Title

Evaluation of the microbial community structure of potable water samples from occupied and unoccupied buildings using16S rRNA amplicon sequencing

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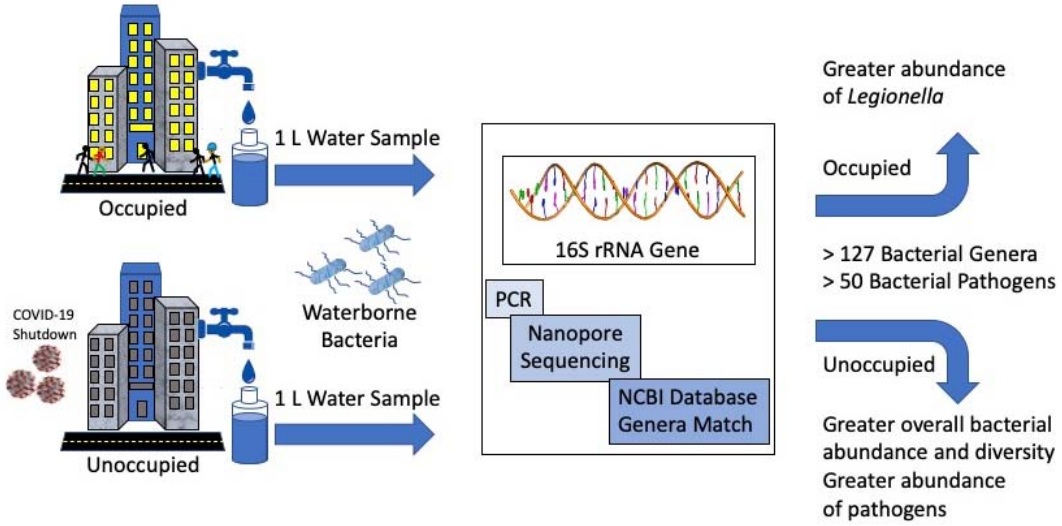
Synopsis

Comparison of microbial community composition in the plumbing of occupied and unoccupied buildings during the COVID-19 pandemic shutdown.



Abstract

We conducted two studies of water samples from buildings with normal occupancy and water usage compared to water from buildings that were unoccupied with little or no water usage due to the COVID-19 shutdown. Study 1 had 52 water samples obtained *ad hoc* from buildings in four metropolitan locations in different states in the US and a range of building types. Study 2 had 36 water samples obtained from two buildings in one metropolitan location with matched water sample types. One of the buildings had been continuously occupied, and the other substantially vacant for approximately 3 months. All water samples were analyzed using 16S rRNA amplicon sequencing with a MinION from Oxford Nanopore Technologies. More than 127 genera of bacteria were identified, including genera with members that are known to include more than 50 putative frank and opportunistic pathogens. While specific results varied among sample locations, 16S rRNA amplicon abundance and the diversity of bacteria were higher in water samples from unoccupied buildings than normally occupied buildings as was the abundance of sequenced amplicons of genera known to include pathogenic bacterial members. In both studies *Legionella* amplicon abundance was relatively small compared to the abundance of the other bacteria in the samples. Indeed, when present, the relative abundance of *Legionella* amplicons was lower in samples from unoccupied buildings. *Legionella* did not predominate in any of the water samples and were found, on average, in 9.6% of samples in Study 1 and 8.3% of samples in Study 2.

Keywords

Legionella, waterborne pathogens, stagnant water, building plumbing systems, 16S rRNA gene, nanopore sequencing

Introduction

Stagnation of water in building plumbing systems can result in deterioration of water quality and proliferation of pathogens causing a major public health concern [1]. Stagnation can also result in low or undetectable levels of residual disinfectant, such as chlorine [2]. During the COVID-19 pandemic, the United States has experienced building shutdowns on a scale and duration not previously experienced. This disuse may present a risk to public health from pathogenic microbes that can grow in building plumbing systems [2,3]. Numerous recently-issued guidance documents address the potential adverse effect on the quality of water that has stagnated in premise

plumbing systems due to reduced occupancy during the current COVID-19 pandemic [2,4–6]. Much of this guidance centers on *Legionella*, a plumbing-associated waterborne pathogen.

Legionella are found throughout the natural aquatic environment [7]. *Legionella* growth in premise plumbing systems is supported by certain physical-chemical conditions that are associated with stagnant water: accumulated sediment, tepid temperatures, excessive water age and absence of residual disinfectant. Many of the recent guidance documents addressing re-occupancy of buildings after COVID-related closures regard *Legionella* as the predominant, if not the only pathogen of concern in stagnant water in under-occupied buildings; some assume that significant *Legionella* contamination is probable if not inevitable, and some suggest that testing only for *Legionella* is sufficient to determine whether or not a building is safe to re-occupy. Implementation of the recommendations in these guidance documents has important financial and public health implications. At the most fundamental level, we believe that the rationale for focusing on *Legionella* merits evidence-based scrutiny. One approach to this scrutiny is to characterize the microbial community structures of potable water from building plumbing systems, in order to identify bacterial genera with at least some members that are known to be pathogenic.

Marker gene amplicon sequencing is commonly used to assess microbial communities and has a nearly 40-year history [8]. Molecular methods have distinct advantages over culturing and isolation; the routine isolation of all pathogens from water samples is impractical and cannot accurately characterize the microbial diversity in natural environments [9,10]. Interestingly, despite public health relevance of potable water, very little information is available on metagenomic analyses of potable water systems [11]. The 16S rRNA gene is a suitable choice for characterizing microbial community structures [8] and when combined with Next Generation Sequencing (NGS) methods, such as the Oxford Nanopore Technologies MinION™, can provide rapid and cost effective results.

The use of amplicon sequencing to assess microbial community composition and diversity is limited to identification at the genus level. In contrast to qPCR, amplicon sequencing does not broadly provide resolution to the species level. Rather, it provides a powerful means of initial screening for genera known to have at least some pathogenic members and can help elucidate the changes in and differences between microbial communities in potable water from different sources, such as occupied vs. unoccupied buildings. Studies like this one using 16S rRNA gene

sequencing of potable water can provide useful and important insights that provide evidence-based observations to inform further, targeted testing necessary for the safe reopening of buildings after prolonged unoccupancy.

In this report we present the results of two studies (Study 1 and Study 2) of microbial communities in water from buildings with normal occupancy and water usage compared to water from buildings that were unoccupied with little or no water usage due to the COVID-19 shutdown. Study 1 had 52 water samples obtained from buildings in four metropolitan locations in different states in the US and a range of building types. Study 2 had 36 water samples obtained from two buildings in one metropolitan location with matched water sample types. All water samples were analyzed using 16S rRNA amplicon sequencing.

Experimental Methods

For Studies 1 and 2 we collected a total of 88 1-liter water samples for analysis and comparison. Samples were taken from (a) buildings with low or no occupancy ("unoccupied") for which flushing had not been implemented and (b) buildings with normal, uninterrupted occupancy ("occupied").

For Study 1 we collected water samples from 52 buildings across 4 states— Michigan, Nevada, New York and Texas. Types of buildings sampled included residential condominiums, office buildings, hotels and dormitories. 1-L bulk water samples were collected from multiple locations in each building sampled. The number of samples and sample type—e.g., hot water or cold water, proximal or distal—varied from building to building.

For Study 2 we collected 36 water samples from two closely situated, very similar dormitory buildings on a college campus; one was occupied and the other unoccupied. Water sampling locations from the two buildings were similar; samples were taken from the main water supply trunks, hot water supply, and from locations distributed spatially throughout the buildings. The number, type and location of samples were matched as closely as possible.

Samples were shipped overnight in coolers to help maintain stable temperature. Sample processing, initiated within 24 hours of receipt, was as follows:

- <u>Concentration</u>

Samples were concentrated through a membrane ultrafilter (*Filpath*™ Ultrafilter, Nephros, Inc., South Orange, NJ, USA)

- Lysis

Sample concentrate remaining on the filter was treated with a lysis buffer solution (Environmental Lysis Solution, Chai, Inc., Santa Clara, CA) and then removed.

- Amplification

Lysate samples were added directly to PCR reaction mixes for amplifying the 16S rRNA genes. Amplification of 16S rRNA genes was conducted using the 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK) containing the 27F/1492R primer set, LongAmp™ Taq 2x Master Mix (New England Biolabs, Ipswich, MA, USA), and Chai Green Dye 20x (Chai, Inc., Santa Clara, CA, USA). Amplification was performed using a Chai Open™ qPCR thermal cycler (Chai, Inc., Santa Clara, CA, USA) with the following PCR conditions: initial denaturation at 95 °C for 30 s, 40 cycles of 95°C for 30 s, 53°C for 30 s, and 65°C for 2 min.

- Library preparation

PCR products were purified using AMPure XP (Beckman Coulter, Indianapolis, IN, USA), two 70% alcohol washes, and quantified by a NanoDrop (Thermo Fischer Scientific, USA). Purified amplicons were pooled (up to 12) and a total of 100 ng DNA was used for library preparation.

- Amplicon sequencing

MinION™ sequencing was performed using R9.4 flow cells (FLO-MIN106; Oxford Nanopore Technologies) according to the manufacturer's instructions. MINKNOW software ver. 19.12.5 (Oxford Nanopore Technologies) was used for data acquisition.

- Bioinformatic analysis

The EPI2ME™ platform 16S Workflow (Metrichor, Ltd., a subsidiary of Oxford Nanopore Technology) was used to classify sequencing reads and perform bacterial genera identification.

- Estimates of bacterial concentrations

Estimates of bacterial concentrations (as CFU/mL) were based on Cq values for each 1-liter water sample by (a) estimating the starting 16S rRNA target DNA copies present in the PCR reaction volume, and then performing a

series of back-calculations estimating the number of CFU per milliliter of bacteria present in each of the 1-liter samples of water concentrated on the filters, assuming one copy of the template target per cell.

Results and Discussion

Table 1 and 2 provide water sample information collected for Study 1 and 2, respectively. The measured DNA concentration (Study 1 only) was measured using the lysate (post- filter concentration and lysis) using a Thermo-Fisher NanoDrop™ 1000. This DNA concentration is all-source (prokaryotic and eukaryotic) in contrast to the 16S rRNA gene sequence abundance which is representative of bacterial DNA presence in the samples.

Table 1 Study 1 Sample Information

Sample #	**Unoccupied/ Occupied**	**State**	**Date Collected**	**Location**	**Description**	**Temp**	**Chlorine ppm**	**DNA ng/ul**	**16S rRNA Gene Amplicon Sequence Abundance (Reads)**
1	Occupied	NY	5/6/20	Bath shower - hot	First draw	110 deg F		6.9	4
2	Occupied	NY	5/6/20	Tub	First draw	110 deg F		24.5	140
3	Occupied	NY	5/6/20	Bath sink - cold	First draw	<60 deg F		5.3	7
4	Occupied	NY	5/6/20	Kitchen sink - Hot	First draw	115 deg F		6.4	17
5	Unoccupied	NV	5/7/20	Top floor tower		Room temp		8.0	97,584
6	Unoccupied	NV	5/7/20	Ground floor sink	First out	Room temp		6.8	54,076
7	Unoccupied	NV	5/7/20	Ground floor sink		Room temp		8.6	52,260
8	Unoccupied	NV	5/7/20	Top floor 7th		Room temp		13.2	154,259
9	Occupied	NV	5/12/20	Mens room	5 story	Room		4.7	63,013

					building	temp			
10	Occupied	NV	5/12/20			Room temp		3.9	14
11	Occupied	NV	2/12/20			Room temp		7.9	92,421
12	Unoccupied	NY	5/13/20	Kitchen sink	36th floor			27.7	671
13	Unoccupied	NY	5/13/20	Kitchen sink	45th floor			14.2	34
14	Unoccupied	NY	5/13/20	Kitchen sink	36th floor			26.6	11,450
15	Unoccupied	NY	5/13/20	Kitchen sink	45th floor			16.1	108,430
16	Unoccupied	NY	5/12/20	Bath sink - cold	Unit 4A	60 deg F		11.9	12
17	Unoccupied	NY	5/12/20	Kitchen sink - Hot	Unit 4E	70 deg F		13.9	6
18	Unoccupied	NY	5/12/20	Shower/tub - hot	Unit 9J	95 deg F		14.2	26
19	Unoccupied	NY	5/12/20	Porter's closet - cold	10th floor	65 deg F		6.4	250,316
20	Unoccupied	NY	5/12/20	Bath sink - hot	4th floor	70 deg F		84.4	20
21	Unoccupied	MI	5/11/20	Drinking fountain	24th floor			6.5	8,686
22	Unoccupied	MI	5/11/20	Drinking fountain	Basement			3.3	447
23	Unoccupied	MI	5/11/20	Drinking fountain	24th floor			5.6	17,151
24	Occupied	MI	5/8/20	Kitchen sink	1st floor	57 deg F		2.3	1,274
25	Occupied	MI	5/8/20	Kitchen sink	1st floor	57 deg F		4.4	558
26	Unoccupied	MI	5/11/20	Drinking fountain	Basement			4.4	32,568
27	Occupied	NY	5/6/20	Kitchen sink	First draw	70 deg F		7.5	3
28	Occupied	NY	5/6/20	Bath sink - hot	First draw	120 deg F		13.3	5
29	Unoccupied	TX	5/15/20	Manual sink - hot Distilled	First floor	110 deg F	0.52	3.2	1,763
30	Occupied	TX	5/15/20	Manual faucet - cold		76 deg F	0.22	5.3	220,957
31	Unoccupied	TX	5/15/20	Shower - cold		79 deg F	0.20	3.7	619
32	Unoccupied	TX	5/15/20		First floor	74 deg F	1.22	5.0	1,360
33	Occupied	TX	5/15/20	Manual faucet	2nd floor	77 deg F	0.29	3.5	64,188

				sink					
34	Occupied	TX	5/15/20	Manual faucet	First floor	75 deg F	0.26	3.1	122
35	Unoccupied	TX	5/15/20	Manual sink - hot proximal	6th floor	112 deg F	0.72	2.7	43
36	Unoccupied	TX	5/15/20	Sink manual	5th floor	73 deg F	1.12	4.6	181
37	Unoccupied	TX	5/15/20	Sink manual - hot proximal	6th floor	111 deg F	0.78	2.5	33
38	Unoccupied	TX	5/15/20	Sink - cold	6th floor	73 deg F	1.21	4.1	64
39	Occupied	TX	5/15/20	sink proximal - hot		114 deg F	0.14	2.4	177,696
40	Occupied	TX	5/15/20	Sink - cold	2nd floor NW corner	77 deg F	0.28	3.0	844
41	Unoccupied	TX	5/15/20	Manual sink - cold ambient	5th floor	74 deg F	1.12	4.6	4
42	Unoccupied	TX	5/15/20	Manual sink	First floor	76 deg F	1.48	3.4	995
43	Unoccupied	TX	5/15/20	Manual sink - hot Distilled	First floor	112 deg F	0.68	3.1	11,604
44	Occupied	TX	5/15/20	Dynamic flow sink - cold		76 deg F	0.24	3.3	586
45	Unoccupied	TX	5/15/20	Manual sink - cold	6th floor	72 deg F	1.32	4.1	304,833
46	Occupied	TX	5/15/20	cold	First floor	76 deg F	0.27	3.1	20,092
47	Occupied	TX	5/15/20	Sink Auto Faucet - hot	First floor	114 deg F	0.13	2.7	5,513
48	Occupied	TX	5/15/20	cold	First floor	78 deg F	0.25	17.2	27
49	Unoccupied	TX	5/15/20	Manual sink - cold dynamic	First floor	74 deg F	1.29	5.5	4,446
50	Occupied	TX	5/15/20			138 deg F	0.14	4.6	2,538
51	Unoccupied	TX	5/15/20	Dynamic	First floor	70 deg F	0.20	4.9	1,526
52	Occupied	TX	5/15/20	Distilled		138 deg F	0.13	3.6	7

Table 2 Study 2 Sample Information

Sample #	Unoccupied/ Occupied	State	Date	Location	Description	Temp	Amplicon Abundance
1	Unoccupied	NY	5/27/20	Boiler Rm	CW@main - hose bib outside	57 deg F	26
2	Unoccupied	NY	5/27/20		HW supply from tank - first draw	124 deg F	62
3	Unoccupied	NY	5/27/20		Cold makeup for boiler	76 deg F	118,250
4	Unoccupied	NY	5/27/20		HW supply flushed draw	124 deg F	203
5	Unoccupied	NY	5/27/20	Suite 1116-1117	Shower head hotside - first draw	102 deg F	40,613
6	Unoccupied	NY	5/27/20	Suite 1116-1117	Shower head hotside - flushed draw	124 deg F	11
7	Unoccupied	NY	5/27/20	Suite 1140-1141	Shower head - flushed draw	122 deg F	218
8	Unoccupied	NY	5/27/20	Suite 1133-1134	Shower head - first draw	122 deg F	170
9	Unoccupied	NY	5/27/20	Suite 705-706	Shower head hotside - first draw	117 deg F	28
10	Unoccupied	NY	5/27/20	Room 735	Shower head hotside - first draw	122 deg F	59
11	Unoccupied	NY	5/27/20	Room 735	Shower head hotside - flushed draw	122 deg F	188
12	Unoccupied	NY	5/27/20	Suite 722-723	Shower head hotside - first draw	70 deg F	711
13	Unoccupied	NY	5/27/20	Suite 311-312	Shower head cold - first draw	73 deg F	0
14	Unoccupied	NY	5/27/20	Suite 311-312	Shower head hotside - first draw	123 deg F	2
15	Unoccupied	NY	5/27/20	Suite 338-339	Shower head hotside - first draw	115 deg F	0
16	Unoccupied	NY	5/27/20	Suite 318-319	Shower head hotside - first draw	75 deg F	0
17	Unoccupied	NY	5/27/20	Suite 318-319	Shower head hotside - flushed draw	121 deg F	1

18	Unoccupied	NY	5/27/20	Suite 1140-1141	Shower head - first draw	121 deg F	58
19	Occupied	NY	5/27/20	City Main	First draw	57 deg F	2
20	Occupied	NY	5/27/20	City Main	1 minute flush	57 deg F	4
21	Occupied	NY	5/27/20	Boiler Rm	HW return top of tank - 10 second flush	130 deg F	374
22	Occupied	NY	5/27/20	Boiler Rm	HW supply Heater #2 - First draw	122 deg F	1,123
23	Occupied	NY	5/27/20	4K?	Shower head hotside - first draw	125 deg F	1
24	Occupied	NY	5/27/20	Apt 6F	Shower head hotside - first draw	125 deg F	1
25	Occupied	NY	5/27/20	Apt 6F	Shower head hotside - flushed draw	130 deg F	571
26	Occupied	NY	5/27/20	Apt 6O	Shower head hotside - first draw		23
27	Occupied	NY	5/27/20	Apt 6O	Shower head hotside - flushed draw	138 deg F	779
28	Occupied	NY	5/27/20	Apt 6J	Shower head hotside - first draw	123 deg F	32
29	Occupied	NY	5/27/20	Apt 4D	Shower head hotside - flushed draw	70 deg F	114
30	Occupied	NY	5/27/20	Unit 4B	Shower head hotside - first draw	98 deg F	2,195
31	Occupied	NY	5/27/20	4B	Shower head hotside - flushed draw	117 deg F	5
32	Occupied	NY	5/27/20	Unit 2F	Kitchen sink hot side - first draw	73 deg F	153,532
33	Occupied	NY	5/27/20	Unit 1E	Shower head hotside - first draw	78 deg F	3,166
34	Occupied	NY	5/27/20	Unit 1G	Shower head hotside - first draw	140 deg F	10
35	Occupied	NY	5/27/20	Unit 1H	Shower head - first draw	126 deg F	15
36	Occupied	NY	5/27/20	Unit 1H	Shower head - flushed draw	137 deg F	54

Bacterial proliferation in stagnant water of unoccupied buildings

In Study 1 we generated a total of 1,765,493 16S rRNA gene amplicon sequences from the 52 water samples. The average amplicon abundance of unoccupied building samples was more than twice that of samples from occupied buildings, with the ratio of 16S rRNA gene amplicon abundance (unoccupied:occupied) ranging 1.5:1 to more than 1400:1. The microbial communities, as indicated by bacterial genera, in unoccupied building samples appear to be more diverse—i.e., 82% more bacterial genera are present. Both unoccupied and occupied samples contain multiple bacterial genera; 10-80% of the identified bacteria genera are putative potential human pathogens, based on the Centers for Disease Control and Prevention (CDC) list of Opportunistic Pathogens of Premise Plumbing [12]. The

amplicon sequence abundance of pathogenic bacteria appears to be generally higher in unoccupied samples, but the percentage proportion of amplicon sequence of pathogenic bacteria appears to be generally lower. In Study 2 we generated a total of 322,601 16S rRNA gene amplicon sequences form the 36 water samples. The average amplicon abundance of unoccupied building samples was almost 1.5 times that of occupied samples, with the ratio of 16S rRNA gene amplicon abundance (unoccupied:occupied) 6:1. Overall the combined 88 samples yield a ratio of amplicon abundance of 1.6:1 and an average amplicon abundance ratio of 1.4:1 (see Table 3).

Table 3: Ratios of Total and Average Amplicon Sequences for All Water Samples Unoccpuied:Occupied

	Unoccupied	Occupied	Ratio of Unoccupied:Occupied
Total Abundance of 16S rRNA gene Amplicon Sequences for All Samples	1,275,448	812,646	1.57:1
Average Abundance of 16S rRNA gene Amplicon Sequences for All Samples	27,137	19,821	1.37:1

In both studies we used an intercalating dye in the PCR reaction when amplifying the 16S rRNA gene target and were only able to obtain a Cq value for 14 of the 52 samples in Study 1and 7 of the 36 samples in Study 2. For the 14 samples in Study 2 with a Cq value we calculated an estimate of the total bacterial bioburden, or the CFU/ml inclusive of all bacteria in the samples. The calculated CFU/ml values ranged from approximately 8 to approximately 2,000 CFU/ml. The average CFU/ml estimate for unoccupied building water samples in Study 1 was approximately 6 times higher than the average CFU/ml for occupied samples. From the 7 Cq values obtained in Study 2 the calculated CFU/ml values ranged from approximately 8 to approximately 1,280 CFU/ml. The number of occupied vs unoccupied samples with Cq values was not sufficient for a comparison. For those samples that did not yield a Cq value, we can only conclude that the bioburden was less than 2 CFU/ml in each of those samples.

<u>Bacterial pathogen proliferation in stagnant water of unoccupied buildings</u>

For simplicity, in both Studies 1 and 2, only the 12 most abundant bacterial genera in each sample were considered for further analysis and comparison. The cutoff of 12 was arbitrarily chosen.

The total abundance of 16S rRNA gene amplicon sequences of in Study 2 for the 12 most abundant bacterial genera was 7,411. Table 4 shows the comparison of gene amplicon sequence proportions for pathogenic genera and nonpathogenic genera in occupied and unoccupied building samples.

Table 4: Proportions of 16S rRNA gene amplicon sequences among pathogen and nonpathogen genera in occupied and unoccupied building samples

	Unoccupied	Occupied	Total
Pathogen	33%	2%	35%
Nonpathogen	64%	1%	65%
Total	97%	3%	100%

The shift observed here of relative dominate proportion from occupied to unoccupied between pathogen and nonpathogen is similarly seem in Study 1. While pathogens seem to predominate in proportion in occupied building water samples when compared to nonpathogen genera, this is inverted in unoccupied building water samples. This phenomenon would seem to indicate a complex microbial community dynamic that merits further examination. Additionally, the proportions of specific bacterial genera gene amplicon sequences show changes or shifts between occupied and unoccupied building water samples (see Figure 1) also indicative of a dynamic microbial community composition.

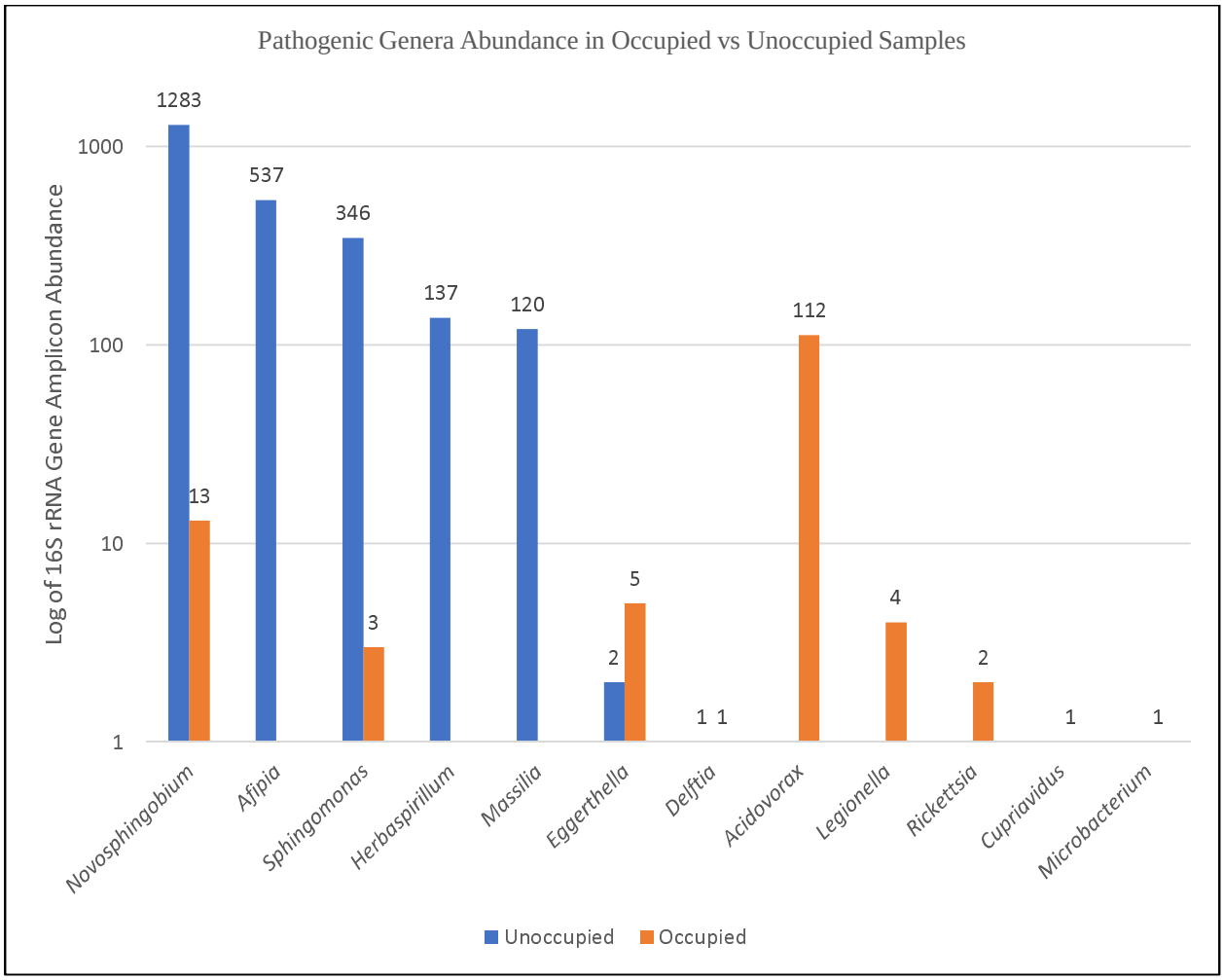


Figure 1: Log of putative pathogenic genera abundance in occupied vs unoccupied building water samples in Study 2 indicating a shift in microbial community composition.

<u>Presence and abundance of *Legionella* in the study samples</u>

Legionella sp. was detected in 9.6% of all samples, including in 16.1% of unoccupied building water samples and 4.7% of non-stagnant samples. In samples where *Legionella sp.* was detected, it was less abundant than 59% of other pathogenic bacteria genera detected.

Study 1 had 5 samples that contained *Legionella*. Figure 2 shows the 16S rRNA gene amplicon percent abundance for the putative pathogen genera in those water samples.

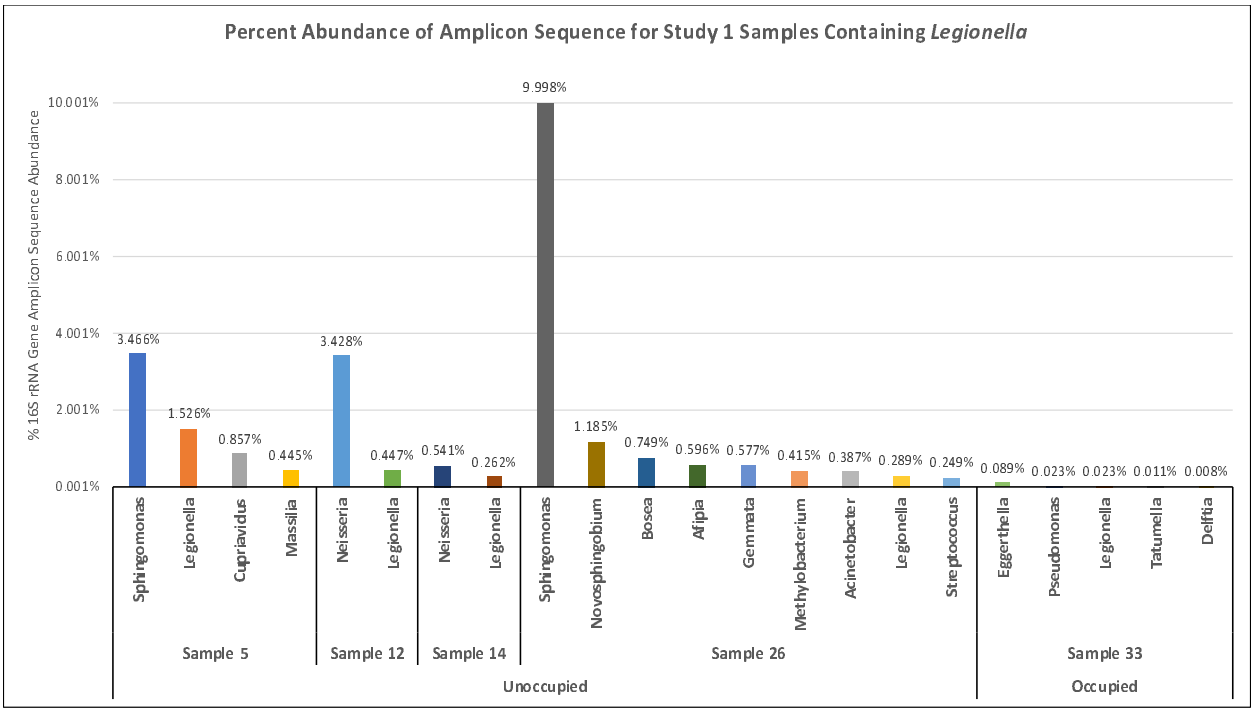


Figure 2: Percent abundance of 16S rRNA gene amplicon sequence for 5 samples from Study 1 (4 unoccupied water samples; 1 occupied water sample) which contained Legionella.

In Study 2, 3 samples contained *Legionella* at an exceedingly low level except one. A single occupied building water sample in Study 2 was taken from a currently unoccupied single living space that had a slow, constant leaking fixture (a kitchen sink faucet). This sample was removed from the further combined analyses for reasons given below but, presents a special case and deserves additional consideration. 16S rRNA gene amplicon sequencing of this sample resulted in the largest total number of reads for a single sample in the study (153,532; almost 50% of the grand total study-wide), and the second largest number of reads in the 12 most abundant genera selected for further analysis (81,513). This single sample accounted for almost 80,000 total putative pathogen sequenced amplicon, 320 of which were identified as *Legionella* (the largest abundance of *Legionella* of any sample in the Study by a factor of more than 100), and 2,333 nonpathogen reads.

Conclusions

The results of these studies should be considered in light of the caveats mentioned previously and with consideration of the limitations described below. Notwithstanding the caveats and limitations, we believe the results have

immediate practical implications with respect to the planning for re-occupancy of buildings following low occupancy due to COVID-19 measures (where water has stagnated in the pipes). Conventional wisdom and the scientific literature indicate that when potable water is allowed to dwell in building plumbing for a long period of time that stagnation will occur, and microorganisms will proliferate. It is likely that buildings that have been vacant or experienced low occupancy due to the recent or ongoing shutdowns will have greater levels of bacteria (including potential human pathogens) than expected under normal occupancy. Given the results presented in these studies water safety managers should not assume the water is contaminated with *Legionella* but rather that it likely harbors other pathogenic bacteria which present a public health hazard if exposed to or consumed by humans. Water testing only for *Legionella* is therefore insufficient and may provide false security against other significant health threats. If water samples are tested, they should be screened for multiple genera using technology similar to those described in these studies or equivalent, then tested for specific pathogens of concern based as indicated by the initial screen.

The results of these studies also underscore the complexity of microbial community dynamics that are resident in building premise plumbing systems. The sample taken in Study 2 from the leaky kitchen faucet also remind us that local effects – a constant, slow flow of nutrient with insufficient force to dislodge biofilms – can drastically impact the microbial community and ecosystem locally and lead to a potentially disastrous situation for someone consuming the water and variety of microbe. There are many questions yet to be addressed and answered utilizing evidence-based observations aided by current technologies.

<u>Study Limitations</u>

- The buildings in Study 1 were not graded by degree of water usage or length of reduced occupancy; in some cases, these details are not known. Assigned categories were binary—unoccupied *vs.* occupied—and did not always consider potentially significant differences in building type, usage patterns, quality/variability in the water supply to the building, or other factors that could have a profound effect on test results.
- Sample analysis was not performed in triplicate.
- The precision of estimates of bacterial concentration (as CFU/mL) based on qPCR is inherently limited. Comparisons made of estimated bacterial populations in stagnant *vs.* non-stagnant samples should be considered qualitative. Estimates of bacterial concentrations were made for only 14 of the 52 samples.

- Classification of the microbial community structure based on comparing targeted sequencing data to databases of 16S rRNA can yield bacterial identity to the level of genera—i.e, operation taxonomic units (OTU) or amplicon sequence variants (ASV)—but may not be sufficiently reliable in all cases for identification at the species level.
- Comparisons between marker gene amplified sequence reads can be biased and confounded, particularly when attempting to draw conclusions regarding environmental or ecological effects.

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Conflict of Interest Statement

Kimothy Smith is the Vice President of Pathogen Detection System for Nephros, Inc. Nephros, Inc. provided a portion of the funding for the studies referenced in this manuscript.

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