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RESEARCH ARTICLE

# Microbial diversities (16S and 18S rRNA gene pyrosequencing) and environmental pathogens within drinking water biofilms grown on the common premise plumbing materials unplasticized polyvinylchloride and copper

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## Abstract

Drinking water (DW) biofilm communities influence the survival of opportunistic pathogens, yet knowledge about the microbial composition of DW biofilms developed on common in-premise plumbing material is limited. Utilizing 16S and 18S rRNA gene pyrosequencing, this study characterized the microbial community structure within DW biofilms established on unplasticized polyvinyl chloride (uPVC) and copper (Cu) surfaces and the impact of introducing *Legionella pneumophila* (Lp) and *Acanthamoeba polyphaga*. Mature (> 1 year old) biofilms were developed before inoculation with sterilized DW (control, Con), Lp, or Lp and *A. polyphaga* (LpAp). Comparison of uPVC and Cu biofilms indicated significant differences between bacterial ($P = 0.001$) and eukaryotic ($P < 0.01$) members attributable to the unique presence of several family taxa: *Burkholderiaceae*, *Characeae*, *Epistylidae*, *Goniomonadaceae*, *Paramoebidae*, *Plasmodiophoridae*, *Plectidae*, *Sphenomonadidae*, and *Toxariaceae* within uPVC biofilms; and *Enterobacteriaceae*, *Erythrobacteraceae*, *Methylophilaceae*, *Acanthamoebidae*, and *Chlamydomonadaceae* within Cu biofilms. Introduction of Lp alone or with *A. polyphaga* had no effect on bacterial community profiles ($P > 0.05$) but did affect eukaryotic members (uPVC, $P < 0.01$; Cu, $P = 0.001$). Thus, established DW biofilms host complex communities that may vary based on substratum matrix and maintain consistent bacterial communities despite introduction of Lp, an environmental pathogen.

## Introduction

Biofilms are ubiquitous to drinking water (DW) distribution system surfaces and provide microenvironments for growth of opportunistic bacterial pathogens [reviewed in (Berry *et al.*, 2006)]. The conventional view is that the majority of DW microorganisms are released from biofilms (Ridgway & Olson, 1981; LeChevallier *et al.*, 1987) although bulk water microorganisms may predominate when there is a loss of chlorine residual (Srinivasan *et al.*, 2008). Somewhat more controversial is the impact of the inflowing microbiota on what members establish within DW biofilms (Pinto *et al.*, 2012) vs. the influence of conditions within a particular system (Wang *et al.*, 2013). Nonetheless, public health concerns stem from the increasing number of legionellosis cases in the United States (Hicks *et al.*, 2011) and the significant health burden arising from these *Legionella* and nontuberculous mycobacteria (Collier *et al.*, 2012). Studies evaluating microbial composition of DW biofilms and environmental pathogens have primarily focused on the initial establishment and maintenance of pathogens, such as *Legionella pneumophila* (Lp), within biofilms. Yet the occurrence of free-living amoeba (FLA) is also a health concern as they are reservoirs for Lp and other DW environmental pathogens (Harb *et al.*, 2000; Lau & Ashbolt, 2009) and have been co-isolated from Lp-contaminated DW samples, specifically the FLA *Acanthamoeba* spp. (Yamamoto *et al.*, 1992; Moore *et al.*, 2006) and *Vermamoeba* (*Hartmannella*) *vermiformis* (Thomas *et al.*, 2006;







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Smirnov *et al.*, 2011). Buse *et al.* showed that Lp colonized mature, DW biofilms grown on copper (Cu) more effectively and shed from those biofilms at a higher frequency and duration compared to unplasticized polyvinyl chloride (uPVC)-grown biofilms, suggesting that DW biofilm microorganisms may have an impact on the colonization of water-based pathogens (Buse *et al.*, 2014). Hence, identifying the taxonomic structure of these prokaryotic and eukaryotic microbial communities is crucial to understanding the ecological niches that are permissive to environmental pathogen development.

Microbial community analysis via 16S and 18S rRNA gene sequencing has provided insight into community structure within treated DW and biofilms collected from different points along the treatment process, distribution system, and in-premise plumbing. Specifically, microbial community composition has been assessed for bulk water isolated after granular activated carbon (GAC) treatment (Poitelon *et al.*, 2010), from finished water of chlorinated (Poitelon *et al.*, 2009, 2010; Otterholt & Charnock, 2011) and unchlorinated systems (Valster *et al.*, 2009; Lautenschlager *et al.*, 2013), from several points along the distribution system (Lautenschlager *et al.*, 2013), and from hospital (tinned steel and copper plumbing) (Felföldi *et al.*, 2010) and government (copper plumbing) (Buse *et al.*, 2013b) hot and cold water endpoints. The latter two studies identified amoeba-resisting bacteria (ARB) and putative nosocomial bacteria, *Acinetobacter lwoffi*, *Corynebacterium tuberculostrearicum*, *Escherichia albertii*, Lp, and *Pseudomonas aeruginosa*; and FLA, *Echinamoeba thermarmum*, *Vermamoeba* (*Hartmannella*) *vermiformis*, *Platyamoeba* sp., *Protacanthamoeba bohemica*, *Pseudoparamoeba pagei*, and *Vannella* sp. in the bulk water.

Several studies have assessed bacterial community diversity within mature, chlorinated DW biofilms established on PVC pipes from a 6-year-old water cooler (Silbaq, 2009) and 5-year-old kitchen drain outlets (McBain *et al.*, 2003), stainless steel (SS) plugs from a model DW distribution system from 1 to 1093 days postestablishment (Martiny *et al.*, 2003), galvanized iron pipe wafers from a model system from 1 to 84 days postestablishment (Lee *et al.*, 2005), and a finished concrete and steel water reservoir for 5 years (Zhang *et al.*, 2012). Interestingly, both *Sphingomonas* and *Pseudomonas* were identified within in the iron- and SS-derived biofilms, whereas *Sphingomonas*, but not *Pseudomonas*, was identified within the concrete and steel-derived biofilms and vice versa for PVC-derived biofilms, indicating possible correlations in co-occurrence of these two bacteria. Additionally, putative pathogens were only identified within PVC kitchen drain outlet-derived biofilms (*Aeromonas*, *Afipia*, *E. coli*, *Klebsiella*, and *Pseudomonas* were identified) and the concrete and steel-derived biofilms (*Bosea*, *Shigella*, and *Streptococcus*).

However, compared to 16S rRNA gene-based community analysis, little data are available on the eukaryotic diversity of DW biofilms, being largely limited to sand and GAC filters in DW treatment plants and for distal points along the DW distribution system (DWDS) (Thomas *et al.*, 2008; Corsaro *et al.*, 2010) where 18S rRNA gene sequencing was performed postamoebal enrichment with the DWDS samples and thus did not represent the entire eukaryotic community. Those studies did report the occurrence of ARB, *Chlamydiae* (with identification of a new *Parachlamydia acanthamoebae* strain recovered from GAC biofilm), *Legionellae*, and *Mycobacteria*, and the putative FLA hosts, *Acanthamoeba*, *Echinamoeba*, *Vermamoeba* (*Hartmannella*), and *Naegleria*.

Despite all the above work, in-depth characterization of these complex DW communities, as well as the tandem use of both 16S and 18S rRNA gene sequencing, is still a relatively unexplored area that may be alleviated with the use of next-generation sequencing technologies. The aim of this study was to determine the microbial diversity and relative abundance within DW biofilms that develop within buildings (in-premise plumbing), to better understand the putative ecological associations between environmental (water-based) pathogens, their *in situ* hosts, and other community members. Moreover, this study builds on previous observations of differences in Lp colonization within Cu and uPVC DW biofilms by evaluating differences in community structure, which might have underlying significance on their colonization properties. This study examined the prokaryotic and eukaryotic community composition on > 1-year-old DW biofilms grown on common in-premise plumbing materials (uPVC and Cu coupon) surfaces and the effect of introducing Lp and the FLA species, *A. polyphaga*, to overall microbial community structure was evaluated.

## Materials and methods

### Center for disease control biofilm reactor setup and sample collection

Six Center for disease control (CDC) biofilm reactors were set up as previously described (Buse *et al.*, 2014). Briefly, three reactors containing copper (Cu) or unplasticized polyvinylchloride (uPVC) coupons (surface area of 1.27 $cm^2$) were fed with ambient (20.8 ± 1.6 °C) DW that was held in a light-protected storage tank (23 L) to allow for natural dechlorination and fed at 40 mL $h^{-1}$ (10 h hydraulic resident time) using a peristaltic pump and Norprene™ food-grade tubing waste line. In-premise stagnation periods were simulated by placing reactors on

a magnetic stir plate which was activated every 2 h for 30 min at *c.* 100 r.p.m., and mature biofilms were allowed to establish on the coupon surfaces for 1 year before Lp strain Philadelphia-1 (ATCC 33152) and *A. polyphaga* (ATCC 30462) inoculation. Three days prior to inoculations, all CDC reactor inlets were turned off and the six Norprene™ effluent lines clamped off and replaced with new sterile sections. Each reactor was placed in a biologic safety cabinet for microbial inoculation where the DW was removed and replaced with 400 mL of 0.22 μm filtered, autoclaved tap water (fat$H_2O$).

Prior to inoculation, duplicate coupons from each reactor were collected to record established biofilm characteristics as a baseline comparison to postinoculation samples. Two reactors containing uPVC or Cu coupons were inoculated with either fat$H_2O$ (designated uPVC-Control and Cu-Control), $10^6$ CFU $mL^{-1}$ of Lp (designated uPVC-Lp and Cu-Lp) or $10^6$ CFU $mL^{-1}$ of Lp and 500 cells $mL^{-1}$ of *A. polyphaga* (designated uPVC-Lp/Ap and Cu-Lp/Ap). Reactors were moved back onto the magnetic stir plates where microbial adhesion was allowed to occur for 24 h with the above-described mixing scheme. After the 24-h incubation, each reactor was connected back to the peristaltic feed pump at the previous flow rate, which resulted in *c.* 2.4 volume changes per day. Two coupons (replicate 1, R1, and replicate 2, R2) were removed from each reactor at −3, 14, 32, 60, 91, and 123 days postinoculation. To collect the biofilm off each coupon surface, a sterile wooden stick was used to scrape the surface and was then rinsed in 300 μL of fat$H_2O$. The coupon surface was washed twice with 100 μL fat$H_2O$ resulting in a final volume of 500 μL suspended biofilm material.

## DNA extraction, PCR, and pyrotag sequencing

DNA was extracted from biofilm suspensions using the T&C buffer and MasterPure Complete DNA Purification Kit™ (Epicentre Biotechnologies) as described previously (Buse *et al.*, 2014). The 16S rRNA gene V4-V6 region and 18S rRNA gene V1-V3 region were amplified with universal bacterial primers B530F and B1100R [modified from (Turner *et al.*, 1999)] and universal eukaryotic primer Euk1aF and Euk516R [modified from (Sogin & Gunderson, 1987; Amann *et al.*, 1990)], respectively. Gene primer sequences were as follows: B530F 5′-GTG CCA GCM GCN GCG G-3′, B1100R 5′-GGG TTG CGN TCG TTG-3′, Euk1aF 5′-AAC CT G GT T GAT CCT GCC AGT-3′, and Euk516R 5′-ACC AGA CTT GCC CTC C-3′. Fourteen forward primers were constructed for both the 16S and 18S rRNA gene amplifications to allow for pooling of samples for pyrotag sequencing (i.e. for 16S and 18S rRNA gene sequencing, six and four pyrosequencing runs, respectively, were performed for all of the samples collected as described above). The forward primers consisted of a 10-bp unique barcode (unique sample tag) downstream of a 26-bp adaptor A and gene primer sequences (i.e. 16S rRNA gene primer: 5′-adaptor_A-sample tag-B530F-3′ and 18S rRNA gene primer: 5′-adaptor_A-sample tag-Euk1aF-3′). Reverse primers included only adaptor B at the 5′ end (i.e. 16S rRNA gene primer: 5′-adaptor B-B1100R-3′ and 18S rRNA gene primer: 5′-adaptor B-Euk516R).

PCR conditions for 16S rDNA amplification consisted of an initial denaturation step of 10 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C with a final elongation step at 72 °C for 10 min. For 18S rRNA gene amplification, PCR conditions consisted of an initial denaturation step of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 56 °C, 30 s at 72 °C with a final elongation step at 72 °C for 6 min. PCR amplicons were verified for size by electrophoresis in 1% agarose gels. The amplicons were excised from the agarose gel and purified with the MoBio UltraClean® GelSpin® DNA Extraction Kit (MoBio Laboratories, Inc., Carlsbad, CA). Amplicons were further purified using the Agencourt® AMPure® XP system (Beckman Coulter, Inc., Brea, CA). The quantity of purified PCR amplicons was determined using the Quant-iT™ PicoGreen® ds DNA Assay Kit (Life Technologies™ Corporation, Grand Island, NY). The same amount (in moles) of PCR amplicons of different samples was pooled together and prepared with the Roche Lib-L kit for sequencing on a 454 GS Junior™ sequencer (454 Life Sciences, Branford, CT) with the Titanium chemistry as per the manufacturer's protocol.

## *Acanthamoeba* spp. qPCR

Taqman qPCR assay for *Acanthamoeba* detection was performed using the genus-specific primers (TaqAcF1: 5′-CGA CCA GCG ATT AGG AGA CG-3′, TaqAcR1: 5′-CCG ACG CCA AGG ACG AC-3′) that amplified position 1267–1362 of the *Acanthamoeba* 18S rRNA gene sequence (Riviére *et al.*, 2006). The Taqman probe (5′-TGA ATA CAA AAC ACC ACC ATC GGC GC-3′) was labeled at the 5′ end with FAM reporter dye and TAMRA quencher at the 3′ end. A total reaction volume of 20 μL for each reaction mix contained 10 μL 2x Environmental Master Mix (Applied Biosystems), primer and probe mix at a concentration of 0.5 μM, and 2 μL template DNA. The thermal cycling conditions performed on the AB 7900HT (Applied Biosystems) included a predenaturation step at 50 °C for 2 min, 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min; and a dissociation stage for confirming amplification of desired product.

### Sequence analysis

A total of 272 232 and 218 926 uPVC DW biofilm-derived 16S and 18S rRNA gene sequences, respectively, were obtained. For Cu DW biofilm-derived 16S and 18S rRNA gene sequences, a total of 333 618 and 168 600 sequences were obtained (Supporting Information, Tables S1 and S2). The raw data was quality filtered using QIIME (Caporaso *et al.*, 2010) to exclude reads < 200 nt; reads with low quality scores (< 25), ambiguous bases (> 0), and homopolymer runs (> 8 nt); and those having > 0 nt mismatches in the primer or uncorrectable barcodes. The 10-bp barcode was used to assign reads to samples. Chimeric sequences were identified using UCHIME which detected chimeras *de novo* by exploiting sequence abundance data (Edgar *et al.*, 2011). The resulting nonchimeric sequences (Tables S1 and S2) were *de novo* clustered into operational taxonomic units (OTUs) using the UCLUST (Edgar, 2010) module from QIIME with a pairwise identity threshold of 97%. For taxonomic assignments of 16S rDNA sequences, the RDP Classifier v2.5 of the Ribosomal Database Project (RDP) (Wang *et al.*, 2007) was used at a confidence threshold of 80%. The 18S rRNA gene sequences were assigned using the BLASTN software (Altschul *et al.*, 1990) and a subset of the Eukaryota silva SSU rRNA reference database (http://www.arb-silva.de/) (Quast *et al.*, 2013) r114 (71 672 eukaryotic sequences with the ambiguous annotations 'uncultured or unidentified/eukaryote or eukaryotic/clone or isolate' removed, resulting in 56 563 eukaryotic sequences). Taxonomic assignments were visualized using MEGAN 4 (Huson *et al.*, 2011) with the 'min-score' filter, corresponding to a bit score cutoff value, set at 30; the top-percent filter used to retain hits, whose scores lie within a given percentage of the highest bit score, set at ten; the min-support core filter, used to set a threshold for the minimum number of sequences that must be assigned to a taxon, was set to five. To correct for varying sampling efforts (number of sequences analyzed per sample), subsampling was performed in MEGAN to randomly select a subset of sequences per set to compare relative differences in OTU-level abundance across time points and sample sets. The pyrosequencing reads have been deposited in the National Center for Biotechnology Information database BioProject number PRJNA214912: SRA accession #SAMN02353900–SAMN02353935.

### Statistical analyses

All statistical analyses were performed using the QIIME software package. To compare microbial diversity (Faith's Phylogenetic Diversity) and richness (Chao1) between different treatments and time points, QIIME was used to randomly choose a series of subsets of each sequence library to calculate respective diversity and richness indices. This procedure was repeated 10× for each subset size, and the average value of 10 replicates of each subset was used to construct the rarefaction curve analyses (Fig. S1). Co-occurrence analysis between prokaryotic and eukaryotic members within the uPVC and Cu biofilms was performed via calculation and plotting of Spearman's rank correlations using MS EXCEL. The differences in overall community composition between uPVC and Cu Control, Lp, Lp & *A. polyphaga* (LpAp) bacterial and eukaryotic sequences were determined using the unweighted UNIFRAC metric (Lozupone & Knight, 2005), which calculates the distance between any pair of communities based on the fraction of unique (nonoverlapping) branch length of their sequences in the tree and were visualized using nonmetric multidimensional scaling (NMDS). A two-way crossed, nonparametric ANalysis Of SIMilarities (ANOSIM) significance test was used to identify statistical differences (Bonferroni corrected *P*-values) between uPVC and Cu biofilms across time points and between Con, Lp, and LpAp samples based on the unweighted UNIFRAC metric. ANOSIM included the R statistic test, where an *R*-value near 1 indicates dissimilarity between groups, while an *R*-value near zero indicates a true null hypothesis of no difference between groups (Clarke, 1993).

## Results

Tables S1 and S2 detail the number of sequences obtained from 454 pyrosequencing of 16S and 18S rRNA gene sequences derived from uPVC and Cu DW biofilms after removal of low-quality and chimeric sequences (Tables S1 and S2). The nonchimeric sequences for each of the six time points and replicates for control (Con), Lp-inoculated, and LpAp co-inoculated biofilm reactors containing either uPVC or Cu coupons are summarized in Table S3. Figure S1 illustrates the rarefaction curves generated to assess microbial diversity (Faith's phylogenetic diversity) and richness (Chao1) and shows that, for both the uPVC and Cu samples, 16S rRNA gene sequences appeared to cluster into two groups indicating slight differences in species richness. This was likely due to the differences in the number of sequences that were derived between the R1 and R2 replicates of those samples (Table S3, mean difference of 5155 sequences between replicates). However, the observed number of OTU, with 3% species cutoff, indicated that the sampling effort was sufficient (Fig. S1) as well as the Good's coverage index (estimator of sampling completeness) showing 95–99% mean coverage for each sample set (Table S3).

*De novo* OTU clustering was performed separately for each time point and replicate for both 16S and 18S rRNA

gene uPVC and Cu biofilm-derived sequences and taxonomically assigned as described in Methods. Family-level taxonomic assignments revealed different compositions within uPVC and Cu biofilms for both prokaryotic and eukaryotic communities (Fig. 1). There were 16 and 22 major microbial OTU groups identified in the uPVC and Cu biofilm communities, respectively, that represented > 1% of the total diversity based on relative abundance (Table 1) with more uniquely identified family members within Cu than uPVC biofilms (Fig. 1, Table 1). For 16S rRNA gene-derived sequences, the order *Burkholderiales* was the dominate member within in the uPVC biofilms, while the genus *Lacibacter* and *Sediminibacterium* were the dominate members within the Cu biofilms (Table 1). Furthermore, the order *Rhizobiales* within the *Alphaproteobacteria* class and the genus *Gemmatimonas* of the *Gemmatimonadetes* phylum were more abundant within uPVC biofilms, while the families *Chitinophagaceae* (*Bacteroidetes*), *Comamonadaceae* (*Betaproteobacteria*), *Microbacteriaceae* (*Actinobacteria*), and *Rhodobacteraceae* (*Alphaproteobacteria*) and the genus *Hydrogenophaga* (*Betaproteobacteria*) and *Singulisphera* (*Planctomycetes*) were more abundant within Cu biofilms (Table 1).

Both uPVC and Cu biofilm-derived 18S rRNA gene sequences taxonomically assigned to the phylum *Tracheophyta*, which is a group comprised of higher land plants such as ferns, flowering plants, and grasses, were not considered indigenous biofilm community members but rather 'contaminants' within the distribution system and were removed from the datasets/further analysis. For the remaining 18S rRNA gene sequences, the relative abundance of family-level-assigned taxa common to both uPVC and Cu biofilms (*Cryptosporidiidae* [Apicomplexa] and *Hartmannellidae* [amoebae]) was higher for the uPVC biofilm samples with *Chromulinaceae* [algae] more abundant in uPVC biofilms and Stremenopiles more abundant in Cu biofilms (Fig. 1, Table 1), possibly due to the higher biofilm biomass on uPVC surfaces (Buse *et al.*, 2014). In contrast to the results obtained for the 16S rRNA gene sequences, many more unique eukaryotic members were identified within uPVC biofilms compared to the Cu biofilm samples (although relative abundances were too low for inclusion in Table 1): the *Characeae* (algae), *Epistylidae* (protozoa), *Goniomonadaceae* (algae), *Paramoebidae* (amoebozoa), *Plasmodiophorida* (protist), *Plectidae* (nematodes), *Sphenomonadidae* (protist), and *Toxariaceae* (algae) families (Table S6). The *Acanthamoebidae* (amoebae), *Mallomonadaceae* (algae), and *Chlamydomoadaceae* (algae) families were unique to the Cu biofilms (Table S7). However, for the Lp-only inoculated reactor, the overriding difference between eukaryotic members developed on Cu vs uPVC coupons was the absence of *Cryptosporidiidae*-like sequences (Fig. 1 and Table S7).

Microbial co-occurrence patterns within the uPVC and Cu biofilms prior to inoculation were analyzed to highlight differences in microbial community structure between biofilms grown on different in-premise plumbing surfaces. Overall, more positive correlations were observed for microbial members within both uPVC (5 of 7 strong correlations) and Cu (10 of 13 strong correlations) biofilms ($R > 0.95$ [Spearman's rank correlation coefficient]; Fig. 2). Correlations between the co-occurrence of prokaryotes and eukaryotes were observed only in the uPVC biofilms, specifically between *Burkholderiales* (*Betaproteobacteria*) and *Plectidae* (Metazoa) ($R = 0.98$); *Microbacteriaceae* (*Actinobacteria*) and *Cryptosporidiidae* (Apicomplexa) ($R = 0.96$); and one between two eukaryotes *Paramoebidae* (amoebozoa) and *Toxariaceae* (algae) ($R = 1$) (Fig. 2a). Within Cu biofilms, strong positive correlations were mostly between the Delta- and Gamma-Proteobacteria and *Chlamydidae* bacterial members ($R = 1$; Fig. 2b). Interestingly, a strong positive correlation between the occurrence of the bacterial families, *Puniceicoccaceae* and *Sorangiineae*, was observed in both biofilm communities although the correlation was not as strong for the Cu biofilms (Fig. 2a, uPVC $*R = 1$; Fig. 2b, Cu $*R = 0.93$). Collectively, the co-occurrence analysis, based on relative abundance, revealed correlative differences between microorganisms within uPVC and Cu-derived DW biofilms even prior to Lp and LpAp inoculation indicating the impact the biofilm substratum has on microbial community structure.

Figure 3 illustrates the NMDS plot ordination of the pairwise unweighted UNIFRAC distances with no distinct and separate clustering of the Con-, Lp-, and LpAp-treated uPVC and Cu biofilm samples. However, *Legionella*-like sequences were only detected in the replicate 1 day 123 (R1.123) of the uPVC-control (Con) sample, in R1.60 and R1.123 of the uPVC-Lp sample, and in replicate 2 day −3 (R2.−3) and R2.123 of the uPVC-LpAp sample and in low relative abundances (Table S4). *Legionella*-like sequences were only detected in one of the Cu biofilm samples, Cu-Con R1.−3 (Table S5), and *Acanthamoeba*-like sequences were also only detected once in the Cu-LpAp day 123 sample (Table S7) and in low abundance. However, using genus-specific qPCR, *Acanthamoeba* spp. were detected more frequently in both uPVC-and Cu-LpAp reactor samples, but also in Cu-Con and uPVC- and Cu-Lp samples (Table 2). These observations indicated the presence of indigenous, low background levels of *Acanthamoeba* within biofilms, but for the LpAp samples, the majority of the positive samples with high *Acanthamoeba* levels can be attributed to the inoculation of *A. polyphaga* into those reactors.

The differences in the phylogenetic distances (as determined by unweighted UNIFRAC analysis) within the bacterial

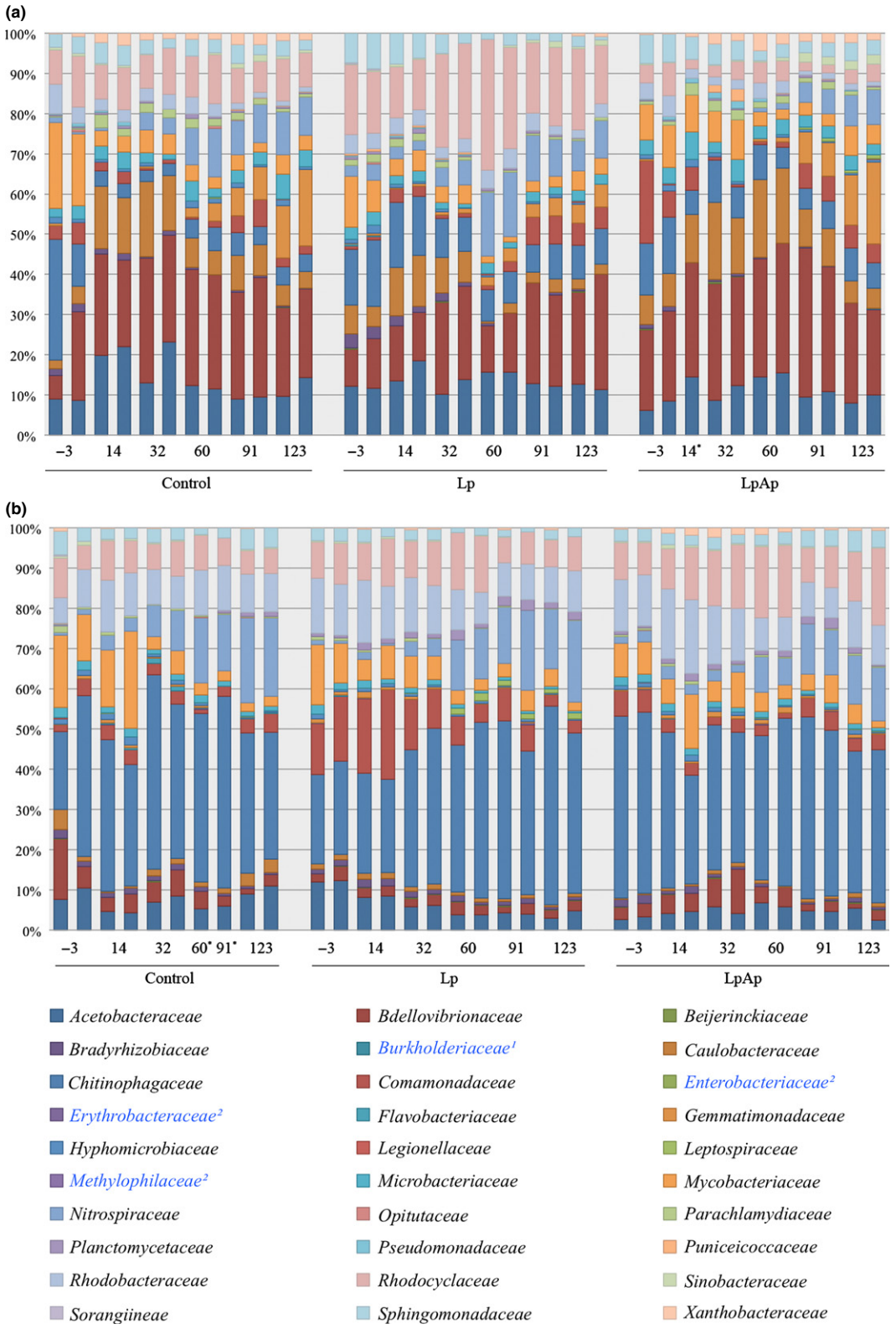


**Fig. 1.** Family-level taxonomic assignments expressed as a percentage of total assigned sequences for each sample. 16S rRNA gene sequences derived from (a) uPVC and (b) Cu DW biofilms; and 18S rRNA gene sequences derived from (c) uPVC and (d) Cu DW biofilms. For blue highlighted text: [1], assignments made only for uPVC-derived sequences; [2], assignments made only for Cu-derived sequences.

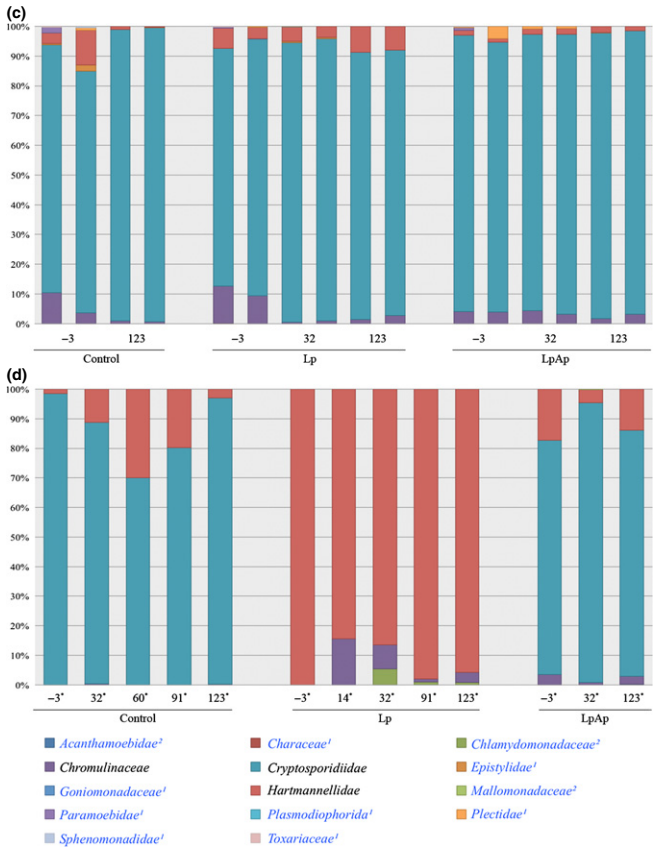


**Fig. 1.** (Continued).

and eukaryotic communities in the uPVC and Cu-Con, -Lp, and -LpAp biofilm samples were most likely related to the presence of unique taxa, sometimes in only one of the two replicate samples (Fig. 3 and Tables S4–S7). For example, in the uPVC-Con group, the day 90, replicate 1 sample (R1.90) clustered away from R2.90 because of the two unique taxa assigned to sequences obtained in that replicate and not the other, *Opitutaceae* and *Plactomycetaceae*, with R1.90 being the only sample in the uPVC-Con group where the genus *Sorangiineae* was identified (Fig. 3a and Table S4). For the eukaryotic sequences derived from the uPVC biofilm samples, the sporadic presence of several unique taxa for the day −3 and 32 Con, Lp, and LpAp samples, consistent between replicates, contributed to the patterns observed in the NMDS plots (Fig. 3c and Table S6). The pattern for the Cu biofilm-derived bacterial sequences of the Con, Lp, and LpAp groups were distinctly clustered together except for the R1 samples for both the Con and Lp groups where the presence of *Beijerinckiaceae*, *Erythrobacteraceae*, *Leptospiraceae*, and *Xanthobacteraceae*, in only one of the replicates, more than likely contributed to the patterns observed (Fig. 3b and Table S5). Similarly, for the Cu biofilm-derived eukaryotic sequences, the unique presence of *Mallomonadaceae* in only the day 32 LpAp sample and the presence of *Acanthamoebidae* in only the day 123 LpAp and the lack of *Cryptosporidiidae*-like sequences in the entire Lp sample group contributed to the clustering pattern observed (Fig. 3d and Table S7).

Overall, uPVC vs. Cu substratum had a strong influence on DW biofilm microbial community composition (Fig. 4). ANOSIM revealed significant differences between bacterial communities within uPVC and Cu biofilms: uPVC-Con vs Cu-Con ($P = 0.001$, $R = 0.634$), uPVC-Lp vs Cu-Lp ($P = 0.001$, $R = 0.662$), and uPVC-LpAp vs Cu-LpAp ($P = 0.001$, $R = 0.566$) with $R$-values further indicating dissimilarities between those groups (Fig. 5). Similar results were obtained for the eukaryotic community comparisons: uPVC-Con vs Cu-Con ($P < 0.01$, $R = 0.588$), uPVC-Lp vs Cu-Lp ($P < 0.01$, $R = 0.994$), and uPVC-LpAp vs Cu-LpAp ($P < 0.01$, $R = 0.0706$) (Fig. 5). Interestingly, the composition of both the bacterial and eukaryotic communities was relatively similar over the 4-month study period ($P > 0.05$, $R$-values between −1 and 0.406) except for the bacterial communities in the biofilms from the uPVC-Con and uPVC-LpAp reactors ($P < 0.05$, $R = 0.467$ and 0.632, respectively)

(Fig. 5). Because DW biofilms were allowed to develop on the uPVC and Cu surfaces for more than a year before inoculation, this could indicate the resiliency of the established biofilms. Nonetheless, inoculation of LpAp did appear to impact eukaryotic community members within Cu DW biofilms ($P < 0.01$, $R = 0.346$) and uPVC DW biofilms ($P < 0.01$, $R = 0.307$) (Fig. 5).

## Discussion

The key finding from this study was the importance of the substratum to subsequent bacterial and eukaryotic biofilm community structure (Figs 2, 4, and 5) despite common feed water from a large building copper pipe in-premise plumbing system. Interestingly, Cu biofilms were composed of more *Betaproteobacteria* genera and displayed less eukaryotic diversity than uPVC biofilms, while bacterial composition was less diverse in uPVC biofilms (Fig. 1 and Table 1). This suggests that the uPVC substratum may be more favorable to eukaryotic colonization, presumably due to the higher biomass productivity (Buse *et al.*, 2014). Additionally, was it the presence of *Betaproteobacteria* or other biofilm members limiting eukaryotic diversity within Cu biofilms or the absence of the antimicrobial/toxic properties of copper within the uPVC biofilms? Confounding these observations is the notion that certain biofilm members can readily adhere and stably colonize biofilms grown on any surface such as the equally strong colonization of *Mycobacterium avium*, *M. intracellulare*, and *M. abscessus* within glass, stainless steel, PVC, and zinc-galvanized steel coupons-grown DW biofilms (Mullis & Falkinham, 2013). Nonetheless, from this study, there were clear differences in bacterial and eukaryotic composition within uPVC vs. Cu DW biofilms grown on a common in-premise source drinking water.

Similar to the results from this study, bacterial composition of finished water from a chloraminated DW system was dominated by the phyla *Actinobacteria*, *Bacteriodetes*, and *Proteobacteria* (Zeng *et al.*, 2013). Specifically, sequences with high homologies to the families *Mycobacteriaceae* and *Microbacteriaceae* (*Acitinobacteria*), *Chitinophagaceae* (*Bacteriodetes*), *Rhodobacteraceae* and *Sphingomonadaceae* (*Alphaproteobacteria*), *Comamonadaceae* and *Rhodocyclaceae* (*Betaproteobacteria*), and *Bdellovibrionaceae* (*Deltaproteobacteria*) were the most abundant in the uPVC and Cu biofilms (Tables S4 and S5). Moreover, the bacterial phyla identified in the current study (Table 1), with the exception of *Spirochaetes,* have previously been reported for finished water samples from a nonchlorinated system (Lautenschlager *et al.*, 2013). The same nonchlorinated system also contained the bacterial phyla *Chlorflexi*, *Chlorobi*, and *Elusimicrobia*, which were not identified in the current study where the tank feed water was derived from a chlorinated (with maintained residual) distribution system. Thus, not surprisingly, the phyla *Chlamydiae*, *Verrucomicrobia*, *Acidobacteria*, *Gemmatimonadetes*, and *Nitrospirae* were absent in finished water from a chlorinated treatment plant but were present upstream in water samples from the GAC beds, except *Gemmatimonadetes* (Poitelon *et al.*, 2010), suggesting that introduction of disinfectants decreases bacterial diversity. However, biofilms isolated from a concrete/steel-finished water storage tank immediately downstream of a DW treatment plant contained bacteria across 33 unique genera (Zhang *et al.*, 2012), 11 of which were identified in this study, indicating that biofilms provide microbial protection from disinfectants and may be a potential source of environmental pathogens in DW (Wingender & Flemming, 2011).

Environmental pathogens identified in DW systems are usually also ARB that exploit the similarities amoebae have with macrophages to cause respiratory diseases in humans (Lau & Ashbolt, 2009). The pathogen-containing families *Bradyrhizobiaceae*, *Mycobacteriaceae*, and *Legionellaceae*, all of which were identified in this study (Fig. 1 and Tables S4 and S5), have been previously isolated from DW and DW biofilms (Thomas *et al.*, 2006, 2007; Feazel *et al.*, 2009). *Mycobacterium* and *Legionella* are well-studied human opportunistic pathogens that can cause severe respiratory diseases (Collier *et al.*, 2012); however, for the other ARB genera, their common isolation from DW systems and ability to resist amoeba predation make them likely candidates for agents of sporadic disease outbreaks whose etiology is unknown (Yoder *et al.*, 2008).

Both uPVC and Cu biofilms contained sequences identified as the *Afipia*, *Bosea*, and *Bradyrhizobium* genera belonging to the family *Bradyrhizobiaceae* with a relatively higher abundance of sequences found within the Cu biofilms (Tables S4 and S5). This trend was also true for the genus *Leptospira* belonging to the family *Leptospiracaea*, members of which are human pathogens and have been associated with waterborne outbreaks (Cann *et al.*, 2013). Members of the genera *Afipia* and *Bosea* are ARB that have been associated with human disease (La Scola *et al.*, 2003; Pagnier *et al.*, 2012) and have been previously identified in kitchen PVC pipe biofilms (McBain *et al.*, 2003) and concrete/steel biofilms isolated from DW reservoirs (Zhang *et al.*, 2012). *Legionellaceae* sequences, members that are also known human pathogens and ARBs (Lau & Ashbolt, 2009), were also identified in the uPVC-Con, -Lp, and -LpAp, and Cu-Con biofilms; however, neighbor-joining phylogenetic tree analysis revealed those sequences to be non-Lp-like indicating low background levels of indigenous (probably nonpathogenic) *Legionella* within DW biofilms (data not shown). It is possible that Lp were in such low abundance that they were not

**Table 1.** Percent microbial distribution (%) and OTU classification (97% cutoff level) for uPVC and Cu biofilms

| Lowest common ancestor | | Control | | | | | | | | | | | | Lp | | | | | | | | | | | | LpAp | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxonomy | Level | −3 | | 14 | | 32 | | 60 | | 91 | | 123 | | −3 | | 14 | | 32 | | 60 | | 91 | | 123 | | −3 | | 14 | | 32 | | 60 | | 91 | | 123 | |
| uPVC | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 16S rRNA gene sequences | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Bacteroidetes* | Phylum | 2 | 5 | 6 | 4 | 6 | 6 | 4 | 5 | 3 | 3 | * | * | 19 | 16 | 16 | 13 | 7 | 7 | 7 | 6 | 3 | 3 | 1 | 2 | 19 | 16 | 19 | – | 15 | 12 | 10 | 7 | 5 | 7 | 4 | 5 |
| *Burkholderiales* | Order | 8 | 24 | 24 | 32 | 26 | 28 | 22 | 20 | 12 | 13 | 8 | 9 | 15 | 17 | 23 | 28 | 29 | 26 | 13 | 20 | 14 | 11 | 17 | 15 | 21 | 25 | 32 | – | 25 | 26 | 21 | 27 | 7 | 7 | 4 | 6 |
| *Rhizobiales* | Order | 2 | 2 | 2 | 1 | 2 | 3 | 3 | 2 | 3 | 2 | 5 | 3 | 4 | 2 | 2 | 2 | 2 | 1 | 3 | 2 | 3 | 3 | 6 | 4 | 3 | 2 | 2 | – | 3 | 3 | 3 | 2 | 3 | 2 | 4 | 3 |
| *Acetobacteraceae* | Family | 6 | 4 | 9 | 9 | 6 | 10 | 5 | 5 | 5 | 5 | 6 | 8 | 6 | 6 | 5 | 7 | 4 | 5 | 8 | 8 | 7 | 7 | 6 | 6 | 3 | 4 | 4 | – | 3 | 5 | 7 | 6 | 5 | 5 | 4 | 5 |
| *Rhodocyclaceae* | Family | 6 | 6 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 6 | 5 | 8 | 7 | 5 | 5 | 9 | 9 | 17 | 12 | 10 | 10 | 10 | 7 | 2 | 3 | * | – | * | 1 | 2 | 2 | * | * | 2 | 2 |
| *Gemmatimonas* | Genus | * | * | * | * | * | * | 1 | 2 | 4 | 4 | 8 | 11 | * | * | * | * | * | * | 1 | 2 | 2 | 2 | 2 | 3 | * | * | * | – | * | * | * | * | 4 | 4 | 7 | 10 |
| *Mycobacterium* | Genus | 14 | 9 | 2 | 2 | 3 | 2 | 2 | 1 | 2 | 1 | 3 | 2 | 6 | 4 | 2 | 2 | 2 | 2 | 1 | 1 | 2 | 1 | 2 | 2 | 4 | 5 | 3 | – | 3 | 4 | 2 | * | 2 | 2 | 4 | 3 |
| *Nitrospira* | Genus | 1 | 0 | 0 | 0 | 2 | 2 | 4 | 5 | 4 | 5 | 6 | 5 | 1 | 2 | 1 | 1 | 2 | 2 | 8 | 8 | 6 | 5 | 4 | 5 | * | * | * | – | * | * | * | * | 3 | 3 | 4 | 4 |
| *Phenylobacterium* | Genus | 1 | 2 | 7 | 6 | 9 | 6 | 3 | 3 | 5 | 4 | 3 | 2 | 3 | 2 | 5 | 5 | 3 | 3 | * | 1 | 1 | 2 | 1 | 1 | 3 | 4 | 3 | – | 7 | 5 | 9 | 7 | 5 | 5 | 3 | 3 |
| *Rhodobacter* | Genus | 3 | * | * | * | * | 1 | 2 | * | * | * | * | * | 2 | * | 1 | 1 | * | * | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | * | – | * | * | 2 | * | * | * | * | * |
| *Sediminibacterium* | Genus | 17 | 4 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | 1 | * | 2 | 4 | 5 | * | – | * | * | * | * | * | * | * | 2 |
| *Sphingopyxis* | Genus | 2 | 2 | 2 | 2 | 2 | * | 2 | 1 | 2 | 1 | 2 | 1 | 3 | 4 | 3 | 2 | 2 | * | * | * | * | * | * | * | 3 | 2 | 1 | – | 2 | 2 | * | 1 | 1 | 2 | 1 | 1 |
| *Vampirovibrio* | Genus | 4 | 10 | 11 | 9 | 15 | 11 | 13 | 13 | 14 | 16 | 13 | 13 | 4 | 6 | 5 | 5 | 9 | 9 | 6 | 7 | 14 | 12 | 12 | 14 | 9 | 10 | 8 | – | 11 | 10 | 13 | 13 | 20 | 16 | 13 | 11 |
| Total (%) | | 66 | 68 | 67 | 70 | 74 | 73 | 65 | 62 | 58 | 58 | 60 | 60 | 71 | 67 | 69 | 71 | 67 | 65 | 66 | 67 | 64 | 59 | 64 | 62 | 72 | 77 | 73 | – | 70 | 68 | 67 | 65 | 55 | 52 | 49 | 54 |
| 18S rRNA gene sequences | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chromulinaceae* | Family | 9 | 4 | – | – | – | – | – | – | – | – | * | * | 12 | 9 | – | – | * | * | – | – | – | – | 1 | 3 | 4 | 4 | – | – | 4 | 3 | – | – | – | – | 2 | 3 |
| *Cryptosporidiidae* | Family | 73 | 79 | – | – | – | – | – | – | – | – | 98 | 99 | 76 | 82 | – | – | 93 | 94 | – | – | – | – | 89 | 88 | 88 | 89 | – | – | 92 | 94 | – | – | – | – | 96 | 95 |
| *Hartmannellidae* | Family | 3 | 11 | – | – | – | – | – | – | – | – | * | * | 6 | 4 | – | – | 5 | 4 | – | – | – | – | 9 | 8 | 1 | 1 | – | – | 2 | 2 | – | – | – | – | 2 | 1 |
| Total (%) | | 86 | 94 | – | – | – | – | – | – | – | – | 98 | 99 | 95 | 95 | – | – | 98 | 98 | – | – | – | – | 99 | 99 | 93 | 94 | – | – | 99 | 99 | – | – | – | – | 99 | 99 |
| Cu | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 16S rRNA gene sequences | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Bacteroidetes* | Phylum | 7 | * | * | * | 2 | 2 | – | 1 | – | * | * | * | * | * | 2 | 2 | * | * | 1 | 2 | 1 | 1 | * | * | 3 | 2 | 3 | 2 | 4 | 3 | 3 | 4 | 4 | 4 | 4 | 3 |
| *Burkholderiales* | Order | 15 | 3 | 5 | 3 | 4 | 4 | – | 3 | – | 3 | 3 | 2 | 5 | 6 | 5 | 5 | 4 | 4 | 3 | 3 | 4 | 4 | 3 | 5 | 2 | 2 | 3 | 3 | 4 | 4 | 3 | 4 | 3 | 3 | 4 | 5 |
| *Acetobacteraceae* | Family | 4 | 7 | 3 | 3 | 5 | 5 | – | 4 | – | 4 | 7 | 8 | 8 | 9 | 5 | 6 | 4 | 4 | 3 | 3 | 3 | 3 | 2 | 3 | 2 | 3 | 3 | 3 | 3 | 2 | 4 | 4 | 3 | 3 | 4 | 2 |
| *Chitinophagaceae* | Family | * | * | 2 | * | * | * | – | * | – | * | 1 | * | * | * | * | * | * | * | * | * | 2 | * | 3 | * | 2 | * | 2 | * | 2 | * | * | * | 2 | * | 2 | * |
| *Comamonadaceae* | Family | * | * | 1 | 1 | * | * | – | * | – | 2 | 1 | 2 | 1 | 2 | 1 | 1 | * | * | * | * | 3 | 2 | * | * | 3 | 3 | 1 | * | * | * | * | * | 3 | 3 | 2 | 2 |
| *Microbacteriaceae* | Family | 1 | 2 | * | 1 | * | * | – | 1 | – | * | * | * | 2 | * | 1 | * | * | * | * | * | * | * | * | * | 2 | 1 | 1 | * | * | * | * | * | * | * | * | * |
| *Rhodobacteraceae* | Family | 2 | 2 | 7 | 3 | 5 | 2 | – | 3 | – | 5 | 6 | 4 | 7 | 4 | 8 | 3 | 7 | 3 | 3 | 3 | 5 | 2 | 6 | 4 | 7 | 3 | 10 | 4 | 7 | 2 | * | 1 | 5 | 2 | 7 | 3 |
| *Rhodocyclaceae* | Family | 4 | 4 | 6 | 5 | 4 | 5 | – | 6 | – | 5 | 4 | 5 | 6 | 7 | 6 | 7 | 6 | 7 | 9 | 8 | 4 | 5 | 5 | 6 | 6 | 6 | 6 | 8 | 7 | 8 | 10 | 11 | 6 | 7 | 8 | 13 |
| *Hydrogenophaga* | Genus | * | 2 | * | 1 | 1 | 2 | – | * | – | * | 1 | 2 | 7 | 10 | 11 | 13 | 8 | 6 | 4 | 3 | 3 | 2 | 1 | 1 | 1 | * | * | * | * | * | * | * | * | * | * | * |
| *Lacibacter* | Genus | 2 | 4 | 11 | 7 | 11 | 9 | – | 9 | – | 17 | 12 | 10 | 6 | 7 | 7 | 6 | 11 | 15 | 12 | 16 | 22 | 16 | 27 | 20 | 19 | 24 | 19 | 13 | 16 | 14 | 21 | 23 | 27 | 25 | 21 | 24 |
| *Mycobacterium* | Genus | 8 | 8 | 9 | 16 | 2 | 4 | – | 2 | – | 2 | 2 | 2 | 10 | 7 | 3 | 5 | 5 | 4 | 2 | 2 | 2 | 3 | 3 | 2 | 6 | 6 | 4 | 8 | 3 | 5 | 3 | 2 | 3 | 5 | 3 | 1 |
| *Nitrospira* | Genus | * | * | 2 | 2 | 5 | 6 | – | 11 | – | 10 | 16 | 14 | 1 | 1 | 1 | 0 | 2 | 3 | 8 | 9 | 10 | 13 | 11 | 14 | 1 | 2 | 2 | 2 | 1 | 1 | 6 | 4 | 9 | 8 | 9 | 9 |
| *Phenylobacterium* | Genus | 2 | * | * | * | 1 | * | – | * | – | * | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | * | * | * | * | * | * | * | * | * | * | * | * |
| *Piscinibacter* | Genus | * | 5 | * | 5 | * | 5 | – | 3 | – | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | 1 | * | * | * | * | * | * | * | * |
| *Rhodobacter* | Genus | * | 4 | 1 | 5 | * | 3 | – | 4 | – | 4 | * | 3 | 2 | 5 | 2 | 6 | 2 | 5 | 3 | 3 | 1 | 4 | 1 | 3 | 2 | 6 | 2 | 8 | 1 | 5 | 4 | 4 | 1 | 3 | 1 | 3 |
| *Sediminibacterium* | Genus | 7 | 23 | 13 | 13 | 21 | 16 | – | 20 | – | 18 | 13 | 13 | 9 | 9 | 8 | 9 | 10 | 12 | 12 | 13 | 7 | 7 | 7 | 7 | 11 | 10 | 4 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 2 |

**Table 1.** Continued

| Lowest common ancestor | | Control | | | | | | | | | Lp | | | | | | | | | LpAp | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxonomy | Level | −3 | | 14 | | 32 | | 60 | | 91 | | 123 | | −3 | | 14 | | 32 | | 60 | | 91 | | 123 | | −3 | | 14 | | 32 | | 60 | | 91 | | 123 | |
| *Singulisphaera* | Genus | * | * | * | * | * | * | – | * | – | * | * | * | * | * | 1 | * | * | * | 2 | * | 1 | 2 | 1 | 1 | * | * | * | * | * | * | 1 | 2 | * | * |
| *Sphingopyxis* | Genus | 3 | 2 | 1 | 2 | 2 | 2 | – | 1 | – | 2 | 4 | 3 | 2 | 2 | 2 | 1 | 1 | 2 | * | * | * | 1 | * | 2 | 1 | 1 | 2 | 2 | 1 | 1 | * | 2 | 2 | 3 | 3 |
| *Vampirovibrio* | Genus | 7 | 4 | 2 | 3 | 3 | 4 | – | 3 | – | 2 | 1 | 2 | 1 | 3 | 1 | 2 | 1 | 2 | 2 | 2 | * | 2 | 2 | 2 | 2 | 3 | 3 | 4 | 6 | 2 | 3 | 1 | 2 | * | 2 |
| Total (%) | | 36 | 59 | 53 | 62 | 57 | 57 | – | 61 | – | 64 | 62 | 59 | 53 | 56 | 52 | 54 | 55 | 59 | 58 | 59 | 57 | 57 | 65 | 60 | 57 | 63 | 51 | 53 | 43 | 45 | 50 | 54 | 57 | 58 | 55 | 61 |
| 18S rRNA gene sequences | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stramenopiles | Phylum | 4 | – | – | – | 5 | – | – | 8 | – | 7 | 1 | – | 1 | – | 7 | – | 2 | – | – | – | 2 | – | 3 | – | 4 | – | – | – | 3 | – | – | – | – | 9 | – |
| *Cryptosporidiidae* | Family | 51 | – | – | – | 28 | – | – | 19 | – | 36 | 57 | – | – | – | – | – | – | – | – | – | – | – | – | – | 4 | – | – | – | 28 | – | – | – | – | 19 | – |
| *Hartmannellidae* | Family | * | – | – | – | 4 | – | – | 8 | – | 9 | 2 | – | * | – | 2 | – | 2 | – | – | – | 6 | – | 7 | – | * | – | – | – | 1 | – | – | – | – | 3 | – |
| Total (%) | | 55 | – | – | – | 36 | – | – | 34 | – | 52 | 60 | – | 1 | – | 9 | – | 4 | – | – | – | 7 | – | 10 | – | 8 | – | – | – | 33 | – | – | – | – | 30 | – |

Only OTUs with abundances > 1% of the total community are shown. 16S rRNA gene sequences: after subsampling: 1183 uPVC- and 2328 Cu-derived sequences. 18S rRNA gene sequences: after subsampling: 1676 uPVC- and 1091 Cu-derived sequences.
–, no data.
*Percent value below 1% of total community.

detected via pyrosequencing, even though Lp-specific qPCR analysis detected it postinoculation in the biofilm samples analyzed in the study (Buse *et al.*, 2014). Buse *et al.* (2014) showed that Lp colonized Cu biofilms more efficiently, especially with the co-inoculation of *A. polyphaga*, than within uPVC biofilms. Thus, coupled with the observation of higher relative abundances of *Bradyrhizobiaceae* sequences in Cu biofilms compared to uPVC biofilms from the current study (Tables S4 and S5), ARBs and other potential environmental pathogens seem to prefer colonization within Cu biofilms than uPVC, which is counterintuitive to the well-documented antimicrobial properties of Cu (Thurman *et al.*, 1989).

ANalysis Of SIMiliarity (ANOSIM) statistical tests revealed that inoculation of Lp and co-cultures of Lp and its permissive host, *A. polyphaga*, only significantly impacted eukaryotic communities ($P < 0.01$, global $R = 0.3$) but induced no significant changes in bacterial communities ($P > 0.05$, global $R$-value $< 0.1$) (Fig. 5). The relatively low abundance of *Legionella* and *Acanthamoeba* detected via pyrosequencing in the Lp and LpAp-inoculated biofilms (Tables S4–S7) and also by qPCR analysis [Table 2 and (Buse *et al.*, 2014)] suggests their poor and inefficient colonization into well-established, mature biofilms which ultimately contributed to the lack of community differences seen in the bacterial profiles across coupon materials. It is possible that the colonization of eukaryotes within DW biofilms is not as stable and prolific as bacterial cells. Eukaryotic taxa identified in this study have also been identified in previous studies examining community composition within DW samples (Poitelon *et al.*, 2009; Valster *et al.*, 2009; Otterholt & Charnock, 2011; Buse *et al.*, 2013b). The major eukaryotic groups within DW are *Amoebozoa*, *Metazoa* (nematodes, rotifers, and copepods), *Alveolata* (*Myzozoa*, dinoflagellates, *Ciliphora*, *Cercozoa*, *Choanozoa*), fungi, and algae (*Heterokontophyta*). However, large-scale pyrosequencing of 18S rRNA genes from DW biofilms is underperformed, and further characterization of these eukaryotes needs to be pursued to fully understand their biofilm colonization dynamics.

Except for 16S rDNA sequences derived from the uPVC-Con and uPVC-LpAp biofilms, ANOSIM indicated that within each reactor, the 16S and 18S rRNA gene sequences for each time group were not significantly different, with $P > 0.05$ and global $R$-values between −1.00 to 0.406 (Fig. 5). Similar to a previous report, during the early stages of DW biofilm development on glass beads and polycarbonate coupons, bacterial composition fluctuated from 0 to 3 months with the stable colonization of *Mycobacteriaceae* occurring at 3 months through to the last sampling at 8 months (Revetta *et al.*, 2013) indicating that after establishment of a quasi-stable microbial structure, composition within biofilms do not significantly change with time, as

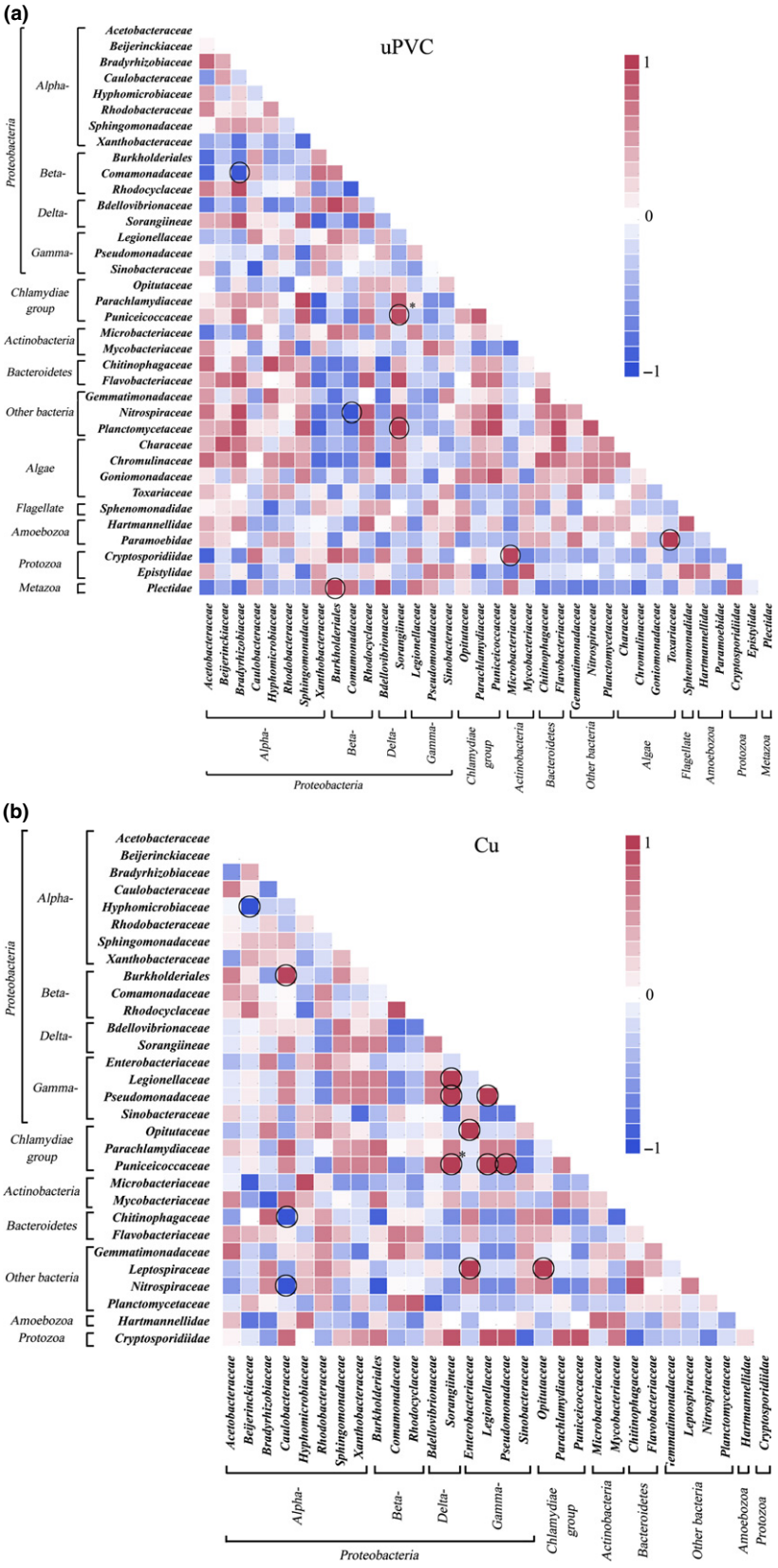


**Fig. 2.** Spearman's rank correlation matrix of microbial populations across domains in uPVC and Cu biofilms prior to inoculation. The colors of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark red), 0 indicating no linear correlation (white), and −1 indicating perfect negative correlation (dark blue) between two microbial populations. Correlations marked with circles and those highlighted with an asterisk (*) are discussed in the text.

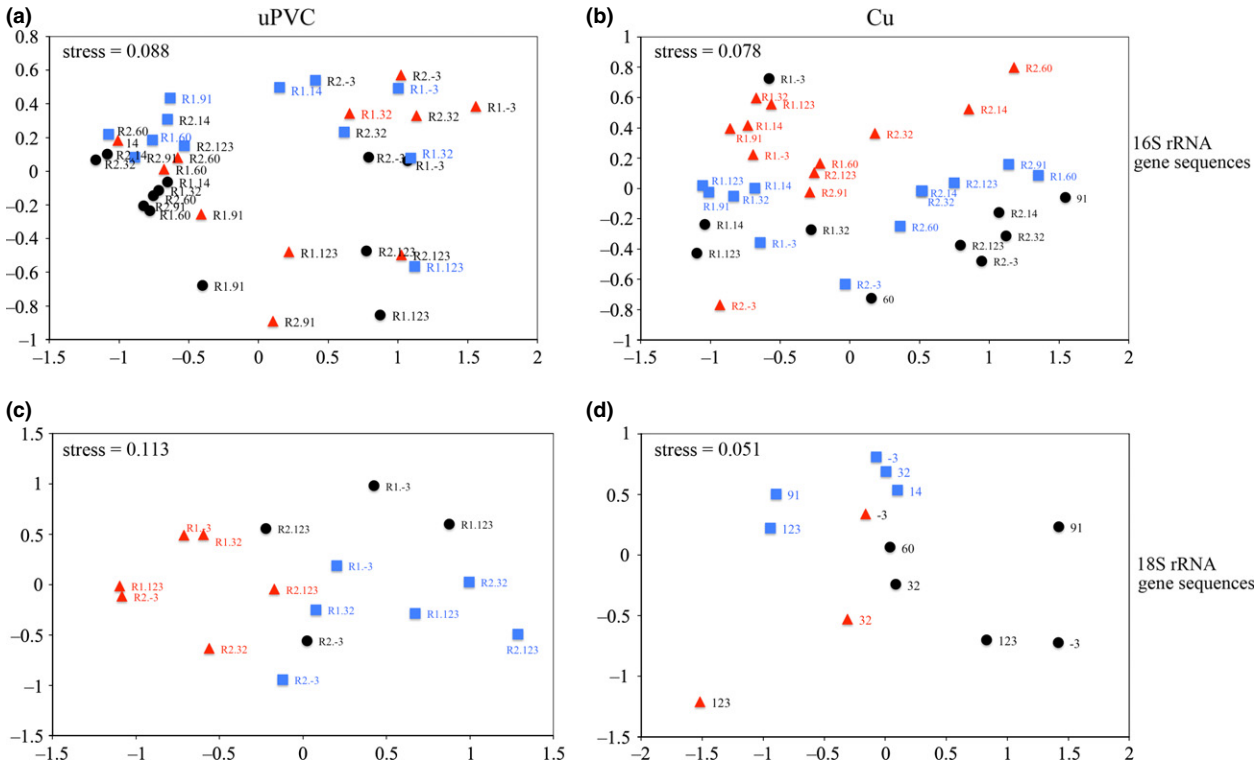


**Fig. 3.** NMDS plots derived from unweighted UNIFRAC distances. Control (black circles), Lp (blue squares), and LpAp (red triangles) samples were compared for uPVC-derived 16S (a) and 18S (c) rRNA gene sequences and for Cu-derived 16S (b) and 18S (d) rRNA gene sequences.

**Table 2.** qPCR quantification of *Acanthamoeba* spp. in uPVC and Cu biofilms*

| Samples | Time point | uPVC | | Cu | |
|---|---|---|---|---|---|
| | | R1 | R2 | R1 | R2 |
| Control | −3 | – | – | – | 5.5† |
| | 14 | – | – | – | 5.5† |
| | 32 | – | – | – | – |
| | 60 | – | – | – | – |
| | 91 | – | – | – | – |
| | 123 | – | – | – | 2.7† |
| fLp | −3 | 22.7 | – | – | 1.3† |
| | 14 | – | – | – | 2.6† |
| | 32 | – | – | – | – |
| | 60 | – | – | – | – |
| | 91 | – | – | – | – |
| | 123 | – | 2.6† | 2.7† | 13.2 |
| LpAp | −3 | – | – | – | 4.3† |
| | 14 | 5.1† | 11.1 | 29.3 | 29.4 |
| | 32 | 6.6† | 7.2 | 28.8 | 15.1 |
| | 60 | 4.7† | 8.5 | 3.0† | 4.8† |
| | 91 | 3.2† | 7.8 | 2.9† | 3.5† |
| | 123 | – | 9.5 | 7.6 | 7.6 |

–, not detected.
*Cell equivalents (CE) $cm^{-2}$.
†Less than limit of detection (LOD) 7.2 CE $cm^{-2}$.

observed in the current study. It is difficult to determine which bacterial members and what relative abundance trends contributed to the statistical significance of time groups within the uPVC-Con and -LpAp biofilms that were absent from all the other biofilm samples (Fig. 5); however, as stated above, these differences can most likely be attributed to reactor-to-reactor differences rather than any direct or indirect effect of inoculating LpAp.

In the current study, the uPVC eukaryotic biofilm communities were more diverse than Cu biofilms, but the majority of sequences in both biofilms were identified as *Cryptosporidiidae*-like (Table 1, Tables S6 and S7). Cu-Lp biofilms did not contain any *Cryptosporidiidae*-like sequences even in the day −3 samples, suggesting that the organisms may not have initially colonized this particular reactor. A majority of the Cu-Lp sequences were not assigned to any taxon under the parameters used for the BLAST assignments; thus, it is possible that no reference sequences are available for this particular group of sequences and represents novel DW biofilm microeukaryotes. The high abundance of the *Cryptosporidiidae*-like sequences is nonetheless concerning as members of this family are human DW pathogens. However, phylogenetic analysis indicates these sequences are taxonomically

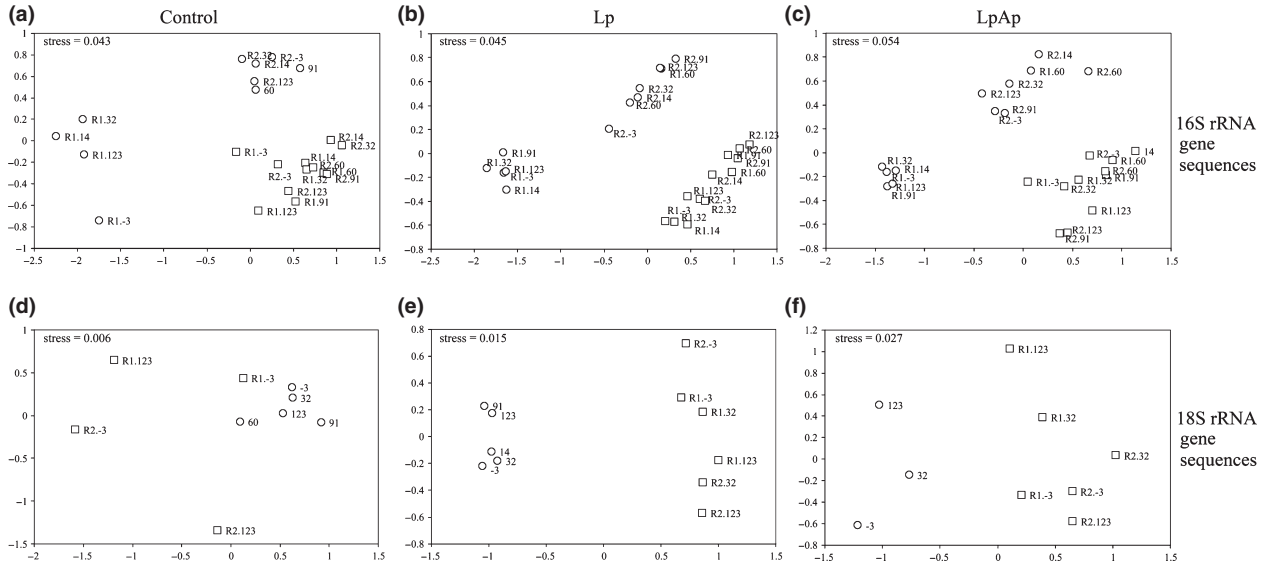


**Fig. 4.** NMDS plots derived from unweighted UNIFRAC distances. 16S and 18S rRNA gene sequences derived from uPVC (squares) and Cu (circles)-grown biofilm samples were compared between the Control (a, d), Lp (b, e), and LpAp (c, f) inoculation groups, respectively.

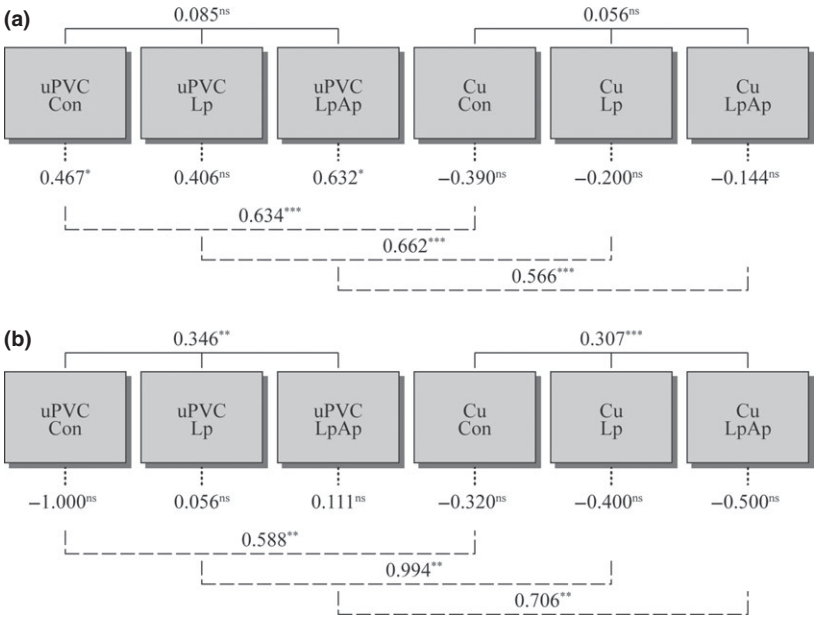


**Fig. 5.** Summary of statistical comparative analysis between the uPVC and Cu community assemblages using ANOSIM. Tests were performed for 16S (a) and 18S (b) rRNA gene sequences from uPVC and Cu biofilms. *P* and global *R*-values were generated for comparisons between Con, Lp, LpAp inoculation groups (solid lines), between time groups (dotted lines), and between substratum groups (dashed lines). Superscript annotation: ns, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P = 0.001$.

distinct from *C. parvum* and *C. hominis*, but are a sister genus to those groups within the family *Cryptosporidiidae* (data not shown). Analysis of these putative DW and DW biofilm indicator sequences is being pursued further as they were also found to have a high homology to uncultured eukaryotic sequences previously identified in DW and DW treatment plants (Kasuga *et al.*, 2007; Valster *et al.*, 2009, 2011) and cooling tower water (R.M. Valster, B.A. Wullings and D. van der Kooij, unpublished data, NCBI GenBank sequence JF774955).

The uPVC and Cu biofilms also contained a high abundance of *Vermamoeba* (*Hartmannella*)-like sequences (Table 1, Table S6 and S7). *V. vermiformis* is commonly found in DW and is susceptible to *Legionella* infectivity where intracellular replication has been observed in DW samples collected from in-premise plumbing (Nahapetian *et al.*, 1991). Thus, there were high expectations for Lp to colonize the uPVC and Cu biofilms postinoculation via parasitization of the permissive indigenous *Vermamoeba* hosts. However, previous

reports suggest that *V. vermiformis* may not be permissive to all *Legionella* strains with temperature affecting infectivity (Wadowsky *et al.*, 1991; Buse & Ashbolt, 2011) as well as secreted *V. vermiformis* factors that could inhibit *Legionella* activity (Buse *et al.*, 2013a). Future studies will examine the DW biofilm colonization potential of Lp strain Chicago-2 (previously shown to be *V. vermiformis* submissive at 32 °C) (Buse & Ashbolt, 2011).

Collectively, this work utilized pyrosequencing to describe both bacterial and eukaryotic communities on established, mature DW biofilms grown on common in-premise plumbing materials, uPVC and Cu. Microbial composition and relative abundance of each group varied greatly depending on the biofilm growth substrata, with Cu surfaces seemingly more conducive for colonization by putative environmental pathogens. Overall, the results imply upstream microbial composition impacts downstream community composition, which has also been previously observed when evaluating the bacterial composition for the various DW treatment steps (Zeng *et al.*, 2013). Notwithstanding individual reactor differences, however, the introduction of LpAp appeared to result in significant differences in the eukaryotic communities within the DW biofilms. The results from this study further the understanding of DW biofilms and their potential impacts on human health by beginning to address questions, such as, what microorganisms are indigenous within DW biofilms, are certain DW materials more prone to harbor potential environmental pathogens, and can introduction of a pathogen/host inoculum perturb the microbial community?

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Summary of uPVC and Cu DW biofilm derived 16S rRNA gene raw, quality filtered, and chimera removed sequences.

**Table S2.** Summary of uPVC and Cu DW biofilm derived 18S rRNA gene raw, quality filtered, and chimera removed sequences.

**Table S3.** Summary of uPVC and Cu DW biofilms derived from 16S and 18S rRNA gene nonchimeric sequences and Good's coverage indices.

**Table S4.** Number of uPVC-derived 16S rRNA gene sequences assigned to each bacterial family.

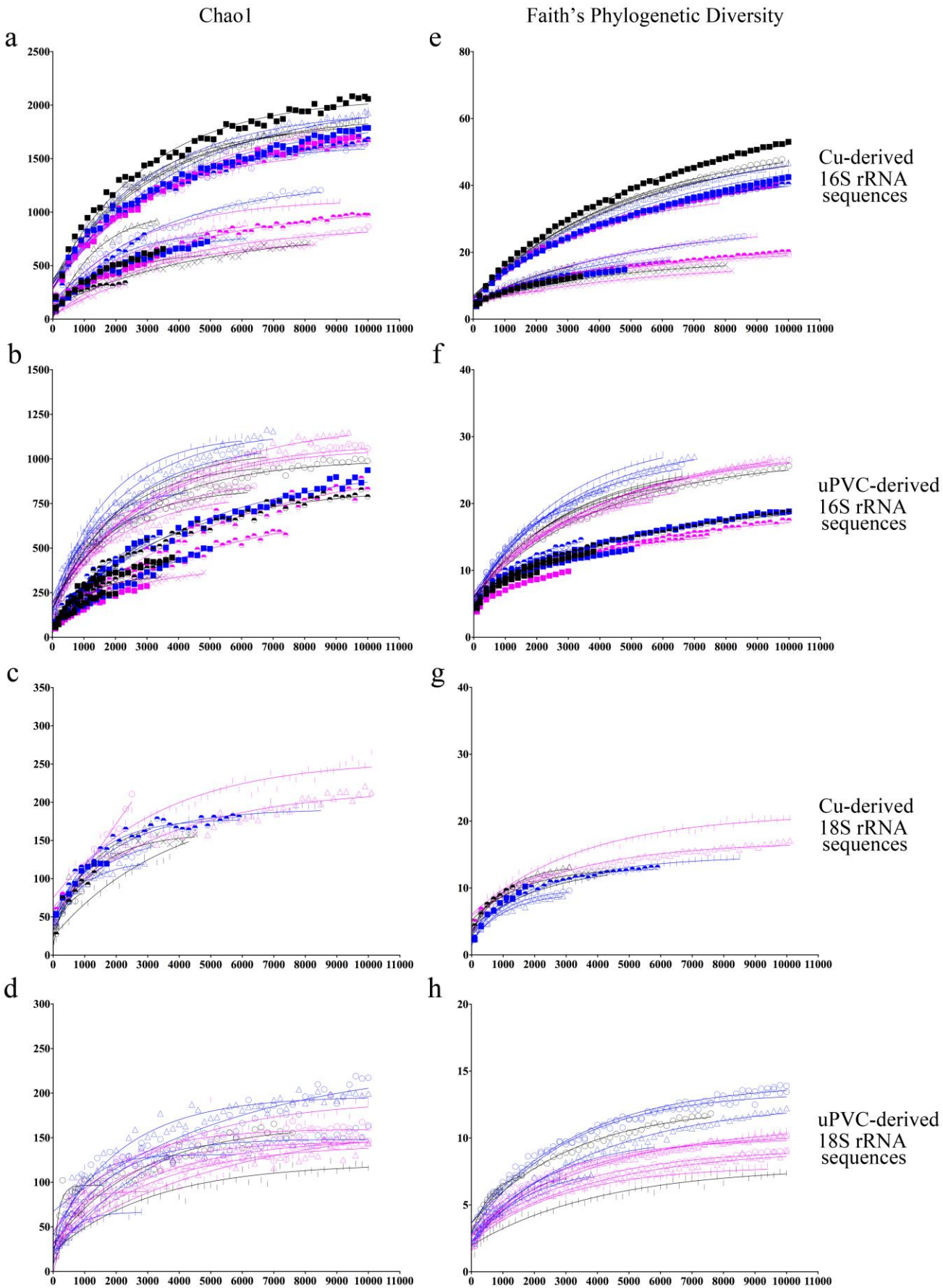
**Table S5.** Number of Cu-derived 16S rRNA gene sequences assigned to each bacterial family.

**Table S6.** Number of uPVC-derived 18S rRNA gene sequences assigned to each eukaryotic family.

**Table S7.** Number of Cu-derived 18S rRNA gene sequences assigned to each eukaryotic family.

**Fig. S1.** Rarefaction analysis for uPVC and Cu biofilm derived 16S and 18S rRNA gene sequences.

**Figure S1.** Rarefaction analysis for uPVC and Cu biofilm derived 16S and 18S rRNA gene sequences.



## Supporting Information files

**Table S1.** Summary of uPVC and Cu DW biofilm derived 16S rRNA gene raw, quality filtered, and chimera removed sequences.
application/msexcel, 17K

**Table S2.** Summary of uPVC and Cu DW biofilm derived 18S rRNA gene raw, quality filtered, and chimera removed sequences.
application/msexcel, 13K

**Table S3.** Summary of uPVC and Cu DW biofilms derived from 16S and 18S rRNA gene nonchimeric sequences and Good's coverage indices.
application/msexcel, 14K

**Table S4.** Number of uPVC-derived 16S rRNA gene sequences assigned to each bacterial family.
application/msexcel, 18K

**Table S5.** Number of Cu-derived 16S rRNA gene sequences assigned to each bacterial family.
application/msexcel, 18K

**Table S6.** Number of uPVC-derived 18S rRNA gene sequences assigned to each eukaryotic family.
application/msexcel, 13K

**Table S7.** Number of Cu-derived 18S rRNA gene sequences assigned to each eukaryotic family.
application/msexcel, 13K