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Effect of Disinfectant, Water Age, and Pipe Materials on Bacterial and Eukaryotic Community Structure in Drinking Water Biofilm

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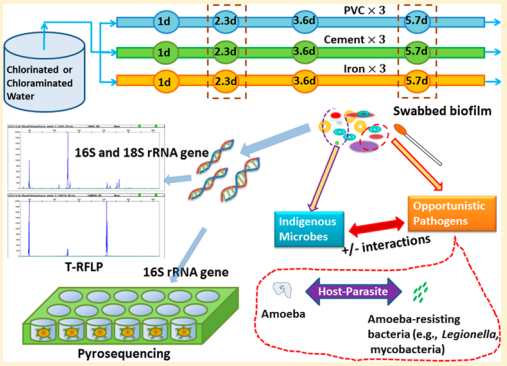
Supporting Information

ABSTRACT: Availability of safe, pathogen-free drinking water is vital to public health; however, it is impossible to deliver sterile drinking water to consumers. Recent microbiome research is bringing new understanding to the true extent and diversity of microbes that inhabit water distribution systems. The purpose of this study was to determine how water chemistry in main distribution lines shape the microbiome in drinking water biofilms and to explore potential associations between opportunistic pathogens and indigenous drinking water microbes. Effects of disinfectant (chloramines, chlorine), water age (2.3 days, 5.7 days), and pipe material (cement, iron, PVC) were compared in parallel triplicate simulated water distribution systems. Pyrosequencing was employed to characterize bacteria and terminal restriction fragment polymorphism was used to profile both bacteria and eukaryotes inhabiting pipe biofilms. Disinfectant and water age were both observed to be strong factors in shaping bacterial and eukaryotic community structures. Pipe material only influenced the bacterial community structure (ANOSIM test, $P < 0.05$). Interactive effects of disinfectant, pipe material, and water age on both bacteria and eukaryotes were noted. Disinfectant concentration had the strongest effect on bacteria, while dissolved oxygen appeared to be a major driver for eukaryotes (BEST test). Several correlations of similarity metrics among populations of bacteria, eukaryotes, and opportunistic pathogens, as well as one significant association between mycobacterial and proteobacterial operational taxonomic units, provides insight into means by which manipulating the microbiome may lead to new avenues for limiting the growth of opportunistic pathogens (e.g., *Legionella*) or other nuisance organisms (e.g., nitrifiers).



INTRODUCTION

Availability of potable water devoid of pathogens is fundamental to public health. However, drinking water systems are not sterile; rather they harbor a variety of microorganisms including bacteria, protozoa, and viruses in bulk water and biofilm.[1] While the vast majority of microbes in drinking water are likely harmless, opportunistic pathogens can also become established as part of the drinking water microbiome and are of growing concern.[2] These include agents such as *Legionella*, *Mycobacterium*, *Pseudomonas aeruginosa*, and *Acanthamoeba*. For example, *Legionella* was the most frequently reported causative agent of drinking water-associated disease outbreaks in the U.S. from 2007 to 2008.[3] Increased prevalence of *Mycobacterium* spp., *P. aeruginosa* and amoeba infections were also reported.[4−6] The values are likely underestimated, as there is no requirement for reporting disease by these agents. Free-living amoebae in drinking water are of particular concern as some members can cause severe eye and brain infections and support intracellular growth of *Legionella*, *Mycobacterium*, and other bacterial pathogens.[7]

With the onset of the "microbiome" era, it is now readily apparent that the drinking water environment is a robust ecological niche for microbes. Acceptance of this reality leads to the logical conclusion that if engineers wish to control pathogens, then they must work toward desirably controlling the drinking water microbiome. Previous studies have found several important factors that can affect the composition of drinking water microbial communities, including water treatment processes,[8] disinfection,[9−11] pipe materials,[12,13] and climate.[14] However, there is still a need for controlled, parallel, and replicated measurements of the effect of changes in factors influencing the drinking water microbiome. Further, recent advances in next-generation DNA sequencing technologies, including pyrosequencing, provide a new opportunity to extensively and systematically compare the effects of engineering manipulations on the drinking water microbiome, and may reveal interactions among microbes that can be exploited to control opportunistic pathogens. While awareness is growing of the potential importance of free-living amoebae in controlling opportunistic pathogens,[7] few molecular surveys have been carried out on eukaryotes in drinking water systems.[15−17] To date, triggers for opportunistic pathogen proliferation and

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effectiveness of engineering controls for remediating and preventing outbreaks remain unclear.

The first objective of this study was to investigate the effects of engineering factors applied in water mains, including disinfectant (chlorine and chloramine), pipe material (cement, iron and PVC), and water age (2.3 days and 5.7 days) on the drinking water microbiome using triplicate, parallel simulated drinking water distribution systems (SDSs). Pyrosequencing and terminal restriction fragment length polymorphism (T-RFLP) were applied together to provide insight into effects on the bacterial (pyrosequencing and T-RFLP) and eukaryotic (T-RFLP) components of the microbiome. The second objective was to explore potential associations among various microbial groups and opportunistic pathogens. We have previously found and measured naturally occurring *Mycobacterium* spp., *Pseudomonas aeruginosa*, *Acanthamoeba* spp., and *H. vermiformis* within these systems using quantitative polymerase chain reaction (q-PCR).[18] Our broad hypothesis is that water main conditions select for distinct microbiomes, which, in turn, influence opportunistic pathogens and possibly other nuisance organisms.[19−23] Overall, research in this area could lead to new approaches to pathogen control in drinking water systems.

MATERIALS AND METHODS

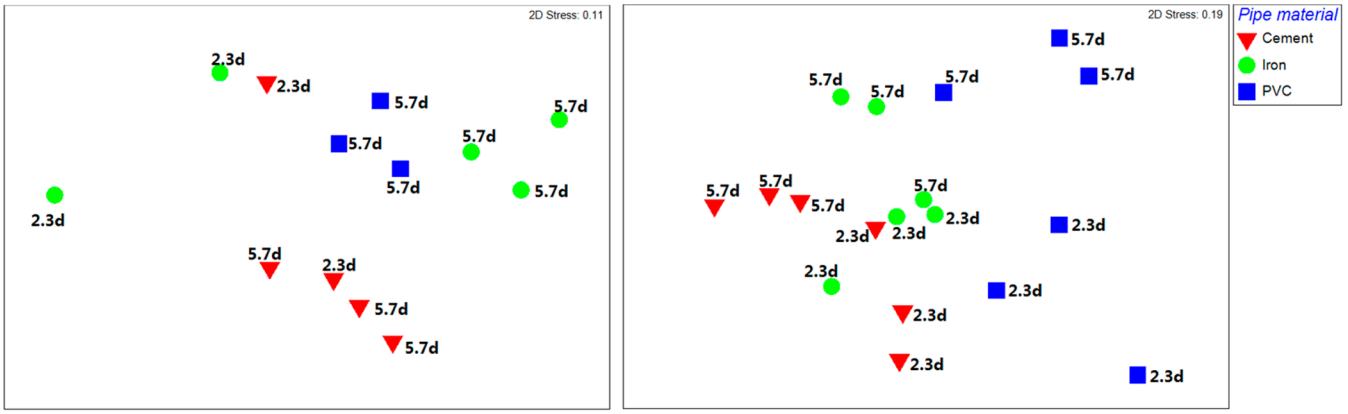
Simulated Distribution System (SDS) Set-Up and Operation. The details of the SDSs, which were constructed using new materials (Supporting Information (SI) Figure S1), have been described previously.[18] In brief, six SDSs comparing cement, iron, and PVC main pipe materials were fed with either 4.0 mg/L chlorinated or 4.8 mg/L chloraminated water from a 20L Hedwin cubitaner (Hedwin, Baltimore, MD). Iron, PVC, and cement were joined with epoxy into a total cumulative length of 62 inches that were inserted into 1.5 in.-diameter PVC pipe. The flow rate was maintained at 0.40 ± 0.005 mL/min using two 12-channel low-speed digital peristaltic pumps (Ismatec, Vancouver, WA) in all six SDSs, resulting in four water ages of 1, 2.3, 3.6, and 5.7 days from the cubitaner to the sampling ports. All six SDSs were disinfected with 500 mg/L sodium hypochlorite (30 s contact time for pipes and 24 h for tubing) prior to operation, to simulate new main disinfection practices according to AWWA standard C651,[24] and thereafter operated in parallel at ∼20 °C for about 6 months prior to biofilm sampling. Disinfectant concentrations in the cubitaner were maintained by replacing the water daily or every other day. Gloves and sterile materials were used during SDS sampling in order to minimize the introduction of contaminants.

Biofilm Sampling and DNA Extraction. Biofilm samples were collected by swabbing the inside area of the sampling port (surface area = 60 cm^2) with a sterile cotton swab and sampling downstream first to avoid contamination (SI Figure S1). This approach provided a consistent biofilm sample from the same smooth surface of PVC (a material commonly used in water mains and premise plumbing) corresponding to the water chemistry (influenced by disinfectant type, water age, and pipe material) at that point in the SDS. This was in lieu of swabbing the pipe coupons themselves, which varied in roughness and surface chemistry (e.g., iron pipe corrosion, SI Figure S1) and could not be accessed without compromising the samples. Therefore, this study investigated how the distinct water chemistry imposed by the pipe materials influenced the biofilm microbiome, which is of particular interest and practical value as pipe materials and conditions in the water main or upstream might affect nearby and downstream (e.g., premise plumbing) biofilm densities[25] and opportunistic pathogen occurrence.[26] Spatial separation between main service lines and premise plumbing may vary roughly from 0 to 100 m, and thus sampling biofilm on the most proximal PVC surface was expected to best capture potential influence of pipe main material on the downstream biofilm microbiome. DNA was recovered from cotton swabs using a FastDNASPIN Kit (MP Biomedicals, Solon, OH) according to manufacturer's protocol.[18]

Water-Quality Analysis. Disinfectant concentrations (free chlorine in chlorinated SDSs, total chlorine in chloraminated SDSs), pH, dissolved oxygen (DO), and total organic carbon (TOC) were measured every two weeks as reported previously.[18]

Pyrosequencing of Bacterial 16S rRNA Genes. Biofilm samples corresponding to water ages of 2.3 days (i.e., relatively high disinfectant residual, SI Table S1) and 5.7 days (i.e., depletion of disinfectant residual, SI Table S1) were selected for bacterial community profiling by pyrosequencing, targeting 16S rRNA genes, which was performed at the Research and Testing Laboratory, Lubbock, TX, USA (http://www.researchandtesting.com/). Diluted biofilm DNA samples (20 μg/μL) were amplified with primers 341f and 907r as follows: initial denaturation at 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 54 °C for 45s, 72 °C for 60s, followed by a final extension 72 °C for 10 min. The concentration of PCR product from each reaction was measured and mixed proportionally with each other prior to pyrosequencing on a Roche 454 FLX Titanium platform (Roche, Nutley, NJ). Resulting sequences were subject to noise-reduction and quality improvement following 454 SOP using Mothur v1.28.0.[27] Operational taxonomic units (OTUs) were generated from all samples using average-neighbor clustering with cutoff value of 3%. The taxonomic affiliation of the OTUs/sequences was determined against ribosomal database project (RDP) references using the Mothur version of the "Bayesian" rRNA classifier with a confidence threshold of 80%. Normalized OTU and phylotype outputs generated by subsampling (i.e., n = 1120 sequences for each sample) were used to calculate relative abundance, the Simpson diversity index, and Bray−Curtis similarities using Mothur or Primer-E (Plymouth, United Kingdom). The get.oturep command of Mothur was used to select one representative sequence for each *Legionella* spp., *Mycobacterium* spp., and *Pseudomonas* OTUs, which were subject to the Basic Local Alignment Search Tool (BLAST) search against 16S rRNA sequences (Bacteria and Archaea) in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find the closest species match.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) of 16S and 18S rRNA Genes. 16S rRNA genes and 18S rRNA genes from DNA samples were amplified on a Bio-Rad (Hercules, CA) C1000 thermal cycler using bacterial primers 8f/926r[28] and eukaryote primers Euk20f/Euk1179r[29] using nested PCR, respectively. Forward primers, 8f and Euk20f, were labeled with florescence FAM and VIC at the 5′ ends, respectively. Each 50 μL PCR reaction contained 1 × PCR buffer (5 prime, Gaithersburg, MD), 0.2 μM of each primer, 0.8 mM dNTPs (Promega, Madison, WI), 1.5 mM $MgCl_2$, 1 unit of *pfu* Turbo polymerase (Agilent, Santa Clara, CA) and 2 μL DNA template. The reaction for 16S rRNA genes consisted of an initial denaturation at 94 °C for 3 min, followed by 15 (first round) or 35 (second round) cycles of 30 s at 94 °C, 45 s at an annealing temperature of 60 °C, 60 s at 72



(a) Chlorinated SDSs (Pyrosequencing) (b) Chloraminated SDSs (Pyrosequencing)

Figure 1. Multidimensional scaling analysis (MDS) of SDS biofilm bacterial communities derived from pyrosequencing as a function of water age, pipe material, and (a) chlorine or (b) chloramine disinfectant. Data points are labeled with the water ages (2.3 days versus 5.7 days) at sampling ports.

°C, and a final extension step for 5 min at 72 °C. The reaction conditions for the 18S rRNA genes was: initial denaturation at 94 °C for 5 min, followed by 15 (first round) or 35 (second round) cycles of 30 s at 94 °C, 30 s at an annealing temperature of 55 °C, 90 s at 70 °C, and a final extension step for 7 min at 72 °C. PCR products were visualized using 2% agarose gel to ensure yield of sufficient PCR products.

Bacterial 16S rRNA gene and eukaryotic 18S rRNA gene amplicons for each individual sample were combined prior to being purified using a GeneClean spin kit (MP Biomedicals, Solon, OH). Ten microliters of purified PCR products were digested with 20 U *Hha*I (Promega) in a 20 uL reaction system. One microliter digested DNA was subjected to T-RFLP analysis on an ABI 3130 genetic analyzer (Applied Biosystems (ABI), Foster, CA) using previously described methods.[30] T-RFLP profiles were analyzed using GeneMapper V 4.0 (ABI) to identify valid T-RFs and bin peaks with similar size as described elsewhere.[30]

PCR, Cloning and Sequencing. Six 5.7d biofilm samples were selected for cloning/sequencing analysis based on visual inspection of T-RFLP profiles in order to identify dominant peaks. 16S rRNA gene and 18S rRNA gene amplicons were generated by PCR using same primer sets without florescence labeling and protocols described above (35 cycles). Amplicons were cloned into TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Inserts were amplified from colonies by PCR using vector-specific primers M13F and M13R. M13 PCR products were reamplified with florescence-labeled primers followed by restriction digest and T-RFLP analysis to match the clone to the closest T-RFs (< ± 1 bp difference). M13 DNA products from 128 bacterial clones and 53 eukaryotic clones were sequenced by the Virginia Bioinformatics Institute (http://www.vbi.vt.edu/). Bacterial sequences were classified to different taxonomic levels using RDP classifier with a bootstrap cutoff of 80% (http://rdp.cme.msu.edu/classifier/classifier.jsp); while eukaryotic sequences were subject to a BLAST search against the NCBI nucleotide collection database.

Quantitative Polymerase Chain Reaction (q-PCR). q-PCR methods and results for quantification of *Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba*, and *H. vermiformis* in the SDS biofilm were reported previously[18] and data were reanalyzed in this study to consider relationship to the broader microbiome. Ammonia-oxidizing bacterial and archaeal numbers were measured in 2.3 and 3.6 day biofilm samples by targeting *amo*A genes using primer sets amoA1-F/amoA2-R[31] and Arch-amoAF/Arch-amoAR[32] with an annealing temperature of 60 °C and 64 °C, respectively; while *Nitrospira* q-PCR targets *Nitrospira* 16S rDNA using primers EUB338f and Ntspa0685 M[33] with an annealing temperature of 68 °C.

Data Analysis and Statistics. Multidimensional scaling (MDS) and analysis of similarity (ANOSIM) based on Bray–Curtis similarity matrices derived from pyrosequencing and T-RFLP were performed using Primer-E.[30] Euclidean distance matrices were calculated based on physicochemical data using Primer-E. RELATE command of Primer-E was used to explore the correlation between any two similarity matrices. An exploratory tool, Biota and/or Environment matching (BEST) analysis, which is based on rank correlation between biological data and environmental physiochemical data dissimilarity resemblances was used to identify the most important environmental variables for driving microbial community composition shifts. Nonparametric Spearman Rank Correlation (r_s) analysis was used to identify the correlation between pyrosequencing and q-PCR outputs. Two-way ANOVA was used to evaluate the effects of disinfectant and pipe materials on the number of OTUs (species richness) and Simpson index at each investigated water age. The maximal information coefficient (MIC, ranging from 0 to 1),[34] a type of maximal information-based nonparametric exploration (MINE) statistic, was used to detect potential linear and nonlinear associations between OTUs representative of mycobacteria and other dominant OTUs (the first 300 OTUs) retrieved by pyrosequencing. Statistical significance was set at $p < 0.05$. Benjamini and Hochberg false discovery rate (FDR) was used for p-value correction for MIC analysis.[34]

RESULTS

Characterization of the Drinking Water Microbiome. *Pyrosequencing.* A total of 65 993 pyrosequences were obtained from 31 biofilm samples, with an average sequence yield of 2128 ± 832 for each sample (range 1120–5117). 1541 OTUs were defined based on clustering with a similarity cutoff of 97%, with 754 singleton OTUs. Sample coverage ranged between 93–97%, suggesting that the medium-depth pyrosequencing captured the majority of unique bacterial OTUs. Five

samples (i.e., chlorinated iron 2.3 day pipe 1, chlorinated PVC 2.3 day pipes 1−3, chlorinated cement 2.3 day pipe 1) were excluded from pyrosequencing analysis due to insufficient sequences (i.e., <700) likely as a result of high chlorine levels. These samples also yielded relatively lower 16S rRNA gene copy numbers (i.e., <7500 gene copies/cm^2 [18]) and low intensity PCR products, despite optimization efforts.

Pyrosequences were assigned to 14 phyla, of which *Proteobacteria* was the most abundant, accounting for 66−98% of total pyrosequences across all samples (SI Figure S2). The other two dominant phyla were *Bacteroidetes* (0.4−24%) and *Actinobacteria* (0.3−8.4%), the former mainly were comprised of *Sphingobacteria* and *Flavobacteria*. About 0.2−8.6% of pyrosequences were unclassified at phylum level. In general, *α-Proteobacteria* (25−96%), and *β- Proteobacteria* (1.2−69%) were the most dominant *Proteobacteria*, followed by *γ- Proteobacteria* (0.2−10%), and *δ- Proteobacteria* (0−0.4%). Less than 1.2% of pyrosequences could not be identified beyond *Proteobacteria*.

Comparison of T-RFLP with Pyrosequencing. As expected, the number of T-RFs retrieved from T-RFLP (7−27) was generally less than the number of OTUs obtained by pyrosequencing (62−132) (SI Tables S2 and S3). The identities of the thirty most abundant pyrosequencing OTUs (i.e., descending abundance from OTU1 to OTU 30 across all samples) are compared with those of the most abundant T-RFs in SI Table S4. Corresponding T-RFs were identified for 19 out of 22 pyrosequencing OTUs classified at the genus level. The most abundant T-RFs (i.e., 78.8 bp, 202.2 bp, 337.1 bp, and 341.1 bp) also corresponded with the dominant pyrosequencing OTUs. However, some genera identified by sequencing T-RFs clones were not found by pyrosequencing, which is likely a result of different primers.[35] MDS plots of T-RFLP profiles of biofilm bacteria demonstrated similar patterns with those generated from pyrosequencing data (Figure 1, SI Figure S3). RELATE analysis further confirmed agreement between results from pyrosequencing and T-RFLP, based on significant correlations between the Bray−Curtis matrices derived by these two methods (chlorinated SDSs: ρ = 0.413, P = 0.008; chloraminated SDSs: ρ = 0.639, P = 0.001). Thus, pyrosequencing and T-RFLP provided similar snapshots of the bacterial community composition.

Effect of Disinfectant, Pipe Material, and Water Age on the Drinking Water Microbiome. Comparison of biofilm samples corresponding to water ages of 2.3 and 5.7 days provided insight into the effects of contrasting water chemistries created by different disinfectant, pipe material, and water ages. As described previously,[18] lower disinfectant residuals were observed in 2.3 days chloraminated systems (0.15−0.36 mg/L, SI Table S1) compared to 2.3 days chlorinated systems (0.75−2.57 mg/L, SI Table S1) due to faster chloramine decay triggered by nitrification. This was supported by detection of *amo*A genes in chloraminated SDSs. The average disinfectant concentrations were <0.1 mg/L in all 5.7 day samples, except for higher chlorine residuals in chlorinated PVC SDSs (i.e., 0.40 ± 0.13 mg/L, SI Table S1). Faster disinfectant depletion compared to real-world drinking water distribution systems was likely associated with a higher (room) temperature and low flow rate (0.40 mL/min) used in this study, which was necessary to achieve a wide range of water ages (i.e., 1−5.7 days) in manageable lab-scale pipe systems. In chlorinated SDSs, chlorine decreased faster in iron SDSs compared to cement and PVC SDSs. Decreased DO with water age was observed in all SDSs, while an increase of pH was only observed in cement SDS due to lime leaching (SI Table S1). Thus, SDSs generated a range of water chemistry scenarios occurring in real-world drinking water systems that might have various influence in drinking water biofilm composition, although precise effect of shear stress that are typically imposed on biofilms in distribution mains[36,37] could not be simulated because of the low flow rate selected to achieve varying water ages.

Effect on the Bacterial Microbiome. MDS plots comparing pyrosequencing of biofilm bacteria in chlorinated versus chloraminated SDSs are shown in Figure 1. In most cases samples from triplicate pipes clustered, especially at 5.7 day water age. Higher variance in community structures of 2.3 day samples is likely due to greater differences in physiochemical conditions (e.g., disinfectant concentrations[18]) among triplicate pipes as well as relatively lower DNA yield.[38] Two-way ANOSIM demonstrated significant effects of pipe materials (r = 0.654−0.884, P = 0.001) and water age (r = 0.494−0.583, P < 0.05) on the bacterial microbiome in both chlorinated and chloraminated SDSs (Table 1). The effect of disinfectant on biofilm bacteria was examined by comparing chlorinated and chloraminated SDS biofilm samples at both water ages (SI Figure S4). ANOSIM tests demonstrated that the bacterial biofilm community in chlorinated SDSs was distinct from that in chloraminated SDSs (r = 0.568−0.667, P < 0.01, Table 2).

Table 1. Effects of Water Age and Pipe Materials on Bacterial Communities Using Two-Way ANOSIM

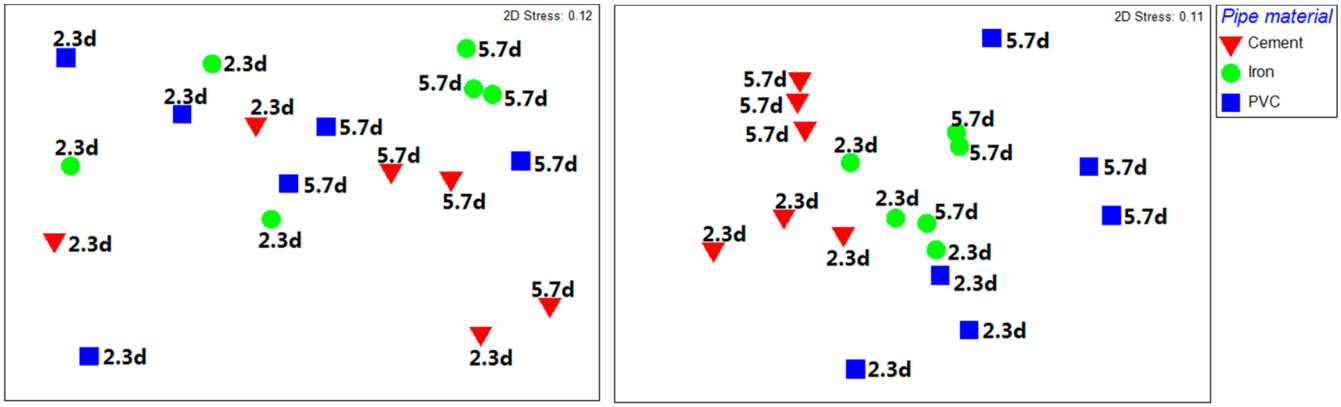
		pyrosequencing		T-RFLP	
SDSs	factors	global r value	p value	global r value	p value
chlorinated SDSs					
	water age	0.583	**0.04**	0.531	**0.003**
	pipe material	0.884	**0.001**	0.189	0.051
	Pairwise Tests (Across All Water Age Groups)				
	iron vs cement	0.886	**0.033**	0.259	0.07
	iron vs PVC	0.889	0.1[a]	0.259	0.1
	cement vs PVC	1	0.1[a]	0.074	0.3
chloraminated SDSs					
	water age	0.494	**0.008**	0.605	**0.001**
	pipe material	0.654	**0.001**	0.663	**0.001**
	Pairwise Tests (Across All Water Age Groups)				
	iron vs cement	0.704	**0.01**	0.63	**0.02**
	iron vs PVC	0.667	**0.01**	0.574	**0.01**
	cement vs PVC	0.574	**0.02**	0.815	**0.01**

[a]The p value was limited by the possible permutations (n = 10).

No effects of disinfectant, pipe material, or water age were observed on the total number of OTUs (i.e., species richness) (P > 0.05) (SI Table S2). No effect of these factors was observed on $1/\lambda$ (Inverse of the Simpson index) either, except that higher $1/\lambda$ values (higher diversity) were observed in chlorinated iron 2.3 day samples compared to chlorinated cement 2.3 days samples, and in chlorinated iron 5.7 days biofilm samples compared to 2.3 day samples (P < 0.05). Similarly, effects of disinfectant, pipe material, and water age on

Table 2. Effect of Disinfectant on Bacterial Communities (Two-Way ANOSIM, Across All Pipe Material Groups)

	bacterial community				eukaryotic community	
	pyrosequencing		T-RFLP		T-RFLP	
chlorinated SDSs vs chloraminated SDSs	global r value	*p* value	global *r* value	*p* value	global *r* value	p value
2.3 days	0.667	**0.001**	0.654	**0.003**	0.457	**0.016**
5.7 days	0.568	**0.004**	0.506	**0.008**	0.37	**0.008**



(a) Chlorinated SDSs (T-RFLP) (b) Chloraminated SDSs (T-RFLP)

Figure 2. Multidimensional scaling analysis (MDS) of SDS biofilm eukaryotic microbial communities derived from T-RFLP as a function of water age, pipe material, and (a) chlorine or (b) chloramine disinfectant. Data points are labeled with the water ages (2.3 days versus 5.7 days) at sampling ports.

number of T-RFs and $1/\lambda$ were not apparent with only two exceptional cases (i.e., higher $1/\lambda$ in chloraminated iron 5.7 day vs chloraminated iron 2.3 day samples, higher $1/\lambda$ in chlorinated iron 2.3 day vs chloraminated iron 2.3 day samples; $P < 0.05$; SI Table S3).

Effect on the Eukaryotic Microbiome. The eukaryotic microbiome of biofilm samples was profiled using T-RFLP. Figure 2 compares the MDS plots of the eukaryotic microbiomes of chlorinated and chloraminated systems. Different disinfectants resulted in distinct eukaryotic microbiomes ($r = 0.37–0.457$, $P < 0.05$, Table 2). ANOSIM analysis indicated that water age was also an important factor shaping the biofilm eukaryotes (Table 3). A significant pipe effect was only observed upon comparison of cement and PVC in the chloraminated SDSs ($P < 0.05$, Table 3).

Table 3. Effects of Water Age and Pipe Materials on Eukaryotic Communities Derived from T-RFLP

	chlorinated SDSs		chloraminated SDSs	
factors	global *r* value	*p* value	global *r* value	*p* value
water age	0.494	**0.005**	0.42	**0.022**
pipe material	0.082	0.232	0.239	**0.047**
Pairwise Tests				
iron vs cement	0.056	0.33	0.315	0.11
iron vs PVC	0.241	0.20	0	0.42
cement vs PVC	0.037	0.45	0.389	**0.03**

At water age of 2.3 days, fewer eukaryotic T-RFs were observed in chlorinated iron and PVC systems compared to corresponding chloraminated systems ($P < 0.05$). Otherwise, pipe materials and disinfectant type appeared to have little effect on the total number of T-RFs or $1/\lambda$ (SI Table S5). A significant water age effect on the number of T-RFs was only observed in the chlorinated PVC systems, while effect of water age on $1/\lambda$ was only observed in chlorinated iron systems ($P < 0.05$).

Seven OTUs were found from forty-six successfully sequenced eukaryote clones, corresponding to thirteen T-RFs found in T-RFLP profiles (Table 4). Based on a BLAST search of the GenBank database, the sequences exhibited highest similarity with those of *Fusarium oxysporum*, *Colpoda steinii*, *Sarocladium strictum*, *Hartmannella vermiformis*, *Acanthamoeba* spp., *Cercozoa* spp., and an *Eimeriidae* environmental sample clone, respectively, in an order of descending abundance. Presence of *Hartmannella vermiformis* and *Acanthamoeba* spp. was previously confirmed by q-PCR.[18]

Relationships between SDS Microbiomes and Physiochemical Data. RELATE analysis demonstrated a significant correlation between similarity matrices derived from microbial communities and environmental physiochemical data (SI Table S1) [$\rho = 0.356$, $P = 0.001$ for bacterial community (pyrosequencing); $\rho = 0.22$, $P = 0.001$ for eukaryotic community (T-RFLP)]. This implies that the drinking water microbiome was significantly influenced by the water chemistry. BEST indicated that disinfectant concentration and DO were the most important individual factors driving bacterial ($\rho_w = 0.274$, pyrosequencing) and eukaryotic ($\rho_w = 0.263$, T-RFLP) community compositions, respectively. However, relatively lower ρ and ρ_w values (i.e., <0.5) imply that there are other uninvestigated factors that also influenced the microbial communities in drinking water biofilms.

Relationships between Bacteria, Eukaryotes, and Opportunistic Pathogens. Bacterial similarity matrices derived from pyrosequencing (RELATE analysis: $\rho = 0.198$, $P = 0.001$) and T-RFLP ($\rho = 0.339$, $P = 0.001$) demonstrated a moderate correlation with eukaryotic similarity matrices derived from T-RFLP. Similarity matrices derived based on opportunistic pathogens (i.e., *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba* spp., *H. vermiformis*[18]) were correlated with overall bacterial (RELATE analysis: $\rho = 0.27$, $P = 0.004$

Table 4. Identities of Eukaryotic Clones/T-RFs

T-RFs from T-RFLP[1]	clone ID	blast results of sequences	max identity	accession No.
180	V69, V58	*Acanthamoeba* spp.[4]	99%	GU320589.1, GU808288.1
205.4[2]	V72	*Cercozoa* sp. xt43 18S rRNA gene	91%	EU709247.1
255.1	V66	NA[3]		
262.4	V70, V75, V77, V78, V82, V84, V88	*Sarocladium strictum* strain CBS 346.70 18S rRNA gene[5)]	99%	HQ232211.1
405.1, 406.1	V41, V44,V45, V59, V60, V62, V63, V64, V65, V67, V76,V89, V93,V94	*Colpoda steinii* strain Sp1 small subunit rRNA gene[6)]	99%	DQ388599.1
411.9, 412.4, 413.3	V42, V43, V46, V47, V48, V49, V51,V52, V53,V55,V56, V68, V73, V91,V92,	*Fusarium oxysporum* strain OUCMDZ-630 18S rRNA gene[5)]	100%	JN604549.1
420.7, 422.7, 423.9	V71, V74, V79,V85, V80, V81	*Hartmannella vermiformis*[4] partial 18S rRNA gene	99−100%	FR832469.1
475.6	V83	*Eimeriidae* environmental sample clone	80%	EF023130.1

[1]Length of T-RFs for each clone were rounded up to the T-RFs found in biofilm sample T-RFLP profiles if available. [2]T-RF found for clone, but not found in biofilm sample T-RFLP profiles. [3]Sequencing failed. [4]amoeba. [5)]fungi. [6)]ciliate.

(pyrosequencing)) and eukaryotic (ρ = 0.228, P = 0.007(T-RFLP) communities. MIC analysis[34] identified 74 pairs of significant associations among the first 300 abundant pyrosequencing OTUs, with an association found between one mycobacterial OTU (OTU20) and one unclassified proteobacterial OTU (OTU123) (MIC = 0.44, P = 0.019).

Detection of *Legionella*, *Mycobacterium*, and *P. aeruginosa*, by Pyrosequencing. A total of 483 sequences (out of 65 993 sequences) were classified as *Mycobacterium* spp., clustering into six OTUs (SI Table S6). Analysis of representative sequences revealed closest matches with *Mycobacterium mucogenicum* (Identity (I) = 100%, n = 474), *Mycobacterium avium/Mycobacterium colombiense* (I = 99%, n = 3), *Mycobacterium intermedium/Mycobacterium saskatchewanense* (I = 98%, n = 3), *Mycobacterium mucogenicum/Mycobacterium pallens* (I = 97%, n = 1), *Mycobacterium branderi* (I = 97%, n = 1), and *Mycobacterium abscessus/Mycobacterium massiliense* (I = 98%, n = 1). Seven sequences were classified as *Legionella* spp., clustering into three OTUs, which most closely matched *Legionella pneumophila* (I = 98%, n = 5), *Legionella waltersii* (I = 99%, n = 1), and *Legionella maceachernii/Legionella micdadei* (I = 95%, n = 1). There were 78 sequences with highest similarity to *P. aeruginosa* (I = 100%).

Pyrosequencing and q-PCR provided complementary and consistent information in terms of opportunistic pathogen detection. The number of pyrosequences classified as *Mycobacterium* was positively correlated with the gene copy numbers detected by q-PCR (r_s = 0.5996, P < 0.001). Rare detection of *Legionella* spp. was also consistent with their qPCR results. *P. aeruginosa* was more readily detected by q-PCR than pyrosequencing (84% detection rate vs 32%), which is not surprising due to the use of species-specific primers; while pyrosequencing employed general primers for 16S rRNA genes, and strongly depends on sequencing depth in terms of detection of rare groups.

Detection of Nitrifiers by Pyrosequencing and q-PCR. Only eight pyrosequences affiliated with nitrification (all *Nitrospira* genus) were recovered across all chloraminated and chlorinated SDS biofilm samples. *Nitrospira* was not detected by q-PCR. No sequences affiliated with other nitrifiers (e.g., *Nitrosomonas*, *Nitrosospira*, *Nitrobacter*) were found by pyrosequencing, while q-PCR demonstrated the presence of bacterial *amo*A genes in chloraminated systems, with the highest number of 783 ± 100 gene copies/cm^2 detected in one iron 2.3 day biofilm sample. The relationship between detection of nitrifiers by q-PCR versus by pyrosequencing was consistent with *P. aeruginosa* detection and is likely driven by primer specificity and sequencing depth. No known archaeal nitrifiers were detected by q-PCR.

DISCUSSION

Engineering Factors Impacting Drinking Water Bacteria in SDSs. This study demonstrated clearly that engineering factors, such as disinfectant type and concentration, pipe material, and water age strongly influence the composition of the microbiome. However, specific effects of disinfectant, pipe material, and water age may vary in different distribution systems with various source water qualities. Repeatability among triplicate pipe materials is an encouraging indicator that such engineering controls could be applied with some confidence to intentionally manipulate the microbiome, as suggested in prior field research.[26,39] For example, a range of conditions could be identified that inhibits opportunistic pathogens by encouraging the establishment of competing microorganisms and/or eliminates host organisms that enhance their proliferation (i.e., amoebae).[40]

Effect of Disinfectant. Different disinfection modes of chlorine and chloramine likely played a major role in shaping the corresponding differences in the microbiome. Chloramine has been reported to penetrate biofilm more strongly, while chlorine may better inactivate microorganisms near the biofilm surface.[41] Others have similarly reported distinct bacterial communities in chlorinated versus chloraminated drinking water systems. For example, it was found that *Mycobacterium* and *Dechloromonas* were dominant components in chloraminated SDS biofilms, while a variety of α- and β- *Proteobacteria* dominated the chlorinated SDS biofilms.[11] Another metagenomic analysis of drinking water samples demonstrated *Mycobacterium*, *Acidovorax*, *Burkholderia*, *Pseudomonas*, and *Dechloromonas* were dominant in chloraminated water, while *Caulobacter*, *Rhodopseudomonas*, *Synechococcus*, *Bradyrhizobium*, and *Pseudomonas* were the most abundant members in chlorinated water.[10] However, in our study, biofilm from chlorinated and chloraminated SDSs shared same major components including *Novosphingobium*, *Bradyrhizobium*, *Hyphomicrobium*, *Acidovorax*, *Methylobacterium*, *Sphingomonas*, and *Afipia*, though their relative abundances differed in most cases. Source water quality is a powerful influence and may

have contributed to different microbial compositions in biofilm and the greater similarity observed between the two disinfectants in this study relative to published studies[10,11] and likely to real-world distribution systems. Note that the present study used breakpoint-chlorination-treated Blacksburg chloraminated tap water after treating surface water by prechlorination, flocculation, sedimentation and dual-medium filtration to prepare chlorinated and chloraminated feedwater, which imposed a double disinfection toward source water microbes; while the two other studies[10,11] directly used ammonia-amended chlorinated water. Though double disinfection is not an uncommon practice in actual water systems, intense disinfection in the present study (i.e., break-point chlorine plus relative high dose of disinfectants in reservoir) might influence the microbiome by selecting some viable disinfectant-resistant and/or biofilm-forming microbes in the source water. However, this approach also helped maintain a relatively consistent influent microbiome, regardless of temporal variations. Also, the relatively lower chloramine residuals for 2.3 day and 5.7 day samples, compared to chlorinated SDSs, likely reduced the differences between chlorinated and chloraminated SDSs in this study.

Effect of Pipe Material. Significant effects of pipe materials on bacterial communities in chlorinated and chloraminated systems, together with higher 16S rRNA genes (indicative of total bacteria) and lower *Mycobacterium*, *P. aeruginosa*, and *Acanthamoeba* gene copy numbers in the iron systems (mainly in chloraminated systems [18]), imply that pipe material was a major factor governing the drinking water microbiome. Distinct bacterial communities have also been observed on steel, copper, stainless steel, and PVC coupons exposed to same drinking water in annular reactors.[12] Note, however, that the present study focused on how the distinct water chemistries imposed by the pipe materials influenced biofilm microbiome on nearby PVC surface, as the representative distribution system pipe material coupons themselves were not directly swabbed. In addition to surface roughness, chemical properties of pipe materials have been observed to play an important role in shaping biofilm communities.[42] In the present study, iron and cement have higher surface area compared to PVC due to high porosity, which is favorable for biofilm establishment.[43] Further, the iron-corrosion observed in chlorinated and chloraminated coupons is a complex process involving chemical (e.g., iron react with disinfectant, O_2) and biological (e.g., microbial corrosion) reactions, which can result in lower disinfectant residual, dissolved oxygen (SI Table S1), and bioavailable nutrients (e.g., fixed C, N, P[44]).

Effect of Water Age. Significant shifts among microbes were observed to be associated with water age and could have been a direct effect of changes in water chemistry, including depletion of disinfectant residual, increased TOC, and decreased DO with increased water age. Increased gene copy numbers of 16S rRNA, *Mycobacterium*, *P. aeruginosa*, *H. vermiformis*, *Acanthamoeba* were consistently observed with increased water age, demonstrating negative associations with disinfectant concentrations.[18] Importantly, water age, disinfectant, and pipe material do not act independently. Rather, they interact with each other to create distinct physiochemical conditions and ecological niches, in which various microbes can be selected and enriched in drinking water systems. Combined effects of these factors have been observed on the relative abundance of various drinking water microorganisms in other studies,[45,46] and specifically on numbers of total bacteria and opportunistic pathogens in our previous study of these same systems.[18]

Comparison of Molecular Methods Employed. Generally, the taxonomic phylotype distributions of the systems were similar to two previous pyrosequencing studies of bulk water in drinking water treatment plant[8] and water-meter biofilms.[47] Comparison of pyrosequencing and T-RFLP provided robust characterization of the microbiome and also is useful for interpreting past studies employing T-RFLP as next-generation DNA sequencing tools become more widely adopted. Similarity of T-RFLP and pyrosequencing has also been reported in studies investigating human throat and gut microbiome[48] and streamwater and biofilm samples.[49] However, T-RFLP is more susceptible to biased microbial diversity estimation due to generation of pseudo-T-RFs[50] as well as low resolution of T-RF (i.e., one T-RF may represent multiple microorganisms from different genus, for example, 78.8 bp in SI Table S4). This may partially explain why a significant effect of pipes on bacterial communities (Table 1) was observed in chlorinated SDSs by pyrosequencing but not T-RFLP.

Factors Impacting Eukaryotes in Simulated Distribution Systems. Eukaryotes were of special interest in this study as they represent potential targets for opportunistic pathogen control and few surveys have been conducted of eukaryote occurrence in drinking water systems.[7,15−17] To the authors' knowledge, this study is the first to demonstrate a distinct shaping force of disinfectant type and water age toward drinking water eukaryotes. Effect of pipe materials on eukaryotic communities was not apparent in chlorinated SDSs, which is consistent to the pipe materials effect on bacterial communities profiled by T-RFLP but not pyrosequencing. In contrast to bacteria, effects of pipe material on eukaryotes were less apparent (only between chloraminated PVC and cement SDSs) in chloraminated SDSs based on T-RFLP profiling of 16S and 18S rRNA genes. Future studies employing deep sequencing of eukaryotic communities will likely help delineate the effect of pipe materials.

Further fine-scale investigation on how environmental factors affect drinking water eukaryotes, especially free-living amoeba, will be of value considering that controlling amoebae may be a viable avenue for regulating numbers of opportunistic pathogens, such as *Legionella* and *Mycobacterium*.[7] Poitelon and colleagues[15] recovered 24 eukaryotic OTUs in finished chlorinated drinking water samples from three different water treatment plants in France based on 122 clones containing 18S rDNA sequence inserts. Ten to 13 OTUs were found in each water sample, which is comparable to 5−17 T-RFs detected in the present study. Clone libraries of water and biofilm samples from two unchlorinated drinking systems revealed highly diverse eukaryotic communities, with 219 OTUs found from 545 sequences.[16] This highlights a potentially important consequence of not disinfecting water, as it is not known how eukaryotic diversity might influence opportunistic pathogen numbers. In contrast to relatively low percentage of fungi clones in the two above-mentioned libraries and another recent constructed clone library from building water[17] (i.e., ≤8.9%), the clone library in this study was dominated by fungi *Fusarium oxysporum* (32.6%) and *Sarocladium strictum* (15.2%), both of which are known plant pathogens.[51,52]

Kinds of protozoa selected could also be of importance, given that opportunistic pathogens vary in their host selectivity.[53] *Colpoda steinii* was the most abundant ciliate found in this study (accounting for 30% of clones). Examination of T-RFLP profile

revealed that T-RFs representative of *C. steinii* were commonly found in chloraminated SDSs, regardless of pipe materials, while they were mainly detected in chlorinated cement SDSs. *C. steinii* are found in soil and freshwater and have been used as a bioindicator for heavy metal pollution.[54,55] Free-living amoebae, *Acanthamoeba* and *H. vermiformis*, accounted for 17% of the clones. Relatively high abundance of *H. verimiformis* was consistent with results from pyrosequencing analysis of 18S rRNA gene in two faucet biofilm samples.[56]

Insight into Ecological Interactions Between Drinking Water Microbes and Opportunistic Pathogens. Pathogens do not exist in isolation, rather, they are vital components of a robust microbiome built upon an array of ecological interactions such as competition, antagonism, symbiosis, and commensalism. The significant associations between 74 pairs of pyroseqeuncing OTUs further illustrated the complexity of drinking water ecological interactions. There is currently no "silver bullet" solution to opportunistic pathogen control with existing engineering controls. For example, disinfection might knock out fast growers and some sensitive predators (e.g., *H. vermiformis*), but not disinfectant-resistant opportunistic pathogens (e.g., *Acanthamoeba*, *Legionella*, mycobacteria)[57] Therefore, intentionally manipulating the microbial ecology represents an avenue worth exploring in order to alter survival, persistence, and virulence of opportunistic pathogens.[40] Previous studies have provided insight into potentially important ecological interactions that could be exploited. *Acidovorax* sp. and *Sphingomonas* sp., two dominant genera found in SDS biofilm samples, have been demonstrated to have a negative effect on *L. pneumophila* cultivability.[58] Similarly, a variety of other aquatic bacteria, including *Pseudomonas* spp., *Acinetobacter* spp., *Flavobacterium* spp., *Bacillus* spp., *Stenotrophomonas maltophilia*, *Aeromonas hydrophila*, *Burkholderia capaciai*, which were commonly detected in this study, are capable of producing toxins (e.g., bacteriocin-like substances (BLSs), proteases) that act against *L. pneumophila*.[21,22,59] Moreover, presence of background microbes is known to play a role in reducing infectivity of *Legionella* and *Mycobacterium* toward amoeba.[19,20] In this study, only one significant association was found between an opportunistic pathogen OTU (OTU 20: *Mycobacterium* spp.) and other microbes (OTU123: unclassified *Proteobacteria*). Lack of associations between opportunistic pathogens and other microbes may be a result of shallow to medium sequencing, which might neglect rare species. Studies employing deeper sequencing of 16S and 18S rRNA genes may help reveal more interactions among different drinking water microbes.

The correlation between eukaryotic and bacterial community similarity matrices (RELATE analysis) highlight the need for deeper understanding of the ecological role of eukaryotes, especially free-living amoeba. On one hand, amoebae can help maintain biofilm density via biofilm grazing.[60] On the other hand, they are involved in a critical relationship with multiplication, virulence, and persistence of *Legionella*, *Mycobacterium*, and other amoeba-resisting bacteria in drinking water.[7] This host-parasite relationship was also intimated in positive correlations between *Mycobacterium* spp., *P. aeruginosa*, and *Acanthamoeba* in biofilms.[18] The important role of free-living amoebae has also been recognized by other researchers claiming that monitoring/controlling them in drinking water will lead to reduction of drinking water pathogen numbers.[7,61] Therefore, complex ecological interactions are vital factors governing ultimate opportunistic pathogen risk in drinking water and cannot be ignored in developing effective strategies for opportunistic pathogen control.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Information includes SDS physiochemical parameters (Table S1), OTU and diversity characterization of pyrosequencing and t-RFLP data (Tables S2, S3, and S5), comparison of pyrosequencing and t-RFLP (Table S4), and BLAST results from sequences most related to *Legionella*, *Mycobacteria*, and *Pseudomonas* (Table S6). Figures include an illustration of the SDS and sampling approach (Figure S1), relative phyla/class abundances (Figure S2), MDS analysis of bacterial t-RFLP profiles (Figure S3), and MDS analysis of bacterial pyrosequences organized by water age (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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