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

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ABSTRACT

Effects of copper at 0, 4, 30, 250, or 2000 µg/L on microbial communities were examined in an 11-month study using triplicate 120-mL water heater microcosms with PEX-b pipes containing mature biofilms to simulate premise plumbing. Effluent total cell counts (TCCs) and *Mycobacterium avium* peaked at 250 µg/L, reflecting the dual role of copper as a nutrient and antimicrobial. TCCs and *M. avium* were relatively consistent among replicate microcosms at each dose, but *Legionella pneumophila (Lp)* diverged among biological triplicates at 250 µg/L, consistently producing high culturable *Lp* (average 2.5 log MPN/mL) in one microcosm and

low/non-detectable levels in the other two. Repeated cross-inoculations and a reinoculation failed to normalize the microbial community composition across 250 µg/L and other triplicate microcosms. 16S rRNA gene amplicon sequencing revealed that the 250 µg/L replicate with high *Lp* was characterized by a distinct microbial community composition relative to the two replicates. At 2,000 µg/L copper, microbial diversity and TCCs initially decreased, but then TCCs subsequently increased and ultimately were not statistically different from the 250 µg/L microcosms. This study provides insight into mechanisms underlying non-linear effects of copper dosing when applied as a disinfectant to premise plumbing for opportunistic pathogen control.

KEYWORDS *Legionella,* mycobacteria, opportunistic pathogens, copper, drinking water, cross-linked polyethylene pipes

SYNOPSIS

Non-linear effects of copper on *Legionella pneumophila* and microbial communities are identified, providing deeper understanding to the efficacy of its use as a premise plumbing disinfectant.

INTRODUCTION

Copper is of interest as a disinfectant for control of opportunistic pathogen growth in premise (i.e., building) plumbing. Copper can be released into drinking water from corrosion of copper alloy pipe or intentionally dosed for this purpose.[1] Broadly, 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] Further, copper has been found to sometimes act as a nutrient to drinking water microbes at low concentrations and an antimicrobial at high concentrations,[2] suggesting that sub-

48 optimal doses could stimulate growth, rather than death, of pathogens. Thus, there is an array of
49 factors to consider in applying copper as a premise plumbing disinfectant.
50 *Mycobacterium avium* and *Legionella pneumophila* (*Lp*) are two key opportunistic
51 pathogens of concern that are prone to growth in premise plumbing.[2] Prior field surveys of
52 *Legionella* and *Lp* occurrence in drinking water suggest antimicrobial thresholds for copper of >
53 50 μg/L,[4] > 400 μg/L,[5] or > 1055 μg/L.[6] However, other studies have reported contradictory
54 results, suggesting positive effects on *Legionella* and *Lp* growth at concentrations > 500 μg/L[7],
55 or *Lp* persistence at concentrations > 2000 μg/L[8]. *M. avium* has been shown to be inactivated by
56 high levels of copper in warm premise plumbing environments,[9,10] but *M. avium* and other
57 nontuberculous mycobacteria (NTM) are generally considered more resilient to copper than
58 *Legionella*.[10,11] The prior studies suggest that copper, if present at sufficiently high
59 concentrations, could sometimes effectively control both NTM and *Lp,* which is the ideal case.
60 Nonetheless, many existing strategies for controlling opportunistic pathogens in drinking water
61 have limitations and, in some instances, may inadvertently promote pathogen proliferation.[11,12]
62 Controlled studies are needed to resolve discrepancies in the efficacy of copper as a disinfectant
63 and to gain deeper mechanistic understanding needed to improve its application for simultaneous
64 reduction of multiple opportunistic pathogens.
65
66 To gain insight into the effects of copper on *Lp* and NTM control in hot water plumbing
67 systems, specifically at temperatures that support opportunistic pathogen growth in premise
68 plumbing, we previously conducted a series of studies at both pilot- (spanning 3 years) and
69 microcosm- (spanning 198 days) scale using the same local Blacksburg water supply.[9,13,14] These
70 studies demonstrated how a complex array of phenomena; including anode rod corrosion,
71 associated hydrogen evolution, and pH shifts, can confound the antimicrobial action of copper.

72 Similar confounding effects were likely at play, but not considered in the interpretation of results
73 derived from prior field-scale research.[2] Comparatively, another relatively long-term study (12
74 months) investigating opportunistic pathogens in water heaters was only able to detect NTM, and
75 not *Legionella* spp., *Lp*, or *Pseudomonas aeruginosa* at any point in the experiment,[15]
76 highlighting challenges in simultaneous study of *Lp* and NTM at pilot scale, especially over
77 extended time periods. In another prior microcosm-scale study, the effect of a 0.8 – 5 mg/L total
78 Cu dose on *Lp* culturability was examined over a 4-week period using a synthetic water at
79 36°C.[16] That work found that different *Lp* strains reacted differently to copper. Furthermore,
80 environmental isolates derived from biofilm in a hot water system exhibited greater resistance to
81 copper compared to the clinical strains tested, suggesting that the environmental isolates (which
82 had a significantly higher expression of the copper resistance gene copA) were better adapted to
83 copper.[16] Finally, a 76 week microcosm-scale study examined the impact of different materials
84 (including pipe coupons) on *Lp* and NTM, finding that high concentrations of copper (≈10
85 mg/L) suppressed established NTM and *Lp* and lowered their concentrations in the
86 microcosms.[17]

87 The objective of this study was to assess the effects of a wide range of copper
88 concentrations on *M. avium* and *Lp* in premise plumbing systems colonized by both organisms.
89 Fifteen 120-mL water heater microcosms equipped with PEX-b pipes containing mature biofilms
90 (>3.5 years old) and colonized with both *Lp* and *M. avium*[9] were acclimated to Blacksburg tap
91 water over a 6-month period and subsequently divided into biological triplicate microcosms
92 subject to copper dosing at 0, 4, 30, 250, or 2,000 µg/L (as total copper) for 11-months.
93 Microbial total cell counts (TCC) were monitored via flow cytometry, *M. avium* was measured
94 via droplet digital polymerase chain reaction (ddPCR), and *Lp* was measured both via ddPCR

95 and Legiolert™. 16S rRNA gene amplicon sequencing was applied to profile microbial
96 communities and provide insight into microbial ecological factors mediating the effects of
97 copper. The goal was to assess doses at which copper acted as a nutrient versus as an
98 antimicrobial for opportunistic pathogens in warm water premise plumbing.
99
100 **MATERIALS AND METHODS**
101
102 *Premise Plumbing Microcosm Design and Establishment*
103
104 Fifteen new 120-mL glass microcosms (referred to as "simulated glass water heaters" in
105 previous works)[18] provided replicable simulation of premise plumbing with mature drinking
106 water biofilm in triplicate. Each replicate microcosm contained two new and two conditioned
107 PEX-b pipe coupons, with conditioned coupons cut from the middle of ~4.6-meter recirculating
108 lines of pilot-scale hot water plumbing systems, one that was previously dosed with phosphate
109 and one that received no added phosphate during a >3.5-year-old experiment.[9,13] All coupons
110 were 2.5 cm long with a ~ 1.7 cm inner diameter. The pilot-scale hot water plumbing systems
111 had been inoculated with two strains of *Lp* serogroup-1 that were isolated from the Quincy,
112 Illinois Veterans Home during a Legionnaires' Disease outbreak.[19] Prior to this experiment, the
113 recirculation line biofilms were confirmed to be colonized by both *M. avium* and *Lp*,[9] serving as
114 an inoculum to the microcosms.
115
116 *Microcosm Influent & Water Changes: Acclimation Phase*
117
118 The water heater microcosms were filled with 110 mL of local tap water (Blacksburg, VA)
119 treated by the granular activated carbon (GAC) filter feeding the recirculating pipe rig from
120 which the pipe coupons were extracted. The influent water was collected in batches at 5-week
121 intervals and stored at 4°C. On the day before a water change, 2L of the water was collected and

122 heated to 37°C overnight then adjusted to pH 6.70 ± 0.05 using H_2SO_4, before addition to the
123 microcosms. The microcosm bulk water was changed 2× weekly, by replacing 75% of the bulk
124 water volume (~82 mL) in each microcosm with new influent, to match the 3.2-day hydraulic
125 retention time of the pilot-scale plumbing system. The 3.2-day hydraulic retention time was
126 selected to simulate common residential hot water usage in the United States.[13] The microcosms
127 were maintained at 37°C, representing the low end of a water heater set point, and gently mixed
128 on a shaker table at 100 rpm. The microcosms were acclimated for 6 months under these
129 conditions prior to commencement of copper dosing.

130 *Cross-Inoculation of the Microcosms*

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

139 *Copper Dosing and Reinoculation*

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

145 Figure 1). Triplicate microcosms subsequently received one of five copper dosages of 0, 4, 30,
146 250, or 2000 µg/L copper over an 11-month period. Copper was dosed directly to the influent
147 using stock solutions prepared with $CuSO_4 \cdot 5H_2O$. After ~ 3.5 months of copper dosing, random
148 testing among the microcosms indicated that *Lp* still had not established in some replicates and,
149 therefore, all microcosms were reinoculated once more. This was achieved using water from the
150 same pilot-scale water heater from which the microcosm pipe coupons were originally derived.
151 The inoculation water was mixed with the normal influent water to target ~50 MPN/mL
152 culturable *Lp* in each microcosm.

153 The influent water preparation protocol was changed throughout the 6-month acclimation
154 (experimental months 0-6) and 11-month copper dosing (experimental months 6-17) phases of
155 the experiment in an attempt to establish a replicable response of *Lp* to copper. Modifications
156 included: 1) passing GAC-filtered water through a ferric oxide filter to decrease phosphate levels
157 to < 0.05 mg/L followed by filter-sterilizing the water using a 0.22-µm pore size mixed
158 nitrocellulose ester membrane (Whatman, Maidstone, United Kingdom) during the experimental
159 months 0-8; 2) dosing GAC-treated influent with phosphate throughout the copper dosing phase
160 to achieve 5 µg/L as P during experimental months 6-17; 3) no longer sterilizing the influent or
161 treating it with a ferric oxide filter during experimental months 8-17; and 4) dosing ferric
162 pyrophosphate (100 µg as Fe) after week 35 of the copper dosing phase to provide bioavailable
163 iron experimental months 14-17.

164

165 *Microbial Analysis*

166

167 Effluent collected from the microcosms during bi-weekly water changes was subject to
168 microbial analysis. TCC was measured on a BD Accuri C6 (BD Bioscience, Franklin Lakes, NJ)
169 using SYBR Green I dye to stain total (intact + damaged) cells.[20,21,22] Gating used the Eawag

170 FL1-A (emission filter 533/30) vs FL3-A (emission filter 670 LP) template for drinking water,
171 with 50 µL of each sample analyzed at medium speed (35 µL/min) and with an acquisition
172 threshold set to 800.[22] Legiolert™ was used to enumerate culturable *Lp*, subjecting 1.0 mL of
173 sample bulk water to the nonpotable procedure per the manufacturer's instructions.
174
175 During regular water changes, ~82 mL of influent or effluent water from each microcosm
176 was filtered through a 0.22-µm mixed-cellulose ester membrane filter (MilliporeSigma,
177 Burlington, MA) and the filter was subject to DNA extraction using a FastDNA SPIN kit (MP
178 Biomedicals Inc., Solon, OH).
179 *Droplet Digital PCR (ddPCR) Analysis*
180 DNA extracts were analyzed for opportunistic pathogens using a QX200 ddPCR
181 instrument (Bio-Rad, Hercules, CA) (SI Table 1). These assays, targeting the *Lp* specific *mip*
182 gene and *M. avium* 16S rRNA gene, had previously been optimized for qPCR[23] and were adapted
183 for ddPCR in this study. Thermal gradients, ranging from 50-60°C, were conducted on each
184 assay to determine the optimal annealing temperature (SI Table 1) to separate negative and
185 positive droplets. Each sample was analyzed in technical triplicate for *Lp* and *M. avium.*
186 Reactions were prepared using a total volume of 22 µL per well, with 20 µL in each well used for
187 analysis. PCR amplification was carried out on a C100 Touch Thermal Cycler (Bio-Rad)
188 (thermocycling conditions listed in SI Table 1). Each 96-well plate included three no-template
189 controls (NTCs) using molecular-grade water, as well as three positive controls containing
190 synthetic gene fragments (gBlocks™, Integrated DNA Technologies, Coralville, IA)
191 corresponding to the targeted gene regions. A sample was classified as positive if a minimum of
192 three positive droplets were detected in at least two of the three of the technical replicates.[24] Only
193 data from wells generating > 10,000 accepted droplets were included in the analysis.

194 *16S rRNA Gene Amplicon Sequencing and Data Analysis*
195
196 To analyze the microbial composition of each microcosm, 16S rRNA gene amplicon
197 sequencing was carried out on the DNA extracts. Amplicon sequence reads targeted the V4-V5
198 hypervariable region of the 16S rRNA gene using the 515f/926r primer set. Samples were
199 sequenced on a MiSeq V3 600 cycle run, yielding an average of 119,910 reads per sample (SD =
200 69,156). Reads were imported and subsequent analysis were carried out using DADA2
201 (v1.18.0)[25] in R (v 4.0.3).[26] Reads were filtered, trimmed, and paired before exact amplicon
202 sequence variants (ASVs) were generated and chimeras removed. Taxonomy of ASVs was
203 assigned using RDP Train Set 18.[27] ASVs were rarefied to the minimum sequencing depth of
204 10,544.[28] Multivariate homogeneity was checked with the betadisper R function ($p > 0.05$)
205 before conducting permutational multivariate analysis of variance (PERMANOVA) with Bray-
206 Curtis distance matrices using the vegan R package (v 2.6.4).[29] Linear mixed-effect models were
207 created on *M. avium* and *Lp* ddPCR measurements, controlling for the three consecutive
208 samplings of each microcosm in the final month of the experiment using the lme4 R package (v
209 1.1-36).[30] ANOVA was run on the linear mixed-effect models to assess the impact of copper
210 dosage and on the Shannon and Simpson diversity (calculated with vegan) using the R stats
211 package (v 4.4.2).[26] Tukey's HSD was used as a post-hoc test to ANOVA to assess the impact of
212 each copper dosage using the multcomp R package (v 1.4-28).[31] Wald's test was used for the
213 log2 fold change between the 250 µg/L Cu condition microcosms using the DESeq2 R package
214 (v 1.46.0).[32]A paired t-test controlling for repeated measures was used to assess differences in
215 TCC before and after copper dosing using the R stats package (v 4.4.2).[26]
216 *Data Availability*

The 16S rRNA gene amplicon 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 cells/mL (SD = 4.16×10^3) (Figure 1, SI Figure 1b). In the short-term after copper dosing began (1-3.5 months post copper dosing), the TCC indicated growth at 250 µg/L. As the experiment progressed, the three replicates dosed with 250 µg/L copper consistently produced the highest cell counts. Replicates dosed with 2000 µg/L copper initially experienced a 0.66 log reduction (a 78% decrease from its final pre-dosing concentration), resulting in the lowest TCCs observed in the study. But by the end of the 11-month experiment, the mean TCC at 2000 µg/L copper gradually recovered and eventually surpassed those at doses ≤ 30 µg/L and were not significantly different than at 250 µg/L (Tukey's HSD $p<.05$) (Figure 1, SI Table 2). In fact, the TCC steadily increased with time in the 2000 µg/L microcosms, over a time period spanning both before and after the re-inoculation attempt that occurred 3.5 months into the copper dosing phase (Figure 1, SI Figure 2).

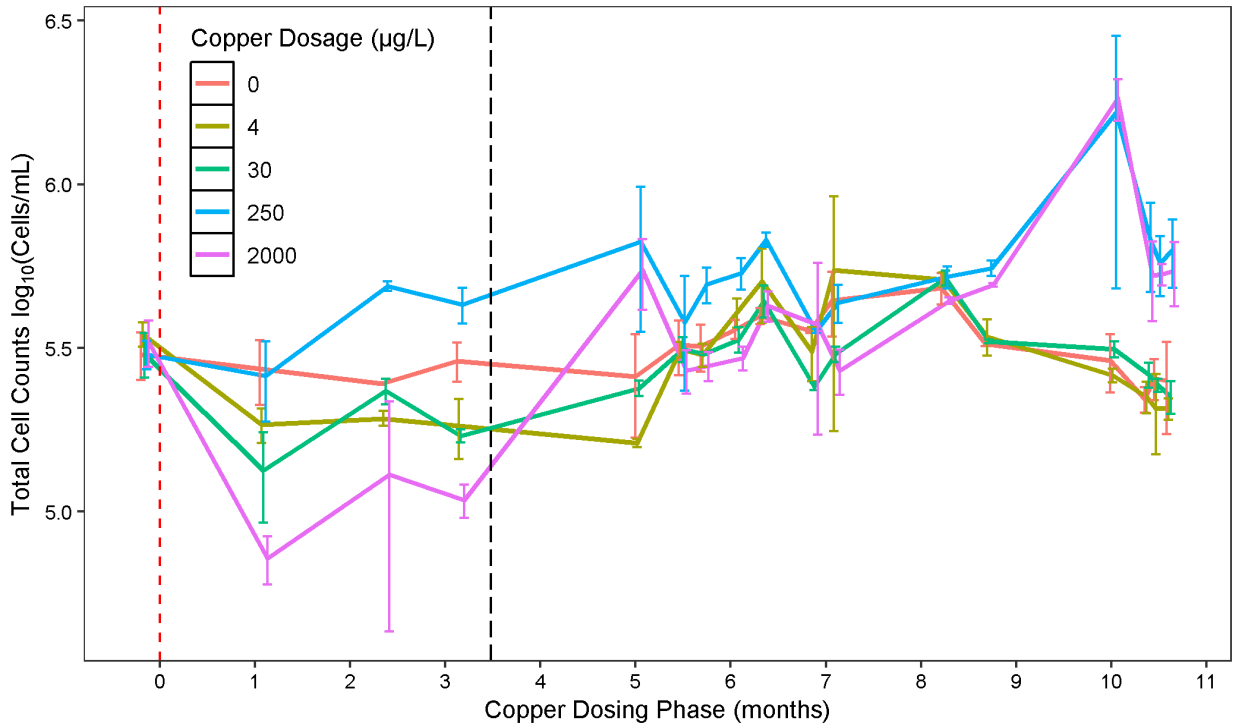

Figure 1. Mean Total Cell Counts (cells/mL) in microcosm bulk water effluent over the course of the experiment. Error bars are the standard deviation of biological triplicate microcosms. Lines connect the data points to guide comparison and are colored by copper concentration. The first measurement (time -0.25) occurred at the end of the acclimation phase, a week 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 to avoid overlapping datapoints.

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 µg/L condition contained higher concentrations of *M. avium* compared to other copper dosages (0.55 – 0.85 logs higher) (Tukey's HSD, $p<0.05$, SI Table 3). *M. avium* was not detectable in the influent water using ddPCR, indicating that the increase in the effluent was the result of growth in the microcosms.

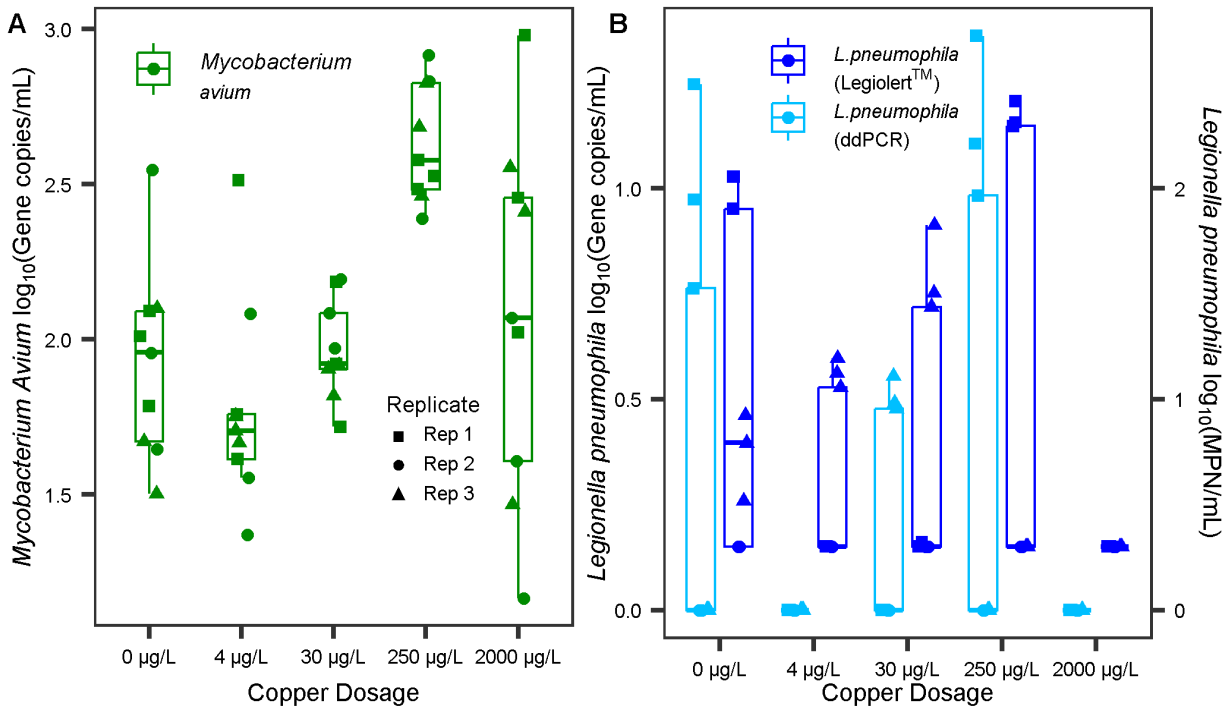

Figure 2. Mean ddPCR technical triplicate gene copies/mL counts of (A) *M. avium* detected through ddPCR and (B) *Lp* detected via ddPCR (mean technical triplicate) and Legiolert™ across the fifteen microcosms over three consecutive samplings in the final (11th) month of the experiment. For (A), the data consists of 5 copper levels × 3 microcosms × 3 sampling events = 45 *M. avium* data points). For (B) the data consists of 5 copper levels × 3 microcosms × 3 sampling events = 45 culturable (MPN/mL) or 45 ddPCR (gc/mL) *Lp* data points. *M. avium* was detectable in all samples. For *Lp*, points plotted at zero (gc/mL or MPN/mL) indicate non-detects for ddPCR (estimated ~detection limit of 1.5 gc/mL) and Legiolert™ (detection limit of 1 MPN/mL for the protocol used).

L. pneumophila response to copper in the microcosms

Lp response to copper dosing was confirmed both by ddPCR and Legiolert™. On dates when both Legiolert™ and ddPCR tests were conducted on aliquots of the same sample (n=45), measurements were strongly correlated ($R^2 = 0.85$) (t-test, $p<0.05$), but the log ddPCR gc/mL values were typically 0.51 times the log of Legiolert™ MPN/mL (SI Figure 3). *Lp* concentrations did not significantly differ across the microcosms as a function of copper condition (Linear Mixed-Effect Model ANOVA, $p > 0.05$), but both Legiolert™ and ddPCR measurements indicated a consistent ranking of average concentration across microcosms as

278 follows: 2000 µg/L ≤ 4 µg/L < 30 µg/L < 0 µg/L < 250 µg/L. *Lp* was never detectable in the
279 influent used for the microcosm water changes using ddPCR or Legiolert™, indicating that the
280 increase in the effluent was the result of growth in the microcosms. It was noted that there was a
281 much wider variance in *Lp* numbers among replicates than there were for *M. avium* (Figure 2).
282 This was most evident for microcosms dosed with 250 µg/L copper, for which one microcosm
283 consistently displayed the highest concentrations of culturable *Lp*, while the second and third
284 replicates produced non-detectable levels of culturable *Lp* over the majority of the sampling
285 events (87% and 73% of events) over the course of the copper-dosing phase of the experiment
286 (Figure 3). *Lp* levels quantified via Legiolert™ ranged from 8 MPN/mL to 1,460 MPN/mL for
287 the first replicate microcosm in the 250 µg/L Cu condition (Figure 3). The stark differences
288 between these microcosms were maintained even though they had been cross-inoculated ten
289 times during acclimation to establish the same baseline microbial community prior to copper
290 dosing, reinoculated ~ 3.5 months into the copper dosing phase, and subject to four adjustments
291 to the water chemistry.

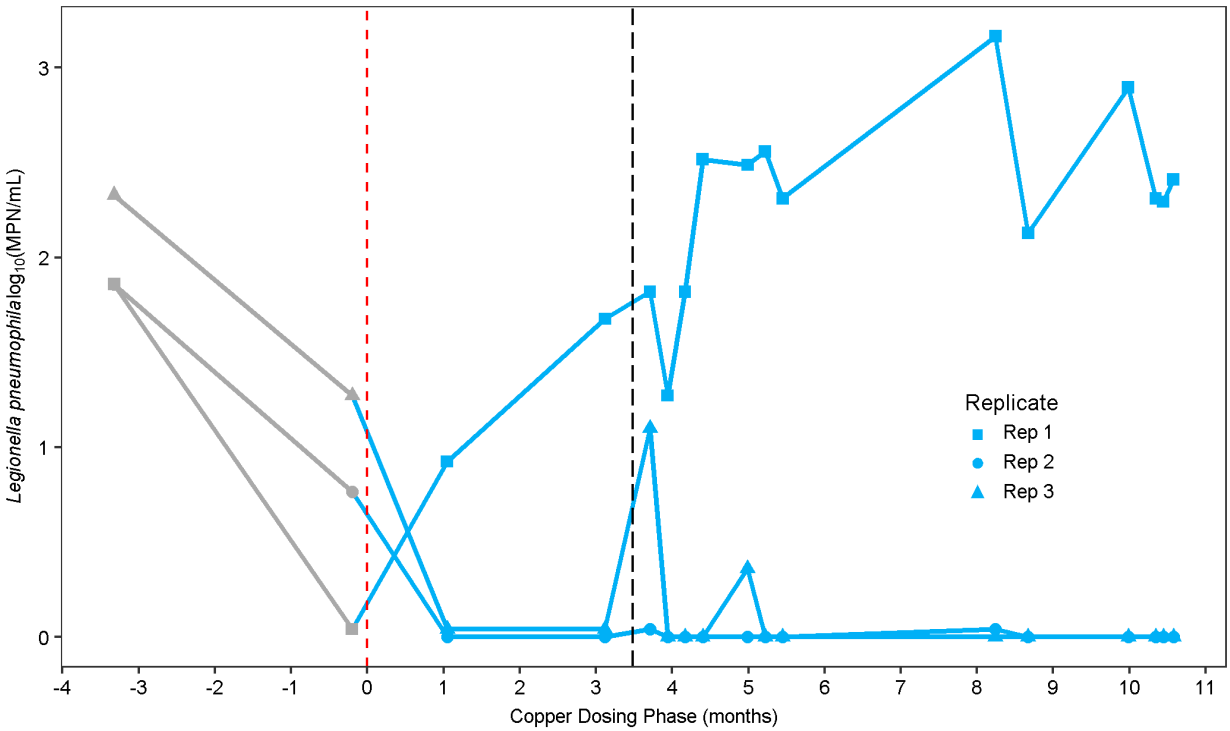

Figure 3. *Lp* MPN/mL, determined via Legiolert™, among the three replicate microcosms dosed with 250 µg/L copper that displayed divergent behavior over the 11-month experimental period. The vertical, dashed line at ~3.5 months represents the point at which all microcosms were reinoculated with *Lp*. Time 0, shown with the red dashed vertical line represents when the copper dosing began. Points before 0 months represent 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 SI Figure 4. Legiolert™ detection limit was 1 MPN/mL. Points plotted at an MPN/mL of zero indicate non-detects for culturable *Lp*.

Consistent with the Legiolert™ results, replicates 2 and 3 of the 250 µg/L 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 gene amplicon Sequencing

16S rRNA gene amplicon 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 (PERMANOVA, p

313 < 0.05) (Figure 5). Copper doses of 0, 4, and 30 µg/L produced a similar microbial profile in
314 terms of the ten most abundant phyla from each replicate microcosm, with a marked shift in
315 composition in the 250 µg/L and 2,000 µg/L conditions (Figure 4). Of note, the microcosms
316 dosed with 0, 4, and 30 µg/L copper were mostly comprised of *Proteobacteria*, while the
317 reactors dosed with 250 µg/L copper contained relatively equal proportions of *Proteobacteria*
318 and *Gemmatimonadetes.* Microcosms dosed with 2000 µg/L were primarily dominated by
319 *Bacteriodetes* and *Proteobacteria* (Figure 4). Within each condition from 0-250 µg/L, the
320 microbial communities appeared relatively consistent among the three replicates. However,
321 there was marked variation in the microbial community among the 2,000 µg/L condition
322 replicates (Figure 5). Copper concentration had a significant effect on Shannon diversity and
323 Simpson diversity (ANOVA, $p < 0.05$) (Figure 6).
324 The microbial community profile of the 250 µg/L microcosm that consistently had the
325 highest level of *Lp* was found to be dominated by the same top ten phyla, but differences in
326 relative abundance stood out (Figure 4, highlighted in orange boxes). Specifically, this
327 microcosm was significantly enriched in *Ignavibacteriae* and depleted in *Chlamydiae* and
328 *Bacteroidetes* (Wald's test, $p<0.05$) (Figure 4, highlighted in orange boxes). Beta diversity
329 analysis, however, did not reveal an obvious difference in the microbial community composition
330 of this replicate beyond the variation that was seen for replicates of other copper concentrations
331 (Figure 5).

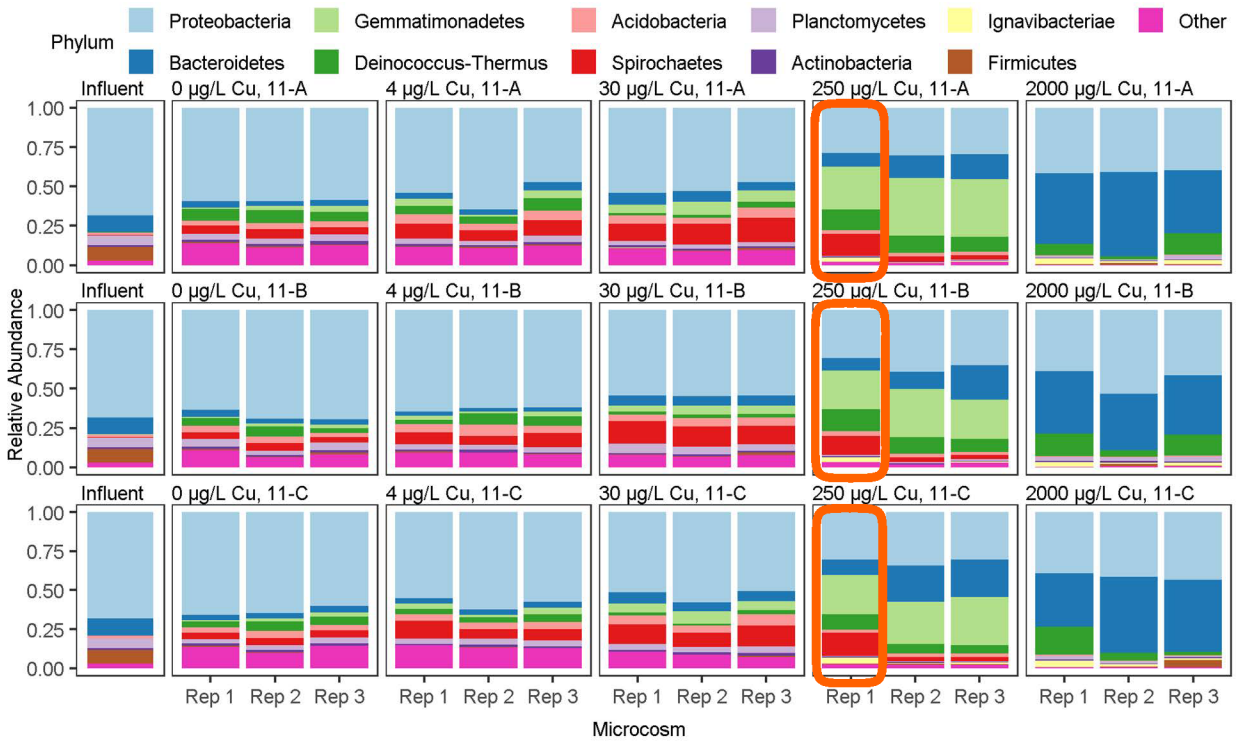

Figure 4. Relative abundances of each of the top ten most abundant phyla over all ASVs in each replicate microcosm grouped by copper concentration and sampling date. The orange boxes indicate the 250 µg/L copper replicate that consistently displayed the highest concentrations of *Lp*. The influent [collected 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.

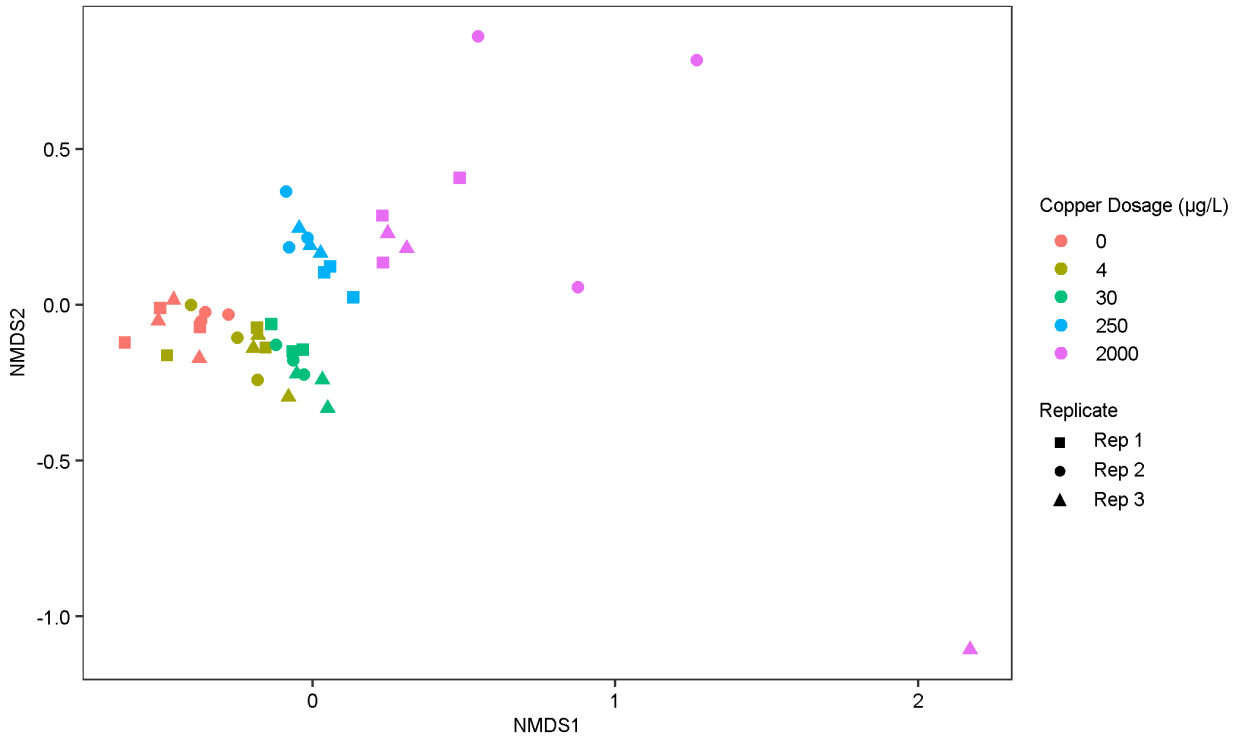

Figure 5. Bray-Curtis-based nonmetric multidimensional scaling of taxa from effluent water of microcosms dosed with 0, 4, 30, 250, and 2000 µg/L of copper over an 11-month period (Stress = 0.08836). The data consists of 5 copper levels × 3 microcosms (per copper level) × 3 sampling events = 45 data points.

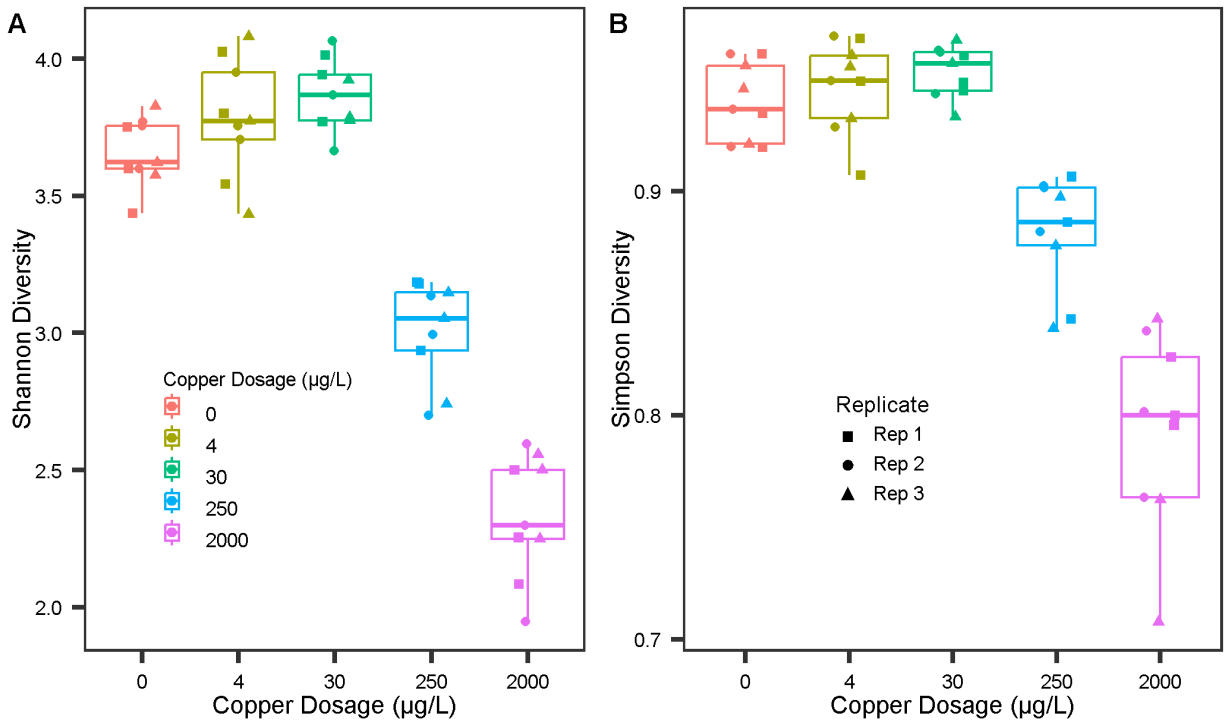

Figure 6. Shannon and Simpson diversity index of replicate microcosms on the final three sampling dates grouped by copper condition. The data consists of 5 copper levels × 3 microcosms (per copper level) × 3 sampling events = 45 data points.

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 µg/L than in the 250 µg/L condition, while *Firmicutes* indicated little change across copper dosages (Figure 7). *Gemmatimonadetes* were enriched from 0 to 250 µg/L, but dramatically decreased at 2000 µg/L (Figure 7). *Deinococcus-Thermus* exhibited a slight decrease in relative abundance from 250 µg/L to 2000 µg/L (Figure 7). *Chloroflexi*, *Acidobacteria*, and *Actinobacteria* markedly decreased with increasing copper (Figure 7).

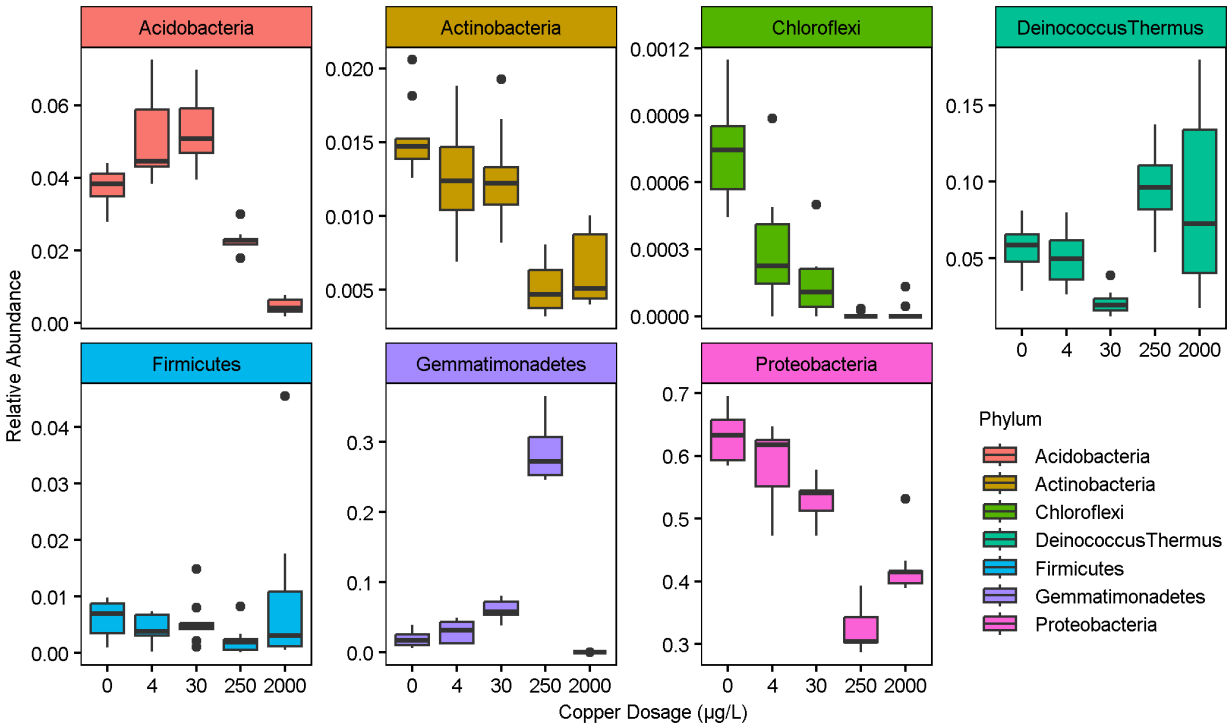

Figure 7. Relative abundance of select phyla across copper dosages in each replicate over the three sequential sampling events carried out in the final (11th) month of the experiment.

DISCUSSION

This study reveals apparent hormetic effects of copper on premise plumbing microbiomes, i.e., acting as a nutrient at low concentrations and as an antimicrobial at higher concentrations. TCCs provided an indicator of the effects on bacterial communities at-large, revealing some surprising patterns over the course of this near year-long experiment. While TCCs initially decreased markedly when the highest dose of 2000 µg/L was commenced, as one would expect, over the course of subsequent months, the microbial community adapted such that TCCs were as high as the 250 µg/L by the end of the experiment. Further, TCC levels in the 2000 µg/L condition had not yet plateaued by the end of the experiment, suggesting that levels could have eventually surpassed those of the 250 µg/L condition.

Interestingly, TCCs initially increased in the 250 µg/L condition and remained elevated throughout the experiment relative to the un-dosed control, suggesting that copper acted largely

382 as a nutrient at this concentration. Doses of 4 and 30 µg/L Cu were initially associated with a
383 significant decrease in TCCs (paired t-test, $p < 0.05$), but remained comparable to the control for
384 the majority of the experiment.

385 *M. avium* concentrations, which were only measured in the 11th month, were also highest in
386 the 250 µg/L condition, but were lowest in the 2000 µg/L condition. This suggests that very high
387 doses of copper are likely needed to effectively control *M. avium*, and lower doses serve as a
388 nutrient or selector for the organism. Overall, the findings for TCC and *M. avium* were consistent
389 with expectations that copper has a dual role of nutrient and antimicrobial.[2] Further, a level of
390 copper that is antimicrobial in the short-term can behave as a nutrient over a period of months to
391 years as microbial communities adapt to the copper.[16]

392 Support for this hypothesis was provided in the microbial community analysis. Even
393 though TCCs were elevated in the 250 µg/L and 2000 µg/L conditions, microbial diversity was
394 diminished. As richness and evenness declined, a subset of the remaining species were enriched
395 and became dominant in the community (Figures 4 and 6). With the significant decrease in
396 diversity, a few taxa prevailed, making the community vulnerable to stochasticity. This was
397 apparent in terms of the wide variance in microbial community composition among the 2000
398 µg/L replicates (Figure 5).

399 Prior research by Song and colleagues using the same municipal tap water supply also
400 assessed the effect of dosed copper on the microbial community in a full-scale water system, but
401 the experimental design differed from the present study in that the dose was incrementally
402 increased from 0, 50, 100, 300, 600, 1200, to 2000 µg/L, while maintaining each concentration
403 for only periods of 4 to 16 weeks.[9] Overall Phyla-level trends in *Proteobacteria*, *Deinococcus-*
404 *Thermus*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria* were similar to those observed in this

405 work. However, Song and colleagues also observed enrichment over 6 weeks at 1200 μg/L in
406 *Firmicutes*, and enrichment of *Gemmatimonadetes* up through 2000 μg/L. The differences in
407 *Firmicutes* and *Gemmatimonadetes* behavior when comparing the results of this study could
408 potentially be attributed to differences in duration of copper dosing, as Song and colleagues
409 maintained the 2000 μg/L copper dose for 12 weeks,[9] whereas the microcosms in this study were
410 acclimated at this dose for 11 months.

411 At 250 μg/L, stochasticity was observed in the concentrations of culturable *Lp*,
412 measurements of which maintained relatively consistent among all replicates at lower copper
413 doses (0, 4, and 30 μg/L total Cu), while almost always being undetectable at 2000 μg/L (SI
414 Figure 4). In a prior study investigating the effect of copper pipe in warm premise plumbing
415 systems, *Lp* numbers were initially lowest in the biofilms on copper pipes, but eventually
416 increased to the same concentrations as PEX and stainless-steel pipes after two years.[33] In such
417 studies, it is important to note that copper pipe aging leads to decreasing release of copper into
418 solution, and thus it is difficult to know if the observed increase in *Lp* was due to lower levels of
419 copper or microbial adaptation.

420 A synthesis of our results over the last decade using Blacksburg tap water reveals two
421 general responses of experimental plumbing systems with respect to *Lp* inoculation. As one
422 would expect, there are many circumstances in which the system response to inoculated *Lp* is
423 deterministic, in which replicate microcosms behave as true replicates. But here, we report a
424 situation in which stochastic behavior clearly occurred, because in some replicate microcosms *Lp*
425 consistently died off and in other reactors it thrived. With benefit of hindsight, we now recognize
426 similar stochastic behavior for *Lp* amongst replicate microcosms during at least two prior
427 experiments in our lab.[34,35] We were reluctant to accept the conclusion that these systems were

428 stochastic, preferring to believe that there were subtle uncontrolled differences between
429 replicates, but we consider the evidence gathered herein to be conclusive. This study
430 demonstrates that even in the most simplistic simulation of premise plumbing, i.e., a glass
431 microcosm containing pipe sections, that there are complexities at play that can determine if
432 effluent *Lp* is extremely high or very low.

433 Interestingly, the replicate microcosms described herein were deterministic and replicable
434 for measured dimensions of cell counts, mycobacteria and community analysis, even as they
435 were stochastic for *Lp*. We speculate that the sensitive predator, prey and parasitic relationships
436 that drive the *Lp* life cycle make it particularly vulnerable to random events that can dictate the
437 trajectory of its ecology in the premise plumbing environment. In a companion paper we report
438 a discovery that *Neochlamydia* was consistently abundant in the replicate microcosms where *Lp*
439 failed to establish.[36] Previous research in pure culture settings has shown that amoeba carrying
440 *Neochlamydia* are resistant to *L. pneumophila* infection,[37] potentially explaining the observed
441 stochastic behavior. If such stochastic behavior for *Lp* is commonplace, as it is in certain other
442 areas of microbial ecology,[38,39] this discovery has profound implications for experimental design
443 and the interpretation of past data from laboratory and field sampling.[40] Overall, the findings of
444 this study reveal both hormesis and stochasticity in microbial ecological succession as key
445 mechanisms underlying the observed non-linear response of *Lp* to copper as a disinfectant in
446 premise plumbing.
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ASSOCIATED CONTENT

Supporting Information

Information regarding ddPCR assays, statistical analysis, initial *Lp* and TCC of the microcosm groups at the end of the acclimation phase, additional TCC information for the 2000 µg/L Cu condition, a comparison between Legiolert™ and ddPCR *Lp* measurements, and the culturable *Lp* for all the copper conditions throughout the study, can be found in the Supporting Information document (PDF).

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NOTES

The authors declare no conflict of interest.

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