Influence of Copper Dose on *Mycobacterium avium* and *Legionella pneumophila* Growth in Premise Plumbing

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ABSTRACT

Copper can be released from pipes or added intentionally to drinking water as an antimicrobial, possibly limiting biofilm and pathogen growth in building (i.e., premise) plumbing systems, but longer-term effects of various doses are poorly defined. Here, triplicate 120-mL water heater microcosms equipped with PEX-b pipes containing mature biofilms were fed influent dosed with copper at 0, 4, 30, 250, or 2000 ppb (as total Cu) and monitored for 11 months. Effluent total cell

counts and *Mycobacterium avium* peaked at 250-ppb copper dosage, reflecting the dual role of copper as a nutrient and antimicrobial. Total cell counts and *Mycobacterium avium* were relatively consistent in replicate microcosms at each dose. While *Legionella pneumophila (Lp)* responded similarly to copper and also peaked at 250 ppb, this dose also caused bifurcating behavior of biological replicates, with one consistently producing high culturable *Lp* (average of 2.5 log (MPN/mL)) and the other two consistently producing low or non-detect levels over an 11-month dosing period. We cross-inoculated the microcosms ten times periodically throughout the 6-month acclimation phase before copper dosing, and reinoculated the microcosms with *Lp* one time ~ 3.5 months into dosing in an attempt to normalize the microbial community composition across replicate microcosms. However, *Lp* levels across the three 250-ppb microcosms remained bifurcated throughout the copper dosing period. 16S rRNA gene sequencing revealed that the 250-ppb replicate with high *Lp* was characterized by a distinct microbial community composition relative to the two replicates with low *Lp*. At the highest dose of 2,000 ppb copper total cell counts initially decreased by about 78%, but then steadily increased to a point where they were not statistically different from the 250-ppb microcosms. Dosing 2000 ppb copper also reduced microbial diversity.

SYNOPSIS

A dose of 250 ppb copper caused peak growth of *Mycobacterium avium* and *Legionella pneumophila* in drinking water microcosms simulating hot water plumbing lines.

INTRODUCTION

Copper can be released into drinking water from corrosion of brass or copper pipe or can be intentionally dosed to control microbial growth in premise (i.e., building) plumbing.[1] Evidence suggests that copper can act as a nutrient to drinking water microbes at low

concentrations and an antimicrobial at high concentrations,[2] suggesting an optimal range for growth of pathogens. Efficacy of copper as an antimicrobial can be dependent on water chemistry, physiology of the target microbes, biofilm characteristics, water flow patterns, levels of other disinfectants (e.g., chlorine), and other factors.[2,3]

Mycobacterium avium and *Legionella pneumophila* (*Lp*) are two key opportunistic pathogens of concern that are prone to growth in premise plumbing.[2] Prior field surveys of *Lp* occurrence in drinking water suggest antimicrobial thresholds for copper of > 50 μg/L,[4] > 400 μg/L,[5] or > 1055 μg/L,[6] whereas other studies have reported contradictory results suggesting positive effects on growth at concentrations > 500 μg/L[7], or *Lp* persistence at concentrations > 1170 μg /L[8]. *M. avium* has been shown to be inactivated by high levels of copper in warm premise plumbing environments,[9,10] but *M. avium* and other nontuberculous mycobacteria (NTM) are generally considered more resilient to copper than *Lp*.[10,11] These prior studies suggest that copper, if present at sufficiently high concentrations, could be an effective means to simultaneously control both NTM and *Lp,* a goal that has remained elusive given that many existing drinking water control strategies (such as chloramination or silver ions), dependent upon disinfection concentration, may have no impact or even favor the growth of the other.[11,12]

To gain insight into the effects of copper on *Lp* and NTM control in real-world simulations of hot water plumbing systems, we conducted a series of studies using local Blacksburg municipal tap water at both large scale pilot (spanning 3 years) and microcosm scale (spanning 198 days).[9,13,14] These studies demonstrated how higher rates of anode rod corrosion, pH shifts, and hydrogen evolution can confound antimicrobial action of copper. Similar confounding effects were likely at play but not considered in the interpretation of results derived from prior field-scale research.[2] Comparatively, another relatively long-term study (12 months)

72 investigating opportunistic pathogens in water heaters was only able to detect NTM, and not
73 *Legionella* spp., *Lp*, or *P. aeruginosa* at any point in the experiment,[15] highlighting challenges in
74 simultaneous study of *Lp* and NTM at pilot scale, especially over extended time periods. In
75 another prior microcosm-scale study, the effect of copper dosed 0.8 – 5 mg/L total Cu to assess
76 the culturability of *Lp* over a 4-week period using synthetic water to mimic tap water at 36°C.[16]
77 That work found that different *Lp* strains reacted differently to copper. Furthermore,
78 environmental isolates derived from biofilm in a hot water system exhibited greater resistance to
79 copper compared to the clinical strains tested, suggesting that the environmental isolates (which
80 had a significantly higher expression of the copper resistance gene copA) were better adapted to
81 copper.[16] In a 76 week microcosm-scale study examining the impact of different materials
82 (including pipe coupons) on *Lp* and NTM, copper coupons released high concentrations of
83 copper (≈10 mg/L) when heated to 55 and 60°C, which in tandem, strongly suppressed
84 established NTM and *Lp* concentrations in the microcosms.[17]
85 The objective of this study was to assess the effects of a wide range of copper
86 concentrations on *M. avium* and *Lp* in premise plumbing systems colonized by both organisms.
87 Fifteen 120-mL water heater microcosms equipped with PEX-b pipes containing mature biofilms
88 (>3.5 years old and carrying both *Lp* and *M.* avium)[9] were acclimated to Blacksburg tap water
89 over a 6-month acclimation period and subsequently divided into biological triplicate
90 microcosms subject to copper dosing at 0, 4, 30, 250, or 2,000 ppb (as total copper) for 11-
91 months. Microbial total cell counts (TCC) were monitored via flow cytometry, *M. avium* was
92 measured via droplet digital polymerase chain reaction (ddPCR), and *Lp* was measured both via
93 ddPCR and Legiolert™. 16S rRNA gene sequencing was applied to profile microbial
94 communities and provide insight into microbial ecological factors mediating the effects of

copper. The goal was to assess doses at which copper was a nutrient and doses where it was antimicrobial for opportunistic pathogens in warm water premise plumbing.

MATERIALS AND METHODS

Premise Plumbing Microcosm Design and Establishment

Fifteen 120-mL glass microcosms, referred to as "simulated glass water heaters" in previous works,[18] were employed in this study to provide replicable simulation of premise plumbing with mature drinking water biofilm. Each replicate microcosm contained two new and two conditioned PEX-b pipe coupons, with the conditioned coupons cut from the middle of ~4.6-meter recirculating lines of pilot-scale hot water plumbing systems, one that was previously dosed with phosphate and one that received no added phosphate.[9,13] All coupons were 2.5 cm long with a ~ 1.7 cm inner diameter. The pilot-scale hot water plumbing systems had been inoculated with two strains of *Lp* serogroup-1 that were isolated from the Quincy, Illinois Veterans Home during a Legionnaires' Disease outbreak.[19] Prior to this experiment, the recirculation line biofilms were confirmed to be colonized by both *M. avium* and *Lp*,[9] serving as an inoculum to the microcosms.

Microcosm Influent & Water Changes: Acclimation Phase

The water heater microcosms were filled with 110 mL of local tap water (Blacksburg, VA) that had been further treated by granular activated carbon (GAC), which was the same water fed to the pilot-scale rig from which the pipe coupons were originally extracted. The influent water was collected in 5-week intervals and stored at 4°C until the day before the water change. On the day before a water change, 2L of the water was collected and heated to 37°C overnight then adjusted to pH 6.70 ± 0.05 using H_2SO_4, prior to being used for the microcosm water changes.

122 Throughout the study, the microcosm bulk water was changed 2× weekly, by replacing 75% of
123 the bulk water volume (~82 mL) in each microcosm with new influent, to match the 3.2-day
124 hydraulic retention time of the pilot-scale plumbing system. 3.2-days was selected to simulate
125 common residential hot water usage in the United States.[13] The microcosms were maintained at
126 37°C, representing the low end of a water heater set point, and gently mixed on a shaker table at
127 100 rpm. The microcosms were acclimated for 6 months to these conditions prior to commencing
128 the copper dosing.

129 *Cross-Inoculation of the Microcosms*

130 A month into the acclimation phase, the effluent from each microcosm was cross
131 inoculated with the aim of establishing baseline *Lp* and microbial populations across the 15
132 replicates. This was achieved by collecting the effluent (75% bulk water volume) from the
133 microcosms into a common reservoir (an autoclaved 2-L polypropylene bottle), vigorously
134 shaking this mixture, returning 25% of the combined effluent to the microcosms, and replacing
135 the remaining 50% with the GAC-treated water. This procedure was repeated over the course of
136 ten sequential water changes, starting a month into the acclimation phase, for five weeks
137 thereafter.

138 *Copper Dosing and Reinoculation*

139 Nearing the end of the 6-month acclimation phase, a week before the copper dosing
140 commenced, the microcosms were sampled for culturable *Lp* levels measured by Legiolert™
141 tests (IDEXX Laboratories, Westbrook, USA) and TCCs. Based on these measurements, the
142 microcosms were grouped into five sets of triplicate microcosms, so that there was no statistical
143 difference in mean values of TCC and *Lp* among experimental conditions (ANOVA, $p > 0.05$)
144 (Supplemental Figure 1). Triplicate microcosms subsequently received one of five copper

145 dosages of 0, 4, 30, 250, or 2000 ppb copper over an 11-month period. Copper was dosed
146 directly to the influent as total Cu using stock solutions prepared with $CuSO_4 \cdot 5H_2O$. Five
147 separate 500 mL glass containers were used for dosing: one contained 280 mL of GAC-treated
148 influent for the 0 ppb Cu control, while the 4, 30, and 250 ppb conditions were prepared by
149 diluting a 25 mg/L total Cu stock solution with GAC-treated influent to achieve the target
150 concentrations, resulting in 280 mL of copper-dosed influent to use per condition. The 2000 ppb
151 condition was prepared by mixing GAC-treated influent with a small volume of a 200 mg/L Cu
152 stock solution. Stock solutions (25 mg/L and 200 mg/L) were verified using a DR3900
153 Spectrophotometer (Hach, Loveland, USA) prior to each water change, and dosing was adjusted
154 accordingly based on spectrophotometric readings. After ~ 3.5 months of copper dosing, random
155 testing among the microcosms indicated that *Lp* still had not established in some replicates and,
156 therefore, all microcosms were reinoculated once more. This was achieved using water from the
157 same pilot-scale water heater from which the microcosm pipe coupons were originally derived.
158 The inoculation water was mixed with the normal influent water to target ~50 MPN/mL
159 culturable *Lp* in each microcosm.

160 The influent water preparation protocol underwent various adjustments, throughout the 6-
161 month acclimation (months 0-6 overall) and 11 month copper dosing (months 6-17 overall)
162 phases of the experiment, in an attempt to establish a replicable response of *Lp* to copper.
163 Modifications included: 1) passing GAC-filtered water through a ferric oxide filter to decrease
164 phosphate levels to < 0.05 mg/L followed by filter-sterilizing the water using a 0.22-μm pore
165 size mixed nitrocellulose ester membrane (Whatman, Maidstone, United Kingdom) throughout
166 the acclimation phase and for the first two months of the copper dosing phase (months 0-8), 2)
167 dosing GAC-treated influent with phosphate throughout the copper dosing phase to achieve 5

168 μg/L as P (months 6-17), 3) no longer sterilizing the influent or treating it with a ferric oxide
169 filter after 8 weeks into the copper dosing phase of the experiment (months 8-17), and 4) dosing
170 ferric pyrophosphate (100 μg as Fe) after week 35 of the copper dosing phase to provide
171 bioavailable iron (months 14-17).
172
173 *Microbial Analysis*
174
175 Effluent collected from the microcosms during bi-weekly water changes was subject to
176 microbial analysis. TCC was measured on a BD Accuri C6 (BD Bioscience, Franklin Lakes,
177 USA) using SYBR Green I dye to stain total (intact + damaged) cells.[20,21,22] Gating used the
178 Eawag FL1-A (emission filter 533/30) vs FL3-A (emission filter 670 LP) template for drinking
179 water, with 50 μL of each sample analyzed at medium speed (35 μL/min) and with an acquisition
180 threshold set to 800.[22] Legiolert™ was used to enumerate culturable *Lp*, subjecting 1.0 mL of
181 sample bulk water to the nonpotable procedure per the manufacturer's instructions.
182
183 During regular water changes, ~82 mL of influent or effluent water from each microcosm
184 was filtered through a 0.22-μm mixed-cellulose ester membrane filter (MilliporeSigma,
185 Burlington, USA) and the filter was subject to DNA extraction using a FastDNA SPIN kit (MP
186 Biomedicals Inc., Solon, USA). DNA extracts were analyzed for opportunistic pathogens using a
187 QX200 ddPCR (Bio-Rad, Hercules, USA) (Supplemental Table 1). These assays had previously
188 been optimized for qPCR[23] and were adapted for ddPCR in this study. Thermal gradients, ranging
189 from 50-60°C, were conducted on each assay to determine the optimal annealing temperature
190 (Supplemental Table 1) to optimally separate negative and positive droplets. Samples for all
191 targets were analyzed in triplicate and deemed as positive if at least 3 positive droplets were
192 found per reaction.[24]

16S rRNA Sequencing and Data Analysis

To analyze the microbial composition of each microcosm, 16S rRNA sequencing was carried out on the DNA extracts. Amplicon sequence reads targeted the V4-V5 hypervariable region of the 16S rRNA gene using the 515f/926r primer set. Samples were sequenced on a MiSeq V3 600 cycle run, yielding an average of 119,910 reads per sample (SD = 69,156). Reads and all subsequent analysis were carried out using DADA2 (v1.18.0)[25] in R (v 4.0.3).[26] Reads were filtered, trimmed, and paired before exact amplicon sequence variants (ASVs) were generated and chimeras removed. Taxonomy of ASVs was assigned using RDP Train Set 18.[27] ASVs were rarefied to the minimum sequencing depth of 10,544.[28] Permutational multivariate analysis of variance (Permanova) using Bray-Curtis distance matrices was conducted using the vegan R package (v 2.6.4).[29]

Data Availability

The 16S rRNA Sequencing gathered from the microcosms in this experiment has been uploaded to the National Center for Biotechnology Information Sequence Read Archive database under Accession number PRJNA1229645.

RESULTS

Total Cell Counts

The effluent mean TCC for all the microcosms during the end of the acclimation phase (month 6), before copper addition, was 3.18×10^5 events/mL (SD = 4.16×10^3) (Figure 1, Supplemental Figure 1b). In the short-term after copper dosing began (1-3.5 months post copper dosing), the total cell counts indicated optimal growth at 250-ppb, and lower growth at lower and higher copper levels. As the experiment progressed, the three replicates dosed with 250 ppb

copper consistently produced the highest cell counts. Replicates dosed with 2000 ppb copper initially had a 0.66 log reduction (a 78% decrease from its final pre-dosing value), causing the lowest cell counts observed in the study. But by the end of the 11- month experiment the mean cell counts at 2000 ppb copper gradually recovered and eventually surpassed those at doses ≤ 30 ppb and were not significantly different than at 250 ppb (Tukey's HSD $p<.05$) (Figure 1, Supplemental Table 2). In fact, the TCC steadily increased with time in the 2000 ppb microcosms both pre-and post-re-inoculation attempt 3.5 months into the copper dosing phase (Figure 1, Supplemental Figure 2).

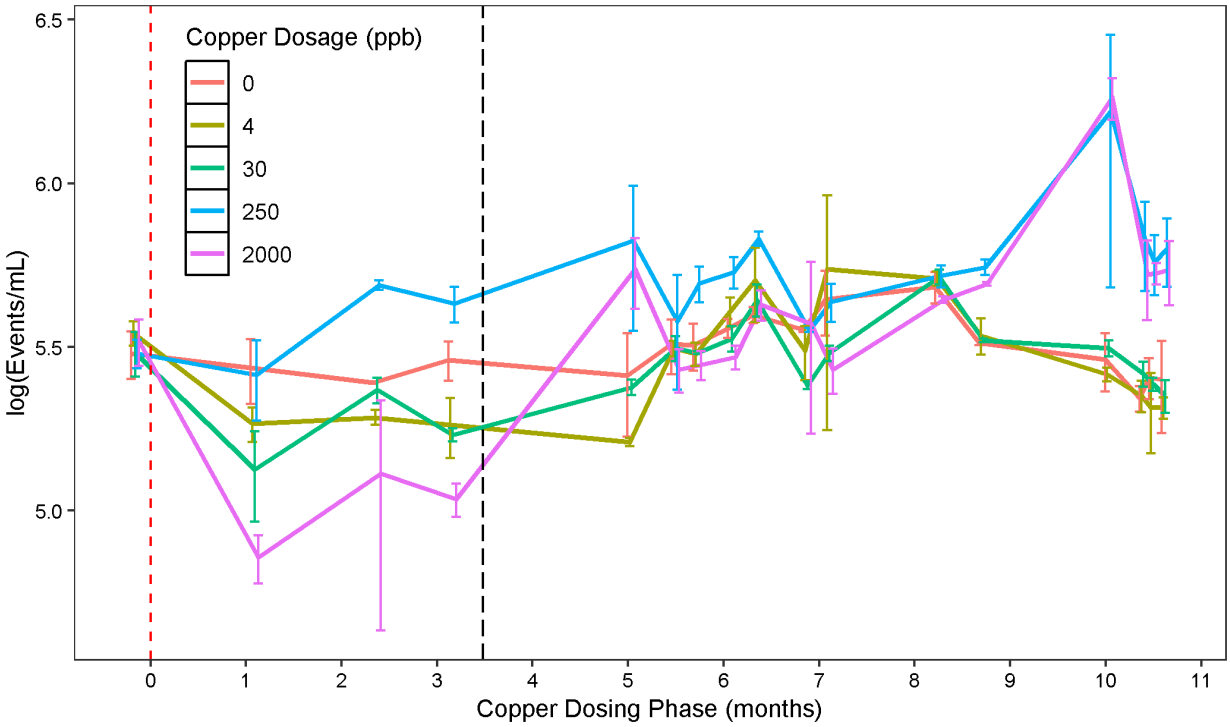

Figure 1. Mean Total Cell Counts (events/mL) in microcosm bulk water effluent over the course of the experiment. Error bars are the standard deviation of biological triplicate microcosms. Lines connect the data points to guide comparison and are colored by copper concentration. The first measurement (time -0.25) occurred at the end of the acclimation phase, before copper dosing began (time = 0) represented by the red dashed vertical line. The black dashed vertical line at ~ 3.5 months represents the point at which all reactors were reinoculated with *Lp*. Time points have a set jitter for visual purposes.

Mycobacterium avium response to copper in the microcosms

When tested over three sequential samplings in the final (11th) month of the experiment, significant differences in *M. avium* gene copies/mL were found in the microcosm bulk water as a function of copper concentration (Linear Mixed-Effect Model ANOVA, $p<0.05$) (Figure 2). The 250-ppb condition had higher concentrations of *M. avium* compared to other copper dosages (0.55 – 0.85 logs higher) (Tukey's HSD, $p<0.05$, Supplementary Table 3). *M. avium* was not detectable in the influent water using ddPCR so the increase in the effluent represents growth in the microcosms.

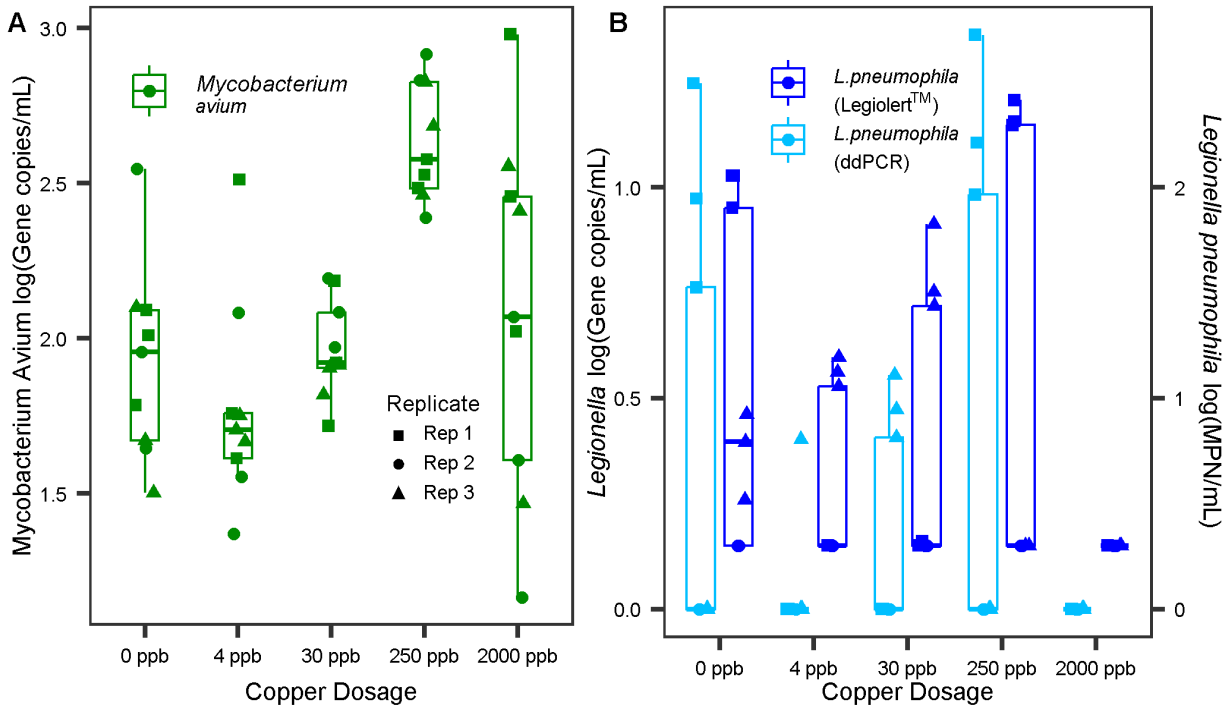

Figure 2. Mean gene copies/mL counts of (A) *M. avium* detected through ddPCR and (B) *Lp* detected via ddPCR and Legiolert™ across the fifteen microcosms over three consecutive samplings in the final (11th) month of the experiment.

L. pneumophila response to copper in the microcosms

257 *Lp* response to copper dosing was confirmed both by ddPCR and Legiolert™. On dates
258 when both Legiolert™ and ddPCR tests were conducted on aliquots of the same sample (n=45),
259 results were strongly correlated ($R^2 = 0.86$) (t-test, $p<0.05$), but the log ddPCR gc/mL values
260 were typically 0.51 times the log of Legiolert™ MPN/mL measurements (Supplemental Figure
261 3). *Lp* concentrations did not significantly differ across the microcosms as a function of copper
262 condition (Linear Mixed-Effect Model ANOVA, $p > 0.05$), but both Legiolert™ and ddPCR
263 measurements indicated a consistent trend ranking as follows: 2000 ppb < 4 ppb < 30 ppb < 0
264 ppb < 250 ppb. *Lp* was never detectable in the influent used for the microcosm water changes
265 using ddPCR or Legiolert™ so the increase in the effluent represents growth in the microcosm. It
266 was noted that there was a much wider variance in *Lp* numbers within replicates than there were
267 for *M. avium* (Figure 2). This was most evident for microcosms dosed with 250 ppb copper, for
268 which one microcosm consistently displayed the highest concentrations of culturable *Lp*, while
269 the second and third replicates produced non-detectable levels of culturable *Lp* for 87% and 73%
270 of the sampling events over the course of the copper-dosing phase of the experiment (Figure 3).
271 *Lp* levels quantified via Legiolert™ ranged from 8 MPN/mL to 1460 MPN/mL for the first
272 replicate microcosm in the 250-ppb Cu condition (Figure 3). The stark differences between these
273 microcosms were maintained even though they had been cross-inoculated ten times during
274 acclimation to establish the same baseline microbial community prior to copper dosing as well as
275 in addition to a post-copper dosing reinoculation event ~ 3.5 months into the copper dosing
276 phase and four adjustments to the water chemistry.

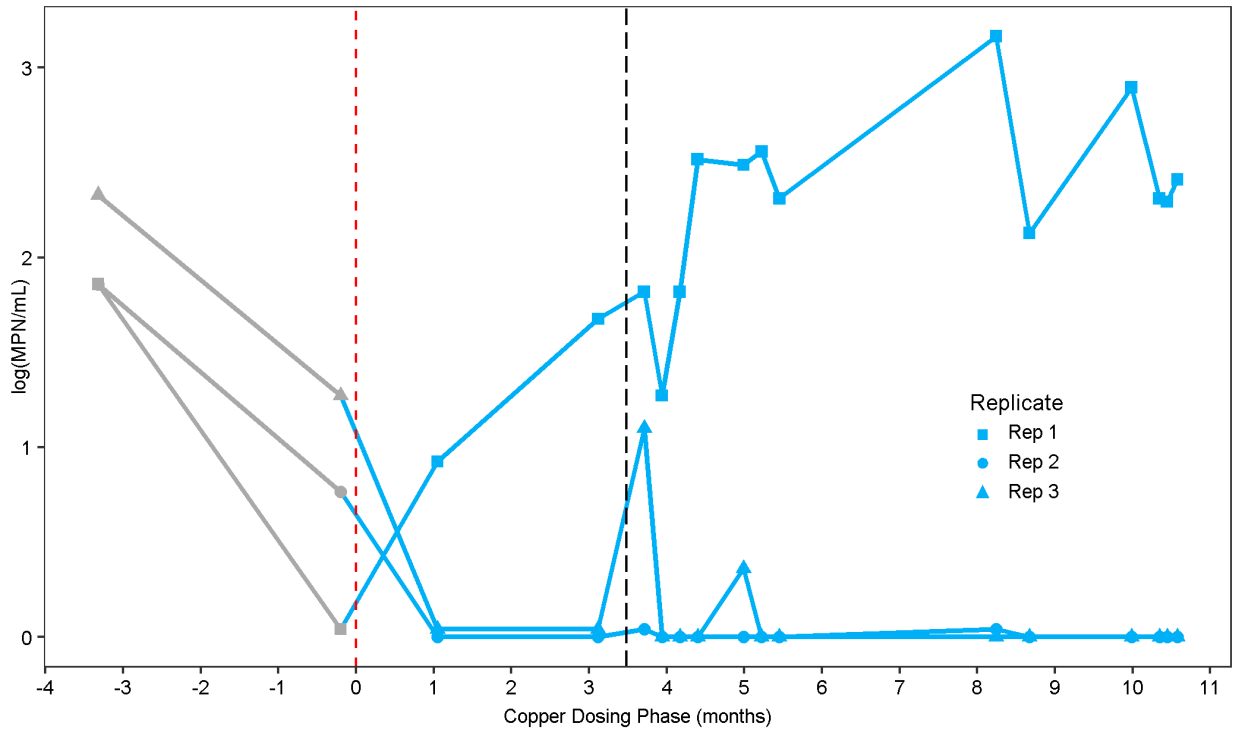

Figure 3. *Lp* MPN/mL, determined via Legiolert™, among the three replicate microcosms dosed with 250 ppb copper that displayed divergent behavior over the 11-month experimental period. The vertical, dashed line at ~3.5 months represents the point at which all microcosms were reinoculated with *Lp*. Time 0, shown with the red dashed vertical line represents when the copper dosing began. Points before 0 months represents culturable *Lp* in the microcosms prior to copper dosing, during the acclimation phase. Culturable *Lp* concentration measured with time for the microcosms receiving other copper concentrations can be found in Supplemental Figure 4. Legiolert™ detection limit was 1 MPN/mL.

Consistent with the Legiolert™ results, replicates 2 and 3 of the 250-ppb copper condition consistently yielded undetectable gene copies/mL of *Lp*, while up to 22 gene copies/mL were measured in replicate 1 (Figure 2).

16S rRNA Sequencing

16S rRNA sequencing allowed comparison of the microbial community profiles across the microcosms and influent water (Figure 4). The microbial communities were significantly different from each other as a function of copper concentration (ANOSIM, $p < 0.05$) (Figure 5). The ten most abundant phyla from each replicate microcosm produced a similar profile for

298 copper doses of 0, 4, and 30 ppb, with a marked shift in composition in the 250 ppb and 2000
299 ppb conditions (Figure 4). Of note, the microcosms dosed with 0, 4, and 30 ppb copper were
300 mostly comprised of *Proteobacteria*, while the reactors dosed with 250 ppb copper contained
301 relatively equal proportions of *Proteobacteria* and *Gemmatimonadetes.* Microcosms dosed with
302 2000 ppb were primarily dominated by *Bacteriodetes* and *Proteobacteria* (Figure 4). Within
303 each condition from 0-250 ppb, the microbial communities were relatively consistent among the
304 three replicates. However, there was marked variation in the microbial community among the
305 2000 ppb condition replicates (Figure 5). Copper concentration had a significant effect on
306 Shannon diversity and Simpson diversity as measured by analysis of variance (ANOVA, $p <$
307 0.0001) (Figure 6).

308 The microbial community profile of the 250-ppb microcosm that consistently had the
309 highest level of *Lp* was dominated by the same top ten phyla, but differences in relative
310 abundance stood out (Figure 4, highlighted in orange boxes). Specifically, this microcosm was
311 statistically enriched in *Ignavibacteriae* and depleted in *Chlamydiae* and *Bacteroidetes* (Wald's
312 test, $p<0.05$) (Figure 4, highlighted in orange boxes). Beta diversity analysis, however, did not
313 reveal an obvious difference in the microbial community composition of this replicate beyond
314 the variation that was seen for replicates of other copper concentrations (Figure 5).

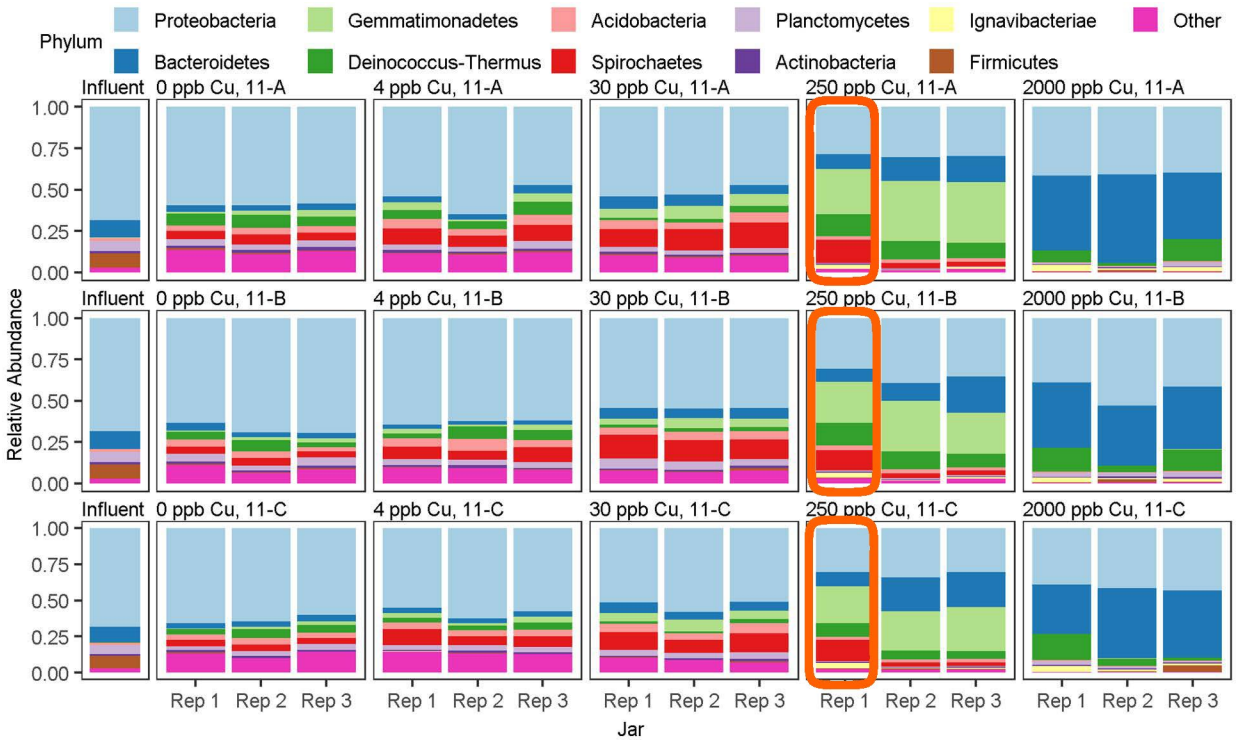

Figure 4. Relative abundances of each of the top ten most abundant phyla over all ASVs in each replicate microcosm grouped by copper concentration and sampling date. The orange boxes indicate the 250-ppb copper replicate that consistently displayed the highest concentrations of *Lp*. The influent [collected at the start of month 10 for water changes (n=1)] represents the water used throughout three sequential sampling events during water changes carried out in the final (11th) month of the experiment. 11-A/B/C, represent the first (A), second (B), and third (C) sequential water changes.

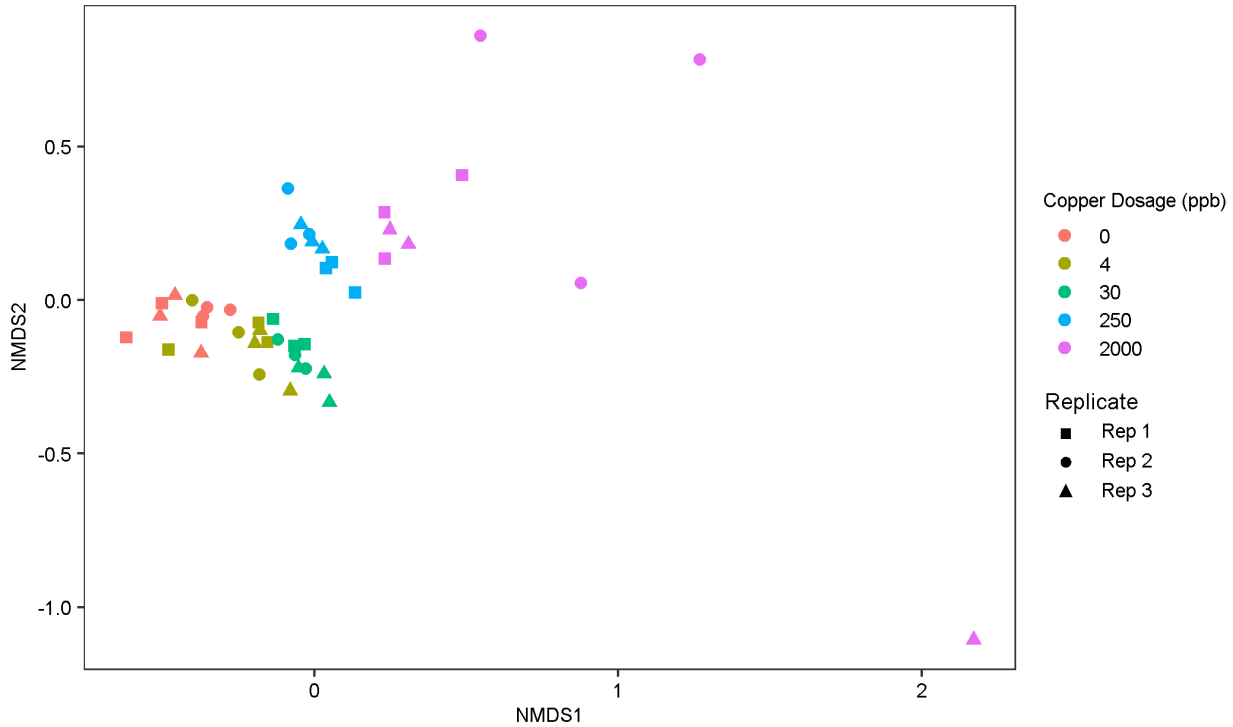

Figure 5. Bray-Curtis-based nonmetric multidimensional scaling of taxa from effluent water of microcosms dosed with 0, 4, 30, 250, and 2000 ppb of copper over an 11-month period (n=3 (Stress = 0.08836)

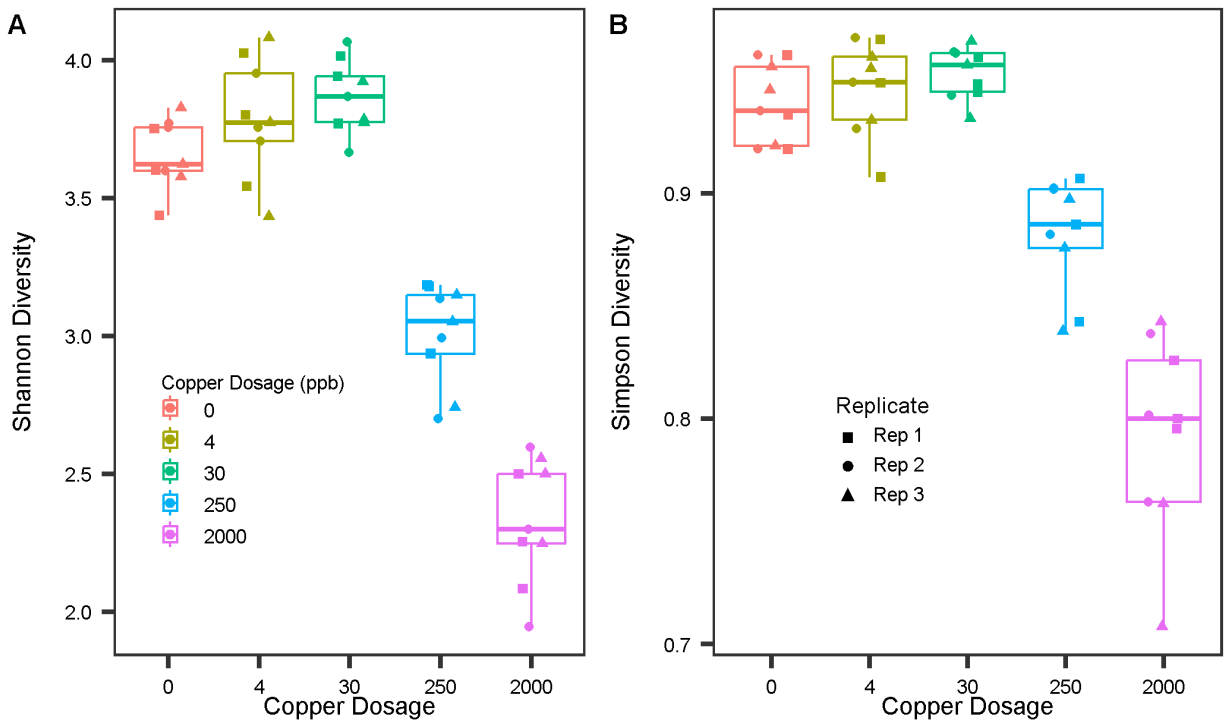

Figure 6. Shannon and Simpson diversity index of replicate microcosms on the final three sampling dates grouped by copper condition (n = 3 microcosms × 3 sampling events = 9 for each copper condition).

Relative abundances of individual classes of bacteria were further compared across microcosm conditions revealing some notable differences as a function of copper concentration (Figure 7). For example, *Proteobacteria* was higher in the 2000 ppb than in the 250 ppb condition, while *Firmicutes* indicated little change across copper dosages (Figure 7). *Gemmatimonadetes* were enriched from 0 to 250 ppb, but dramatically decreased at 2000 ppb (Figure 7). *Deinococcus-Thermus* indicated a slight decrease in relative abundance from 250 ppb to 2000 ppb (Figure 7). *Chloroflexi*, *Acidobacteria*, and *Actinobacteria* markedly decreased with increasing copper (Figure 7).

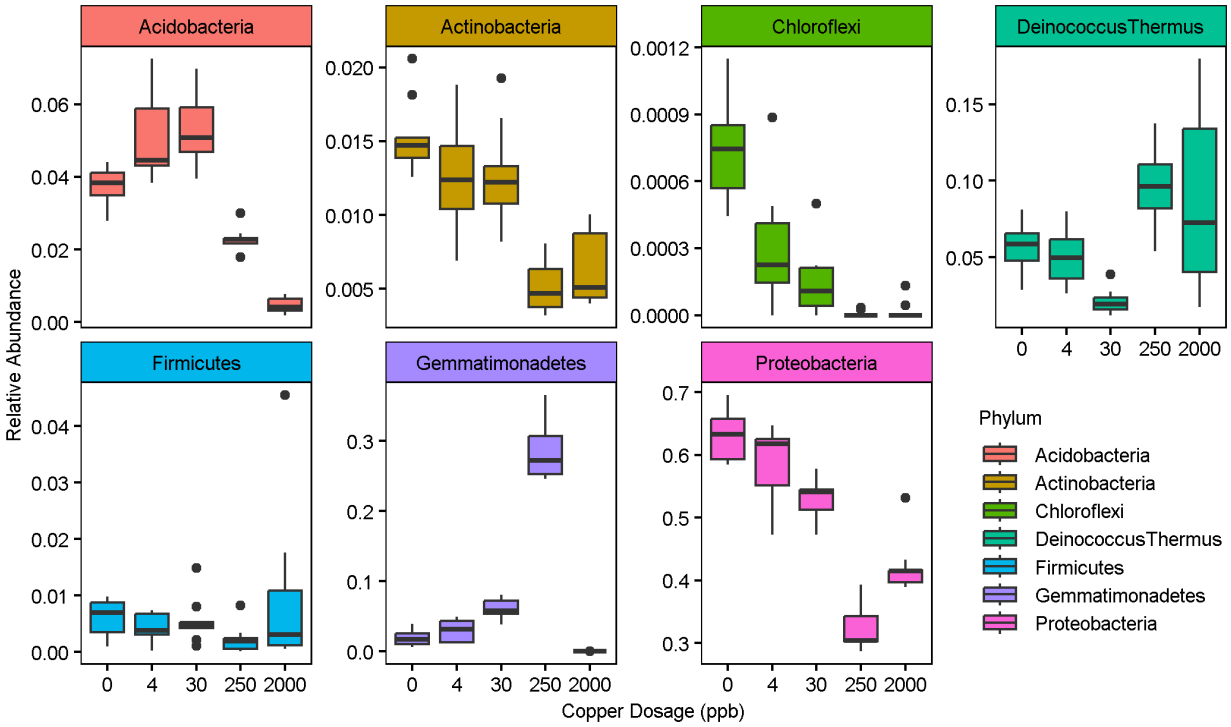

Figure 7. Relative abundance of select phyla across copper dosages in each replicate over the three sequential sampling events carried out in the final (11th) month of the experiment.

DISCUSSION

This study provided new insights into the effects of copper in premise plumbing as a possible nutrient and antimicrobial. TCCs provided an indicator of broad-scale effects on bacterial communities at-large, revealing some surprising long-term patterns. While TCCs initially decreased markedly when the highest dose of 2000 ppb was added as expected, over the course of subsequent months, the microbial community adapted such that TCCs were as high as 250 ppb by the end of the 11-month experiment. The rise in total cell counts at 2000 ppb did not plateau by 11 months, suggesting it might eventually rise above the 250 ppb dose after years or decades in premise plumbing.

Interestingly, TCCs initially increased in the 250-ppb condition and remained elevated throughout the experiment relative to the un-dosed control, suggesting that copper acted largely as a nutrient at this concentration. Doses of 4 and 30 ppb Cu were initially associated with a

364 significant decrease in TCCs (paired t-test, $p < 0.05$), but remained comparable to the control for
365 the majority of the experiment.

366 *M. avium* concentrations, which were only measured in the 11th month, were also highest in
367 the 250-ppb condition, but were lowest in the 2000 ppb condition. This suggests that very high
368 doses of copper are likely needed to effectively control *M. avium*, and lower doses serve as a
369 nutrient or selector for the organism. Overall, the findings for TCC and *M. avium* were consistent
370 with expectations that copper has a dual role of nutrient and antimicrobial,[2] or that a level of
371 copper that is antimicrobial in the short-term can behave as a nutrient over a period of months to
372 years as microbial resistance to copper develops.[16]

373 Support for this hypothesis was provided in the microbial community analysis. Even
374 though TCCs were elevated in the 250 ppb and 2000 ppb conditions, microbial diversity was
375 diminished. As richness and evenness dropped, a subset of the remaining species asserted
376 dominance over the community (Figures 4 and 6). With the significant decrease in diversity, a
377 few taxa began dominating the community, causing stochasticity as per the wide variance in
378 microbial community composition among the 2000 ppb replicates (Figure 5). Prior research
379 using the same municipal tap water supply also assessed the effect of dosed copper on the
380 microbial community in a full-scale water system, but the experimental design differed from the
381 present study in that the dose was incrementally increased from 0, 50, 100, 300, 600, 1200, to
382 2000 ppb, while maintaining each concentration for only periods of 4 to 16 weeks.[9] Song and
383 colleagues observed similar trends as those reported in this work for *Proteobacteria*,
384 *Deinococcus-Thermus*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*. However, Song observed
385 enrichment over 6 weeks at 1200 ppb in *Firmicutes*, and enrichment of *Gemmatimonadetes* up
386 through 2000 ppb. The differences in *Firmicutes* and *Gemmatimonadetes* behavior when

387 comparing the results of this study could potentially be attributed to differences in duration of
388 copper dosing, as Song and colleagues maintained the high copper dose for 12 weeks,[9] whereas
389 the microcosms in this study were acclimated at this dose for 11 months.

390 At 250 ppb, stochasticity was observed in the concentrations of culturable *Lp*, which was
391 relatively consistent in all replicates at lower copper doses (0, 4, and 30 ppb total Cu) and was
392 almost always undetectable at 2000 ppb copper (Supplementary Figure 4). In a prior study
393 investigating the effect of copper pipe in warm premise plumbing systems, *Lp* numbers were
394 initially lowest in the biofilms on copper pipes, but eventually increased to the same
395 concentrations as PEX and stainless-steel pipes after two years.[30] In such studies, it is important
396 to note that copper pipe aging leads to decreasing release of copper into solution, and thus it is
397 difficult to know if the observed increase in *Lp* is due to lower levels of copper or microbial
398 adaptation.

399 A synthesis of our results over the last decade using Blacksburg tap water reveals two
400 general responses of experimental plumbing systems with respect to *Lp* inoculation. As one
401 would expect, there are many circumstances in which the system response to inoculated *Lp* is
402 deterministic, in which replicate reactors behave as true replicates. But here, we report a situation
403 in which stochastic behavior clearly occurred, because in some replicate reactors *Lp* consistently
404 died off and in other reactors it thrived. With benefit of hindsight, we now recognize similar
405 stochastic behavior for *Lp* amongst replicate reactors during at least two prior experiments in our
406 lab.[31,32] We were reluctant to accept the conclusion that these systems were stochastic, preferring
407 to believe there were subtle uncontrolled differences between replicates, but we consider the
408 evidence gathered herein to be conclusive. This study demonstrates that even in a relatively

409 simple simulation of premise plumbing, such as a glass microcosm containing pipe sections, that
410 there are complexities at play that can determine if effluent *Lp* is extremely high or very low.
411 Perhaps even more interestingly, the replicate reactors described herein were deterministic
412 and replicable for measured dimensions of cell counts, mycobacteria and community analysis,
413 even as they were stochastic for *Lp*. We speculate that a specific biological factor profoundly
414 affects the predator, prey and parasitic relationships of the *Lp* life cycle. If such stochastic
415 behavior for *Lp* is commonplace, as it is in certain other areas of microbial ecology,[33,34] this
416 discovery has profound implications for experimental design and the interpretation of past data
417 from laboratory and field sampling.[35]
418

419 **ASSOCIATED CONTENT**
420 **Supporting Information**

421 Information regarding ddPCR assays used, statistical analysis (on the total cell count and *M.*
422 *avium*.), initial *Lp* and TCC of the microcosm groups at the end of the acclimation phase,
423 additional total cell count information for the 2000 ppb Cu condition, a comparison between
424 Legiolert™ and ddPCR *Lp* results, and the culturable *Lp* for all the copper conditions throughout
425 the study, can be found in the attached supplemental document (PDF).

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NOTES

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