyoshi et al., 2005), a reduced genome (<1Mb) with reduced functionality compared to cells with large genomes (e.g., lacking ATP synthase (Suzuki et al., 2017)), and ectosymbiosis or parasitism towards other organisms (Nelson and Stegen, 2015; Wrighton et al., 2012). Gracilibacteria (GN02) have also been reported to possess small genomes (Rinke et al., 2013). Saccharibacteria (TM7) recently achieved one cultivated representative bacteria from a human host. It had a small coccus shape, small genome (with reduced capacity), and was an epibiont of Actinomyces odontolyticus with parasitic tendencies (He et al., 2015). Metagenomic reconstructions of Saccharibacteria genomes from activated sludge and other sources confirm small genomes (< 1 Mb), and indicate a fermented microaerophilic lifestyle and small cell size (< 0.7 µm) (Albertsen et al., 2013; Kantor et al., 2013). Dependentiae (TM6), has been suggested as an LNA-content bacterial taxa previously (Longnecker et al., 2005). This phylum is thought to contain widespread parasitism and endosymbiosis, as it has been associated with small genomes (0.5-1.5 Mb), a lack of complete essential synthetic pathways, and endosymbiosis with
amoebae (Yeoh et al., 2016; McLean et al., 2013; Delafont et al., 2015). Many of these CPR bacteria have high and variable abundance reported across freshwater ecosystems (e.g., tap water dominated by Parcubacteria, with more diversity in CPR in groundwater) (Ludington et al., 2017; Borrel et al., 2010; Bruno et al., 2017). The consistency in size and features within this superphylum may indicate that cellular size, on a log-scale, is a complex and deeply conserved phylogenetic trait.

Deltaproteobacteria deviated from the rest of the Proteobacteria phylum, with many OTUs identified as small. Some belonged to the Spirobacillales order, so named because they are associated with a spiral shape (Rodrigues et al., 2008). This could indicate a bias in our results, as only the smallest dimension determines filterability, and these cells may otherwise be considered large. However, many others belonged to Bdellovibrionales, including the predatory genus *Bdellovibrio*, which is known to be small (e.g., 0.2 x 0.5 µm) (Rendulic et al., 2004). Other orders, including Myxococcales, did not follow the trend of the class and were identified as HNA. Interestingly, it has been speculated that Deltaproteobacteria have a close evolutionary relationship with Omnitrophica (OP3), a candidate phylum associated with small bacteria in this study, due to similar metabolic capabilities and genes (Glöckner et al., 2010).

Although only 20 OTUs in the Actinobacteria phylum could be identified as small, this represented a large proportion of the community (Figure S12), especially in lakes. Actinobacteria and Microbacteriaceae were previously associated with LNA-content bacteria (Read et al., 2015; Hahn et al., 2003). While the AC1 lineage of Actinobacteria was not specifically found in high abundance, this association may be interesting, as the AC1 lineage of Actinobacteria has many similarities to the CPR (Neuenschwander et al., 2017). For the AC1 lineage, dependencies on metabolites from other organisms (auxotrophies) are proposed to develop through genome-streamlining (Neuenschwander et al., 2017), and thus
the small cell-size may be linked to a more recently evolved and less conserved trait than for
the CPR.

Other taxa that were identified as predominantly small include SR1, Mollicutes, Endomicrobia, and Fibrobacteria. A previously suggested LNA-content bacterium, *Polynucleobacter* (Wang et al., 2009), was confirmed as small in this study, even though it was classified as an HNA-content bacterium in a cell-sorting study (Martinez-Garcia et al., 2012). Other suggested LNA taxa, including AC1, Alphaproteobacteria – LD12 (Neuenschwander et al., 2015), SAR11 (Hill et al., 2010; Mary et al., 2006), SAR86 (Fuchs et al., 2005), Katanobacteria (WWE3), and Microgenomates (OP11) (Luef et al., 2015) were not abundant enough for analysis in this study. Some of these taxa are not expected in this freshwater data (e.g., SAR 11 is predominantly marine), and others may have had specific primer bias against their identification (e.g., LD12 only had low coverage with the selected primers). It can not be excluded that that other particular phylotypes were biased against with the primers.

### 3.5.2 - Phyla associated with large OTUs

Phyla associated with large size had diverse descriptions, perhaps consistent with the much larger size range associated with HNA-content bacteria. Predominantly large phyla included Bacteroidetes, Proteobacteria (with the exception of Deltaproteobacteria), Planctomycetes, Firmicutes, Chlorobi, Verrucomicrobia, and Fusobacteria.

These taxa often overlapped with taxa suggested to be HNA-content bacteria in literature (e.g. Bacteroidetes (Read et al., 2015; Longnecker et al., 2005; Schattenhofer et al., 2011; Vila-Costa et al., 2012) and Gammaproteobacteria (Bouvier et al., 2007)). Several Proteobacteria previously identified as LNA were identified as large here, including Methylbacteriaceae, *Pseudomonas*, and Alteromonodaceae (Longnecker et al., 2005).
3.5.3 – Taxa associated with non-exclusive OTUs

Only 38 OTUs were identified as non-exclusive, meaning they were sometimes categorized as large and sometimes small. These belonged to Bacteroidetes (8), Actinobacteria (6), Nitrospirae (2), TM6 (3), Verrucomicrobia (3), Chlamydiae (1), and Proteobacteria (Betaproteobacteria (11), Gammaproteobacteria (3), and Alphaproteobacteria (1)). Interestingly, Bacteroidetes has previously also been identified as recovering from a starved form that can pass 0.2 µm filter (Haller et al., 2000), which may indicate its ability to change sizes across a wide range. Our results suggest that environment-dependent variations in cell size are not common, but appear to be present in certain bacteria.

3.6. Implications

In this study we showed that FCM clusters identified as LNA-content bacteria are found across diverse natural and engineered aquatic ecosystems at varying relative and absolute abundances. Moreover, we link the concepts of LNA-content bacteria (Bouvier et al., 2007), USB (Luef et al., 2015), small genome size, and UMB (Nakai et al., 2011) to 0.4 µm filterability, small cell size, and low green fluorescence. Individual OTUs could be classified as exclusively small or large based on filterability, even across five diverse ecosystems. This data strongly supports previous suggestions that LNA-content bacteria are viable microorganisms relevant to our understanding of microbial communities in natural and engineered ecosystems. The fact that individual OTUs exclusive to large and small sizes classified distinctively on phylum level, suggests that bacteria’s size and classification as LNA- or HNA-content bacteria is part of a fundamental and evolutionarily well-preserved trait.

Additionally, since many OTUs exclusively filterable through the 0.4 µm filter were members of clades with non-culturable or parasitic bacteria, this may point to a limited capacity for independent life for some of these species. Finally, observing LNA with FCM, for example by using FCM fingerprinting to track spatio-temporal dynamics in engineered (Prest et al., 2013,
or natural freshwater (Besmer et al., 2014; Amalfitano et al., 2016) systems, offers an easy way to quantify these abundant small bacteria that are otherwise rather difficult to culture and study.

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Author Contributions

CP led data analysis and interpretation with respect to sequencing data, and writing. MB contributed to experiment planning, data acquisition, data interpretation (especially flow cytometry data), and writing. TL contributed to data analysis. KB contributed to data acquisition. J-CW contributed to sequencing data processing. MA contributed to writing. HB contributed to data analysis, interpretation, and writing. FH contributed to experiment planning, data analysis, interpretation, and writing.
References


Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems


Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems


Prest EI, Hammes F, Kötzsch S, van Loosdrecht MCM, Vrouwenvelder JS. (2013). Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric


Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems


Supplementary information

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1. Method Details

Table S1: Site information

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</tr>
<tr>
<td>WWTP 2 (Fällanden)</td>
<td>1</td>
</tr>
<tr>
<td>Pilot Scale Treatment Plant (Dübendorf)</td>
<td>2</td>
</tr>
<tr>
<td>WWTP 3 (Niederglatt)</td>
<td>1</td>
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</tbody>
</table>
## Table S2: Filtration Volumes

<table>
<thead>
<tr>
<th></th>
<th>Groundwater</th>
<th>River water&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Lake water&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Tap water</th>
<th>Wastewater&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of sampling sites</strong></td>
<td>4</td>
<td>5 (+11)</td>
<td>9 (+11)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td><strong>Sampled volume (mL)</strong></td>
<td>25'000</td>
<td>500</td>
<td>500</td>
<td>5'000</td>
<td>500</td>
</tr>
<tr>
<td><strong>Volume per filter (mL)</strong></td>
<td>5'000</td>
<td>100</td>
<td>100</td>
<td>1'000</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>1</sup> One river and lake each were sampled 12 times to assess temporal dynamics

<sup>2</sup> Secondary effluent from a wastewater treatment plant
Table S3: Details for Illumina sequencing

<table>
<thead>
<tr>
<th>Amphlicon PCR Primers</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Bakt_805R (S-D-Bact-0785-a-A-21)</td>
<td>GACTACHVGGGTATCTAATCC</td>
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</table>

| Nextera adapter tail before forward | TCG-TCG-GCA-GCG-TCA-GAT-GTGTAT-AAG-AGA-CAG-GA |
| Nextera adapter tail before reverse | GTC-TCG-TGG-GCT-CGG-AGA-TGTGTA-TAA-GAG-ACA-GAG |

<table>
<thead>
<tr>
<th>PCR Details</th>
<th>Assay</th>
<th>Holding</th>
<th>Cycling Reps</th>
<th>Cycling</th>
<th>Kti/Mix and Reaction Chemistry</th>
<th>Template/Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicon PCR</td>
<td>95 °C</td>
<td>95 °C 0:30</td>
<td>19 X</td>
<td>54 °C 0:30</td>
<td>1 U KAPA 2G robust HotStart Polymerase (KAPA Biosystems, Boston, USA), 1 x reaction buffer B, and 0.4 µM of each primer in a final volume of 25 µL. Sensoquest Labcycler Basic used.</td>
<td>2 µL DNA template (0.8-50 ng) Two sets of frame-shifted primer sets were used on each replicate extraction per sample: Sets 0 and 2 for replicate A and sets 1 and 3 for replicate B</td>
</tr>
<tr>
<td>Index PCR</td>
<td>95 °C</td>
<td>95 °C 0:30</td>
<td>10 X</td>
<td>55 °C 0:30</td>
<td>1 X KAPA HiFi HotStart Ready Mix and 5 µl of each of the respective Nextera index primers in a total reaction volume of 50 µl</td>
<td>Pooled amplicon PCR product</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Additional Step</th>
<th>Step</th>
<th>System</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purification of Amplicon PCR product</td>
<td>Agencort AMPure beads XP sytem (Beckman Coulter)</td>
<td>Supplier's protocol</td>
</tr>
<tr>
<td></td>
<td>Purification of Index PCR product</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quality Control of Index PCR product</td>
<td>Agilent Bioanalyzer</td>
<td>Supplier's protocol</td>
</tr>
<tr>
<td></td>
<td>Quantification of Index PCR product</td>
<td>KAPA library quantification kit</td>
<td>Supplier's protocol</td>
</tr>
</tbody>
</table>
Table S4: Details for Bioinformatics

<table>
<thead>
<tr>
<th>Step</th>
<th>Algorithm/Version</th>
<th>Parameters</th>
<th>Citation</th>
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</thead>
<tbody>
<tr>
<td>Quality Control</td>
<td>FastQC v.0.11.2</td>
<td></td>
<td></td>
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<tr>
<td>Merge Reads</td>
<td>FLASH v1.2.9</td>
<td>minimum overlap: 15 max overlap: 250 max mismatch density: 0.25</td>
<td>(Magoc and Salzberg, 2011)</td>
</tr>
<tr>
<td>Trim adaptor sequences and sort frame shifts</td>
<td>Cutadapt v1.5</td>
<td>error rate: 0 full-length</td>
<td>(Martin, 2011)</td>
</tr>
<tr>
<td>Quality Filtering</td>
<td>PRINSEQ-lite v0.20.4</td>
<td>size range: 390-440 bp minimum mean quality score: 25 max 1 ambiguous nucleotides GC range: 30-70 low complexity filter dust/25</td>
<td>(Schmieder and Edwards, 2011).</td>
</tr>
<tr>
<td>OTU clustering</td>
<td>USEARCH v7.0.1090</td>
<td>identity cutoff: 97% abundance sorting: 2 chimera filtering</td>
<td>(Edgar, 2010)</td>
</tr>
</tbody>
</table>
2. Diversity of total cell concentrations and %LNA content bacteria between and different freshwater ecosystems

![Graph showing the diversity of total cell concentrations and %LNA content bacteria between different freshwater ecosystems.](image)

**Figure S1**: Overview of investigated freshwater ecosystems with respect to flow cytometry total cell concentration and percentage LNA content bacteria. Clusters are only for visualization purposes. Note that the x-axis is on logarithmic scale.
Figure S2: Staining and Typical flow cytometric density from an array of samples from four ecosystems (Lake water, River water, Tap water, and Wastewater) stained with SYBR Green I. Dotted black lines indicate electronic gates separating bacteria from background. Red gates/dotted lines indicate electronic gates separating LNA and HNA content bacteria.
3. Flow cytometry comparison measurements with different staining methods and flow cytometer models

Figure S3: Comparison of SG staining measurements of TCC measured on two different flow cytometer models with two operators. Panels A and B are from the same river water.
sample, and panels C and D are from the same wastewater sample. Measurements for panels B and D were performed with a BD Accurri with measurement as described (Methods). Measurements for panels A and C were performed as described below. Both operators and instruments identified a clear separation between the two main clusters, although more sophisticated optics revealed more subpopulations. The LNA cluster was similarly distinguished by low values for mean fluorescence.

Measurements for panels A and C were performed with a BD Influx v7 Sorter USB, (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) equipped with a 488 nm Sapphire OPS laser (400 mW) and a 355 nm Genesis OPS laser (100 mW, both Coherent, Santa Clara, CA, USA). The 488 nm laser light was used for the analysis of the forward scatter (FSC, 488/10) and the side scatter (SSC, 488/10, trigger signal). The SybrGreenI induced fluorescences were collected in the FL1 channel (616/23, green fluorescence) and the FL3 channel, respectively (670/30, red fluorescence). The DAPI-fluorescence was detected in the FL9 channel (460/50). The fluidic system was run at 33 psi using a 70 µm nozzle. The sheath fluid consisted of 0.5 x FACSFlow buffer (BD). For optical calibration of the cytometer in the linear range, 1 µm blue fluorescent FluoSpheres (Molecular Probes, F8815, Eugene, OR, USA) and 2 µm yellow-green fluorescent FluoSpheres (ThermoFisher Scientific, F8827, Waltham, MA, USA) were used. For calibration in the log range, 0.5 µm UV Fluoresbrite Microspheres (Polysciences, 18339, Warrington, PA, USA) were applied. Data were collected in log mode.
Figure S4: Comparative analysis with different dyes and instrumentation. River water and wastewater were stained with SYBR Green I (A, B) and DAPI (C, D) and analyzed on a BD Influx v7 Sorter USB, (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Bacteria fall within the orange gate. The SYBR Green I data show high similarity with the SYBR Green data in Figure 2 and Figure S2. Moreover, the data show clear separation of the LNA cluster on the Forward Scatter, suggesting a clear size separation between HNA and LNA cells. While the LNA cluster was clearly discernable on the DAPI stained samples, it is interesting to note that the fluorescence separation with this dye is much less obvious compared to SYBR Green, and in contrast more HNA clusters are observed. Both stains (DAPI and SYBRGreen) similarly identified the LNA cluster by low values of forward scatter, similar to the identification by low fluorescence (Figure S3). The DAPI staining revealed more sub-communities for the HNA cluster. Although forward scatter is not directly related to cell since in the range of 0.5 to 5 µm (Shapiro, 2003), the trend points to similar low estimates for LNA.

The flow cytometer was with a 488 nm Sapphire OPS laser (400 mW) and a 355 nm Genesis OPS laser (100 mW, both Coherent, Santa Clara, CA, USA). The 488 nm laser light
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems

was used for the analysis of the forward scatter (FSC, 488/10) and the side scatter (SSC, 488/10, trigger signal). The SybrGreenI induced fluorescences were collected in the FL1 channel (616/23, green fluorescence) and the FL3 channel, respectively (670/30, red fluorescence). The DAPI-fluorescence was detected in the FL9 channel (460/50). The fluidic system was run at 33 psi using a 70 µm nozzle. The sheath fluid consisted of 0.5 x FACSFlow buffer (BD). For optical calibration of the cytometer in the linear range, 1 µm blue fluorescent FluoSpheres (Molecular Probes, F8815, Eugene, OR, USA) and 2 µm yellow-green fluorescent FluoSpheres (ThermoFisher Scientific, F8827, Waltham, MA, USA) were used. For calibration in the log range, 0.5 µm UV Fluiorsbrite Microspheres (Polysciences, 18339, Warrington, PA, USA) were applied. Whereas SybrGreenI stained samples were spiked with 0.5 µm UV Fluiorsbrite Microspheres of known concentration in order to calculate the absolute number of cells, 1 µm yellow-green fluorescent FluoSpheres (ThermoFisher Scientific, F13081, Waltham, MA, USA) were added to DAPI stained cells. Data were collected in log mode.
4. Filterability of LNA and HNA content bacteria

**Figure S5**: Example of the filterability of LNA and HNA content bacteria. River water was analyzed before (A,C) and after (B,D) filtration with 0.4 µm pore size filters. All samples were stained with SYBR Green I. Dotted red lines indicate electronic gates separating bacteria from background and LNA from HNA content bacteria. FL1-A = green fluorescence intensity; FL3-A = red fluorescence intensity.
5. Agreement with qPCR measurement of bacteria

Figure S6: Correlation between flow cytometry (FCM) and qPCR data. Total cell concentration determined by FCM with Sybr Green1 staining. qPCR is a measure of 16S rRNA gene copies (see methods below). Symbol and color determined by one of three size groups determined by filtration method, including: ‘All bacteria’ (gray triangles), the total community, directly filtered onto a 0.2 µm filter; Large bacteria (red circles), the HNA-dominated community filtered onto a 0.4 µm filter; and Small bacteria (blue squares), the LNA-dominated filtration of 0.4 µm filtrate onto a 0.2 µm filter.

Linear models calculated for each group had distinct slopes, suggesting a difference in the average number of gene copies in each size of cell. With intercept set to 0, slopes for each group were as follows - small: 1.2 gc/cell, large: 4.4 gc/cell, all: 2.4 gc/cell, with R² of 0.34, 0.23, and 0.26 respectively.

Quantitative polymerase chain reaction (qPCR) for 16S rRNA gene copies were done as described previously (Proctor et al., 2016). Briefly, DNA extracts were diluted either 10 or 50 times prior to qPCR depending on the DNA concentration of the extract. qPCR
Chapter 7 – Supplementary Information

Quantifications were performed on a Roche LightCycler II with the following temperature program: 10 min at 95°C, followed by 45 cycles of 40 sec at 95°C and 53°C, and extension 1 min at 72°C. Reactions were performed with Light Cycler 480 probes master (Roche), Bact349F/Bact806R or Arch 349F/Arch806R primers and probe Bac516F23 (for bacteria) (Takai and Horikoshi, 2000) (Details, below). 16S rRNA gene copy numbers were quantified against standards created from dilutions of plasmids with a matching insert. Concentrations were calculated by normalizing over the filtered sample volume. Data were assembled and prepared for further analysis in R using Excel.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bact 806R</td>
<td>GGACTACYVGGGTATCTAAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bac516F_FAM</td>
<td>FAM-TGCCAGCACGCCGCG GTAAACRDA-TAMRA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arch349F</td>
<td>GYGCASCAGKCGMGAAW</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arch806R</td>
<td>GGACTACVSGGGGTATCTAAT</td>
<td></td>
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</tbody>
</table>


6. Relative importance of various factors explaining community variations within each ecosystem.

Table S5: Relative importance of various factors in bacterial communities within each ecosystem, calculated by Adonis. For each explanatory variable (Sampling site, size, and interactions between these two factors), the percent of variation explained by the variable and the p-value for statistical significance of the factor are expressed. Sampling site refers to the specific location for each sample (i.e. River A vs River B). Size refers to one of three groups of bacteria determined by filtration method, including: ‘All bacteria’ is the total community, directly filtered onto a 0.2 µm filter. Large bacteria is the HNA-dominated community collected on a 0.4 µm filter, and Small bacteria is the LNA-dominated community in the 0.4 µm filtrate, collected on a 0.2 µm filter.

<table>
<thead>
<tr>
<th>Ecosystem</th>
<th>Sampling Site</th>
<th>Size</th>
<th>Site*size</th>
<th>Number in analysis (Filters) - Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake water</td>
<td>0.31 (0.001)</td>
<td>0.27 (0.001)</td>
<td>0.07 (1.00)</td>
<td>(48) - 16</td>
</tr>
<tr>
<td>River water</td>
<td>0.17 (0.001)</td>
<td>0.30 (0.001)</td>
<td>0.07 (0.459)</td>
<td>(54) - 18</td>
</tr>
<tr>
<td>Groundwater</td>
<td>0.50 (1.00)</td>
<td>0.22 (1.00)</td>
<td>0.28 (1.00)</td>
<td>(12) - 4</td>
</tr>
<tr>
<td>Wastewater</td>
<td>0.42 (0.001)</td>
<td>0.25 (0.001)</td>
<td>0.14 (0.858)</td>
<td>(18) - 6</td>
</tr>
<tr>
<td>Tap water</td>
<td>0.57 (1.00)</td>
<td>0.25 (1.00)</td>
<td>0.18 (1.00)</td>
<td>(12) - 4</td>
</tr>
</tbody>
</table>
7. Time-series data in a river indicate consistent LNA content and communities within each faction.

Extended discussion on temporal variation: As a test for temporal stability within a sampling site, samples taken over four months (June - September) from River Site A were further analyzed. Both community composition by size (as measured with 16S amplicon sequencing) and the percentage of LNA-content bacteria (as measured with FCM) remained relatively stable in this period.

While the community composition shifted moderately through time (Figure S7), it still remained distinct from other sampling sites (Site A, Figure 5). Moreover, the vectors of temporal change in the ordination space are similar across bacterial groups (Figure S7), indicating that the groups responded similarly to environmental conditions. Looking at FCM data, the percentage of LNA-content bacteria remained stable between approximately 50% and 60% with no clear temporal shift (Figure S8). A more detailed time-series dataset from the same sampling site did show dramatic short-term dynamics linked to rainfall, but these varied around a stable baseline (Besmer et al., 2014). Together with the comparable stability of the time-series on lakes (data not shown), this temporal stability indicates that while we often relied on single samples from a site, these were likely representative in terms of their size groups, and our data was not strongly influenced by temporal variations.

**Figure S7:** Non-metric multidimensional scaling (NMDS) of bacterial communities (characterized with 16S amplicon sequencing) calculated with Bray-Curtis dissimilarity in a river (River Site A in Figure 5) over four months (June, July, August, September) in the three size-based groups based on filter pore-size: ‘All bacteria’ is the total community, directly filtered onto a 0.2 µm filter. Large bacteria is the HNA-dominated community collected on a 0.4 µm filter, and Small bacteria is the LNA-dominated community in the 0.4 µm filtrate, collected on a 0.2 µm filter.
Figure S8: Flow cytometry-based comparison of temporal evolution of the percentages of LNA (blue bars) and HNA (red bars) content bacteria as well as total cell concentration (grey circles) in river water over different time scales: (A) time series of river water sampled 11 times at the same sampling site (River water - site A, Figure 5, Figure S7) over 4 months with low frequency and (B) time series of river water sampled approximately 1'400 times at the same sampling site (River water - site A) over 2 weeks with high frequency and influenced by two precipitation events (panel B is adapted from Besmer et al. (2014)). The percentage of LNA content bacteria remained stable between approximately 45% and 55% with no clear temporal shift.

8. SEM images of possible cross-contamination on filters

**Figure S9:** Scanning electron microscopy (SEM) image of bacteria from a river (Site A), filtered onto a 0.4 μm pore-size filter. Filter pores are visible as black holes, bacteria are highlighted in blue/purple/orange/pink shades and extracellular filaments are highlighted in green (all colors were artificially added). Note the difference in size between A and B. (A) Several large bacteria and large extra-cellular matrix which visual inspection suggests would not pass through 0.4 μm pores. A single small bacterium (blue) is seen entering the pore, but not passing through. (B) A single small bacterium (pink), while of filterable size, is stuck on the filter. Original image without artificial coloring available in Figure S14.
9. Detail phylogenetic trees and relative abundances

**A**

Phylum
- [Thermi]
- Acidobacteria
- Actinobacteria
- Armatimonadetes
- Bacteroidetes
- Chlamydiae
- Chlorobi
- Chloroflexi
- Cyanobacteria
- Elusimicrobia
- Fibrobacteres
- Firmicutes
- Fusobacteria
- GN02
- NC10
- Nitrospirae
- OD1
- OP11
- OP3
- Planctomycetes
- Proteobacteria
- Spirochaetes
- SR1
- Tenericutes
- TM6
- TM7
- Verrucomicrobia
- WWE1

**B**

- Exclusively Large OTUs
- Exclusively Small OTUs
- Non-exclusive OTUs
- Unclassifiable OTUs
- Eliminated OTUs
Figure S10: Phylogenetic trees to give more detail about total diversity measured across freshwater ecosystems. Phylogenetic trees were created using FastTree, including maximum likelihood options. (A) Phylogenetic tree used in Figure 6 with phyla marked with color, (B) Phylogenetic tree Figure 6, expanded to include 3,805 “Eliminated” OTUs. (C) Figure S10B with phyla marked with color. Phyla are marked only with “old names”. For example, Parcubacteria are OD1 and OP3 are now Omnitrophia.
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems

Bacteroidetes
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems

Alphaproteobacteria labeled with family (where available)
**Figure S11:** Detailed trees of various phyla (Bacteroidetes, Parcubacteria (OD1), and classes (Alphaproteobacteria, Deltaproteobacteria)), including boot-strap values from maximum-likelihood construction and labeled with order or family. Colors of OTUs match Figure 6.
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems
Figure S12: Detail relative abundance of each phyla and OTU type, averaged across all Large (0.4µm filters) and Small (0.2µm filters of filtrate) in each ecosystem. OTUs with abundance <20 OTUs in <2 filters are left out of analysis, but included in the total relative abundance proportions (Low abundance/Rare). OTU types are used as defined in Figure 1 - -Large OTU, small OTU, non-exclusive (two types, detailed below), unclassifiable, eliminated and rare). Non-exclusive OTUs are further divided here as to whether this occurred in duplicate matching filters (I) or without matching duplicates (II). On the second page, the abundance of each of these OTU types is shown within each phylum.
10. Original SEM images

Figure S13: Grayscale (original) image of Figure 3. Scanning electron microscopy (SEM) image of bacteria from a stagnant pond sample rich in LNA content bacteria (> 90%), filtered onto a 0.2 µm pore-size filter. Filter pores are visible as black holes, bacteria are oblong light gray and extracellular filaments as light-gray strands.
**Figure S14:** Figure S9 without artificial coloring.
Phylogenetic clustering of small LNA-content bacteria across diverse freshwater ecosystems
Chapter 8

Quantifying bacterial abundance in water and biofilms:
correlation between 16S gene copy and total cell concentrations

To be submitted for publication as a mini-review by:
Caitlin R. Proctor, Helmut Bürgmann, and Frederik Hammes
Abstract

Accurate bacterial abundance measurements are critical in both applied and fundamental studies of natural and engineered aquatic ecosystems. As background data to this mini-review, two popular methods for quantification of total bacterial abundance were compared using 887 data-points from 10 separate studies, including both water and biofilm samples. Total cell concentration (TCC) as measured with flow cytometry (FCM) and 16S rRNA gene copy (gc) concentration with qPCR had strong and positive correlations for both planktonic (n = 609) and biofilm (n = 278) samples (Pearson’s r = 0.75 and 0.43, respectively). For planktonic samples, a linear regression unexpectedly indicated a nearly 1:1 ratio of 16S gc and TCC (m = 0.96, R² = 0.57). Here we critically consider and review (1) theoretical differences between methods (e.g., difference in targets), (2) methodological bias (e.g., DNA extraction efficiency for qPCR and cell clumps for FCM), and (3) sample and experiment-specific biases (e.g., oxidative stress). Thus, one must carefully consider and choose the appropriate quantitative method for a specific study. However, on the log-scale, these positive correlations clearly enable broad cross-study comparisons of bacterial abundances in diverse environments.

Keywords: flow cytometry, qPCR, cultivation-independent methods, bacterial quantification, total cells, 16S rRNA gene copies
1. Introduction

Several cultivation-independent methods are used to quantify or estimate planktonic (i.e., water, suspended) and biofilm (i.e., sessile) bacterial concentrations in natural and engineered aquatic ecosystems. These include, for example, microscopy (Zeder and Pernthaler, 2009), enzyme based assays (Zhang et al., 2013, 2017), total DNA, total cell concentrations (TCCs) or intact cell concentrations (ICCs) as measured with flow cytometry (FCM) (Prest et al., 2013), adenosine tri-phosphate (ATP) concentrations (Nescerecka et al., 2016), and 16S rRNA gene copy (gc) concentrations as measured with quantitative polymerase chain reaction (qPCR) (Hunter et al., 2002; Suzuki et al., 2000; Takai and Horikoshi, 2000). Typically, a laboratory will focus on only one of these methods for quantitative information.

Since laboratories specialize in different quantitative methods, comparing the results from different studies is both challenging and questionable. This methodological barrier can make reproducing another laboratory’s results or synthesizing a growing body of literature that much more difficult. However, correlations of measures that target the same or similar bacterial targets/indicators have been shown, even with extremely different methods. For example, in freshwater samples strong correlations were demonstrated between ICC and ATP concentrations (Van Nevel et al., 2017), which both target viable cells. FCM and microscopy have also been shown to correlate when analyzing indigenous microbial communities (Felip et al., 2007). In pure culture experiments, ICC measured with FCM and specifically targeted organisms measured with qPCR (i.e., not 16S rRNA gc) correlated well (Tamburini et al., 2013).

Standardization to a single method is highly unlikely, or even undesirable, and use of multiple methods is often cost and time prohibitive. Thus, it would be useful to understand if and how these very different measures of bacterial abundance correlate with one another.
For quantification of total bacterial abundance (i.e., both alive and dead), TCC as measured with FCM and 16S rRNA gc concentrations as measured with qPCR are arguably the most popular methods currently used. Additionally, FCM-TCC was recently proposed as a useful measure for translating relative abundance community data obtained with 16S rRNA amplicon sequencing into more quantitative and meaningful measures of absolute abundance (Props et al., 2016; Prest et al., 2014; Zhang et al., 2017). However, 16S rRNA gc concentrations have been used for the same purpose (Proctor et al., 2016; Zhang et al., 2017), and have more methodological similarity to amplicon sequencing than TCC. Thus, it would be useful to understand how FCM data correlates with molecular methods for bacterial quantification.

Both TCC with FCM and 16S rRNA gc concentrations with qPCR target nucleic acids, but quantify with very different methodology. TCC measurements use a fluorescent dye to nonspecifically stain the nucleic acids in all cells (both dead and alive). FCM then enumerates the particles, using fluorescence to differentiate between microbial cells and abiotic particles (Van Nevel et al., 2017). For 16S rRNA qPCR, there are more steps between sample collection and results, including filtration to concentrate cells and DNA extraction. The qPCR itself is performed on the DNA extract by amplifying and quantifying a specific section of the 16S rRNA gene (i.e., nucleic acids), which is common to all bacteria and archaea. This relies on universal primer sets that are designed to achieve a broad coverage of bacteria or all prokaryotes, but do not achieve 100% coverage of the known phylogeny (V. Wintzingerode et al., 1997). Thus, despite similar information and targets, there are many obvious challenges with data comparisons between these methods. While the relationship between FCM and 16S rRNA gc qPCR has been explored minimally within studies (Suzuki et al., 2000; Proctor et al., 2016), to our knowledge, detailed information comparing TCC and 16S rRNA gc from real aquatic environments does not exist.
Here, we review the use of TCC with FCM and 16S rRNA gc with qPCR as measures of bacterial abundance quantification in ecological and engineering studies. We use a large dataset collected from separate studies (Table 1) completed by several scientists on a variety of freshwater ecosystems to investigate correlations between the two measures of bacterial abundance (Table 2) in both biofilm and planktonic samples. Here we consider (1) theoretical differences between methods, (2) methodological biases, and (3) sample and experiment-specific biases. Given these considerations, we provide a critical opinion regarding the value of each method throughout.

2. Total cell concentrations (TCC) correlate well with 16S rRNA gene copy (gc) concentrations

A total of 887 samples from 10 different studies (Table 1) from varied natural and engineered freshwater ecosystems were used to calculate correlations between 16S rRNA gc concentration and TCC. These samples included both planktonic (i.e., tap water [180], treated wastewater [102], river water [84], suspended pure culture [76], lake water [64], groundwater [64], and wastewater influent [41]), and biofilm samples (shower hose biofilms [176], and biofilm traps (Figure S1) in tap [81] and groundwater [21]). TCC for these samples ranged between $4 \times 10^3$ – $4.2 \times 10^8$ cells/mL and $3.4 \times 10^4$ – $5.6 \times 10^8$ cells/cm$^2$, respectively. 16S rRNA gc concentration ranged between $2.5 \times 10^2$ – $1.9 \times 10^9$ gc/mL and $4.6$ – $1.4 \times 10^8$ gc/cm$^2$, respectively.

The correlation between TCC and 16S rRNA gc concentration was strong and positive (Figure 1). For planktonic samples, correlation between the two measures was strong (Pearson’s $r = 0.75$, $p < 0.001$, $n = 609$), and the linear correlation was remarkably close to a 1:1 correlation with zero intercept ($m = 0.96$, $b = -0.03$, $R^2 = 0.57$, $p < 0.001$). Thus, on average for planktonic samples, for each cell detected, approximately one 16S rRNA gc was
detected. For biofilm samples, the correlation was not as strong as for planktonic samples (Pearson’s r = 0.43, p < 0.001, n = 278), and the best linear correlation did not fall exactly on the 1:1 correlation line (m = 0.65, b = 1.5, R² = 0.18, p < 0.001). Thus, samples with TCC in

Table 1: Studies included in analysis

<table>
<thead>
<tr>
<th>Study</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Laboratory shower simulator with six different materials in Switzerland. Stagnated warm water was gently decanted from hoses after 6 hours and 22 hours. Biofilm was extracted after various growth times.</td>
</tr>
<tr>
<td>2</td>
<td>First flush (i.e., stagnant) and fully flushed warm water from real showers in a laboratory office building in Switzerland.</td>
</tr>
<tr>
<td>3</td>
<td>A laboratory water distribution simulator with cold constantly flowing water (low-speed) through biofilm traps then shower hoses in Switzerland. Biofilm samples were collected from 1 month to 12 months of development, and water was collected from various aged biofilm lines after 11 months.</td>
</tr>
<tr>
<td>4</td>
<td>Water collected from five distinct ecosystems in Switzerland.</td>
</tr>
<tr>
<td>5</td>
<td>Biofilm extracted from shower hoses that were collected from all over the world (11 countries).</td>
</tr>
<tr>
<td>6</td>
<td>Water samples collected from before and after drinking water treatment and after distribution from eight drinking water suppliers in Switzerland. Raw water originated from a variety of sources. Treatment varied by source, and three treatments included chlorination and a chlorine residual.</td>
</tr>
<tr>
<td>7</td>
<td>The effect of ozone, a strong oxidant and disinfectant, was tested in environments on E. coli pure cultures and the effluent (i.e. treated) of a wastewater treatment plant. Several real samples of the influent (i.e. sludge) and effluent of a real wastewater treatment plant were also sampled.</td>
</tr>
<tr>
<td>8</td>
<td>Samples collected every hour over 48 hours with auto-sampling devices from raw spring water before and after multi-stage treatment in parallel in a small water utility in Switzerland.</td>
</tr>
<tr>
<td>9</td>
<td>Biofilm collected in biofilm traps installed in three springs used for drinking water in a karstic area and in seven drinking water distribution networks of small water utilities in Switzerland.</td>
</tr>
<tr>
<td>10</td>
<td>Water samples collected from before and after drinking water treatment in Uganda. Raw water originated from a lake. Water was treated with gravity driven membranes.</td>
</tr>
</tbody>
</table>

Citation

(Proctor et al., 2016) [biomass data only, water data not yet published]

Chapter 6: test-system A

not yet published

Chapter 7

Chapter 3

(Imminger et al., 2015)

(Czekalski et al., 2016)

not yet published

not yet published
Table 2: Methods used in various studies. In all studies, 0.2 µm filters were used for filtration of samples to collect cells prior to DNA extraction.

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Studies</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCM</td>
<td>When necessary, samples were diluted in 1:10 serial dilutions with filtered (0.22 µm Millex-GP, Millipore), bottled mineral water (Evian, France). Suspended biofilms were diluted up to 10,000 times. Samples were stained and measured as described previously (Prest et al., 2013) Briefly, a working solution of SYBR® Green I (SG) (Invitrogen AG, Basel, Switzerland) was prepared by 100x dilution in anhydrous dimethylsulfoxide (DMSO) for measuring total cell concentration (TCC). Water samples and biofilm suspensions were stained with SG or SGPI at 10 µL/mL. Samples were stained and incubated at 37 °C in the dark for 13 min before measurement. Flow cytometric measurements were performed on a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium). Data analysis was performed using the BD Accuri CFlow® software with standardized gates.</td>
<td>Group A – Study 1-5, 8-10</td>
<td>(Prest et al., 2013) Group B – Study 6-7</td>
</tr>
<tr>
<td>DNA Extraction</td>
<td>Enzymatic digestion and cetyltrimethyl ammonium bromide (CTAB) extraction. In short, enzymatic cell lysis was performed on filters by subsequent incubations with Lysozyme, Proteinase K, and RNase A (Proteinase K volume was increased to 10 µl, and RNase to 5 µl). DNA was extracted with a CTAB buffer, and purified by chloroform isoamyl alcohol (we used 49:1 instead of 24:1 v:v ratio) extraction. DNA was precipitated with ethanol and DNA redissolved in TE buffer. Power Water DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to manufacturers' instructions. Both biofilm suspensions and water samples were filtered similarly.</td>
<td>4, 7</td>
<td>(Llirós et al., 2008) 1-3, 5, 6, 8-10</td>
</tr>
<tr>
<td>qPCR</td>
<td>qPCR reactions were performed on a LightCycler 480-II (Roche) and analyzed using the LightCycler 480 ver. 1.5.1 software (Roche). Quantification of bacterial 16S rRNA genes was carried out using the primers Bact349F/Bact806R and probe Bac516F; performed using a LightCycler 480 Probes Master hot start reaction mix (Roche). qPCR was performed by an external certified lab (Microsynth, AG, Switzerland) with 341F &amp; 802R primers.</td>
<td>1, 2, 4-7, 10</td>
<td>(Proctor et al., 2016; Takai and Horikoshi, 2000)</td>
</tr>
</tbody>
</table>

the range of most of the samples here had an underestimation from the 1:1 ratio by qPCR. Residuals for calculated linear regressions did not show any trend (i.e., fairly evenly distributed around zero), but were much larger for biofilms, with especially large negative residuals (Figure S2).

These relatively strong correlations are extremely promising for several reasons. (1) Investing in a quantitative method can be expensive, with costs for equipment, reagents and proper training. Beyond those costs, considerable time and effort are needed to properly establish and standardize a method that works in a laboratory and for specific samples. The
correlation presented here indicates that using multiple quantitative methods may be (at least partially) redundant. (2) Many researchers are hesitant to compare between studies that use different methods, due to their different advantages and disadvantages (Douterelo et al., 2014; Props et al., 2016). This limits the body of literature available to put a study into a larger context. While the correlations presented here are not perfect, one can easily appreciate that log differences between samples will be similar with both methods. (3) With advancement of next generation sequencing, highly quantitative methods are sometimes ignored for the sake of relative abundances in the total community, or quantification of specific bacteria. While these specific measures are important, absolute total abundance is critical for understanding natural and engineered aquatic systems. For example, mass balances on ecosystem boundaries and engineering processes requires quantitative microbial information. Rather, absolute abundance can be used in concert with community data, for example for the tracking of pathogen growth or distinguishing the thriving organisms (enriched due to outgrowth) from the survivors (enriched due to disappearance of other organisms) (Props et al., 2016; Prest et al., 2014; Proctor et al., 2016).

Despite these strong correlations and generally positive implications, the correlations reported above must be critically evaluated.

3. There is a fundamental biological difference between the two methods

While each bacterial cell has at least one 16S rRNA gene, each species has a specific number of 16S rRNA gene copies, varying from 1-15 gc/cell (Klappenbach et al., 2001; Stoddard et al., 2015; Kembel et al., 2012). With TCC in these studies, no distinction is made based on the amount of nucleic acid inside a cell. Thus, for a mixed community, a higher ratio than 1 gc/cell would be expected if methods were applied correctly.
Figure 1: Total cell concentration (x-axis) and 16S rRNA gene copy (gc) concentration (y-axis) for 609 water samples (left) and 278 biofilm samples (right). For water, concentration units are per milliliter, while for biofilm, surface area concentrations with unit per cm² are used. A reference line for a 1:1 ratio is added (this is not the linear correlation amongst points). For each system, color represents the ecosystem and symbol represents the study (Table 1) from which the sample originates. Note that symbols and colors do not necessarily match between water and biofilm plots.

Moreover, the average number of 16S gc/cell in a community might be expected to vary based on microbiome composition or with nutrient conditions (Kembel et al., 2012; Roller et al., 2016), but the 1 gc/cell ratio in Figure 1 remained strong across many diverse microbiomes (i.e., clean tap water [Chapter 6,7] to wastewater treatment plant influent (Czekalski et al., 2016)). Cell size may also contribute to the number of gc/cell. In a study that used sequential filtration with 0.4 µm (to capture large cells only) and 0.2 µm (to capture the remaining small cells) filters, a difference in gc/cell ratio was found between small and large cells (Chapter 7). In the studies presented here, size separation was not used. Thus, with a mix of cell sizes, it is possible that those cells with many gene copies essentially normalized those with fewer. Even so, a higher ratio than 1:1 would be expected.
Additionally, qPCR does not distinguish between cellular and extracellular DNA, and will measure both, if they are caught on the filter and extracted. If correctly used, TCC only measures cells, with extracellular DNA appearing as background (Hammes and Egli, 2010). Altogether, these differences in targets would suggest that qPCR would have a higher estimation of bacterial abundances that FCM.

There is, however, one difference in targets that would suggest that FCM would have a higher estimation of bacterial abundance. Despite the best design, qPCR primers are not entirely universal (V. Wintzingerode et al., 1997). For example, they can be optimized for either bacterial or archaeal cells (Takai and Horikoshi, 2000). This targeting could be selecting only a fraction of cells that are detected with FCM. For example, in study 4, archaeal primers were also used (Chapter 7), and these accounted for an average of 13 % of the total 16S gc measured. While experience can help inform bacteria-specific gating (Hammes and Egli, 2010), all cells, including bacteria, archaea, yeast, fungi, protozoa, and viruses are detected with FCM, which may lead to over-estimation of bacterial abundance.

4. Method biases are introduced for both FCM and qPCR

Both qPCR and FCM methodologies can introduce bias that would make a correlation unlikely. These can be either systematic (i.e., based on the protocols and user) or sample specific (e.g., background matrix causing sample-specific interference).

4.1 qPCR Method bias

qPCR introduces several more steps than FCM, and each one potentially introduces contamination, bias, and inefficiency, as has been extensively reviewed previously (Smith et al., 2009; Salter et al., 2014; Albertsen et al., 2015; Schrader et al., 2012; V. Wintzingerode...
et al., 1997; Wang et al., 2008). While filtration-loss (i.e., cells passing through filters meant to capture target bacteria), extraction inefficiency, and PCR inhibition could lead to underestimation with qPCR, contamination of reagents or along the process could lead to an overestimation with qPCR. While these issues must be considered for all samples, contamination prevention is especially important for samples with low bacterial concentrations while maximizing efficiency is more important for samples with high bacterial concentrations.

In our synthesis of studies, the inhibition or inefficiency of qPCR is evident in several biofilm samples. In Figure 1, the biofilm samples in the lower right-hand side of the graph, with high TCC and low 16S rRNA gc concentrations likely suffered from these inefficiencies (also see large negative residuals from the linear correlation in Figure S2). Some biofilms in these studies had extreme turbidity following dispersal and suspension, which could have introduced elements (e.g., calcium, iron) known to inhibit qPCR (Schrader et al., 2012) (i.e., iron in biofilms, Chapter 3). While it is necessary to plan for and optimize around such inefficiency and inhibition, it is often difficult to balance standardization and optimization needs within a study.

Biases are also expected across studies and research groups since systematic biases (e.g., standard quantification, machine efficiencies) can contribute to biases (Smith et al., 2009). Methods for DNA extraction and qPCR were also slightly different across the studies used here (Table 2), and this may have contributed some bias to the correlation.

4.2 FCM Method bias

FCM also faces systematic bias. The user, instrument, staining protocol, and gating in data analysis all affect TCC (Prest et al., 2013; Van Nevel et al., 2017; SLMB, 2012). Across these ten studies, FCM methods were standardized, and while users varied, all were trained
in the same laboratory and used the same instrumentation. Additionally, while qPCR typically utilizes internal standard curves for each round of quantification, standards are not necessarily used directly with FCM-TCC measurements. Rather instruments with absolute quantification hardware are calibrated and quality controlled on a routine basis. Thus, including more variation in groups, instrument types, and protocols would likely introduce both overestimations and underestimations, complicating the correlations observed. For example, when FCM gating is not done correctly, it can include large viruses, micro-algae, auto-fluorescent abiotic particles, and noise, which could all contribute to an overestimation. If flow cytometers are frequently overloaded or not kept clean, then samples could get contaminated by the machine (i.e., sample crossover) leading to an overestimation.

FCM can also face sample-specific bias. Flow cytometers cannot differentiate between one large cell and multiple cells that are stuck together (in both cases one particle would be enumerated). Thus, one might expect an underestimation in scenarios when multiple cells would be expected to cluster together, such as samples from suspended biofilms. qPCR, on the other hand, depends upon an aggressive DNA extraction step which would breaks all cells in aggregates and thus can count each gc individually. For samples where aggregates are expected (e.g., soils, sediments and sludge (Frossard et al., 2016)), dispersal methods are important to consider. In the samples of suspended biofilm studies reported here, a sonication step was added after biofilm detachment and suspension to avoid issues with cell aggregates. Since sonication can also destroy cells, such a dispersal step is not appropriate for all samples and requires demonstrated controls. Amongst biofilm samples here, there was not a systematic underestimation of bacterial concentrations with FCM. Thus, the sonication step used in this data set, together with dilution, were likely appropriate for dispersal. However, DNA extraction and qPCR inefficiencies noted above may have also prevented such an overestimation. One must additionally consider sample pre-treatment for detachment from a solid medium. While these steps can affect both methods (i.e., biofilm
detachment efficiency (Magic-Knezev and van der Kooij, 2004)), the specific method (i.e., sonication), may affect FCM more than DNA extraction.

4.3 Choosing a method

Both methods have clear benefits and drawbacks. While performing both methods (as done in these studies) gives additional information (Zhang et al., 2017), this is not possible in all studies. For example, using buffers or dispersal methods to optimize for one method may prevent application of the other method, or time and cost considerations may prohibit use of both methods. One must consider these biases when choosing a method, since specific samples and experiments are more prone to bias with one method or another. For example, in aquatic samples with low cell concentrations, FCM may be easier and more accurate than qPCR, which requires prevention of contamination through many steps. For samples with many clumps and clusters, extracting DNA for qPCR may be easier and more accurate than adding many dispersal steps to allow FCM. It is also clear that with either method, researchers must continue to consider and address these biases. For example, one can use controls and standards to estimate the recovery rate, and frequent machine calibration can prevent against drift with time.

5. Sample and experiment-specific considerations

Correlations were evident on the aggregate of all data, but were not always strong within individual studies or ecosystems, which spanned a smaller range in bacterial concentrations. For example, in Study 8 with 48 water samples from groundwater and tap water (Figure 2), all samples fall below the typical 1:1 ratio. While there is a correlation between the two measurements in the study (Pearson’s $r = 0.87$, $p < 0.001$, $n = 96$), if the study only included one ecosystem correlations would not be strong (groundwater: Pearson’s $r = 0.59$, $p < 0.001$, $n = 48$). Notably, although triplicates had acceptable reproducibility across these 10 studies.
Quantifying bacterial abundance in water and biofilms: correlation between 16S gc and TC

(Chapter 7, (Czekalski et al., 2016; Proctor et al., 2016)), qPCR data tended to vary more than FCM data (particularly obvious with qPCR variation within an ecosystem in Figure 2). The systematic methodological errors outlined above may create such a bias.

Amongst river samples from three studies, no correlation was found (Pearson’s $r = -0.02$, $p = 0.84$, $n = 84$) (Figure 3). While each of the two primary studies has a more reasonable correlation when considering all samples in the study (Study 4, featuring five freshwater ecosystems: Pearson’s $r = 0.86$, $p < 0.001$, $n = 102$; Study 7, spanning 3 ecosystems and featuring oxidation treatment: Pearson’s $r = 0.69$, $n = 254$), the studies did not fall into the same overall correlation. Study 4 fell largely above the 1:1 ratio, and study 7 fell well below this ratio. While the same methods (i.e., including molecular pipelines) were used in all three of these studies, there were different operators for each, which could contribute to differences. Moreover, studies were completed at different times, so systematic drift in the methods’ bias may contribute (e.g., flow cytometer fluorescence signal drift,
degradation of qPCR standards). The ozone in Study 7 may have affected efficiency for either method (see disinfection discussion below). Additionally, this single ecosystem had a narrow range in TCC. Log-scale differences in both measurements are necessary to see strong correlations, and these do not always occur within a specific study or ecosystem.

The condition of samples is also likely important for this correlation. Amongst only tap water samples (Figure S3), the correlation seems to be driven primarily by Study 1 samples, which had high TCC and 16S gc concentrations. Study 1 comprised only stagnant tap water, while most other samples were flowing tap water. Interestingly, stagnant water typically has more high nucleic acid (HNA)-content bacteria than flowing water [Chapter 6, (Lautenschlager et al., 2010)]. Since HNA-content bacteria are larger and have more 16S gene copies [Chapter 7], it is possible that filtration, DNA extraction, and qPCR were more efficient (i.e., fewer small cells that could pass through a 0.2 um filter (Wang et al., 2008), more targets for qPCR (Chapter 7), more efficient cell lysis during DNA extraction (Moré et al., 1994)). Efficiency of both methods is indeed dependent on microbiome composition. For example, gram-negative and gram-positive bacteria have different lysis efficiency for DNA extraction, and spores and cysts may resist both quantification methods (Hwang et al., 2012; Keserue et al., 2011).

Figure 3: Total cell concentration (x-axis) and 16S rRNA gene copy (gc) concentration (y-axis) for all river water samples used in the analysis, with 34 samples from Study 4, 6 samples from Study 6, and 47 samples from Study 7 (N = 87). A reference line for a 1:1 ratio is added (this is not the linear correlation amongst points).
It is important to note that all of the tap water samples featured in these studies were non-chlorinated. Disinfection of any kind could distort the correlation between bacterial measures, since cell death and destruction is not detected similarly across methods. For example, UV prevents cells from reproducing by damaging DNA, but cell membranes remain intact and nucleic acids remain within the cell. Thus, TCC would appear stable, but 16S rRNA gc concentrations may respond sooner because DNA is damaged. For example, long-amplicon targets were able to distinguish active cells from UV-inactivated ones, even without special DNA extraction methods to target live cells (Banihashemi et al., 2012).

The *E. coli* samples included in this study were subject to oxidation with ozone (Figure 4). Amongst five separate experiments, only one experiment resulted in a large difference to TCC and 16S rRNA gc concentrations (Experiment 2, with secondary effluent), and without this experiment there would be essentially no correlation amongst *E. coli* samples. For another experiment...
(Experiment 1, with PBS), TCC remained unchanged with increasing ozone dose, while 16S rRNA gc concentrations vary nearly 2 orders of magnitude (i.e. variation was higher than response to ozone). The doses of ozone were low in Experiment 1, and did not pass the threshold for TCC (1.3 mg O₃/L) or 16S rRNA gc (1.6 mg O₃/L) response to ozone as reported for Experiment 2 (Czekalski et al., 2016). It should be noted that neither method captured cell death with oxidation, but rather the complete disintegration of cells that occurs with very high doses (i.e. after the death measured with loss of cultivability or intact cell concentrations) (Czekalski et al., 2016). Nonetheless, the difference in threshold concentrations for response using each method further caution against blind comparison of methods with samples under oxidative stress conditions.
6. Conclusions

• Strong correlations were found between two measures of total bacterial abundance, which may allow for broader comparison between different studies.

• Such correlations, especially the near 1:1 ratio are not necessarily expected from a theoretical perspective. Over large concentration ranges and many studies, the biases of the methods thus appear to cancel each other out.

• Correlations are strongest with large order of magnitude differences, and may not be strong within particular studies or ecosystems for a variety of reasons.

• Additionally, the two methods are not interchangeable. Method biases and specific sample and experimental considerations should be taken when choosing a method.

• The relatively large error in the correlations cautions against uncritical use of either approach in quantification or as a basis of normalization of other data, and indicates that bacterial quantification in environmental samples is not a fully solved problem. Microbial ecologists should explore options to estimate recovery, methodological biases, and improve instrument calibration to improve the precision of available methods.

• However, one measure of total bacteria is sufficient for a study (i.e., both are not needed), and comparisons can be made, but only on the broad (log-) scale differences between studies using different methods.
Chapter 8

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Author Contributions

CP contributed to data analysis and interpretation, and took the lead on writing. HB and FH supported writing with expertise in molecular and flow cytometric methods, respectively.
Quantifying bacterial abundance in water and biofilms: correlation between 16S gc and TC

References


Quantifying bacterial abundance in water and biofilms: correlation between 16S gc and TC


Zeder M, Pernthaler J. (2009). Multispot live-image autofocusing for high-throughput microscopy of...


Supplementary Information

Figure S1: Biofilm traps featured in Studies 3 and 9.

The custom-made “biofilm traps” allow the study of biofilm growing based on nutrients present in the water of flow-through systems such as (distribution) pipes. The design allows for (1) convenient sampling of sufficient volumes of biofilm without removing pipes from full-scale systems, (2) exclusion of the influence of pipe material on biofilm growth, and (3) standardized extraction and analysis procedures for biofilms from different locations. The traps consist of a hollow cylinder of approximately 13 cm length with screwing tops at both ends all made of Teflon. The inner space is 4 cm in diameter and 9.4 cm in height and contains glass beads (3 mm in diameter) resulting in a total glass surface of approximately 1'200 cm². Both ends include flow regulation valves and rubber O-rings.

For sampling, the valves were closed on site and the biofilm traps removed, transported in cooled containers to the laboratory, and analyzed within 24 h. The glass beads and the remaining water were poured into sterile glass beakers. The glass beads were then covered with ultrapure water (deionized and 0.22 µm-filtered) and placed in a sonication bath for five minutes to remove the biofilm from the beads and disintegrate aggregates. This step was repeated with fresh ultrapure water another two times. From the pooled solution, subsamples were taken for various analyses.
Figure S2: Residuals of linear regressions calculated for water (left) and biofilm (right) correlations between total cell concentrations (TCC) and 16S rRNA gene copies (gc). Residuals do not have a strong trend, but are larger and less evenly distributed for biofilm than for water. The residual may increase slightly with greater TCC for biofilms as well.
Figure S3: Total cell concentration (x-axis) and 16S rRNA gene copy (gc) concentration (y-axis) for tap water samples from 7 studies. Study 1 includes only stagnant water samples, while the rest include primarily flowing water.
Chapter 9

Conclusions and Outlook
Chapter 9

Historical perspective – quality concerns lag behind supply needs

The first well-known water supply system, the ancient Roman aqueducts, had surprising sophistication, with constantly flowing water that was even delivered directly to homes (Sedlak, 2014). However, as with many great things from Roman society, their engineering knowledge became lost in centuries after their fall. Not until the 13th century would a new system, the Great Conduit of London, be built for a similar purpose. It, too, would have some private pipe connections. However, as late as the 1800’s, water in London was still only supplied to individual homes of the wealthy, and only via intermittent supply (i.e., not constantly available).

In the late 1800’s, several inventions modernized and completely changed the nature of indoor plumbing. These included the popularization and improvements on the S-Bend pipe (invented 1775) for wastewater by Thomas Crapper (Perraudin, 2010), the screw-top faucet for effectively stopping water supply (invented by Guest and Chrimes in 1845), and water heaters (different versions invented by Maughan in 1868 and Ruud in 1889). Moreover, with its increasing affordability, indoor plumbing eventually became a commodity instead of a luxury. The more modern design (i.e., separate hot and cold water delivery, multiple delivery points in the home, multiple-stories connected) provided more extensive water delivery than previously considered possible, and no doubt improved health (e.g., more available toilets, hand-washing).

Around this same time, the idea of maintaining water quality began to slowly take hold (i.e., moving beyond a supply driven perspective). In 1854, John Snow observed a cluster of cholera that traced back to the Broad Street water pump (Sedlak, 2014). While the exact cause was not yet known, improvements to separate drinking water and wastewater were put into place. In the next century, drinking water quality would be improved microbiologically with the prevention of sewage back-siphonage and seeping, and water treatment with sand.
filtration and later chlorination (Sedlak, 2014). Today, we still strive to understand these processes from a microbiological and ecological point of view (e.g., microbial resource management (Read et al., 2011), discovery of new bacteria in water treatment plants (Palomo et al., 2016)).

Nonetheless, despite this shift towards understanding water quality, the fundamentals of drinking water production have not changed in the last centuries, with gravity driven potable water delivery from treatment plants adjacent to the source. For example, many systems comprise decades old pipes that contain lead, which we now know is harmful. Improvements to our understanding of processes and biological stability have been implemented in the form of a patchwork of ad hoc improvements, like treatment plant modifications and chlorine booster stations to ensure growth prevention in the network (i.e., biological stability). Truly new ideas in drinking water production like toilet-to-tap treatment, and separate graywater distribution for non-potable uses are slow to take hold (Schwartz, 2015; Sedlak, 2014), in part due to infrastructure costs.

Only recently, has this quality driven perspective been extended to building plumbing. The Legionnaire’s Disease Outbreak in 1976 sparked interest in new opportunistic pathogens (e.g., *Legionella pneumophila*) that could proliferate in building plumbing, even if not found in the distribution network (Wadowsky et al., 1982). When otherwise safe water sits in the extensive plumbing systems of modern buildings, water quality degradation (e.g., microbiological growth) is inevitable. A single home’s building plumbing system can host $10^{12}$ biofilm bacteria and $10^{10}$ water phase bacteria (estimated with 150 m of 2 cm pipe, $10^7$ cells/cm$^2$, $10^8$ cells/mL, Chapter 2). The long and narrow pipes likely increase the importance of biofilm in buildings compared to the distribution network (i.e., surface-area-to-volume ratios of 200 or more in buildings and 20 or less in distribution networks). Management of these bacteria should thus be a priority. However, while some efforts to reduce microbiological growth in building plumbing systems have been introduced (e.g.,
chlorine boosting for hospitals, hot water recirculation lines to increase temperatures in pipes), these also act like a patchwork of ad hoc improvements.

Several modern developments are even inadvertently inducing microbiological growth in buildings. For example, in developed countries, homes increasingly have more water connections (e.g., rarely used basement bathrooms) and fewer people per house, increasing water age. Green initiatives like reducing water use (e.g., low flush toilets) and reducing energy use (i.e., lower water heater temperatures) serve to increase water age and risk for microbiological growth. An increasingly indoor lifestyle has also led to modern improvements like better insulation and temperature management in homes, which further ensures bacteria friendly temperatures in building pipes year round (Zhang et al., 2015).

In many ways, the Romans had it right the first time. With constant flow, they not only limited chances for microbiological growth, but also protected themselves from lead poisoning from their lead pipes (Sedlak, 2014). Modern considerations like water scarcity and high water production costs will prevent a return to constant flow systems, but that does not prevent one from casting a critical eye on the design of modern building plumbing. Unlike the water distribution network, which would cost trillions to redesign, plumbing in buildings could be creatively redesigned in new buildings or during remodels to improve biological stability. Understandably, radical redesigns also carry a risk (i.e., untested, legal responsibilities).

A legal distinction contributed to knowledge and, ultimately, action gaps.

The responsibility for building water quality falls, perhaps unfairly, on individual building owners. While distribution network operators have a legal responsibility to deliver microbiologically safe water, legal responsibility typically shifts to the building owner at the property line. Utilities drive drinking water research priorities and have the most knowledge
about maintaining water quality (e.g., biological stability), but they usually have neither the ability to sample in homes nor the authority to enact change in building plumbing due to privacy and cost concerns. Moreover, their interests are often aligned with regulations, which typically include only heterotrophic plate counts (HPC) and indicator organisms. Besides the inherent limitations of a culture-based perspective (Van Nevel et al., 2017), the limited focus is not conducive to understanding the entire complex ecology of bacteria in distribution networks, nor the specific risks in building plumbing (e.g., high surface area to volume ratios). Thus, there is relatively little research about the final meters of building plumbing (i.e., research needs explored in Chapter 2), and less that is translated into action properly.

What is already known about maintaining biological stability in building plumbing (e.g., maintaining high temperatures (Bédard et al., 2015) and selecting good materials (Rogers et al., 1994)) is not necessarily communicated well to those that could benefit (i.e., building owners). Only when the legal responsibility might translate into real costs (e.g., getting sued for disease outbreak), do building owners apply precautionary measures. These typically target the opportunistic pathogens (e.g., L. pneumophila, Pseudomonas aeruginosa, and Mycobacterium avium) that can cause disease in immunocompromised populations (Falkinham et al., 2015). Thus, this especially motivates owners of hospitals, spas, and old-age homes, where (1) many building occupants are at high risk, (2) the activities are high-risk (e.g., showering, steam inhalation at spas), (3) the building owner is not the same as the potential litigant (i.e., not private homes), and (4) buildings are large enough to put many at risk.

While this approach protects many vulnerable people, communicating risks effectively to the public, or better yet, designing better building plumbing systems in the first place, will protect more of the population from unnecessary disease. In short, just because legal responsibility is spread thin and doesn’t fall on big organizations (e.g., utilities) capable of research and change, doesn’t mean that the public should go unprotected. There are many regulatory
approaches (e.g., building code changes, material regulations) that could help protect the public from known risks. The work presented here intentionally focused on solutions for common households (rather than large buildings), because average homes are currently underrepresented in the field.

Regulation and government backed recommendations lag behind science in this area for many reasons. For example, there are competing interests for water heater settings, with energy-saving advocates recommending 48 °C and biological safety advocates recommending 60 °C (Lévesque et al., 2004). Other regulatory holes don’t make much sense. For example, in many countries showers are not considered ‘potable water’, and thus materials for shower hoses are not held to standards applied to other pipes (BVer LMG, 2017). However, scientists are also far from consensus concerning the best building plumbing designs, and the contradictory findings prevent wide-scale implementation. Complicating this matter, building plumbing solutions are extremely heterogeneous, so a single silver bullet solution likely does not exist.

**An ecological lens is useful for understanding these engineered systems**

An ecology-based approach was first used to understand wastewater treatment (i.e., microbial resource management (Read et al., 2011)), and was later applied to drinking water treatment and distribution ((Berry et al., 2006), Chapter 2). With an understanding of ecological behavior, one can manage the ecosystem favorably. Rather than changing parameters for the black boxes of various engineering processes until desirable outcomes (e.g., reduction of a pollutant) were achieved, the processes were more properly understood and could be fine-tuned (e.g., balancing a food network to optimize yields). A similar approach would be useful to achieve favorable outcomes in building plumbing (e.g., fostering a benign bacterial community to prevent invasion of unwanted bacteria).
As with any engineering or ecological system, understanding hinges on carefully defined boundaries and conditions. For building plumbing systems, this can be difficult since building plumbing systems are as unique as the buildings they serve. For example, office buildings have very different use patterns than residential buildings. These variations can change the relative importance of particular components in the system (i.e., water heater holds a lower percentage of building system water when there are many connections). In this work the focus was often in the final meters of water distribution network, but to understand the ecology of the system as a whole, boundaries were extended to consider other conditions in the building, distribution network, and treatment plant.

An ecological approach also requires consideration of all the biology occurring in the system. Thus, the planktonic and biofilm phases were both considered, as were interactions between these phases. As drinking water is a fluid and complex microbial habitat, home to thousands of bacterial species (Chapter 2), it is also critical to consider all bacteria (i.e., total cell counts, microbiome sequencing) instead of one or two unwanted bacteria. Focusing on one organism fails to give a complete picture of bacterial behavior, especially since the typically targeted bacteria can have divergent behaviors (Chapter 4, 5). Of course, even the total bacterial focus in this work leaves out important elements, including non-bacterial cells (amoebae, fungi), extra polymeric substances (EPS), and inorganics. These will clearly impact biofilms (e.g., inorganics in Chapter 3), and should be better considered in future work to understand the full ecology of building plumbing.

The simple concepts of community assembly (Kinnunen et al., 2016) – dispersal and selection – were useful for explaining most bacterial behavior in various test-systems. Dispersal, the immigration and emigration events for a particular bacterial community or movement of bacteria across system boundaries, was primarily facilitated by bulk water movement (i.e., into and through the building) and by biofilm attachment/detachment events.
These events occur along spatial scales (i.e., a microbiome continuum across water production and distribution from source to tap, Chapter 2), but also along temporal scales (e.g., intermittent water flow, Chapter 5, Chapter 6). Selection, or the difference in growth conditions that favor some bacteria over others, can also vary with time (e.g., diminishing carbon leaching from pipes over time (Chapter 4), temperature fluctuations (Chapter 5)).

Considering building plumbing as an ecological system allowed for a unified theory (e.g., Figure 8, Chapter 6; Summary, Figure 1). The most important factor in one system does not necessarily hold the same significance in another system, but the same principles apply to all systems. One must examine the boundaries and scale of the system at hand. For example, the two hot water plumbing systems considered in Chapter 5 had similar conditions within the boundaries of the test-system, but upstream selection factors affected dispersal into the system, leading to divergent behavior. While understanding the behavior of bacteria in each component/element of water distribution, or the behavior of one bacterium (e.g., Legionella) is critical, a full understanding of microbiological behavior in building plumbing requires consideration of the entire system as a whole. Below, the elements of selection and dispersal are further discussed.

**Dispersal – movement of bacteria into and through building plumbing**

*From the distribution network into the house:* The vast majority of the bacteria in a building plumbing system originate from the distribution network transported with bulk water flow. Of course, there are many other sources of bacteria when considering the realities of building plumbing systems. For example, end-point contamination can cause back growth along pipes, bacteria are present on pipes during installation, contamination occurs during maintenance, back-siphonage accidents occur, and air gaps are not always properly installed.
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Throughout most studies presented here, the non-chlorinated distribution network water (e.g., fully flushed, cold water) had the highest richness (Chapter 3, 5, 6; all with Swiss non-chlorinated drinking water). This was reduced by elements of selection within the building (discussed below). While this assumes unidirectional flow, this seemed reasonable for buildings, and especially for the test-systems analyzed. Larger buildings may have more complex flow patterns.

All of the factors that affect distribution network microbiome, (e.g., source, chlorine residuals (Bautista-de los Santos et al., 2016; Holinger et al., 2014)) affect the building plumbing bacteria as well. For example, many of the differences in cell concentrations and richness in shower hose biofilms from around the world were traced to the chlorination in the distribution network (Chapter 3). It is unlikely that chlorine residual persisted into the building to act as an active selective force, but the selection upstream most likely limited the microbiome available for dispersal into the building plumbing biofilms.

From the biofilm into water: Water is stagnant for a majority of the time in building plumbing (DeOreo et al., 2016) (Chapter 2, 4). While stagnation likely serves as a selective force, it is perhaps most interesting for its effects on biofilm attachment/detachment dynamics (Chapter 6). Biofilm detachment into water is often thought of during flow (e.g., shear forces, sloughing). However, biofilm detachment also occurs during stagnation, contributing up to 56% of the increase in cells in first flush of water exiting a pipe (Chapter 6). The similarity between biofilm and stagnant water microbiomes is also clear throughout several presented studies (Chapter, 3, 5, 6). The never before possible direct in-pipe measurements of bacteria were critical for exploring these dynamics. Additionally, while many studies only investigate biofilm or water (i.e., due to methodological limitations reviewed previously (Douterelo et al., 2014; Wang et al., 2017)), their interactions are clearly interesting and critical for the understanding of building plumbing.
There is room for much more work related to biofilm water interactions under these unique stagnation and flow conditions. For example, biofilm detachment during stagnation was only observed quantitatively during stagnation (cell counts). Qualitative (i.e., 16S amplicon sequencing) comparisons of in-pipe and end-of-pipe (with flow) water samples might provide more insight about the detachment dynamics. Experiments can also be designed to better quantify the effect of shear forces in shower hoses, for example by utilizing stepped flushing rate experiments.

Of course, studying biofilm and water together in most drinking water systems is difficult because representative biofilm samples are difficult to collect (e.g., swabbing at distal ends, specially designed biofilm coupon systems (Douterelo et al., 2014; Wang et al., 2017)). In this work, the shower hose provided a perfect realistic system to study these interactions. Not only were shower hose biofilms easy to access and analyze (Chapter 3, 4, 7), but unlike hard pipes (Chapter 5), they allowed for a novel perspective from the inside of the pipe (i.e., biofilm pictures Chapter 3, in-pipe measurements, Chapter 6).

**Selection – conditions favoring some bacteria over others**

Many of selective forces discussed here are only active in a specific component of building plumbing (e.g., a specific pipe, water heater). Thus, their effects are constant on the biofilm within that component, but only have a transient effect on the water passing through. The relative strength of a selective factor also depends on the selection factors upstream.

**Material:** Throughout this work, material quality was explored as a potential selective factor in building plumbing (Chapter 2, 4, 5). Synthetic pipes leach organic carbon, a substrate for growth. As carbon is the limiting nutrient in most drinking waters, this additional source critically affected the microbiome, both in terms of absolute numbers and composition.
Since the source of the carbon is also the biofilm substratum, this challenges most biofilm development models, where all nutrients originate from one side of the biofilm (i.e., water phase). Here, while carbon originates from the substratum, other nutrients (oxygen, nitrogen, phosphorous) originate from the water phase. Together with the stagnation and intermittent flow conditions, this creates a counter-diffusion based semi-batch reactor. The carbon in the substratum offers a plentiful, but continuously diminishing source. Nutrients from the water are less plentiful (i.e., controlled during water treatment), but replenished often (i.e., with flow). Additionally, bacteria belong to both a stable population (biofilm) and a semi-transient one (water). Such a biofilm model warrants further exploration, as it is radically different from most drinking water biofilm models studied today.

Given the influence of pipe material as a selective factor, it is bewildering that shower hoses are not regulated as potable water pipes. Potable water pipes must pass leaching tests for both chemical and biological safety reasons. However, many of the modern biological risks for water are linked to inhalation or skin exposure during showers (Falkinham et al., 2015). Since shower hoses are flexible plastics, they leach high amounts of carbon, but better functionally comparable materials are available (Chapter 4). Consumers, however, rely solely on outward appearance when choosing shower hoses. An example solution would be a grading label for performance on the potable water materials tests, which would pressure pipe producers to use high quality materials and inform consumers of potential risks.

*Temperature:* Temperature is very different between the distribution network (e.g., outside, subject to seasons) and building plumbing (e.g., indoor heating, water heaters). Since each bacterium has a different optimal temperature for growth, a shift in the microbiome is not surprising as water enters the home.

The distal ends of pipes, however, all reach a similar temperature regardless of the incoming water (e.g., both cold and hot water pipes, Chapter 5) or outdoor temperature (i.e., stable
indoor temperatures). Thus, selection does not necessarily explain the differences between the hot and cold distribution systems (Chapter 5, (Inkinen et al., 2014, 2016)). A holistic microbiome continuum approach can help explain this discrepancy. The selective conditions of water upstream dictate the microbiome transported into distal pipes (i.e., different hot and cold microorganisms, Chapter 3). Thus, despite similar temperatures during stagnation, hot and cold distribution system distal pipes have different microbiomes (Chapter 5).

Shower hoses are interesting with respect to temperature. Due to their location downstream of mixing valves, shower hoses are seeded with water from both the cold and hot distribution systems, and temperatures in the pipes only range from 20-45 °C. Thus, any bacteria present in small amounts in the cold water distribution system could bypass temperature control mechanisms (i.e., high water heater temperature) and proliferate with the moderate temperatures in shower hoses. Both the post-mixing water (i.e., partial selection) and the fluctuating temperature (i.e., inconsistent selection) conditions within the pipe offer interesting avenues for future research.

*Intentional selection:* Many proposals to improve building plumbing water quality essentially use growth inhibition. For example, recirculating lines for hot water distribution system aim to maintain high temperatures throughout more pipes to control growth. Chlorine booster and copper-silver ionization systems disinfect water within the building. However, these selection-based strategies require that the selective force (e.g., water with chlorine) reach all portions of a building plumbing system, but some distal ends (e.g., shower hoses) inevitably bypass these strategies. Even if distal ends are occasionally opened to introduce selection, chlorine is consumed and temperature drops during stagnation.

Serious consideration of these methods would require a redesign of building plumbing such that selection could be distributed to every point in the system. Constant flow out of all outlets could ensure that hot water or chlorine consistently reaches distal ends, but would
introduce other problems (e.g., high water bills). However, from the beginning of building
design, architects and mechanical, engineering and plumbing/fire protection (MEPFP)
engineers can design to optimize these strategies. They can shorten distal ends by
concentrating water outlets to one location in the building, limit the number of outlets, and
maximize water recirculation to maximize the effectiveness of such selection measures.
More radically, all taps could be automated such that they open simultaneously during
interventions without significant manual effort.

Selection and dispersal act along several scales

Spatial scales: As discussed with respect to dispersal, water delivery occurs along a
continuum, with each component affecting the next (Chapter 2). However, dispersal can also
occur on a smaller scale during stagnation. Both leachate and bacteria diffuse across
component boundaries while water sits still (e.g., convective mixing from recirculating lines
to distal ends (Rhoads et al., 2016)). Thus, heterogeneity, even along a single pipe, might be
expected. In the work presented here, such heterogeneity is disregarded in favor of
capturing a complete picture of biofilms (e.g., 30 cm or more of pipe biofilm measured).
Methods that only capture end-point biofilms (e.g., swabbing a faucet) would be affected by
such spatial scales (e.g., oxygen gradients from the air interface at end points). Shower
hoses offer an improvement, with easy access to long sections (i.e., up to 2 m) of
undisturbed pipe biofilm. A study of heterogeneity along such a shower hose would be very
interesting from an ecological perspective (e.g., demonstrating gradients along a biofilm),
and could furthermore help inform the minimum representative sample size.

Time scales: There are many time scales to consider in building plumbing. The life span of
building plumbing varies (e.g., age of building, time since bathroom/kitchen remodel). The
shower hoses collected in Chapter 3 ranged from 3 months to 40 years old. Thus, building
plumbing studies would ideally span decades, but this is unlikely, given typical funding and personnel structures for research. However, since most research takes place in buildings, researchers can and should take advantage of their own buildings’ mature (or new) building plumbing systems. One can extract pipes during renovations (i.e., analogous to retrieving distribution network pipe cut-outs (Douterelo et al., 2014)) or install monitoring in the building (e.g., online water monitoring, biofilm sampler bypasses). Inkinen et al. took advantage of a new building to monitor biofilm development and water quality during one year (Inkinen et al., 2016, 2014), but such a study could easily be extended for the lifetime of the research group (e.g., getting research group leaders into the lab annually to continue a 20-year experiment).

In the first months to 1 year of biofilm development, most of the relevant changes likely occur. Biofilm ‘stabilization’ in Chapter 5 occurred more quickly in terms of numbers, but the microbiome is continually influenced by dispersal from the distribution network. Even a stable building plumbing system would be vulnerable to changes that occur in the distribution network (e.g., temporary failure of treatment), and these changes are likely long lasting. The principles of bacterial invasion into pipes with an established biofilm/microbiome are not yet understood, and offer a promising avenue for future research.

Building plumbing water is also subject to massive changes on a relatively short time-scale. During stagnation, the water phase bacterial concentrations increase over several orders of magnitude (Chapter 6). These short-term effects were independent of age of the biofilm. However, these changes should inform building plumbing sampling strategies. In the matter of a couple of hours, the nature of the first flush sample will completely change.
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Improved tools and understanding of these tools added a fresh perspective

Understanding the dynamics of building plumbing hinges on appropriate sampling and analysis methods. The new tools explored and implemented in this thesis, as well as a greater understanding of them, are useful for future research efforts.

Shower hoses have been featured in several recent studies (Soto-Giron et al., 2016; Collins et al., 2017; Moat et al., 2016; Whiley et al., 2015), and are heavily featured in this work. The shower hose offers an interesting sampling point due to the unique selection factors at play (e.g., temperature, material) and the easy access to large sections of pipe biofilm. They are also interesting because of their proximity to a primary route for opportunistic pathogen exposure, inhalation and skin exposure in showers. Thus, shower hoses are interesting tools for both scientific and monitoring purposes. While use as a monitoring tool by regulatory agencies will require greater understanding of how these biofilms relate to risk, they offer a practical option for sampling biofilms with limited risk of contamination by sample volunteers. Biofilms are typically hard to access in drinking water (Wang et al., 2017; Douterelo et al., 2014), but offer a valuable insight into the building plumbing microbiome.

Looking inside a pipe, as with the real-time flow cytometry in Chapter 5, is an unbelievably important tool for future research. New dynamics were revealed, and it was possible to see, for the first time, what happens within the pipe before flow began. This tool has many potential applications for future research. For example, one could monitor, directly in-pipe, the effectiveness of building plumbing mitigation methods (i.e., maximum time for chlorine effectiveness before it is consumed).

Throughout this work, both quantitative and qualitative methods were used in concert to improve understanding of the complete ecology of the system. For example, the calculated
absolute abundance (Props et al., 2016a) of potential opportunistic pathogens gave a different understanding of risk than the relative abundance alone (Chapter 3, 4, 5).

For both quantitative and qualitative data, this work employed two complementary methodological approaches: flow cytometry and molecular methods. These were compared quantitatively in Chapter 8 (qPCR for 16S rRNA gene copies and flow cytometry for cell counts), and a reasonable correlation was found despite the many differences between approaches. Every method has drawbacks in specific scenarios and scientists cannot invest the time or capital to master all techniques. This rough comparison will allow for general comparison between many studies, allowing for easier synthesis of literature. Moreover, it points out that blindly accepting a method without understanding the mechanics isn’t wise.

Although flow cytometry was widely applied throughout this work, it was not necessarily used to its fullest potential in every study. Flow cytometric fingerprinting offers additional qualitative data to the highly quantitative cell counts (Koch et al., 2013; Prest et al., 2013; Props et al., 2016b; Besmer et al., 2017). In this work it was clear through basic fingerprinting that during stagnation, high nucleic acid-content (HNA)-content bacteria were favored over their counterpart low nucleic acid (LNA)-content bacteria in drinking water (Chapter 5, 6). This corresponded to a shift in the microbiome. While shifts in flow-cytometric fingerprints have been linked to changes in the microbiome previously (Prest et al., 2013; Props et al., 2016b), the cause for this link is not yet clear.

In Chapter 7, the two approaches were used in concert to help explain this link. Specifically, the unique phylogenetic identity of LNA-content bacteria was revealed and linked to that of ultra-small bacteria and the candidate phyla radiation. These observations offer perhaps the most interesting aspect of this work from a fundamental science perspective. The reasons for the separation of LNA-content bacteria and HNA-content bacteria remain elusive, but this information offers valuable context for interpreting LNA: HNA ratios through many studies.
Practical implications of this work – radical suggestions

After casting a critical eye on the design of building plumbing design, it is clear to me that a complete overhaul, however unlikely, would be the most effective solution to improve building plumbing design. Here, I outline several suggestions, ranging from easily implemented to quite radical.

(1) The hydraulics of building plumbing could be redesigned to reduce or completely prevent water stagnation. By minimizing the length of distal ends and adding recirculation loops to both hot and cold water distribution systems, more water is kept moving. This could be accomplished by influencing architects and MEP/FP engineers, as discussed above. Communication with these groups is anyways necessary. Research is quickly discovering inadvertent effects of plumbing design details. For example, angled pipes encourage convective mixing, inducing water temperatures conducive for growth (Rhoads et al., 2016).

Furthermore, toilets (the most frequent and biggest volume use) could be placed at the end of the line to maximize water turnover (already implemented in some designs). Distal ends could also be designed to completely drain during stagnation. While this latter solution might create new problems (e.g., fungal growth), it warrants investigation.

More radically, the end of the line could rather be an automatic device, which once per hour (or less when water is frequently used) flushes enough water to limit stagnation times. To save water, this water could go to a storage tank for toilet flushing, solar water heating, or graywater uses. This solution would require new plumbing lines for these non-potable uses. Even more radically, a constantly flowing line would go through the home and return to the water distribution network. Water would only be drawn from this constantly flowing line on
demand. This solution would require new solutions for maintaining desired water pressure and charging for water use.

(2) As pipe material and biofilm have a strong impact in building plumbing systems, it may be desirable to increase the diameter of pipes in the home, decreasing the surface-area-to-volume ratio. For example, a single wide-pipe (i.e., 6 inches) could flow through the center of a house, with smaller pipes (i.e., 2 inches) only connecting short distances to distal taps. Typical pipes used in homes are 2 inches or less (i.e. 0.25 inches or 0.6 cm for shower hoses). Such a change might create problems for maintaining water pressure and home design (i.e., thicker walls), but is worth discussing with architects, MEP/FP engineers and plumbers.

(3) As mentioned above, current mediation techniques introduce negative selective factors to the water, but they cannot be consistently effective to all ends. Selective factors should be applied such that they affect the whole building plumbing system. For example, designing better pipe material could limit growth at all points. Caution should be taken with all negative selection methods, however, since limiting the growth of some bacteria may be optimal for other bacteria (e.g., Legionella vs. Mycobacterium in Chapter 4). In any case, a holistic approach, not targeting one opportunistic pathogen at a time, is necessary.

(4) Rather than control growth by limiting it, we should be directing the inevitable growth (i.e., probiotics (Wang et al., 2013)). By adding an initial community directly to pipes during building commissioning, invasion by opportunistic pathogens could be prevented. Such a probiotic strategy has been explored in food sciences to prevent E. coli colonization (Gomes et al., 2017). Particle filters, which are already used at the plumbing entrance to Swiss homes, could be intentionally biologically active to continually seed the building plumbing system with ‘good bacteria’. Such an option needs further research, but has great potential. More radical probiotic approaches, like introducing phages or non-pathogenic bacteria that
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closely resemble and compete with pathogens, can also be explored, but are even less likely to be implemented.

(5) A more digestible solution is to increase monitoring so that problems can be identified before problems or diseases manifest. The rest of the water distribution network is in the hands of engineers, with periodic quality monitoring throughout the distribution network. Buildings could also benefit from regular monitoring. However, to be effective, building samples must be taken in a more meaningful way than currently implemented. The information in this thesis can inform monitoring strategies – for example shower hoses offer easy access to biofilms (Chapter 3), stagnation time for water samples is critical (Chapter 6), and sampling frequency will reveal building dynamics (Chapter 6) (Besmer et al., 2014).

Monitoring all buildings would be a huge undertaking for the distribution network managers. Targeting public buildings could already be effective for identifying the prevalence of problems. However, private buildings could also be monitored using a partially decentralized strategy that involves water customers. For example, volunteers could send shower hose samples to the laboratory, or kits for simple testing could be sent to citizen scientists. Increasing monitoring efforts, whether implemented by utilities or citizen scientists, will require easier microbiological sampling methods (e.g., a stick with colorimetric changes in the presence of harmful bacteria, analogous to pregnancy tests or pH strips). Inline and online monitoring tools could also be developed for public buildings.

(6) Proper control of building plumbing water quality will require an incentive. Monetary and legal actions drive action in large semi-public buildings (e.g., hospitals). While threatening legal action is unnecessary for most buildings, a positive incentive, like inclusion of water quality in home insurance plans, could be beneficial. If a building owner is willing to take preventative steps (e.g., using high quality pipe materials) and monitor building water quality regularly, they could receive a discount on protection from related costs.
Altogether, we are coming towards a better understanding of building plumbing. It is no longer a black-box in which water quality deteriorates. The future of this field includes a better understanding of individual elements (e.g., water biofilm interaction), a holistic approach that synthesizes research about individual components into one system, and, most importantly, communication and implementation of real solutions.

With varying nature of buildings and changing conditions, it is not surprising that a single silver bullet solution does not exist. The solutions for a single-family home (e.g., high temperatures in the water heater) may not scale-up successfully to a multi-family apartment building (i.e., because distance to the water heater increases). Similarly, the solutions for a large building (i.e., adding secondary disinfection) will not be cost-effective for a small building. In this respect, each building plumbing system is a unique engineering and ecological system that requires its own considerations.
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Acknowledgements

In late 2013, I applied for a PhD program on a whim. I was surprised when I was offered a position, and then I was unsure whether I wanted to move to a new country or even continue with research. Luckily, I had many supportive people encouraging me to take what turned out to be the opportunity of a lifetime. These 3.5 years have enriched my life in ways I could not have foreseen.

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Education

Eidgenössische Technische Hochschule (ETH), Zurich, Switzerland
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Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA, USA
M.S. in Civil Engineering, March 2014
  Thesis: Effect of Various Water Chemistry Factors on Legionella Proliferation and the
  Premise Plumbing Microbiome Composition
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B.S. in Civil Engineering, May 2012
  Minors in Green Engineering and Environmental Policy and Planning
  GPA: 3.97 Major Rank: 3 of 287

Experience

Mermaid PhD Fellow – Apr. 2014–present
  -A Marie Skłodowska-Curie Initial Training Network
  -Attended courses for professional development and research skills with a cohort

Corona Environmental Consulting – Internship – Spring 2016
  -Conducted research project related to building plumbing and disseminated results
  -Completed as part of requirements for the Mermaid PhD

Sussman Research Internship – Summer 2013
  -Funding from the Edna Bailey Sussman Foundation for an environmental fellowship
  -Used Illumina sequencing with the EPA in Cincinnati to investigate nitrification

Graduate Research Assistant, Virginia Tech – Aug. 2012–March 2014
  -Worked on experiments related to eventual Masters thesis, managed other students

NSF REU (Research Experience for Undergraduates), Virginia Tech – Summer 2012
  -Learned and applied molecular biology tools to pilot studies in biofilm collection methods

Undergraduate Research Assistant, Virginia Tech – Spring 2012; Spring 2010
  -Worked in two environmental chemistry labs, spending one semester on clam assays, and another semester with microcosm analysis
Honors and Awards

Best Presentation, IBP Congress, ETH (2017)
AWWA Academic Achievement Award, 1st place (2015), national award for master’s thesis
MWH/AEESP Master’s Thesis Award, 2nd place (2015), national award for master’s thesis
AWWA WQTC, Student Best Paper (2013)
NSF GRF, Honorable Mention (2013), for proposal written for fellowship consideration
Charles Via M.S. Fellowship (2012-2014), graduate student fellowship
Via Stewart Scholarship (2008-2012), full tuition scholarship

Publications (♮ PhD Dissertation publications)


Reviewing Activities

Students Mentored

Mauro Reimmann – February – July 2016 – *ETH Bachelors’ Thesis*
Brittany Flittner – Summer 2013 – *NSF REU student*

Outreach Activities and Service

**Organizing Committee,** MEWE 2016, Copenhagen, Denmark (2016)
-Edited and formatted conference proceedings and program
-Participated in planning meetings and contributed to running the conference smoothly

**Workshop Leader,** Flint, Michigan – A water professional’s role in responding to a catastrophic water failure, MEWE 2016, Copenhagen, Denmark (2016)
-Coordinated with fellow workshop leaders, managed logistics planning

**Organizing Committee,** IWA Specialized Conference – Biofilms in drinking water systems: From treatment to tap in Arosa, Switzerland, (2015)
-Edited and formatted conference proceedings, including soliciting entries
-Participated in planning meetings and contributed to running the conference smoothly

**Workshop Leader,** “Microbiohome” – IWA Specialized Conference – Biofilms in drinking water systems – From treatment to tap in Arosa, Switzerland, (2015)
-Coordinated with fellow workshop leaders, managed logistics planning
-Led sub-sessions on “Managing premise plumbing microbiology” and “Probiotics”

**Gymnasium Day Coordinator,** Eawag, Switzerland, (2015)
-Coordinated a day of experiments and lectures for 20 high school students

**Open House Weekend Coordinator,** EWR Department (2013)
-Organized travel, housing, and volunteers for 30 potential students for the department’s Open House

**Punta Cana Study Abroad Program,** Dept. of CEE, Virginia Tech – Summer 2011
-Month study of groundwater in the Dominican Republic with sampling for salinity and microbiological safety and considerations for socio-economic barriers

**Fundraising Director,** Oxfam at Virginia Tech (2009-2010), founding member
-Managed fundraising events for the social justice organization, directing teams of up to 30 students

**Educational Outreach Co-Chair** (2010-2012), Outreach Committee (2008-2009); Society of Women Engineers (SWE)
-Planned and executed two separate days for 40+ girls to perform science and engineering activities on campus