


Biofilms in premise plumbing systems as a double-edged sword: microbial community composition and functional profiling of biofilms in a tropical region

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ABSTRACT

To understand distributions of opportunistic premise plumbing pathogens (OPPPs) and microbial community structures governed by sample location, pipe materials, water temperature, age of property and type of house, 29 biofilm samples obtained from faucets, pipes, and shower heads in different households in Singapore were examined using next-generation sequencing technology. Predictive functional profiling of the biofilm communities was also performed to understand the potential of uncultivated microorganisms in premise plumbing systems and their involvement in various metabolic pathways. Microbial community analysis showed *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Nitrospira*, and *Actinobacteria* to be the most abundant phyla across the samples which was found to be significantly different when grouped by age of the properties, location, and the type of house. Meanwhile, opportunistic premise plumbing pathogens such as *Mycobacterium*, *Citrobacter*, *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* were observed from the samples at 0.5% of the total reads. Functional prediction using 16S gene markers revealed the involvement of the biofilm communities in different metabolic pathways like nitrogen metabolism, biodegradation of xenobiotics, and bacterial secretion implying diverse functionalities that are yet to be studied in this environment. This study serves as a preliminary survey on the microbial communities harboring premise plumbing systems in a tropical region like Singapore.

Key words | biofilm, opportunistic premise plumbing pathogens, premise plumbing pipe

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INTRODUCTION

Premise plumbing refers to the portion of the potable water distribution system connected to the main distribution system via service lines, including both hot and cold water and devices such as water heaters, showers, faucets and filters (Wang *et al.* 2017). Premise plumbing systems differ from the public water supply networks mainly due to conditions such as surface area, low disinfectant residues, periodical flow of water creating a more favourable and ideal niche for microbial growth (Council 2006; Feazel

et al. 2009). These plumbing systems are considered to be the boundary gates before exposure of consumers to the microorganisms that have survived stringent water treatment processes. Presence of microorganisms in residential plumbing systems such as shower heads, faucets, and pipes and how several factors such as pipe material and water temperature affect prevalence of these organisms have been reported in previous studies (Rhoads *et al.* 2015; Proctor *et al.* 2016). These microbial communities were found thriving either in suspended form or as biofilms attached on surfaces with the latter gaining more attention due to their accumulation through time (Buse *et al.* 2014; Wang *et al.* 2014a; Proctor & Hammes 2015).

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Biofilms collected from premise plumbing systems have been shown to contain diverse groups of microorganisms belonging to different genera such as *Sphingomonas*, *Methylobacterium*, and *Novosphingobium* (Kelley et al. 2004; Muñoz Egea et al. 2017; Proctor et al. 2018). While most of these organisms are harmless and do not pose immediate public health risks, the concern regarding the presence of biofilms in oligotrophic or nutrient-depleted environments such as pipes mainly lies in their ability to harbour organisms that may become potentially pathogenic in certain cases (Wingender & Flemming 2011; Wang et al. 2012). Proliferation of these organisms in biofilms on surfaces of pipes is possible due to reactive pipe materials, long retention times of water containing residual nutrients, warmer temperature, and low or no residual disinfectant from the water treatment plants (Feazel et al. 2009; Falkinham 2015).

Due to the ecological niche these organisms are found to be thriving in and their disease-causing nature, they are collectively called opportunistic premise plumbing pathogens (OPPPs) (Buse et al. 2014). Some of the most common genera belonging to OPPPs include *Mycobacterium*, *Legionella*, and *Pseudomonas* (Kusnetsov et al. 2003; Falkinham et al. 2015; Naumova et al. 2016). In contrast to traditional waterborne pathogens, OPPPs naturally colonize, persist, and multiply in the biofilms of potable premise plumbing systems due to the abovementioned conditions (Ren et al. 2015; Richards et al. 2018). Their presence has become a growing concern even in developed countries due to their prevalence, persistence and the eventual healthcare burden these organisms may cause (Feazel et al. 2009; Beer et al. 2015; Falkinham 2015).

Despite these negative reports on biofilms in premise plumbing systems, a study has shown the ability of biofilms to degrade disinfection by-products formed as a result of the reaction between chlorine and natural organic matter in drinking water distribution systems (Pluchon et al. 2013). Since eradication of microbes in premise plumbing systems is practically impossible and unrealistic, another study has hypothesized that a rich microbiome can be harnessed to control good bacteria which might be applicable to control downstream drinking water microbiome by manipulating the microbial community in the filtration process during water treatment (Pinto et al. 2012; Wang et al. 2013). However, limited studies and the lack of knowledge on the microbes

being harboured in biofilms in residential premise plumbing systems as well as their potential functional profiles hinder the applicability of this concept to potable water systems.

In this study, biofilm samples from residential areas in Singapore were collected for the identification of microbial communities in premise plumbing systems using next generation sequencing technology. We also examined how various environmental factors such as location, pipe materials, age of property, and type of house (e.g., public housing, private condominiums, and landed homes) shape microbial community composition in premise plumbing systems. Lastly, functional profiling using 16S gene markers was performed to present different pathways and potential metabolic features of organisms which aim to show a different perspective on the presence of inevitable microbial communities in our plumbing systems at home.

MATERIALS AND METHODS

Sampling sites

A total of 29 biofilm samples were collected from nine different sampling locations from central, southern, and western regions of Singapore using sterile cotton swabs in screw cap tubes (Figure 1). Biofilms from pipes and shower heads that were accessible and did not require extensive dismantling were collected from the residential areas. Samples were kept in a -20°C freezer to preserve the DNA until further processing. The type of house, location, region, age of the property, and the average monthly water usage from the nine sampling sites are listed in Supplementary Table S1. Samples labeled as 'Heated' were collected from shower heads or hoses where the tap water has passed through the heater first while 'Normal' samples are collected from pipes without any installed heating mechanism (Supplementary Table S2).

DNA extraction

DNA was extracted using the FastDNA[®] Spin Kit for Soil (Q-Biogene/MP Biomedicals, Solon, OH, USA) following instructions from the manufacturer. Extraction of DNA from drinking water environmental samples was shown to be most efficient using FastDNA[®] Spin Kit for Soil (Huang et al. 2011). Briefly, the swabs used during sampling were



Figure 1 | Sampling points where the biofilms are collected: A-Redhill (RH); B-Bukit Gombak (BG); C-Jurong West (JW); D-Telok Blangah (TB); E-Bedok North (BN); F-Clementi (CL); G-Yishun (YS); H-Bukit Batok (BB); I-Kampong Arang (KA).

cut allowing only the cotton bud area to be added into tubes containing Lysing Matrix E. 100 μ L of DNA elution solution (DES) was used to elute the extracted DNA and samples were stored in -20°C until before use. DNA concentrations were quantified using Nanodrop[™] (Thermo Scientific, USA).

16S rRNA sequencing and data processing

The 16S rRNA genes were amplified from all the DNA samples using 16S prokaryotic primers used by Takahashi *et al.* (2014) due to its high coverage for species of bacteria and archaea. Initial PCR to amplify 16S rRNA genes from the samples was performed by mixing 12.5 μ L NEB Next[®] Ultra[™] II Q5[®] Master Mix, 400 nM of forward and reverse primers, and 10.5 μ L of DNA template (50 ng) and nuclease-free water. PCR reactions were done in a Veriti 96 Well Fast Thermal Cycler (Applied Biosystems, USA) with the following conditions: 95°C for 3 minutes (initial denaturation), 30 cycles of denaturation at 95°C for 30 secs, annealing at 65°C for 30 secs, and extension at 72°C for 30 secs with a final elongation at 72°C for 5 minutes. PCR products were purified using Agentcourt AMPure XP magnetic beads (Beckman Coulter, Inc., USA). Amplicons were quantified by Qubit[™] Fluorometer (Thermo Fisher Scientific, USA) and only samples that met the required minimum DNA concentration were sent for 16S rRNA amplicon sequencing in a MiSeq 300 \times 300 bp platform to

the Singapore Centre for Environmental Life Sciences Engineering (SCELSE). Paired-end sequences were joined using Pear (Zhang *et al.* 2013) and joined reads were quality filtered, OTU picked, and annotated using SILVA database as a reference (UCLUST algorithm for clustering) with a similarity threshold of 97% in Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.* 2010; Edgar 2010; Quast *et al.* 2012). For the detection of pathogenic microorganisms at the genera level, the paired sequences were clustered against the human pathogenic bacteria 16S rRNA database with default parameters and with a sequence similarity at 99% (Bae *et al.* 2019). OTU tables for potentially pathogenic microorganisms were uploaded in Microbiome analyst for visualization and statistical analysis with the following parameters: low filter count was set at a minimum count of 2 and scaled using the total sum scaling (Dhariwal *et al.* 2017).

Functional profiling using PICRUST

Phylogenetic investigation of communities by reconstruction of unobserved states or PICRUST was used to predict metabolic and functional capabilities of the biofilm samples from the 16S gene markers obtained from the microbial community analysis by a previous study (Langille *et al.* 2013). Briefly, the OTU-containing biom file of the samples (annotated using Greengenes database) was normalized for 16S copy numbers using the default parameters of

normalize_by_copy_number.py script. The resulting biom file was used as input for the *predict_metagenomes.py* script which produces an output with metagenome functional predictions for an OTU table. Thereafter, the biome file output was used for pathway analysis using KEGG pathways which counts the number of genes involved in several pathways per sample using the *categorize_by_function.py* script. KEGG pathways contain manually drawn pathway maps that represent known molecular interaction, reaction, and networks for metabolism, genetic and environmental information processing, cellular processes, organismal systems, human diseases, and drug development (Kanehisa et al. 2016). Data for the functional profiling and the study's metadata (supplementary information Table S2) were uploaded for visualization in statistical analysis of metagenomic profiles (STAMP) using the software's default parameters for group analysis (Parks et al. 2014).

RESULTS AND DISCUSSION

Microbial community structures in premise plumbing systems

A total of around 700,000 reads were used as an input for quality filtering and 176,000 reads with lengths shorter

than the truncation length of 200 bp were also excluded. Of these, 520,000 reads having a median sequence length of 440 bp were processed in QIIME for clustering, OTU picking, and annotation (Caporaso et al. 2010). After quality filtering, 37 various phyla were identified and the top 9 phyla with the highest relative abundance of all samples are shown in Figure 2. The phylum *Proteobacteria* is found to be the most predominant group with a relative abundance of 17% of the total microbial population followed by *Actinobacteria* (4%), *Bacteroidetes*, *Nitrospirae*, *Cyanobacteria*, and *Acidobacteria* with percentages of over 1.0%. The predominance of *Proteobacteria* is consistent with previous reports of microbial communities from biofilm samples collected from water distribution and plumbing systems (Eichler et al. 2006; Hong et al. 2010; Pinto et al. 2012). *Actinobacteria* and *Bacteroidetes* are usually found alongside *Proteobacteria* in biofilms from natural sources such as stone surfaces and biofilms collected from lakes and seawater and engineered systems like activated sludge from wastewater treatment plants and drinking water distribution systems (Revetta et al. 2013; De Sotto et al. 2018). *Cyanobacteria*, a phylum that is primarily recognized for oxygenic photosynthesis and one of the oldest, most morphologically diverse of the phyla, has been seen in chlorinated bulk waters while a study on biofilms from water distribution systems also reported this phylum to be one of the major



Figure 2 | Relative Abundance of the top phyla from the biofilm samples collected from different residential areas across Singapore. The x and y axes are labelled with the sampling points and the top phyla from the biofilm samples, respectively. Sampling points are RH-Redhill; BG-Bukit Gombak; JW-Jurong West; TB-Telok Blangah; BN-Bedok North; CL-Clementi; YS-Yishun; BB-Bukit Batok; and KA-Kampong Arang.

components of the biofilm community (Hwang et al. 2012; Wu et al. 2015). Meanwhile, *Acidobacteria*, aside from its ubiquitous presence in distribution system biofilms, this phylum was reported to be abundant in faucet biofilm communities (Liu et al. 2012). The reported abundant phyla found in biofilms of residential premise plumbing systems in this study are similar to the communities forming the biofilm in the pipes in previous studies (Pinto et al. 2012; Zeng et al. 2013; Liu et al. 2014).

Digging deeper down the taxonomic rank at the genus level, the biofilm samples have a myriad of microorganisms and the genera with greater than 10,000 reads are presented in a stacked bar plot in Figure 3. Five genera with the highest abundances in the biofilm samples included alphaproteobacterial group, namely *Sphingomonas*, *Bradyrhizobium*, *Methylobacterium*, *Novosphingobium*, and *Sphingobium* while *Mycobacterium*, *Nitrospira* and an uncultured

Melainabacteria from *Actinobacteria*, *Nitrospira*, and *Cyanobacteria*, respectively, are the rest of the most abundant species that comprise the biofilm samples collected from the various premise plumbing systems.

In our study, *Sphingomonas* was the most prevalent organism having a range of 0.005–0.38 percentages across all samples with highest prevalence recorded from a sample in YS1 and the least from a heated source. A similar study on biofilms growing on shower curtains showed presence of mainly *Sphingomonas* and *Methylobacterium* in most samples (Kelley et al. 2004). *Sphingomonas* is a Gram-negative, aerobic, polar flagellated, yellow-pigmented organism that contain dihydrosphingosines. These organisms are widely distributed in nature and were seen to play a part in pipe corrosions in water distribution systems and degrade refractory pollutants (White et al. 1996). Also, *Methylobacterium* spp. have been isolated in drinking

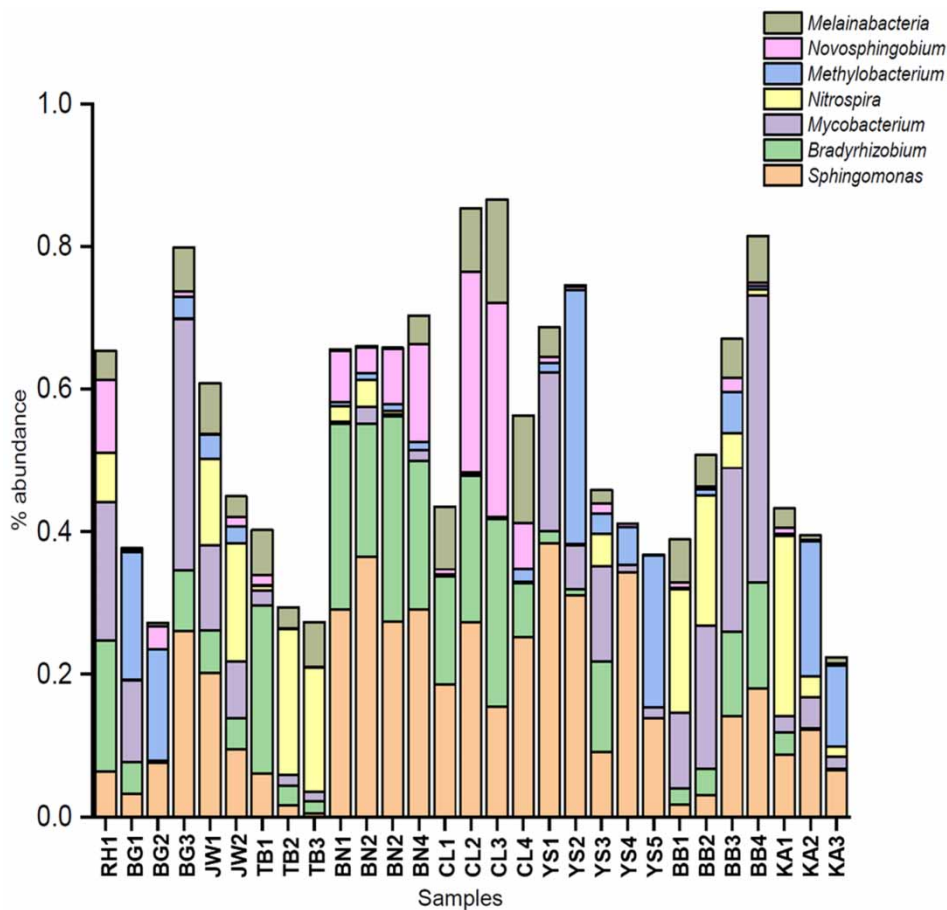


Figure 3 | Predominant genera from the biofilms of the residential premise plumbing system showing the predominance of the genus *Sphingomonas*.

water environment and have been seen to have a strong biofilm-producing ability especially with *Mycobacterium* in biofilms (Simões et al. 2007), while another study demonstrated that the most resistant biofilm to disinfection was that of *Methylobacteria* (Simoës et al. 2010). Here, we have seen relative abundances of this genus at 5×10^{-5} –0.36% from a heated source in TB2 and a non-heated tap water source from YS2, respectively. *Methylobacterium* is a Gram-negative, strictly aerobic, rod-shaped bacteria that is able to grow on one-carbon compounds and may therefore persist in premise plumbing environment due to its oligotrophic nature (Van Aken et al. 2004). *Bradyrhizobium*, which was found at relative abundances from 5×10^{-5} (YS5) and 0.29 (BN3), is a genus within the subphylum *Alphaproteobacteria*. This genus has been found on corroded ductile cast iron pipes in water distribution systems, a regrowth in pure water, and as one of the most abundant genera in drinking water in residential areas (Jang et al. 2012; Proctor et al. 2016; Li et al. 2018). Another organism that was abundant in the biofilm samples is *Nitrospira*, which is widespread in both natural and engineered niches such as freshwater, soils, groundwater, springs, and wastewater treatment plants. This genus plays pivotal roles in nitrification as an aerobic chemolithoautotrophic nitrite-oxidizing bacterium and its presence in premise plumbing systems may be correlated to residual nitrogenous compounds present in drinking water (Sorokin et al. 2012; Wang et al. 2014b; Daims & Wagner 2018). From the samples in this study, this organism's relative abundances were seen within the range of up to 0.25% (KA1). The low abundances of these organisms may be associated with the water temperature where the samples are collected. This was evident from the abovementioned organisms having lower relative percentage abundances from the heated sources (e.g., shower heads, pipe connected to water heater) as compared to the non-heated source following a similar trend with other organisms (e.g., *Legionella*) in previous studies (Kusnetsov et al. 2003; Buse et al. 2017). Most microorganisms in water distributions systems are found to be mesophilic and do not grow optimally at temperatures higher than 37° and their sensitivity to higher temperatures might have affected their abundance in the biofilm samples (Zacheus & Martikainen 1995). In this study, *Sphingomonas* remained the most abundant genus from the heated sources

followed by *Methylobacterium* and *Mycobacterium*, while *Nitrospira* had the least relative abundance at high-temperature water sources.

Permutational analysis of variance (PERMANOVA) of both weighted and unweighted distance matrices of the samples per categories showed no significant difference when the communities are grouped by pipe material and water temperature. However, it revealed that microbial communities grouped by age of properties, location, and type of house were significantly different (p -values <0.05). Since the water that are pumped to residences come from different water treatment facilities with their own raw water influents from several reservoirs across the country, it is possible that the source and the processes involved before the water gets to premise plumbing systems affect the communities that could eventually colonize premise plumbing pipes. With regard to the age of the property and house types (e.g., government-owned housing, condominium, and landed house), microbial communities of premise plumbing systems could be governed by the factor of pipe ages. The differences in how piping systems at home are managed – frequency of cleaning, replacement, renovation, etc, are some of the factors that are not considered in this study and which might have affected the results. Further studies with more samples from various locations, house type (landed, government-owned flats, condominiums), age of property should be used for a more comprehensive analysis on how microbial communities could be affected by the conditions where the biofilm samples are retrieved. The quality of the water being distributed, and other governing factors such as disinfectant used, point of use treatment systems, water retention, and source of water should also be addressed.

Detection of opportunistic premise plumbing pathogens from biofilm samples

From the 520,000 reads that were quality filtered from the paired-end sequences, only about 2,200 reads that were annotated at threshold of 99% sequence similarity with the human pathogenic bacteria 16S rRNA database are from different genera containing pathogenic species and these reads were processed for statistical significance using Microbiome Analyst (Dhariwal et al. 2017). The OTUs from the

pathogenic bacteria and their relative abundance categorized based on location (Figure 4), house type, pipe material, water temperature, and age of household are shown in Supplementary Information Figures S2-S5. The Shannon diversity indices (alpha diversity) of the samples show that of the groups, the only significant one was the house type (public housing known as HDBs, private condominiums, and landed homes) since plumbing systems at each of these house types vary and are handled by different management. Another possibility for the difference in the alpha diversity seen in Figure 4 may be due to the samples collected from the condominium which are relatively new as compared to the ones from landed houses and public housing. Further investigation on the diversity of opportunistic pathogens with a wider sampling coverage is necessary to better understand the influence of house types on these organisms.

Several genera including the most common ones like *Enterococcus*, *Escherichia*, *Mycobacterium*, and *Pseudomonas* were identified from the stringent data analysis. Of these organisms, it was observed that the genus *Mycobacteria* (~2,000 reads) is the most predominant of the OPPPs from

the biofilm samples in all groups except for those from condominium, where *Citrobacter* species dominated. *Mycobacterium* is an aerobic, acid-fast actinomycete that forms slightly curved or nonmotile rod morphologies. This genus has species that are obligate pathogens of humans and animals and the most medically-important species are found in the environment – both aquatic and terrestrial (Saviola & Bishai 2006). Of all organisms identified, only *Mycobacteria* had more than 1,000 reads and its ubiquity and dominance in all samples explains the survivability of *mycobacteria* in biofilm conditions. Mycobacterial biofilms have been shown to survive extreme starvation, resistance to antibiotics, and may also play a role in the pathogenesis of some of its species (Ojha et al. 2005). *Mycobacterium avium*, an OPPPs, is the most abundant species in all potentially pathogenic organisms found in the biofilm samples and the most prevalent *Mycobacterium* in drinking water (Falkinham 2011). *Mycobacterium avium* infections have been observed to originate from environmental sources and are among the nontuberculosis (NTM) mycobacteria. Several reports have linked infections caused by this organism with exposure to contaminated drinking water via

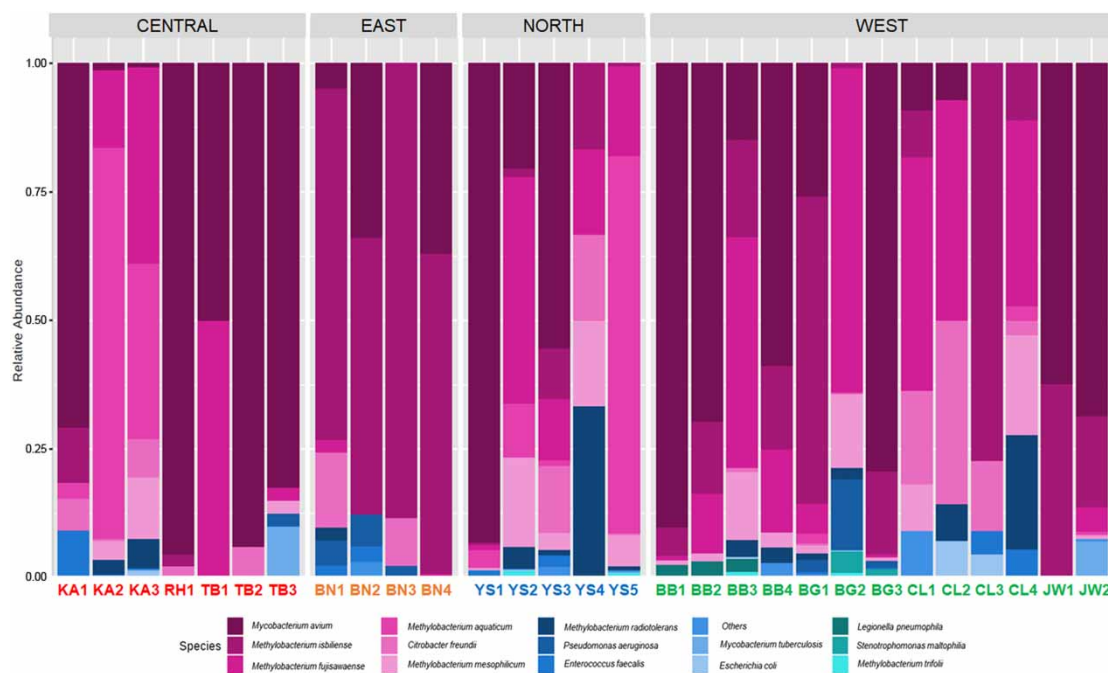


Figure 4 | Relative abundance of genera with potentially pathogenic species grouped by location. Horizontal axis shows the different samples namely KA-Kampong Arang; RH-Redhill; TB-Telok Blangah; BN-Bedok North; YS-Yishun; BB-Bukit Batok; BG-Bukit Gombak; CL-Clementi; and JW-Jurong West.

molecular typing methods including PCR and sequencing (Tobin-D'Angelo et al. 2004; Falkinham et al. 2008). Relative abundances of this organism showed the highest abundance in both the central and north region followed by the west and east regions of Singapore. Meanwhile, *M. avium* showed higher abundances in biofilms collected from non-heated water samples, PVC, the oldest sampling site, and samples from public housing (Supplementary Material S2: house type, S3: pipe material, S4: temperature, and S5: age of household.)

Following *Mycobacteria*, in terms of abundance, are *Pseudomonas* and *Citrobacter* with reads that are greater than 100 reads. *Pseudomonas*, a Gram-negative organism, is known for its members having great metabolic diversity and the ability to colonize a wide range of niches. *Pseudomonas aeruginosa* is one of the leading etiologic agents in nosocomial infections in hospitals and has been observed to be transmitted via water and aerosols, aspiration, indirect transfer from moist environmental surfaces among others (Falkinham et al. 2015). There are no reports, however, that have associated *Pseudomonas* infections from households other than the increased health risk in humans with emphasis on immunocompromised individuals (Völker et al. 2010). In this study, *Pseudomonas* reads were seen mostly from metal pipe material, landed houses, residential houses in the east and west of Singapore, and in heated sources. The occurrence of *Pseudomonas* reads in heated sources in this study is supported by the fact that higher temperatures (<40 °C) tend to be more favourable for bacterial growth. A previous study has reported an increased percentage of this group in warm water than cold water samples (Völker et al. 2010). Meanwhile, since there are no published studies on the microbial community that may be found in water distribution networks in the east and west areas of Singapore (Figure 4), the link between their increased prevalence in these areas cannot be supported by any previous data and therefore needs further investigation. Of the OPPPs, the *Legionella pneumophila* is the least abundant with mean abundance of 0.002%. This organism is the causative agent of a life-threatening pneumonia (Yoder et al. 2008). The presence of this organism in water distribution networks and buildings does not necessarily indicate poor maintenance but rather just their ubiquitous presence totalling 50% in building water systems and about 30% in home water

systems in the United States (Kool et al. 1999). This organism was only found in some of the public housing (Figure S2) and OTU reads in metal (Figure S3), normal water source (Figure S4), and the west region of Singapore (Figure 4).

Citrobacter is a Gram-negative, non-spore-forming facultatively anaerobic genus and is found to be an opportunistic human pathogen which inhabits intestines of animals leading to its presence in soils, water, and sewage as a consequence of fecal shedding (Borenshtein & Schauer 2006). *Citrobacter* was found to be predominant in central and east Singapore, the lone condominium sample, metal pipe material, and biofilm samples collected from sources without a heating mechanism. A study on the effects of different incubation conditions on *Citrobacter werkmanii* biofilm formation revealed that the ability of the organism to form biofilm growth at 37 °C decreased compared to when it was grown at 30 °C (Zhou et al. 2013). Since the heated samples probably had water temperatures more than 40 °C, *Citrobacter* spp. is not expected to dominate in these samples whereas in samples without a heating mechanism, growth of this organism may be supported. While the more common opportunistic pathogenic *Enterococcus* and *Escherichia*, despite their presence in most residential samples, are not the most abundant species in the biofilm samples. *Enterococcus*, *Escherichia*, and *Citrobacter* are usually found in fecal matter and are under the coliform group which are usually common in the aquatic and soil environments and in some cases could persist in water distribution networks (Wingender & Flemming 2011).

The presence of OPPPs in the biofilm samples of premise plumbing systems only accounts for less than 0.5% of the total microbial communities found in the analysis and may be deemed insignificant in terms of public health concerns. However, their prevalence from the samples collected indicates potential health risks of immunocompromised individuals, the very young and the elderly. Also, further studies of microbial ecology and potential regrowth of OPPPs in a premise plumbing pipe system are necessary for better understanding of their ecological roles and potential health risks. Particularly, the relationship between the most frequently identified taxa and the healthy conditions of the residents shall be investigated to develop water treatment strategies and maintenance practices for a premise plumbing pipe system. It is important to note, however,

that the viability of these organisms was not taken into consideration in this study, but only their abundance from extracted DNA from the samples. Monitoring the viability of these opportunistic pathogens and their quantities in biofilm samples collected from the residential areas would be useful for the formulation of risk assessment frameworks that are associated with the presence of these organisms which is considered a health hazard for the public. Furthermore, quantitative PCR (qPCR) using pathogen-specific primers was not performed in this study due to the low-DNA yield from the collected biofilm samples. The authors acknowledge the need to verify the presence of these pathogenic organisms up to the species level using more accurate detection methods to confirm the preliminary results we showed in this study. Culture-dependent methods or quantitative PCR assays with propidium monoazide or DyeTox13 Green C-2 azide could be used as a confirmatory test for the viability of the pathogenic organisms in the premise plumbing systems (Lee & Bae 2018). Amplicon sequencing of 16S rRNA genes was used in this study as an initial survey to know the composition of microbial communities in premise plumbing systems in a tropical region and how this may be influenced by both intrinsic and extrinsic factors.

Functional profiling of microorganisms from residential plumbing system biofilms

Despite the seemingly negative effects of biofilm on human health, the opportunistic pathogenic microorganisms are the minority of the bacterial population in a premise's plumbing system. In this study, we also explored potential functions of the communities from the biofilm via predictive functional profiling using 16S rRNA markers. The top 10 pathways included bacterial transport, secretion systems, proteins involved in motility, and DNA-related processes (e.g. purine metabolism and DNA repair) and their respective gene counts as shown in Supplementary Figure S6. Selected pathways on xenobiotic biodegradation and metabolism, nitrogen metabolism, and bacterial secretion system are also shown in Figure 5 (location of sample) and S6-S9 (age of property, pipe material, house type, and temperature).

Although advanced and stringent physicochemical processes are being used in drinking water treatment plant,

the presence of pharmaceuticals and personal care products in water resources has been reported in previous studies (Webb *et al.* 2003; WHO 2012; Simazaki *et al.* 2015). In this study, the xenobiotic degradation pathway (Figure S8) is significantly different among the groups being compared when the gene counts are grouped per location (p -value <0.05). The xenobiotics biodegradation and metabolism pathway involve processes such as polycyclic aromatic hydrocarbon degradation, toluene degradation, benzoate degradation, and many other maps showing how different compounds are degraded biologically (Kanehisa *et al.* 2016). Studies on the ability of biofilms to degrade complex organic compounds in different environmental matrices have already been shown, however, at trace level concentrations, this ability has not been fully proved yet (Pieper *et al.* 2010; Edwards & Kjellerup 2013). It is surprising that despite our knowledge on the presence of residual xenobiotics and biofilms growing in distribution systems, studies tackling the influence of xenobiotics to biofilm growth and the effects of biofilms on the fate of these organic compounds in premises' plumbing systems have not been delved into. The role of biofilms in the premise plumbing system and their interaction with organic compounds such as xenobiotics should be examined to understand the effect of biofilm on water quality.

Meanwhile, the nitrogen metabolism pathway has also showed a significant difference in the location of samples from the east, west, north, and central Singapore (p -value <0.05). However, when grouped according to the age of property, the pipe material, the temperature and the house type, the results are not statistically different (Figure S9). Nitrogen metabolism is a complex biological process where an interplay of various microorganisms leads to the nitrogen cycle from the fixation of nitrogen to ammonia to the evolution of nitrogen gas via denitrification. The presence of microorganisms in the biofilms capable of performing nitrification/denitrification in a nutrient-limited environment show the adaptability of these organisms to available nutrients which influences their choice of niche (Martens-Habbena *et al.* 2009; Kits *et al.* 2017). Under nutrient-limited environments, biofilms growing in plumbing systems will mainly rely on available organic and inorganic nutrient residues from drinking water distribution systems. Particularly, ammonia produced from the decomposition

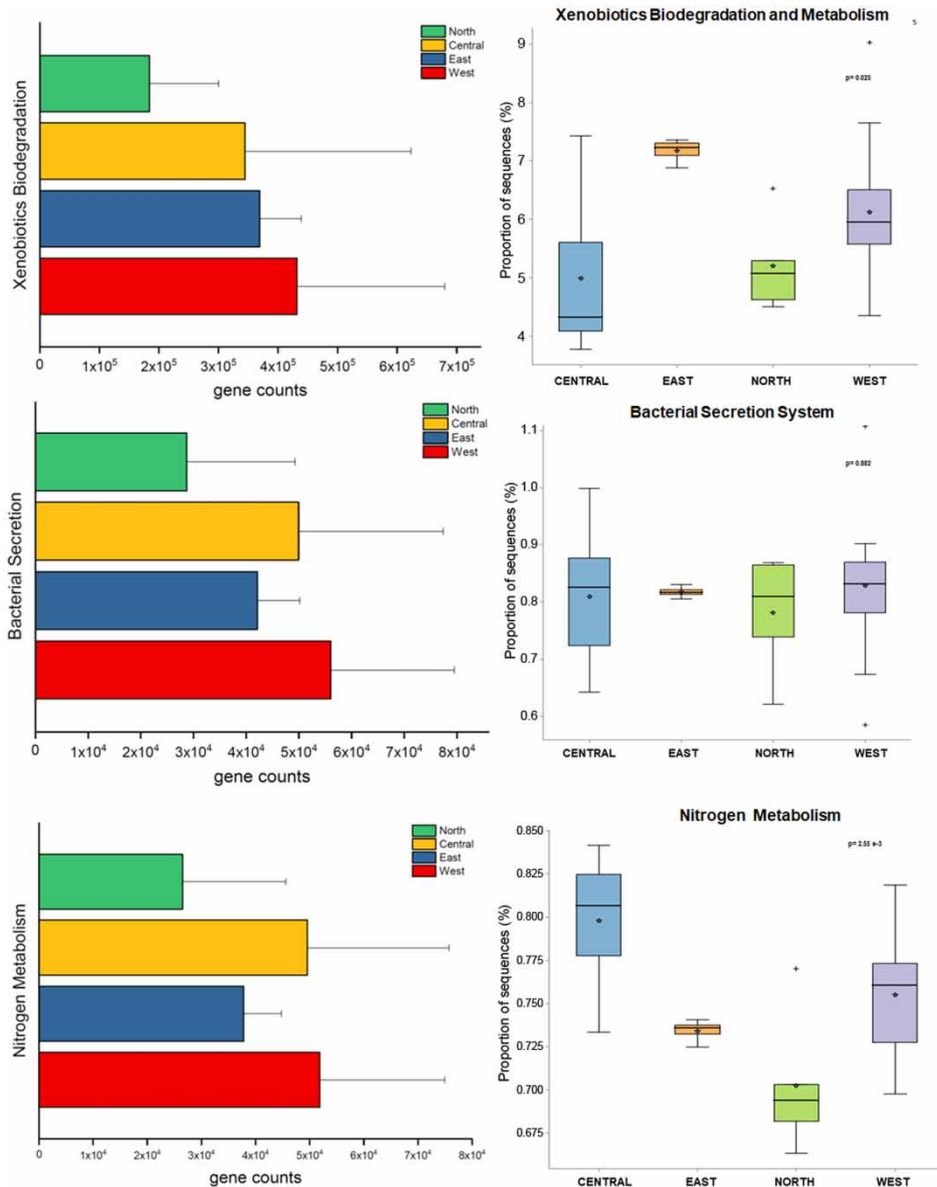


Figure 5 | Counts and sequence proportion of genes involved in xenobiotics biodegradation, bacterial secretion and nitrogen metabolism pathways from the microbial communities in the biofilm samples.

of chloramine leads to nitrification illustrated in previous studies (Sawade *et al.* 2016; Wahman *et al.* 2016). In this case, decay of residual disinfectant might be associated with regrowth of microorganisms and biofilm formation in the premise plumbing system. Despite the importance of nitrogen cycle related, limited studies have been done, so far, on the influence of biofilms growing in residential premise plumbing systems. The relationship between the residual disinfectant concentration and nitrification in the

premise plumbing system should be further investigated to minimize regrowth of unwanted microorganisms.

Contrary to xenobiotic degradation and nitrogen metabolism pathways, the bacterial secretion pathway shows statistically insignificant (p values > 0.05) gene counts in all groupings (Figure S7). The bacterial secretion system is responsible in a wide range of functions including pili and flagella formation, nutrient acquisition, virulence in Gram-negative organisms which is predominantly found in the

collected biofilm samples (Kanehisa *et al.* 2016). Flagellar synthesis and nutrient acquisition are important in biofilms in an oligotrophic environment for biofilm formation and survival, respectively. This may explain the lack of significant change in the gene counts of this pathway because of ubiquitous biofilm in the premise plumbing system. These results only show the potential function of biofilm from the 16S gene markers (OTUs) to generate the predicted gene counts involved in the abovementioned pathways but not the activity of the community. It will be worthwhile to have further research on the role of biofilms and test the hypothesis of the probiotic approach to control microorganisms in premise plumbing systems.

CONCLUSIONS

This study has shown the presence of various microbial communities, their metabolic potential, and potential opportunistic pathogens in the biofilm samples collected from residential areas Singapore. We illustrated that factors such as type of housing, age of property, sampling location, temperature, pipe material may affect the composition of microbial communities and the functional potential of the biofilms thriving in the premise plumbing systems. With regard to opportunistic premise plumbing pathogens (OPPPs), the lack of evidence correlating prevalence of infection and their presence in residential plumbing systems calls for further research on the health risks associated with their occurrence. Furthermore, since the viability of these organisms was not investigated in this study, culture-dependent novel molecular methods such as PMA-PCR or DyeTox13-PCR should be employed in future studies that aim to address any public health-related concerns. Meanwhile, functional profiling of the biofilm communities presented interesting pathways related to membrane transport and DNA regulation and functions like xenobiotics degradation and nitrogen metabolism. The relative abundances of organisms harbouring these traits are significantly higher than the OPPPs which account for only 0.5% of the total sequencing reads. Since biofilms are ubiquitous in premise plumbing systems, it is an imperative to control microbiome to have beneficial effects on both water treatment and residential plumbing systems, while

minimizing regrowth of potential pathogens. The identification of diverse microbial communities from biofilm samples in residential plumbing systems emphasizes the need for further studies that would tackle public health risks from OPPPs as well as the role of biofilms in degrading residual xenobiotic compounds in this kind of environment.

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SUPPLEMENTARY MATERIAL

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