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Article

Premise Plumbing Pipe Materials and In-Building Disinfectants Shape the Potential for Proliferation of Pathogens and Antibiotic Resistance Genes

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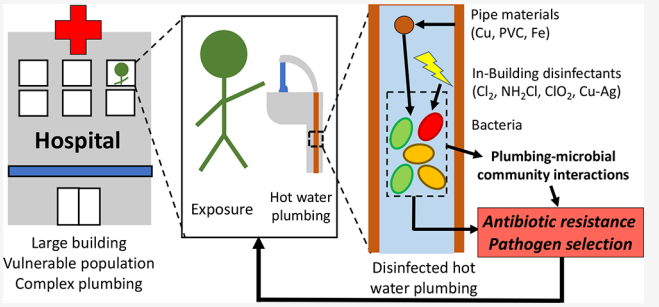
ABSTRACT: In-building disinfectants are commonly applied to control the growth of pathogens in plumbing, particularly in facilities such as hospitals that house vulnerable populations. However, their application has not been well optimized, especially with respect to interactive effects with pipe materials and potential unintended effects, such as enrichment of antibiotic resistance genes (ARGs) across the microbial community. Here, we used triplicate convectively mixed pipe reactors consisting of three pipe materials (PVC, copper, and iron) for replicated simulation of the distal reaches of premise plumbing and evaluated the effects of incrementally increased doses of chlorine, chloramine, chlorine dioxide, and copper−silver disinfectants. We used shotgun metagenomic sequencing to characterize the resulting succession of the corresponding microbiomes over the course of 37 weeks. We found that both disinfectants and pipe material affected ARG and microbial community taxonomic composition both independently and interactively. Water quality and total bacterial numbers were not found to be predictive of pathogenic species markers. One result of particular concern was the tendency of disinfectants, especially monochloramine, to enrich ARGs. Metagenome assembly indicated that many ARGs were enriched specifically among the pathogenic species. Functional gene analysis was indicative of a response of the microbes to oxidative stress, which is known to co/cross-select for antibiotic resistance. These findings emphasize the need for a holistic evaluation of pathogen control strategies for plumbing.



KEYWORDS: *drinking water, opportunistic pathogens, disinfectants, pipe materials, metagenomics*

INTRODUCTION

The portion of the potable water supply delivered in buildings, i.e., premise plumbing, is vulnerable to colonization by opportunistic premise plumbing pathogens (OPPPs).[1−3] OPPPs, such as and *Legionella*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, are the leading cause of drinking water-related illness in the United States.[4,5] In particular, warm temperatures associated with hot water lines, stagnation between water uses,[5] and a wide array of pipe materials shape key chemical features of the water[6] that also can facilitate OPPP growth. A key challenge, at present, is identifying conditions that effectively control multiple OPPPs.[6,7]

In-building disinfectants are commonly applied to limit the growth of OPPPs in the premise plumbing of large buildings, especially hospitals and other facilities, where there is a need to protect at-risk populations. However, in-building disinfectant application has not been well optimized and can have unintended and poorly understood effects.[3] For example, chloramine application in drinking water distribution systems has been shown to reduce the microbial diversity and increase the overall dominance of mycobacteria within the biofilm.[8] Additionally, certain pipe materials may interfere with the effectiveness of disinfectants, dictate the structure of the resident microbial community, or enhance the selection for virulence and antibiotic resistance genes (ARGs) across the microbial community.[6]

Antibiotic resistance is similarly gaining attention as a concern for drinking water systems.[6,9] Antibiotic-resistant OPPPs, such as *P. aeruginosa* and *A. baumannii*, are notorious for multiantibiotic-resistant forms that can colonize premise plumbing in hospitals and that are difficult to treat.[10−19] A challenge in controlling antibiotic-resistant OPPPs with disinfectants is that they also tend to be disinfectant-resistant[2] and that disinfectants

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can selectively enrich for bacteria carrying ARGs.[20−22] This may be a result of selection for bacteria containing colocated ARGs and disinfectant resistance genes (i.e., coselection)[9] or induction of horizontal transfer of ARGs in response to stress.[23,24] One field study of 11 full-scale systems in the United Kingdom and the Netherlands revealed that chlorinated drinking water distribution system microbial communities were more enriched in ARGs compared to nondisinfected systems.[9] Disinfectants, in part, can act as oxidizing agents, which can induce oxidative stress response in microbes, including the expression of virulence genes.[25,26] Disinfectants can also influence the release of metals from pipes and other premise plumbing materials.[27,28] Metals released from such materials or directly added as disinfectants could also hypothetically coselect for ARGs that are colocated with metal resistance genes.[29,30] Zhang et al. observed higher proportions of antibiotic-resistant bacteria in biofilter communities following copper exposure.[31] Various studies have demonstrated associations between copper occurrence and enrichments of ARGs and bacteria in other environments, such as soils and wastewater sediments.[6]

The purpose of this study was to identify optimal combinations of in-building disinfectants and pipe materials that limit the proliferation of multiple OPPPs[7] and ARGs in premise plumbing. We used convectively mixed pipe reactors (CMPRs),[32] consisting of three pipe materials (PVC, copper, and iron), for replicated simulation of the distal reaches of premise plumbing and evaluation of the effects of chlorine, chloramine, chlorine dioxide, and copper−silver disinfectants. *P. aeruginosa* and *A. baumannii* were inoculated into the CMPRs as two representative OPPPs that are prominent agents of antibiotic-resistant[33,34] and nosocomial infections[35−37] that are also resistant to disinfectants.[1,2,38] After incrementally increasing disinfectant doses over a period of several weeks, disinfectants were removed to characterize the regrowth patterns. Shotgun metagenomic sequencing was applied to comprehensively evaluate the responses of multiple taxonomic groups known to contain pathogens ("bacterial pathogen species" or "pathogenic species") and their propensity to propagate ARGs. Additionally, we evaluated other characteristics of the microbial communities, such as functional gene profiles and diversity, to explore the mechanistic drivers of pathogen and ARG propagation. We hypothesized that some disinfectant−pipe-material combinations with known chemical interactions and putative effects on microbial communities, like monochloramine and copper pipe, would increase the relative abundance (i.e., proportion of the microbial community) of pathogenic species markers, ARGs, and mobile genetic elements. In contrast, we expected that other materials would behave more inertly, allowing disinfectants to maintain low absolute (i.e., total numbers per unit volume) and relative abundances.

METHODS AND MATERIALS

Experimental Setup. The general experimental approach was described previously in a study focused on the relationship between water chemistry and the behavior of *P. aeruginosa* and *A. baumannii* in hot water plumbing.[28] Briefly, CMPRs represent three pipe material conditions: PVC, copper, and PVC (copper), and mild steel and PVC (iron). New CMPRs were aged for 10 weeks with dechloraminated Blacksburg, VA, tap water. They were then inoculated with ∼1000 colony forming units per mL of antibiotic-susceptible and multidrug-resistant strains of *P. aeruginosa* (ATCC 2111 and 2795, respectively) and *A. baumannii* (ATCC 1789 and 2801, respectively) prior to the initial sampling at week 0. Influent water was adjusted to a pH of ∼7.5 and a dissolved oxygen (DO) of ∼8.0 mg/L. Internal water temperature was maintained at ∼37 °C to mimic distal portions of hot water plumbing. Water changes (100%) were performed three times per week in a Type A2 BSL-2 cabinet. Over 12 weeks, triplicate sets of each material received a low dose of one of four disinfectants (free chlorine, monochloramine, chlorine dioxide, or copper−silver) followed by incrementally increasing disinfectant doses every 6 weeks up to 100% of the appropriate regulatory limit and then 7 weeks with no disinfectant added (Table 1). Six CMPRs of each material received no disinfectant.

Table 1. Target Influent Water Disinfectant Doses (mg/L) (Modified from Cullom et al.[28])

	no residual	low dose	incrementally increasing doses			no residual
disinfectant	weeks[a] −6 to 0	weeks[a] 7−12	weeks 13−18	weeks 19−24	weeks[a] 25−30	weeks[a] 31−37
Cl_2	0	0.1	0.25	1.0	4.0	0
NH_2Cl	0	0.1	0.25	1.0	4.0	0
ClO_2	0	0.02	0.05	0.2	0.8	0
CSI[b]	0	0.025	0.063	0.25	1.0[c]	0

[a]Samples were collected for metagenomic sequencing in the final week of the indicated experimental phase. [b]Doses as mg/L Cu, with a fixed ratio of Cu/Ag of 10:1 mg/L. [c]Highest dose based on the US EPA secondary maximum contaminant level for silver.

Sampling, DNA Extraction, and qPCR. The protocols for chemical and biological samplings were described previously.[28] Briefly, effluent DO, total organic carbon (TOC), pH, and temperature were measured biweekly. Total and soluble metals were measured at the end of each phase by inductively coupled plasma mass spectroscopy. For each water change, effluent residuals of relevant disinfectants were monitored along with one set of the disinfectant-free control pipes on a rotating basis. Influent disinfectant concentrations were verified before the pipes.

Biological sampling occurred every 6 weeks during weeks 0−30 and week 37. CMPR effluent was filtered through 0.22 μm mixed cellulose-ester filters (Millipore, Billerica, MA) for DNA extraction, which was performed using the FastDNA SPIN Kit (MP Biomedicals Inc., Solon, OH) according to the manufacturer protocol. Extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA) with a dsDNA high-sensitivity kit (Thermo Fisher Scientific, Waltham, MA)

Previous quantitative polymerase chain reaction (qPCR) analyses[28] targeting the 16S rRNA gene as a proxy for total bacteria,[39] the *oprL* gene for *P. aeruginosa*,[40] and the 16S-23S rRNA gene intergenic spacer (ABITS) for *A. baumannii*[41] were used to evaluate the accuracy and quantitation of metagenomic analyses. These analyses included the use of a standard curve, negative controls, analytical triplicates, and targeted $R^2 > 0.97$ and 90−110% efficiencies. A full summary of the protocols was reported previously.[28]

Shotgun Metagenomic Sequencing. One hundred and forty-four unpooled DNA extracts (137 from individual pipe reactors, Table S1) were subject to shotgun metagenomic sequencing across six 2 × 150 bp Illumina NextSeq 500 runs (Sand Diego, CA), targeting 20 million reads per sample. Sequencing was carried out by the Duke Center for Genomic and Computational Biology for library preparation and

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sequencing. Library preparation was performed by using the Kapa HyperPrep kit (Roche Sequencing Solutions, Santa Clara, CA). Samples were selected for sequencing in a manner that captured disinfectant−pipe-material combination at each time point. As controls, three replicates of water used to seed the CMPRs during aging, influent waters from weeks 6 and 12, and three replicates of ZymoBIOMICS Microbial Community DNA Standard II (Zymo Research, Irvine, CA) were also sequenced. A summary of samples sequenced is available in Table S1.

Read-Based Analyses. Reads were trimmed and quality filtered using Trimmomatic.[42] Metagenomic coverage was evaluated using Nonpareil estimation on the filtered forward reads.[43] Filtered reads were annotated to elucidate the effects of premise plumbing conditions on the resident microbiota, including Kraken2 v 2.0.7[44] for general microbial taxonomy and community structure, MetaPhlAn 3.10[45] for annotation of bacterial pathogen species markers (see Sections SI-1, SI-2, and Tables S2, S3), DeepARG v 1.0.1[46] and CARD 3.0.7[47] for ARGs, BacMet[48] for metal resistance genes, and HUMAnN 3.0[49] for functional protein annotation to UniRef50 clusters. Generally, the default parameters were passed to the annotation tools. For CARD annotation, the blastx DIAMOND[50] function was used, with filtering for a minimum identity of 80%, minimum length of 25, and a minimum e-value of 1×10^{-10}. ARGs and GO functional pathway genes were normalized to the number of 16S rRNA genes identified via Greengenes 13 to estimate relative abundance[51] (see Section SI-3), whereas taxonomic annotations were normalized to the total number of annotated reads per sample. We further estimated the absolute abundance of bacterial pathogen species (cells/mL) and ARGs (estimated genes/mL) (Section SI-2). ARGs that target WHO classified "critically important" antimicrobials, i.e., those for which the development of widespread resistance would have the most serious effects for human health,[52] were examined using CARD-annotated reads.

Gene ontology (GO) annotations for corresponding UniRef accessions were extracted using the GO.db package.[53] Custom GO term databases corresponding to functional categories of interest (e.g., oxidative stress resistance) were developed using keyword searches of GO terms and descriptions followed by manual curation of GO terms (Table S4). These were used to filter GO term results and examine the relative abundance of genes targeting specific processes.

Assembly-Based Analyses. Reads were assembled to evaluate the occurrence of ARGs, mobile genetic elements (MGEs), and virulence factors within taxa of interest. Assembly of contigs was performed using MEGAHIT[54] and default parameters. PathoFact[55] was used to annotate virulence factors and toxin genes (see Section SI-4 for results). CARD was used to annotate ARGs as above. MMseqs2[56] was used to annotate contigs against the Genome Taxonomy Database. Kraken2 taxonomy annotations were used to compare results to read-based annotations and to evaluate contig count normalization. MobileOG-db v 1.5[57] was used to annotate MGEs, which were then filtered for e-value $< 1 \times 10^{-10}$ and percent identity >80%. To normalize contigs for semiquantitative analysis, the single copy gene *rpoB*[58] was identified in contigs by comparing contigs against *rpoB* profiles available in the Pfam database using the hmmsearch function HMMER version 3.1b2[59] with a maximum e-value of 0.001. This was done with the understanding that contigs, being longer sequences, offer more precise annotation of translated protein sequences relative to direct annotation of short reads. Subsequently, counts in a sample derived from assembly analyses were divided by the number of *rpoB* genes identified in that sample. For example, the counts of contigs containing a fosfomycin ARGs in one sample would be divided by the number of *rpoB* annotated across the entirety of the corresponding assembly sequences for that sample, regardless of whether the genes were colocalized, as has been done previously.[60] Assembly statistics generated from MEGAHIT were used to evaluate additional normalization methods. To evaluate the biological plausibility of assembly, reads mapping back to contigs were identified and compiled using bbmap[61] with kfilter = 22, subfilter = 15, and maxindel = 80, as well as pileup and samtools[62] using default parameters. ARGs and MGEs were considered colocalized if they were found on the same contig. To evaluate this assumption, the distances between ARG and MGE hallmarks were further examined in one of the samples (Section SI-5).

Statistical Analysis. Two-group univariate comparisons were evaluated using the Wilcoxon rank sum test, whereas 3+ group univariate comparisons employed Dunn's test. When appropriate (e.g., when using Dunn's test or splitting ARG data by resistance and testing classes independently), p values were adjusted and reported using the Benjamini−Hochberg multiple comparison correction. Alpha diversity was evaluated by using the Chao1 diversity index. For some comparisons, the abundances of all examined pathogenic species were summed ("aggregated") into a single value for univariate comparisons between conditions. This also minimized the influence of variance in the quantifiability of one target, affecting the overall comparisons. Procrustes and Mantel tests were used to explore the relationship between taxonomic and functional features. Tests for centroidal and dispersion differences in multivariate data sets were performed on Bray−Curtis transformed data using PERMANOVA and PERMDISP. Spearman rank correlation was used to evaluate the agreement between Kraken2- and MetaPhlAn-annotated reads and qPCR measurements. Relationships among basic water quality parameters (e.g., DO, pH, and TOC) and ARG or pathogen relative abundance were examined using Spearman rank correlation. For these analyses, data were grouped by pipe material, as the pipe materials themselves were a substantial driver of these water quality parameters. Normalization methods for assembled contigs (counts, counts divided by *rpoB* counts, counts divided by *rpoB* counts and N50, and counts divided by *rpoB* counts and average contig length) were evaluated by comparing to Kraken2 annotated short reads (fraction of annotated reads and fraction of all reads) and using the Mantel test and Procrustes PROTEST. Plots and analyses were generated in RStudio, R version 4.2.2, using the tidyverse package.[63] Nonmetric multidimensional scaling (NMDS) plots were generated using the metaMDS function in vegan and a maximum of 900 iterations. Differential abundance analysis of short-read derived taxonomy, GO-term, and ARG profiles was performed using metagenomeSeq[64] (Section SI-6).

RESULTS AND DISCUSSION

Overview of Key Contextualizing Findings from a Prior Study. A prior study of the CMPRs focused on the relationship between water chemistry and the fate of *P. aeruginosa* and *A. baumannii* measured by qPCR and culture and demonstrated expected water chemistry phenomena for each disinfectant−pipe-material conditions.[28] Chlorine-based disinfectants rapidly decayed within the CMPRs, only remaining detectable after 48 h if applied at half the regulatory limit and to

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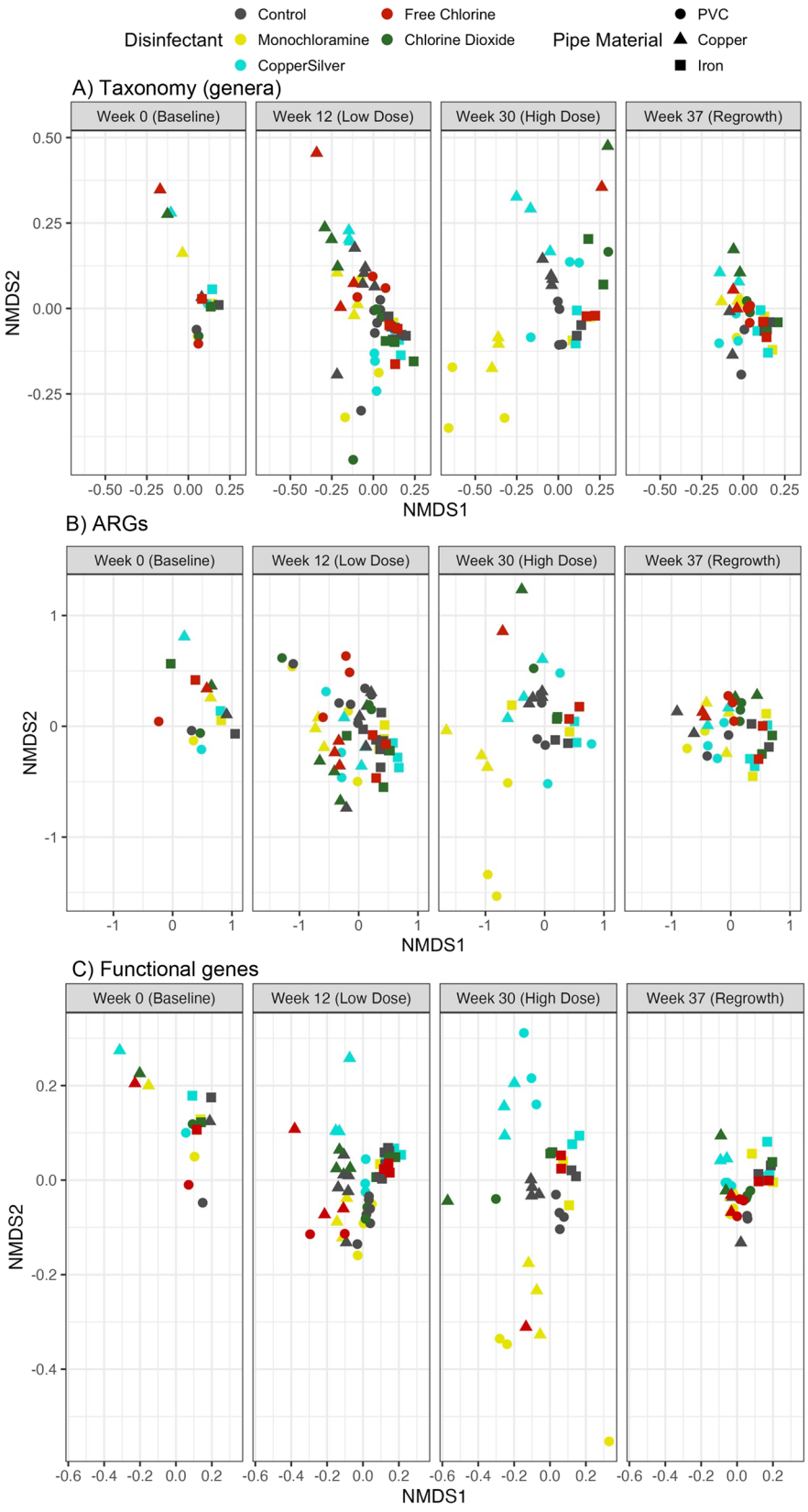

Figure 1. Bray−Curtis-based NMDS ordination of (A) bacterial genera (stress = 0.165), (B) ARGs annotated using CARD v 3.0.7 (stress = 0.157), and (C) functional gene pathways annotated with the gene ontology database (stress = 0.141) illustrating the effect of pipe material and disinfectant. NMDS coordinates were generated for all samples simultaneously.

PVC, the least reactive pipe material. Copper and silver doses were normally detectable but largely removed from solutions. CMPRs maintained consistent and replicable water quality conditions, e.g., temperature and DO. Disinfectants began to

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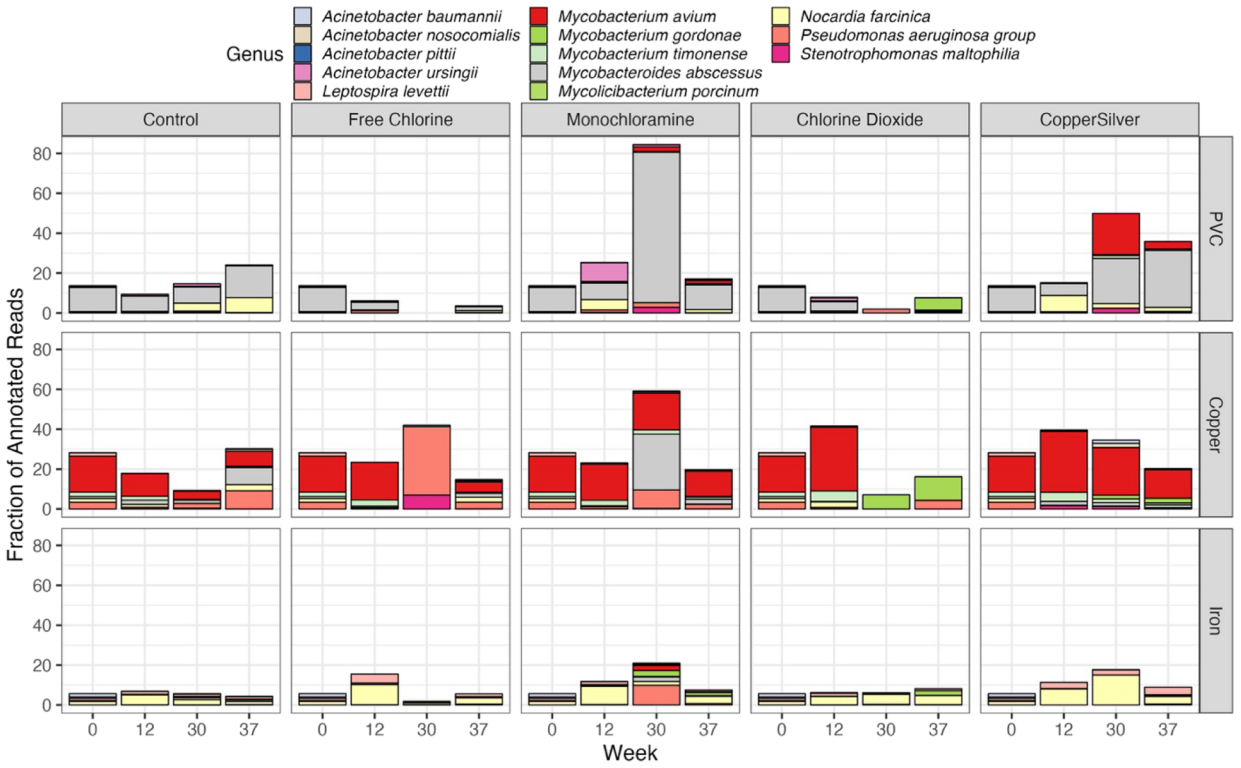

Figure 2. Relative abundance (percentage of total annotated reads per sample) of pathogenic bacterial species annotations in the metagenomic data for each pipe material and disinfectant dosing condition (Table 1). Samples were annotated using MetaPhlAn 3.1.0 using the default parameters. Weeks 0, 12, 30, and 37 represent disinfectant doses equal to 0, 2.5, 100, and 0%, respectively, of the appropriate regulatory limit of each disinfectant being applied. See Section SI-2 for a list of bacterial pathogen species analyzed. The numbers of replicate samples for each condition are reported in Table S1 ($n = 1-6$). Samples from before disinfectant dosing (week 0) for each pipe material are replotted in aggregate for each disinfectant condition to aid in comparison. Insufficient DNA was obtained for sequencing the PVC week 30 chlorine condition.

decrease 16S rRNA gene copy numbers at low doses (e.g., 0.25 mg/L free chlorine) but needed to be applied at levels equal to at least half of their respective regulatory limits to reduce numbers of *P. aeruginosa* and *A. baumannii*.

Assessment of Metagenomic Sequencing and Assembly Quality. Sequencing yielded 19.23 ± 2.99 M (average ± standard deviation) paired raw reads and 19.19 ± 2.95 M quality-filtered paired reads per sample. This resulted in an estimated coverage of 93.2 ± 3.38%, allowing for representative analysis of gene and taxonomic diversity, as well as differential abundances.[65] Samples from the condition with high-dose (4.0 mg/L) free chlorine and a PVC pipe contained DNA below the fluorometric detection limit (0.2 ng, with a maximum of 10 μL analyzed) and failed library preparation. This may be due to chlorine-based degradation of DNA.[66] Assembly resulted in an average of 116,000 ± 49,200 assembled scaffolds per sample. Contigs exhibited an average N50 of 8040 bp, 4 to 9 times greater than other recent studies examining antibiotic resistance and pathogens in drinking water,[67] recycled water,[68] or manure.[69] The high coverage and sequencing depth resulted in 93.37 ± 4.77% (average ± standard deviation) of reads being assembled.

MetaPhlAn accurately and quantitatively annotated the mock community (Figure S1). Further, reads and contigs annotated as *A. baumannii* and *P. aeruginosa* were significantly correlated with *A. baumannii* and *P. aeruginosa* gene copies measured via qPCR[27] (Spearman rank correlation; Figure S2, Table S3). Correlations between assembled and short read beta-diversity distances via Mantel and PROTEST were significant ($p < 0.001$) for all comparisons. These results supported subsequent quantitative comparisons of metagenomic data, including assembled contigs. Counts divided by *rpoB* counts proved to be the most robust normalization method, with further normalization for contig lengths generally reducing the Mantel *r*, PROTEST correlation, or Spearman correlation coefficients (Table S5). Thus, *rpoB*-normalized counts were selected for the downstream analysis of contigs.

Pipe Material Shaped Microbial Community Composition in the Absence of a Disinfectant. NMDS analysis indicated tight grouping of microbial community composition as a function of pipe material in disinfectant-free control pipes (Figure 1A, Figure S3; PERMANOVA, $p < 0.001$, PERMDSIP, $p = 0.45$). Chao1 diversity was lower in copper than iron CMPRs (Dunn's test, $p = 0.003$), with diversity within PVC CMPRs falling in between. NMDS ordination indicated that the iron pipe was the single strongest driver of microbial community structure (Figure 1A). Interestingly, when only disinfectant-free CMPRs were considered, PVC clustered in between the two metallic materials. The greatest variance in community composition was observed among copper CMPRs (Figure 1A, Figure S3), possibly due to the variable nature of copper release both over time and between any two sections of copper pipe.[6] *Bradyrhizobium*, a genus known for nitrogen fixation,[70] was notably enriched by the copper pipe (Figure S4, Wilcoxon rank sum test, $p < 0.001$), especially when compared to the iron pipe (Figure S5, metagenomeSeq). This is consistent with known associations of copper with nitrogen fixation in rhizospheric communities[71] and the critical role copper plays in nitrogenase.[69]

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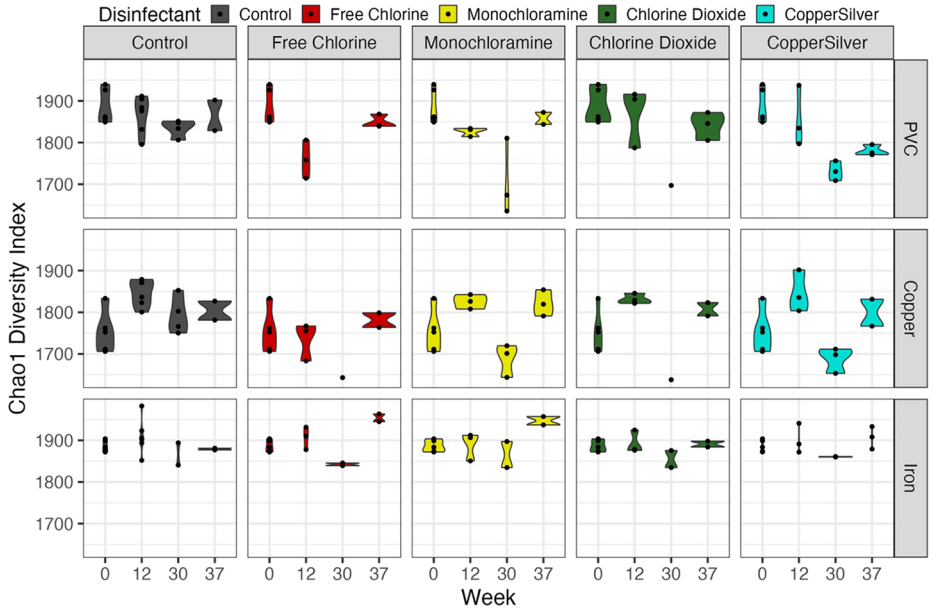

Figure 3. Violin plot distribution of bacterial diversity (Chao1) observed in CMPR effluent with individual sample values represented as dots. The numbers of replicate samples for each condition are reported in Table S1 (range of $n = 1-6$). Samples from before disinfectant dosing (week 0) for each pipe material are aggregated and repeated for each disinfectant condition to aid in comparison. Insufficient DNA was obtained for sequencing the PVC week 30 chlorine condition.

Annotated Pathogenic Bacterial Species in CMPRs in the Absence of a Disinfectant. Among disinfectant-free CMPRs, the percentage of annotated reads associated with bacterial pathogenic species was lower in iron than PVC or copper (Dunn's test, $p = 0.0017$ and 0.0015, respectively) (Figure 2). Several genera known to contain pathogens (*Nocardia, Mycobacteroides, Acinetobacter*) were 1.4 to 20 times more abundant on a relative basis in PVC than in metallic pipes, whereas *Meiothermus* was favored by metallic pipes (Figure S5, metagenomeSeq). Other genera were encountered at a lower relative abundance in iron CMPRs than copper (*Mycobacterium, Mycobacteroides, Stenotrophomonas*) or PVC (*Acinetobacter, Burkholderia, Mycobacterium, Mycobacteroides, Stenotrophomonas*) (Dunn's test, $p < 0.05$) CMPRs. Absolute abundances (estimated cells/mL, see Section SI-2 for calculation) aggregated across all examined pathogenic species demonstrated greater numbers in PVC than in copper or iron CMPRs at weeks 0 and 30 (Dunn's test, $p < 0.024$), whereas in week 12, we found PVC > copper > iron (Dunn's test, $p < 0.023$). However, these differences were eliminated after further aging of the pipes by week 37 (Dunn's test, $p > 0.05$). We also observed that *A. baumannii, P. aeruginosa, Stenotrophomonas maltophilia,* and pathogenic NTM were the most prominent pathogenic bacteria detected among the CMPRs.

Effect of Disinfectant Addition on Microbial Community Composition. Addition of the disinfectants at low doses (week 12) increased the variability in microbial community composition across the CMPR replicates relative to the disinfectant-free control conditions (PERMDISP, $p = 0.005$) (Figure 1A). At the highest dose (week 30), groupings of taxonomic compositions by disinfectant type were apparent (Figure 1A) and significantly affected the taxonomic features of the microbial communities (PERMANOVA, $p < 0.001$). Disinfectants interacted substantially with pipe materials in shaping the taxonomic composition, especially when dosed into iron CMPRs (Figure 1A). Whereas the variability in taxonomic composition increased with disinfectant application, this trend was much more modest in iron CMPRs (Figure 1A). This is likely due to disinfectant consumption by the iron.[28] Copper–silver had the most limited effects in terms of taxonomic structure and diversity, whereas free chlorine had the strongest effects (Figure 1A). This is consistent with the observed effects of these disinfectants on 16S rRNA gene copy numbers.[28] After cessation of disinfectant dosing, the general structure of microbial communities converged, bearing even more similarity across conditions than observed at week 0, with no apparent legacy effects of disinfectants (PERMANOVA, $p = 0.108$).

Diversity indices decreased markedly with disinfectant addition, i.e., by ∼5−9% in PVC and copper CMPRs compared to controls (Figure 3, Wilcoxon rank sum test, $p = 0.01$ and $p = 0.004$, respectively). All disinfectants selected for *Meiothermus.* All disinfectants but monochloramine selected for *Blastomonas* (Figure S6, metagenomeSeq). Addition of monochloramine enriched genera within Mycobacteriaceae (*Mycobacterium* and *Mycobacteroides*) compared to other disinfectant conditions in PVC (Wilcoxon rank sum test, $p = 0.036$) and copper (Wilcoxon rank sum test, $p = 0.002$) but not iron. *Bradyrhizobium* was further enriched in the copper pipe conditions when copper–silver was added at the highest dose (1.0:0.1 mg/L Cu/Ag), as shown in Figure S4 (Wilcoxon rank sum test, $p = 0.036$). Iron CMPRs exhibited more nitrifiers than copper CMPRs (Dunn's test, $p = 0.046$). Monochloramine at the highest dose (4.0 mg/L) tended to result in fewer nitrifiers and denitrifiers relative to control pipes (Dunn's test, $p < 0.05$).

After removing disinfectants, diversity recovered and microbial community structures converged within 7 weeks across all conditions (Wilcoxon rank sum test, $p > 0.05$). Chao1 diversities in CMPRs that had received disinfectants increased at week 37 compared to week 30 (Wilcoxon rank sum test, $p < 0.001$) and were not significantly different than those of the baseline condition (Wilcoxon rank sum test, $p = 0.88$).

Effect of Disinfectant Addition on Annotated Bacterial Pathogen Species. Disinfectant application reduced the absolute abundance of pathogenic species markers if a

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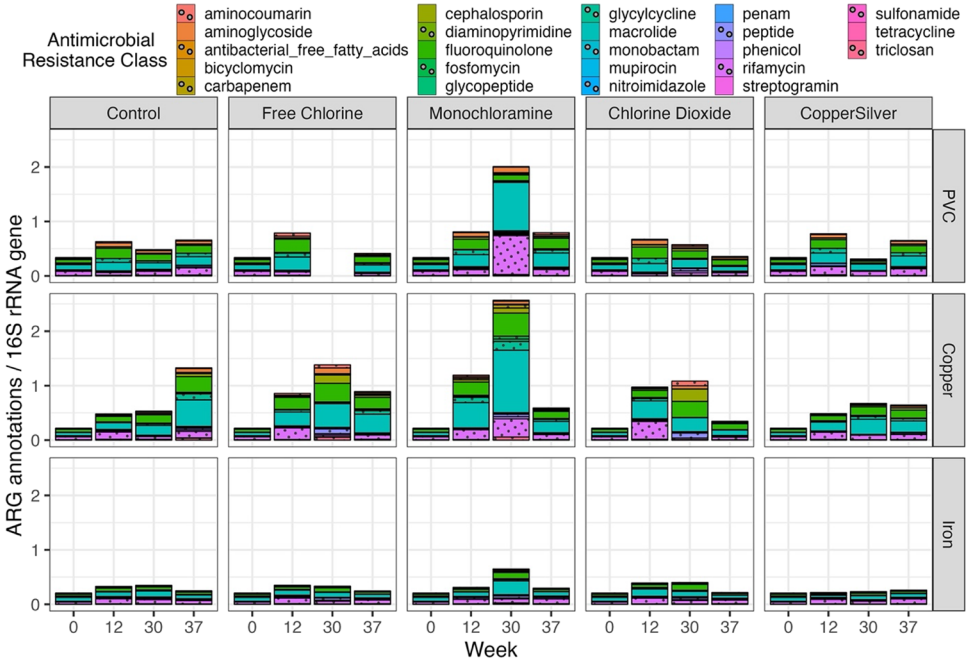

Figure 4. Average relative abundance of ARGs (ARGs/16S rRNA gene) as annotated by CARD v 3.0.7 averaged across replicate CMPRs. The numbers of replicate samples for each condition are reported in Table S1 (n = 1−6). Samples from before disinfectant dosing (week 0) for each pipe material are replotted in aggregate for each disinfectant condition to aid in comparison. Insufficient DNA was obtained for sequencing the PVC week 30 chlorine condition.

sufficiently high dose was applied (Figure S7). However, the low-dose (0.1 mg/L) application of monochloramine in PVC CMPRs resulted in the single highest aggregated absolute abundance of annotated bacterial pathogens among all conditions, 8 times that of the corresponding control. In this condition, most of these annotations corresponded to *Acinetobacter* spp. and *Mycobacteroides abscessus*. Chlorine dioxide proved to be the most consistent and effective in reducing numbers of these taxa and did not appear to enrich numbers when applied at the low dose (0.25 mg/L).

Addition of disinfectants generally did not increase the aggregated relative abundance (percentage of annotated reads) of the examined pathogenic species (Section SI-2), at either low or high doses, compared to the control (Dunn's test, $p > 0.05$), with one exception: in copper CMPRs receiving monochloramine in week 30 (Dunn's test, $p = 0.018$). However, the relative abundance of reads annotated as the pathogens of interest in week 12 was lower with the low dose of chlorine dioxide than in the control within copper CMPRs (Dunn's test, $p = 0.024$). No differences relative to the control were observable after removal of disinfectants (Dunn's test, $p > 0.05$).

Spearman rank correlation analysis was used to evaluate the relationships among annotated bacterial pathogen species, water chemistry parameters, and 16S rRNA gene copy numbers as a proxy for total bacteria (Figure S8). *Mycobacterium gordonae* correlated positively with pH within PVC and copper CMPRs and with DO in PVC CMPRs. The only positive correlation with 16S rRNA gene copy numbers was with *A. baumannii* in PVC CMPRs (Spearman rho = 0.489, $p = 0.003$). Five taxa (*M. gordonae, Mycobacteroides abscessus, Mycolicibacterium porcinum, P. aeruginosa,* and *S. maltophilia*) correlated negatively with 16S rRNA gene copy numbers in at least one pipe material. None of the examined taxa correlated positively with the TOC. Together, this indicates that conditions conducive to growth of OPPPs likely differ from those of drinking water microbiota generally. This is consistent with the known limitations of TOC as an indicator of OPPP regrowth potential.[72,73]

Effects of Pipe Material and Disinfectants on ARGs. Total ARG relative abundance negatively correlated with taxonomic Chao1 diversity (Spearman rho = −0.20, p = 0.017). The distribution of ARGs further shifted when the disinfectant doses were increased to the highest level (PERMANOVA, p = 0.017) and disinfectants appeared to induce heteroskedasticity in ARG profiles (PERMDISP, $p <$ 0.001) (Figure 1B). Taxonomic and ARG beta-diversity matrices correlated strongly (PROTEST $p < 0.001$, correlation = 0.75, Mantel $p < 0.001$, $R = 0.71$), whereas functional (UniRef 50) and ARG profiles were more moderately correlated (PROTEST $p < 0.001$, correlation = 0.48, Mantel $p < 0.001$, R = 0.27). Chemical water quality parameters (pH, DO, temperature, and TOC) generally did not correlate with ARG relative abundance except for DO and TOC in pipes dosed with monochloramine (Spearman rank correlation, rho = 0.82, 0.74; p = 0.002, 0.027, respectively).

ARG profiles were distinctly shaped by pipe material in CMPRs without disinfectant (PERMANOVA, $p < 0.015$). The relative abundance of total ARGs (ARGs/16S rRNA genes) generally decreased from copper to PVC to iron (Figure 4, CARD; Figure S9, DeepARG). As the copper pipes aged, Chao1 diversity of ARGs increased, eventually reaching levels greater than in PVC and iron by week 37 (Dunn's test, $p = 0.013$ and $p <$ 0.001, respectively). ARGs were 42% higher in relative abundance in both PVC and copper CMPRs than in iron CMPRs (CARD, Dunn's test, $p < 0.001$ and $p = 0.0019$, respectively).

Monochloramine was the only disinfectant to individually reduce ARG absolute abundance relative to the control pipes and only when applied to PVC pipes at the regulatory limit (Dunn's test, $p = 0.031$); however, when applied at 0.1 mg/L, it also induced the greatest increase (~4× greater than in the

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corresponding control) in ARG concentration (Figure S10). Monochloramine application also generated the greatest increases in ARG relative abundance, whereas copper−silver had no noticeable impact. For PVC CMPRs, CARD-annotated ARGs within the monochloramine condition were 6.5 times more abundant than in copper−silver (Dunn's test, $p = 0.0094$). For copper CMPRs, ARGs were 4.8 times more abundant in the monochloramine condition than the disinfectant-free condition (Dunn's test, $p = 0.042$). Monochloramine applied at the high dose also exhibited higher ARG Chao1 diversity than those in copper−silver conditions in PVC and copper CMPRs (Dunn's test, $p = 0.034$ and 0.032, respectively). Within iron CMPRs, the rapid neutralization of disinfectants[28] likely ameliorated potential effects of these disinfectants on ARG profiles.

For individual ARGs, log-fold differences were modest when comparing among conditions (max = 0.1235), as revealed by differential abundance analysis. The multidrug efflux pump gene *adeF*, which has been shown to be common in clinical isolates of multidrug-resistant *A. baumannii*,[74] exhibited the greatest difference among all three pipe material comparisons, with the highest relative abundance in PVC followed by copper and iron (Figure S11, metagenomeSeq). Comparisons between metallic and PVC CMPRs were similar, with 8 out of 10 of the most differentially abundant ARGs being consistent across the three pipe materials. Carbapenem resistance-associated OXA genes represented at least 1 of the 10 most differentially abundant ARGs across all disinfectant conditions (Figure S12, metagenomeSeq). Conversely, some ARGs were found at a higher relative abundance in control CMPRs compared to any of the disinfected CMPRs, such as *ade* genes and *QepA4*.

ARGs associated with critically important antibiotics (i.e., as classified by the WHO) exhibited trends similar to those that were observed for total ARGs (Figure S13, Dunn's test, $p < 0.002$). Profiles of critically important ARGs in disinfectant-free conditions differed as a function of pipe material (PERMANOVA, $p < 0.015$), but the within-condition variability of ARG profiles was similar across all pipe materials (PERMDISP, $p > 0.12$), implying differential selection[75] for these genes between the different pipe materials. Similar pipe-material associated trends were observed for ARGs encoding resistance to critically important antibiotics in PVC (6.2 times more in monochloramine than copper−silver) and copper (5.9 more in monochloramine than copper−silver) (Dunn's test, $p = 0.042$ and 0.024, respectively) conditions.

In general, ARG encoding resistance to glycycline and macrolides was the most prominent across all conditions. Several beta-lactamase families, such as those of OXA, TEM, NDM, ADC, and PDC, featured prominently among the identified critically important ARGs (Table S6). The PDC family is notably associated with *P. aeruginosa*.[47] No water quality parameters were correlated with the relative abundance of critically important ARGs among any set of disinfectant-free CMPRs when analyzed within each pipe material (Spearman rank correlation, $p > 0.05$).

Effects of Pipe Material and Disinfectants on ARGs, MGES, and Bacterial Hosts Identified in Contigs. Plasmids dominated the CMPR mobilomes compared to conjugative elements, phages, and transposable elements (Figure S14A). Across all CMPR conditions, the prominence of MGE classes ranked as follows: integration/excision > transfer ≈ replication/recombination/repair > stability/transfer/defense > phage (Dunn's test, $p < 0.001$) (Figure S14B). Across all CMPRs, copper CMPRs contained more MGEs than PVC ($p = 0.017$) or iron ($p < 0.001$) CMPRs, and PVC CMPRs contained more than iron CMPRs ($p = 0.009$). No disinfectant condition appeared to affect MGE relative abundances (Dunn's test, $p > 0.05$). This indicates that cross-selection, rather than increased horizontal gene transfer, drove the observed ARG enrichment with disinfectant application.

Across CMPRs, ARGs were prominent within *P. aeruginosa* (Figure S15). ARGs associated with bacterial pathogens tended to be associated with multiple drug classes (Figure S16). The classes of ARGs associated with pathogenic species differed somewhat from those that tended to be harbored by the general microbial community. Whereas ARG-containing contigs were moderately correlated with ARG reads (Mantel's test, $r = 0.417$, $p < 0.001$), ARG-containing contigs associated with pathogenic species were only weakly so (Mantel's test, $r = 0.239$, $p < 0.001$). However, ARG contigs were well correlated with ARG contigs within pathogenic species (Mantel's test, $r = 0.662$, $p < 0.001$).

Mobile ARGs were defined as assembled contigs containing both an ARG and MGE. Among disinfectant-free conditions, iron CMPRs contained a lower relative abundance of mobile ARGs than PVC and copper CMPRs (Dunn's test, $p < 0.0001$ and $p = 0.0018$, respectively) (Figure S17). Individual comparisons of disinfected conditions to the controls did not reveal an effect (Dunn's test, $p > 0.05$). However, when all pipe materials were tested simultaneously, mobile ARG relative abundance was higher in monochloramine conditions than in free chlorine and chlorine dioxide conditions (Dunn's test, $p = 0.033$ and 0.0472, respectively). We also observed a distinct increase in mobile ARG relative abundance with increased copper−silver dose in PVC and copper CMPRs. These levels continued to increase in the PVC condition even after the copper−silver disinfectant was removed.

Mobile ARGs carried by pathogenic bacterial species were operationally defined as contigs that contained both an ARG and MGE that were annotated with mmseq2 and gtdb as one of the pathogenic bacterial species listed in Section SI-2. Mobile ARGs were found to be associated with five of these pathogens: *A. baumannii*, *Mycobacterium avium*, *P. aeruginosa*, *M. abscessus*, and *Stenotrophomonas maltophilia* (Figure S18). *M. absessus*, *A. baumannii*, and *P. aeruginosa* appeared to be the most common carriers of mobile ARGs. Mobile ARGs were commonly located within one of these organisms (on average, 45% of mobile ARGs).

Functional Gene Profiling of CMPRs. Functional gene profiles were notably altered by both pipe material and the high dose of disinfectants, as apparent at week 30 (Figure 1C, PERMANOVA, $p < 0.05$). Functional and taxonomic profiles across CMPR pipe and disinfectant conditions were moderately correlated (PROTEST $p < 0.001$, correlation = 0.35). In general, functional profiles of the two metallic CMPRs differed from those of the PVC CMPRs in similar ways, sharing 9 of their 10 most underenriched GO terms (i.e., genes sharing a molecular function) and 6 out of their 10 most enriched GO terms (Figure S19, metagenomeSeq). Both metals enriched the "methane monooxygenase complex", the most of any GO term, with "traversing start control point of mitotic cell cycle" the second most. "Cell−cell adhesion" and "single-species biofilm formation on inanimate substrate" were also prominently enriched by both metals. PVC selected for oxygen metabolic processes (GO: 0072592, pathways associated diatomic oxygen), which agrees with the greater DO loss observed in the metallic CMPRs.[28]

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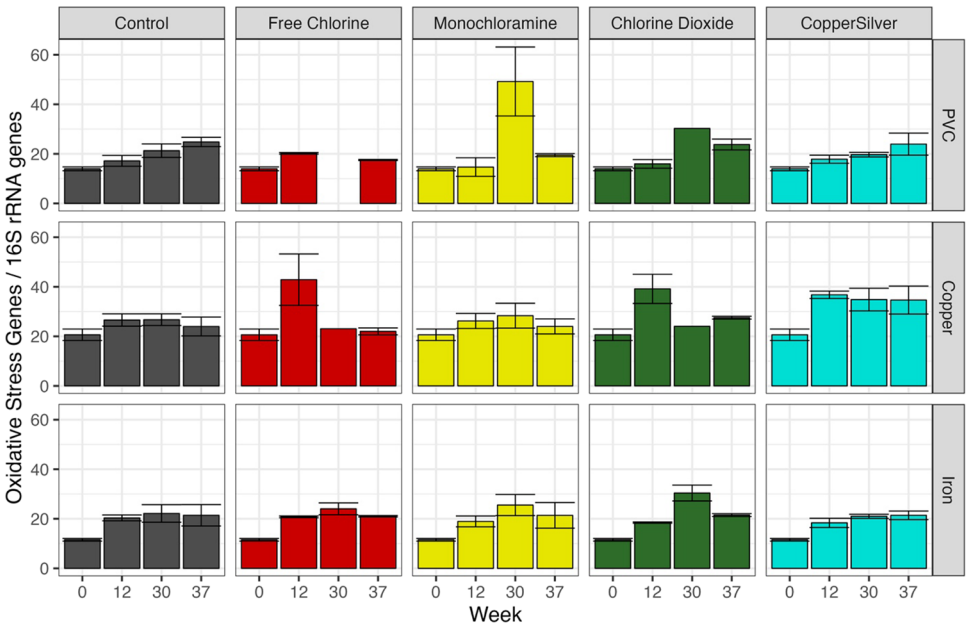

Figure 5. Relative abundance (genes per 16S rRNA gene) of oxidative stress related genes. Error bars represent the standard error. The numbers of replicate samples for each condition are reported in Table S1 ($n = 1-6$). Samples from before disinfectant dosing (week 0) for each pipe material are replotted in aggregate for each disinfectant condition to aid in comparison. Insufficient DNA was obtained for sequencing the PVC week 30 chlorine condition.

Table 2. Summary of Key Results

	disinfectants	pipe material
taxonomic composition	-Disinfectants tended to alter microbial community structure and decrease taxonomic diversity. Copper−silver had the most limited overall effect.	-Pipe material was a strong driver of microbial community composition.
	-Absolute abundances of annotated pathogenic species were reduced when high doses were applied, but sometimes increased at low doses.	-Microbial community compositions remained the most consistent across different disinfectant conditions when iron pipe was used, and estimated abundances of OPPPs were also lowest.
antibiotic resistance and mobile genetic elements	-Disinfectants tended to enrich ARGs and only reduced absolute abundances when applied at the highest levels.	-Iron pipe held ARGs and MGEs at lower relative abundance.
	-Disinfectants did not enrich MGEs but enriched some mobile ARGs.	-Copper pipe enriched ARGs and MGEs.
other functional genes	-Some disinfectants enriched oxidative stress resistance genes.	-Copper and iron selected for more similar functional genes compared to PVC.
	-Disinfectants appeared to select for similar processes.	-Copper enriched metal resistance genes.

Selection for functional genes appeared to be much more similar across disinfectant conditions than what was observed in the taxonomic analysis (Figure S20, metagenomeSeq). Programmed cell death (apoptotic) processes were the most enriched process for all disinfectant conditions. This may be a result of disinfected conditions favoring biofilm lifestyles, in which programmed cell death provides communal benefits by strengthening the biofilm structure and enriching the extracellular matrix.[76,77] Oxygen metabolic processes were enriched in the control condition compared to those in each disinfectant.

Genes involved in mediating oxidative stress were 35.2% more abundant in the copper than in the iron conditions (Dunn's test, $p < 0.001$) and 23.8% more abundant than in the PVC (Dunn's test, $p = 0.004$) control conditions (Figure 5). At week 30, monochloramine addition increased the normalized abundance of oxidative stress genes relative to all other disinfected conditions (Dunn's test, $p < 0.013$). This is perhaps a result of the selective pressure incurred by monochloramine that favors mycobacteria, which tend to be disinfectant resistant[1,2] and to carry robust oxidative stress resistance mechanisms.[78,79] This effect was reversed by week 37 when disinfectants were removed. Nitrification/denitrification genes were more abundant in copper than PVC (Dunn's test, $p = 0.0031$) and iron (Dunn's test, $p < 0.001$) conditions (Figure S21), which are consistent with nitrogen-fixing *Bradyrhizobium* also being more prevalent in the copper conditions and indirectly stimulating nitrifiers/denitrifiers. Additionally, these genes were higher in CMPRs dosed with monochloramine, which also results in the introduction of nitrogen-containing compounds, than any other disinfection condition (Dunn's test, $p < 0.013$, week 30).

Copper, but not iron, increased the MRGs in disinfectant-free conditions (Figure S22). Copper contained MRGs at a relative abundance 334% higher than PVC (Dunn's test, $p = 0.0033$) and 40% higher than iron (Dunn's test, $p < 0.001$). All disinfectants increased MRG relative abundance compared to control pipes at week 30 (Dunn's test, $p < 0.049$). There were no significant differences among disinfectants in terms of apparent selection of MRGs.

Key Observations and Recommendations. The controlled, replicated conditions of the CMPRs provided for the first time a systematic understanding of how in-building disinfectants might be selected and dosed in a manner that effectively controls the potential for multiple OPPPs and antibiotic resistance to proliferate (Table 2). A key finding

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was that the effects of disinfectant addition are strongly influenced by the pipe material. Iron is highly reactive and incurs a high disinfectant demand, consequently eliminating the antimicrobial capacity of the disinfectants. However, iron CMPRs tended to house the lowest relative and absolute abundances of ARGs and organisms annotated as OPPPs. Iron largely exists in buildings as a legacy material because of associated water quality problems incurred by corrosion, and it is also associated with the occurrence of *Legionella* in field studies (though not in this study).[80,81] By contrast, copper is inherently antimicrobial and, even in the absence of a disinfectant, was associated with a higher relative abundance of ARGs by the end of the study. This situation was markedly exacerbated by monochloramine addition to copper CMPRs, wherein the highest relative abundances of ARGs were found. Copper CMPRs also contained the highest relative abundance of mobile ARGs, especially when disinfectants were added. Iron, on the other hand, appears to ameliorate the effects of disinfectants and potentially other oxidizing agents in the water..

If supplied at a sufficient dose, disinfectants decreased the absolute abundance of OPPPs and ARGs. However, the application of low doses of monochloramine to PVC CMPRs resulted in elevated absolute abundances of both ARGs and the annotated pathogens, underscoring the importance of maintaining residuals above biocidal levels, below which they may simply provide selective pressures that favor ARGs and some pathogens. Among the four disinfectants examined, monochloramine was revealed to be the most problematic, as it enriched both pathogen and ARG gene markers. This poses a challenge given that in-building chloramine disinfection is one of the most proven approaches for *Legionella* control.[82,83] Even when considering absolute abundance, although most pathogenic species were reduced by disinfectants, low-dose application of monochloramine resulted in the single highest aggregated abundance of pathogenic species among any of the conditions. In particular, monochloramine addition tended to enrich Mycobacteriaceae, which are associated with nontuberculous lung disease and are known to be relatively tolerant of disinfectants and intrinsically resistant to antibiotics.[84] The fact that almost half of mobile ARGs were found within bacterial pathogens suggests that it should be possible to identify unified strategies to control both. Excepting the iron condition, chlorine dioxide and copper−silver addition stood out as the most effective for maintaining low relative and absolute abundances of both ARG and OPPP annotations.

There are a number of limitations to this study that should be considered. First, although the operation of the CMPRs here is representative of distal, infrequently used, hot water plumbing, this is perhaps the most challenging setting to successfully control microbial growth with residual disinfectants. This is distinct from the large-diameter pipes, cooler temperatures, and more consistent flow conditions typical of a municipal water distribution system. Second, although powerful for providing a broad overview of the behavior of numerous OPPPs, ARGs, MGEs, MRGs, and other functional genes of interest, there are a number of inherent limitations to metagenomic analysis. These include limited resolution for identifying bacterial species, hence the evaluation and use of multiple approaches explained in Section SI-1, and for verifying pathogen genotypes, which typically requires culture and whole genome sequencing. Additionally, the inherent generation of relative abundance data is insightful for characterizing ecological phenomena but less so for quantifying exposures for the purpose of risk assessment. Thus, the trends for bacterial pathogen species observed via metagenomics should ideally be verified with a culture-based assessment. Assembly of metagenomic data provides useful insight into the context in which a gene or marker occurs but introduces uncertainty[85] and reduces the quantitative capacity (Table S3). Additional inherent limitations to metagenomics include the fact that genotype is not equivalent to phenotype (e.g., in the context of functional gene analysis) and that it does not directly distinguish live from dead organisms. However, by applying metagenomic sequencing after the experimental condition of interest was held for at least 6 weeks, following several dump and fill cycles to flush out dead cells, and by comparing multiple replicates representing the experimental conditions, controls, and time points, here we assumed that metagenomics captured fundamental adaptions of the microbial communities to the conditions of interest. We also note that the 16S rRNA gene, used here for normalization, is a common proxy for total bacteria, but copy numbers of this gene differ among taxa.[86] Finally, combinations of residual disinfectants are sometimes utilized for microbial growth control,[87,88] whereas this study only evaluated their individual effects. Simultaneous application of disinfectants will likely induce further interactions between water quality and the microbial community.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c05905.

Methodological details on taxonomic and functional pathway annotation, normalization, and differential abundance analysis; description of results from colocalization analysis, as well as annotation for virulence factors; results used to evaluate the accuracy of sequencing approach and taxonomic annotation; and additional figures describing results from annotations of taxonomy, antibiotic resistance genes, mobile genetic elements, and functional pathways, as well as differential abundance analysis (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Falkinham, J.; Pruden, A.; Edwards, M. Opportunistic Premise Plumbing Pathogens: Increasingly Important Pathogens in Drinking Water. *Pathogens* **2015**, *4*, 373−386.

(2) Falkinham, J. Common Features of Opportunistic Premise Plumbing Pathogens. *Int. J. Environ. Res. Public Health* **2015**, *12*, 4533−4545.

(3) Rhoads, W. J.; Pruden, A.; Edwards, M. A. Anticipating Challenges with In-Building Disinfection for Control of Opportunistic Pathogens. *Water Environment Research* **2014**, *86*, 540−549.

(4) Collier, S. A.; Stockman, L. J.; Hicks, L. A.; Garrison, L. E.; Zhou, F. J.; Beach, M. J. Direct Healthcare Costs of Selected Diseases Primarily or Partially Transmitted by Water. *Epidemiol Infect* **2012**, *140*, 2003−2013.

(5) Rhoads, W. J.; Pruden, A.; Edwards, M. A. Convective Mixing in Distal Pipes Exacerbates Legionella Pneumophila Growth in Hot Water Plumbing. *Pathogens* **2016**, *5*, 29.

(6) Cullom, A. C.; Martin, R. L.; Song, Y.; Williams, K.; Williams, A.; Pruden, A.; Edwards, M. A. Critical Review: Propensity of Premise Plumbing Pipe Materials to Enhance or Diminish Growth of Legionella and Other Opportunistic Pathogens. *Pathogens* **2020**, *9*, 957.

(7) Proctor, C.; Garner, E.; Hamilton, K. A.; Ashbolt, N. J.; Caverly, L. J.; Falkinham, J. O., III; Haas, C. N.; Prevost, M.; Prevots, D. R.; Pruden, A.; Raskin, L.; Stout, J.; Haig, S. Tenets of a Holistic Approach to Drinking Water-Associated Pathogen Research, Management, and Communication. *Water Res.* **2022**, *211*, No. 117997, DOI: 10.1016/j.watres.2021.117997.

(8) Waak, M. B.; Lapara, T. M.; Hallé, C.; Hozalski, R. M. Nontuberculous Mycobacteria in Two Drinking Water Distribution Systems and the Role of Residual Disinfection. *Environ. Sci. Technol.* **2019**, *53*, 8563−8573.

(9) Sevillano, M.; Dai, Z.; Calus, S.; Bautista-de los Santos, Q. M.; Eren, A. M.; van der Wielen, P. W. J. J.; Ijaz, U. Z.; Pinto, A. J. Differential Prevalence and Host-Association of Antimicrobial Resistance Traits in Disinfected and Non-Disinfected Drinking Water Systems. *Science of The Total Environment* **2020**, *749*, No. 141451.

(10) Muscarella, L. F. Contribution of Tap Water and Environmental Surfaces to Nosocomial Transmission of Antibiotic-Resistant Pseudomonas Aeruginosa. *Infect Control Hosp Epidemiol* **2004**, *25*, 342−345.

(11) Exner, M.; Kramer, A.; Lajoie, L.; Gebel, J.; Engelhart, S.; Hartemann, P. Prevention and Control of Health Care-Associated Waterborne Infections in Health Care Facilities. *Am. J. Infect Control* **2005**, *33*, S26−S40.

(12) Trautmann, M.; Lepper, P. M.; Haller, M. Ecology of Pseudomonas Aeruginosa in the Intensive Care Unit and the Evolving Role of Water Outlets as a Reservoir of the Organism. *Am. J. Infect Control* **2005**, *33*, S41−S49.

(13) Lavenir, R.; Sanroma, M.; Gibert, S.; Crouzet, O.; Laurent, F.; Kravtsoff, J.; Mazoyer, M.-A.; Cournoyer, B. Spatio-Temporal Analysis of Infra-Specific Genetic Variations among a Pseudomonas Aeruginosa Water Network Hospital Population: Invasion and Selection of Clonal Complexes. *J. Appl. Microbiol.* **2008**, *105*, 1491−1501.

(14) Debast, S. B.; Meis, J. F. G. M.; Melchers, W. J. G.; Hoogkamp-Korstanje, J. A. A.; Voss, A. Use of Interrepeat PCR Fingerprinting to Investigate an Acinetobacter Baumannii Outbreak in an Intensive Care Unit. *Scand J. Infect Dis* **1996**, *28*, 577−581.

(15) Jefferies, J. M. C.; Cooper, T.; Yam, T.; Clarke, S. C. Pseudomonas Aeruginosa Outbreaks in the Neonatal Intensive Care Unit − a Systematic Review of Risk Factors and Environmental Sources. *J. Med. Microbiol* **2012**, *61*, 1052−1061.

(16) Blanc, D. S.; Nahimana, I.; Petignat, C.; Wenger, A.; Bille, J.; Francioli, P. Faucets as a Reservoir of Endemic Pseudomonas Aeruginosa Colonization/Infections in Intensive Care Units. *Intensive Care Med.* **2004**, *30*, 1964−1968.

(17) La Forgia, C.; Franke, J.; Hacek, D. M.; Thomson, R. B.; Robicsek, A.; Peterson, L. R. Management of a Multidrug-Resistant Acinetobacter Baumannii Outbreak in an Intensive Care Unit Using Novel Environmental Disinfection: A 38-Month Report. *Am. J. Infect Control* **2010**, *38*, 259−263.

(18) Umezawa, K.; Asai, S.; Ohshima, T.; Iwashita, H.; Ohashi, M.; Sasaki, M.; Kaneko, A.; Inokuchi, S.; Miyachi, H. Outbreak of Drug-Resistant Acinetobacter Baumannii ST219 Caused by Oral Care Using Tap Water from Contaminated Hand Hygiene Sinks as a Reservoir. *Am. J. Infect Control* **2015**, *43*, 1249−1251.

(19) Breathnach, A. S.; Cubbon, M. D.; Karunaharan, R. N.; Pope, C. F.; Planche, T. D. Multidrug-Resistant Pseudomonas Aeruginosa Outbreaks in Two Hospitals: Association with Contaminated Hospital Waste-Water Systems. *Journal of Hospital Infection* **2012**, *82*, 19−24.

(20) Sanganyado, E.; Gwenzi, W. Antibiotic Resistance in Drinking Water Systems: Occurrence, Removal, and Human Health Risks. *Science of The Total Environment* **2019**, *669*, 785−797.

(21) Tan, Q.; Li, W.; Zhang, J.; Zhou, W.; Chen, J.; Li, Y.; Ma, J. Presence, Dissemination and Removal of Antibiotic Resistant Bacteria and Antibiotic Resistance Genes in Urban Drinking Water System: A Review. *Front Environ. Sci. Eng.* **2019**, *13*, 36.

(22) Shi, P.; Jia, S.; Zhang, X.-X. X.; Zhang, T.; Cheng, S.; Li, A. Metagenomic Insights into Chlorination Effects on Microbial Antibiotic Resistance in Drinking Water. *Water Res.* **2013**, *47*, 111−120.

(23) Vikesland, P. J.; Pruden, A.; Alvarez, P. J. J.; Aga, D.; Bürgmann, H.; Li, X.; Manaia, C. M.; Nambi, I.; Wigginton, K.; Zhang, T.; Zhu, Y. Toward a Comprehensive Strategy to Mitigate Dissemination of Environmental Sources of Antibiotic Resistance. *Environ. Sci. Technol.* **2017**, *51*, 13061−13069.

(24) Zhang, Y.; Gu, A. Z.; He, M.; Li, D.; Chen, J. Subinhibitory Concentrations of Disinfectants Promote the Horizontal Transfer of Multidrug Resistance Genes within and across Genera. *Environ. Sci. Technol.* **2017**, *51*, 570−580.

(25) Wargo, M. J. Is the Potable Water System an Advantageous Preinfection Niche for Bacteria Colonizing the Cystic Fibrosis Lung? *mBio* **2019**, *10*, No. e00883-19.

(26) Kim, J.; Park, H. J.; Lee, J. H.; Hahn, J. S.; Gu, M. B.; Yoon, J. Differential Effect of Chlorine on the Oxidative Stress Generation in Dormant and Active Cells within Colony Biofilm. *Water Res.* **2009**, *43*, 5252−5259.

(27) Hu, J.; Gan, F.; Triantafyllidou, S.; Nguyen, C. K.; Edwards, M. A. Copper-Induced Metal Release from Lead Pipe into Drinking Water. *CORROSION* **2012**, *68*, 1037−1048.

(28) Cullom, A.; Spencer, M. S.; Williams, M. D.; Williams, M. D.; Falkinham, J. O., III; Pruden, A.; Edwards, M. A. Influence of Pipe Materials on In-Building Disinfection of P. Aeruginosa and A. Baumannii in Simulated Hot Water Plumbing. *Water Res. X* **2023**, *21*, No. 100189, DOI: 10.1016/j.wroa.2023.100189.

(29) Alonso, A.; Sanchez, P.; Martinez, J. L. Environmental Selection of Antibiotic Resistance Genes. *Minireview. Environ. Microbiol* **2001**, *3*, 1−9.
(30) Alonso, A.; Sanchez, P.; Martínez, J. L. Stenotrophomonas Maltophilia D457R Contains a Cluster of Genes from Gram-Positive Bacteria Involved in Antibiotic and Heavy Metal Resistance. *Antimicrob. Agents Chemother.* **2000**, *44*, 1778−1782.
(31) Zhang, M.; Chen, L.; Ye, C.; Yu, X. Co-Selection of Antibiotic Resistance via Copper Shock Loading on Bacteria from a Drinking Water Bio-Filter. *Environ. Pollut.* **2018**, *233*, 132−141.
(32) Spencer, M. S.; Cullom, A. C.; Rhoads, W. J.; Pruden, A.; Edwards, M. A. Replicable Simulation of Distal Hot Water Premise Plumbing Using Convectively-Mixed Pipe Reactors. *PLoS One* **2020**, *15*, No. e0238385.
(33) Liu, W. J.; Fu, L.; Huang, M.; Zhang, J. P.; Wu, Y.; Zhou, Y. S.; Zeng, J.; Wang, G. X. Frequency of Antiseptic Resistance Genes and Reduced Susceptibility to Biocides in Carbapenem-Resistant Acinetobacter Baumannii. *J. Med. Microbiol* **2017**, *66*, 13−17.
(34) Pang, Z.; Raudonis, R.; Glick, B. R.; Lin, T. J.; Cheng, Z. Antibiotic Resistance in Pseudomonas Aeruginosa: Mechanisms and Alternative Therapeutic Strategies. *Biotechnol Adv.* **2019**, *37*, 177−192.
(35) Gonzalez-Villoria, A. M.; Valverde-Garduno, V. Antibiotic-Resistant Acinetobacter Baumannii Increasing Success Remains a Challenge as a Nosocomial Pathogen. *J. Pathog* **2016**, *2016*, 1−10.
(36) Bédard, E.; Prévost, M.; Déziel, E. Pseudomonas Aeruginosa in Premise Plumbing of Large Buildings. *Microbiologyopen* **2016**, *5*, 937−956.
(37) Hidron, A. I.; Edwards, J. R.; Patel, J.; Horan, T. C.; Sievert, D. M.; Pollock, D. A.; Fridkin, S. K. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Annual Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006−2007. *Infect Control Hosp Epidemiol* **2008**, *29*, 996−1011.
(38) Karumathil, D.; Yin, H.-B.; Kollanoor-Johny, A.; Venkitanarayanan, K. Effect of Chlorine Exposure on the Survival and Antibiotic Gene Expression of Multidrug Resistant Acinetobacter Baumannii in Water. *Int. J. Environ. Res. Public Health* **2014**, *11*, 1844−1854.
(39) Suzuki, M. T.; Taylor, L. T.; DeLong, E. F. Quantitative Analysis of Small-Subunit RRNA Genes in Mixed Microbial Populations via 5′-Nuclease Assays. *Appl. Environ. Microbiol.* **2000**, *66*, 4605−4614.
(40) Le Gall, F.; Le Berre, R.; Rosec, S.; Hardy, J.; Gouriou, S.; Boisramé-Gastrin, S.; Vallet, S.; Rault, G.; Payan, C.; Héry-Arnaud, G. Proposal of a Quantitative PCR-Based Protocol for an Optimal Pseudomonas Aeruginosa Detection in Patients with Cystic Fibrosis. *BMC Microbiol* **2013**, *13*, 143.
(41) Chang, H. C.; Wei, Y. F.; Dijkshoorn, L.; Vaneechoutte, M.; Tang, C. T.; Chang, T. C. Species-Level Identification of Isolates of the Acinetobacter Calcoaceticus - Acinetobacter Baumannii Complex by Sequence Analysis of the 16S-23S RRNA Gene Spacer Region. *J. Clin Microbiol* **2005**, *43*, 1632−1639.
(42) Bolger, A. M.; Lohse, M.; Usadel, B. Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* **2014**, *30*, 2114−2120.
(43) Rodriguez-R, L. M.; Gunturu, S.; Tiedje, J. M.; Cole, J. R.; Konstantinidis, K. T. Nonpareil 3: Fast Estimation of Metagenomic Coverage and Sequence Diversity. *mSystems* **2018**, *3*, No. e00039-18, DOI: 10.1128/mSystems.00039-18.
(44) Wood, D. E.; Lu, J.; Langmead, B. Improved Metagenomic Analysis with Kraken 2. *Genome Biol.* **2019**, *20*, 257.
(45) Beghini, F. *MetaPhlAn 3* 2020.
(46) Arango-Argoty, G.; Garner, E.; Pruden, A.; Heath, L. S.; Vikesland, P.; Zhang, L. DeepARG: A Deep Learning Approach for Predicting Antibiotic Resistance Genes from Metagenomic Data. *Microbiome* **2018**, *6*, 23.
(47) Alcock, B. P.; Raphenya, A. R.; Lau, T. T. Y.; Tsang, K. K.; Bouchard, M.; Edalatmand, A.; Huynh, W.; Nguyen, A.-L. V.; Cheng, A. A.; Liu, S.; Miroshnichenko, A.; Tran, H.; Werfalli, R.; Nasir, J.; Oloni, M.; Speicher, D.; Florescu, A.; Singh, B.; Faltyn, M.; Hernandez-Koutoucheva, A.; Sharma, A.; Bordeleau, E.; Pawlowski, A.; Zubyk, H.; Dooley, D.; Griffiths, E.; Maguire, F.; Winsor, G.; Beiko, R.; Brinkman, F.; Hsiao, W.; Domselaar, G.; McArthur, A. CARD 2020: Antibiotic Resistome Surveillance with the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Res.* **2020**, *48*, D517−D525.
(48) Pal, C.; Bengtsson-Palme, J.; Rensing, C.; Kristiansson, E.; Larsson, D. G. J. BacMet: Antibacterial Biocide and Metal Resistance Genes Database. *Nucleic Acids Res.* **2014**, *42*, D737−D743.
(49) Franzosa, E. A.; McIver, L. J.; Rahnavard, G.; Thompson, L. R.; Schirmer, M.; Weingart, G.; Lipson, K. S.; Knight, R.; Caporaso, J. G.; Segata, N.; Huttenhower, C. Species-Level Functional Profiling of Metagenomes and Metatranscriptomes. *Nat. Methods* **2018**, *15*, 962−968.
(50) Buchfink, B.; Reuter, K.; Drost, H.-G. Sensitive Protein Alignments at Tree-of-Life Scale Using DIAMOND. *Nat. Methods* **2021**, *18*, 366−368.
(51) DeSantis, T. Z.; Hugenholtz, P.; Larsen, N.; Rojas, M.; Brodie, E. L.; Keller, K.; Huber, T.; Dalevi, D.; Hu, P.; Andersen, G. L. Greengenes, a Chimera-Checked 16S RRNA Gene Database and Workbench Compatible with ARB. *Appl. Environ. Microbiol.* **2006**, *72*, 5069−5072.
(52) World Health Organization *Critically Important Antimicrobials for Human Medicine: Ranking of Antimicrobial Agents for Risk Management of Antimicrobial Resistance Due to Non-Human Use*; World Health Organization, 2017; ISBN 978−92−4-151552−8.
(53) Carlson, M. *GO.Db: A Set of Annotation Maps Describing the Entire Gene Ontology*; 2019.
(54) Li, D.; Liu, C.-M.; Luo, R.; Sadakane, K.; Lam, T.-W. MEGAHIT: An Ultra-Fast Single-Node Solution for Large and Complex Metagenomics Assembly via Succinct de Bruijn Graph. *Bioinformatics* **2015**, *31*, 1674−1676.
(55) de Nies, L.; Lopes, S.; Busi, S. B.; Galata, V.; Heintz-Buschart, A.; Laczny, C. C.; May, P.; Wilmes, P. PathoFact: A Pipeline for the Prediction of Virulence Factors and Antimicrobial Resistance Genes in Metagenomic Data. *Microbiome* **2021**, *9*, 49.
(56) Steinegger, M.; Söding, J. MMseqs2 Enables Sensitive Protein Sequence Searching for the Analysis of Massive Data Sets. *Nat. Biotechnol.* **2017**, *35*, 1026−1028.
(57) Brown, C. L.; Mullet, J.; Hindi, F.; Stoll, J. E.; Gupta, S.; Choi, M.; Keenum, I.; Vikesland, P.; Pruden, A.; Zhang, L. MobileOG-Db: A Manually Curated Database of Protein Families Mediating the Life Cycle of Bacterial Mobile Genetic Elements. *Appl. Environ. Microbiol.* **2022**, *88*, No. e00991-22, DOI: 10.1128/aem.00991-22.
(58) Ogier, J.-C.; Pagès, S.; Galan, M.; Barret, M.; Gaudriault, S. RpoB, a Promising Marker for Analyzing the Diversity of Bacterial Communities by Amplicon Sequencing. *BMC Microbiol* **2019**, *19*, 171.
(59) Eddy, S. *HMMER: Biosequence Analysis Using Profile Hidden Markov Models*; Available online: http://hmmer.org/.
(60) Sevillano, M.; Dai, Z.; Calus, S.; los Santos, Q. M. B.; Eren, A. M.; van der Wielen, P. W. J. J.; Ijaz, U. Z.; Pinto, A. J. Disinfectant Residuals in Drinking Water Systems Select for Mycobacterial Populations with Intrinsic Antimicrobial Resistance. *bioRxiv* **2019**, No. 675561, DOI: 10.1101/675561.
(61) Bushnell, B. *BBMap: A Fast, Accurate*; Splice-Aware Aligner: United States, 2014.
(62) Danecek, P.; Bonfield, J. K.; Liddle, J.; Marshall, J.; Ohan, V.; Pollard, M. O.; Whitwham, A.; Keane, T.; McCarthy, S. A.; Davies, R. M.; Li, H. Twelve Years of SAMtools and BCFtools. *Gigascience* **2021**, *10*, giab00 DOI: 10.1093/gigascience/giab008.
(63) Wickham, H. *Tidyverse: Easily Install and Load the "Tidyverse"*; 2017.
(64) Jonsson, V.; Österlund, T.; Nerman, O.; Kristiansson, E. Statistical Evaluation of Methods for Identification of Differentially Abundant Genes in Comparative Metagenomics. *BMC Genomics* **2016**, *17*, 78.
(65) Rodriguez-R, L. M.; Konstantinidis, K. T. Estimating Coverage in Metagenomic Data Sets and Why It Matters. *ISME J.* **2014**, *8*, 2349−2351.

(66) He, H.; Zhou, P.; Shimabuku, K. K.; Fang, X.; Li, S.; Lee, Y.; Dodd, M. C. Degradation and Deactivation of Bacterial Antibiotic Resistance Genes during Exposure to Free Chlorine, Monochloramine, Chlorine Dioxide, Ozone, Ultraviolet Light, and Hydroxyl Radical. *Environ. Sci. Technol.* **2019**, *53*, 2013−2026.
(67) Zhang, H.; Chang, F.; Shi, P.; Ye, L.; Zhou, Q.; Pan, Y.; Li, A. Antibiotic Resistome Alteration by Different Disinfection Strategies in a Full-Scale Drinking Water Treatment Plant Deciphered by Metagenomic Assembly. *Environ. Sci. Technol.* **2019**, *53*, 2141−2150.
(68) Zhu, N. J.; Ghosh, S.; Edwards, M. A.; Pruden, A. Interplay of Biologically Active Carbon Filtration and Chlorine-Based Disinfection in Mitigating the Dissemination of Antibiotic Resistance Genes in Water Reuse Distribution Systems. *Environ. Sci. Technol.* **2021**, *55*, 8329−8340.
(69) Guron, G. K. P.; Arango-Argoty, G.; Zhang, L.; Pruden, A.; Ponder, M. A. Effects of Dairy Manure-Based Amendments and Soil Texture on Lettuce- and Radish-Associated Microbiota and Resistomes. *mSphere* **2019**, *4*, No. e00239-19, DOI: 10.1128/mSphere.00239-19.
(70) Hara, S.; Morikawa, T.; Wasai, S.; Kasahara, Y.; Koshiba, T.; Yamazaki, K.; Fujiwara, T.; Tokunaga, T.; Minamisawa, K. Identification of Nitrogen-Fixing Bradyrhizobium Associated With Roots of Field-Grown Sorghum by Metagenome and Proteome Analyses. *Front Microbiol* **2019**, *10*, 1−15.
(71) González-Guerrero, M.; Matthiadis, A.; Sáez, Á.; Long, T. A. Fixating on Metals: New Insights into the Role of Metals in Nodulation and Symbiotic Nitrogen Fixation. *Front Plant Sci.* **2014**, *5*, 1−6.
(72) Williams, K.; Pruden, A.; Falkinham, J. O.; Edwards, M. Relationship between Organic Carbon and Opportunistic Pathogens in Simulated Glass Water Heaters. *Pathogens* **2015**, *4*, 355−372.
(73) Edwards, M.; Pruden, A.; Falkinham, J. O., III; Brazeau, R.; Williams, K.; Wang, H.; Martin, A.; Rhoads, W. J.Relationship Between Biodegradable Organic Matter and Pathogen Concentrations in Premise Plumbing. *Water Research Foundation*; Denver, CO, USA, 2013, 111
(74) Tyumentseva, M.; Mikhaylova, Y.; Prelovskaya, A.; Tyumentsev, A.; Petrova, L.; Fomina, V.; Zamyatin, M.; Shelenkov, A.; Akimkin, V. Genomic and Phenotypic Analysis of Multidrug-Resistant Acinetobacter Baumannii Clinical Isolates Carrying Different Types of CRISPR/Cas Systems. *Pathogens* **2021**, *10*, 205.
(75) Anderson, M. J.; Walsh, D. C. I. PERMANOVA, ANOSIM, and the Mantel Test in the Face of Heterogeneous Dispersions: What Null Hypothesis Are You Testing? *Ecol Monogr* **2013**, *83*, 557−574.
(76) Allocati, N.; Masulli, M.; Di Ilio, C.; De Laurenzi, V. Die for the Community: An Overview of Programmed Cell Death in Bacteria. *Cell Death Dis* **2015**, *6*, e1609−e1609.
(77) Ma, L.; Conover, M.; Lu, H.; Parsek, M. R.; Bayles, K.; Wozniak, D. J. Assembly and Development of the Pseudomonas Aeruginosa Biofilm Matrix. *PLoS Pathog* **2009**, *5*, No. e1000354.
(78) Choo, S. W.; Rishik, S.; Wee, W. Y. Comparative Genome Analyses of Mycobacteroides Immunogenum Reveals Two Potential Novel Subspecies. *Microb Genom* **2020**, *6*, 1−12.
(79) Saikolappan, S.; Das, K.; Dhandayuthapani, S. Inactivation of the Organic Hydroperoxide Stress Resistance Regulator OhrR Enhances Resistance to Oxidative Stress and Isoniazid in Mycobacterium Smegmatis. *J. Bacteriol.* **2015**, *197*, 51−62.
(80) States, S. J.; Conley, L. F.; Ceraso, M.; Stephenson, T. E.; Wolford, R. S.; Wadowsky, R. M.; McNamara, A. M.; Yee, R. B. Effects of Metals on Legionella Pneumophila Growth in Drinking Water Plumbing Systems. *Appl. Environ. Microbiol.* **1985**, *50*, 1149−1154.
(81) Rakić, A.; Perić, J.; Foglar, L. Influence of Temperature, Chlorine Residual and Heavy Metals on the Presence of *Legionella Pneumophila* in Hot Water Distribution Systems. *Ann. Agric. Environ. Med.* **2012**, *19*, 431−436.
(82) Moore, M. R.; Pryor, M.; Fields, B.; Lucas, C.; Phelan, M.; Besser, R. E. Introduction of Monochloramine into a Municipal Water System: Impact on Colonization of Buildings by Legionella Spp. *Appl. Environ. Microbiol.* **2006**, *72*, 378−383.
(83) Flannery, B.; Gelling, L. B.; Vugia, D. J.; Weintraub, J. M.; Salerno, J. J.; Conroy, M. J.; Stevens, V. A.; Rose, C. E.; Moore, M. R.; Fields, B. S.; Besser, R. Reducing Legionella Colonization of Water Systems with Monochloramine. *Emerg Infect Dis* **2006**, *12*, 588−596.
(84) Falkinham, J. O., III Surrounded by Mycobacteria: Nontuberculous Mycobacteria in the Human Environment. *J. Appl. Microbiol.* **2009**, *107*, 356−367.
(85) Brown, C. L.; Keenum, I. M.; Dai, D.; Zhang, L.; Vikesland, P. J.; Pruden, A. Critical Evaluation of Short, Long, and Hybrid Assembly for Contextual Analysis of Antibiotic Resistance Genes in Complex Environmental Metagenomes. *Sci. Rep* **2021**, *11*, 3753 DOI: 10.1038/s41598-021-83081-8.
(86) Větrovský, T.; Baldrian, P. The Variability of the 16S RRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses. *PLoS One* **2013**, *8*, No. e57923.
(87) Silva Martínez, S. Electrolytically Generated Silver and Copper Ions to Treat Cooling Water: An Environmentally Friendly Novel Alternative. *Int. J. Hydrogen Energy* **2004**, *29*, 921−932.
(88) Modol, J.; Sabria, M.; Reynaga, E.; Pedro-Botet, M. L.; Sopena, N.; Tudela, P.; Casas, I.; Rey-Joly, C. Hospital-Acquired Legionnaires Disease in a University Hospital: Impact of the Copper-Silver Ionization System. *Clinical Infectious Diseases* **2007**, *44*, 263−265.

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