

# Natural Organic Matter, Orthophosphate, pH, and Growth Phase Can Limit Copper Antimicrobial Efficacy for *Legionella* in Drinking Water

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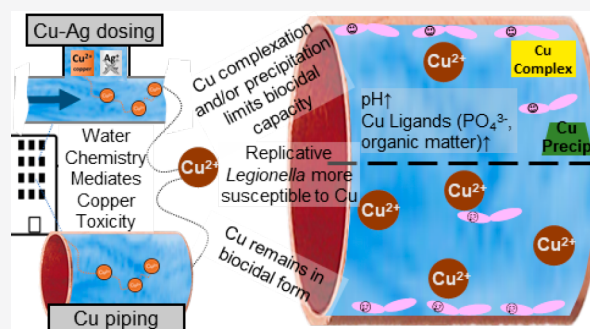
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**ABSTRACT:** Copper (Cu) is a promising antimicrobial for premise plumbing, where ions can be dosed directly via copper silver ionization or released naturally via corrosion of Cu pipes, but Cu sometimes inhibits and other times stimulates *Legionella* growth. Our overarching hypothesis was that water chemistry and growth phase control the net effect of Cu on *Legionella*. The combined effects of pH, phosphate concentration, and natural organic matter (NOM) were comprehensively examined over a range of conditions relevant to drinking water in bench-scale pure culture experiments, illuminating the effects of Cu speciation and precipitation. It was found that cupric ions ( $\text{Cu}^{2+}$ ) were drastically reduced at  $\text{pH} > 7.0$  or in the presence of ligand-forming phosphates or NOM. Further, exponential phase *L. pneumophila* were 2.5× more susceptible to Cu toxicity relative to early stationary phase cultures. While  $\text{Cu}^{2+}$  ion was the most effective biocidal form of Cu, other inorganic ligands also had some biocidal impacts. A comparison of 33 large drinking water utilities' field-data from 1990 and 2018 showed that  $\text{Cu}^{2+}$  levels likely decreased more dramatically (>10×) than did the total or soluble Cu (2×) over recent decades. The overall findings aid in improving the efficacy of Cu as an actively dosed or passively released antimicrobial against *L. pneumophila*.

**KEYWORDS:** copper, *Legionella pneumophila*, corrosion control, natural organic matter, premise plumbing



## INTRODUCTION

With a >500% increase in reported incidence in the U.S. over the last 20 years, Legionnaires' disease increasingly burdens building owners and operators who seek its control. Water management programs provide broad guidelines on how to ensure the safety of a given building's water,<sup>1</sup> and specific strategies depend on the technology applied, local laws and code, water quality parameters, and building-level treatment and operation.<sup>2–4</sup> Selecting pipe materials that are less amenable to *Legionella* growth is one avenue of interest. In particular, copper (Cu) pipes can exert natural biocidal properties when Cu is released via corrosion. Intentionally dosing Cu ions via Cu–silver ionization (CSI) systems is also a common on-site disinfection technology for large institutional buildings.<sup>5–8</sup> However, conflicting efficacies of both Cu pipes and CSI systems in controlling *Legionella* growth have been reported.<sup>7,9–12</sup>

The main biocidal form of Cu is believed to be cupric ion ( $\text{Cu}^{2+}$ ),<sup>13</sup> which interacts with negatively charged cell membranes and causes cell lysis.<sup>5</sup> In many drinking waters,  $\text{Cu}^{2+}$  concentrations are expected to be relatively low and strongly influenced by water chemistry. However, little research has been carried out to establish the practical range of water chemistry parameters that ensure efficacy of Cu as a

biocide in building water systems. Monitoring plans for CSI systems tend to focus mainly on maintaining total Cu levels in the range of 200–800  $\mu\text{g/L}$  and do not consider soluble Cu or  $\text{Cu}^{2+}$  ions.<sup>3,14–16</sup> The negative impacts of elevated pH on the biocidal capacity of Cu toward *Legionella* have been reported,<sup>10</sup> but only at the extremes of typical drinking water system pH (7 vs 9). A higher resolution examination of pH effects between these two extremes, and considerate of other potential mediating factors, is needed.

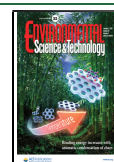
Other water chemistry attributes that are likely to influence Cu corrosion, speciation, and bioavailability in tap water are also important to consider. The use of phosphate-based corrosion inhibitors has increased nationally over the last 20 years and is likely to reduce the toxic effects of Cu on *Legionella* based on existing knowledge of cuprosolvency and speciation.<sup>17,18</sup> However, to our knowledge, this has never

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been demonstrated or studied. In addition, baseline levels of natural organic matter (NOM) have been increasing in some U.S. source waters as an indirect consequence of climate change, land use alteration, and improved sulfur and nitrogen air pollution controls under the Clean Air Act,<sup>19,20</sup> which might also be decreasing Cu bioavailability in potable water systems.<sup>21–24</sup>

The physiological state of *Legionella* is also important to consider in evaluating the efficacy of Cu disinfection. Within host amoeba cells, *Legionella* are typically viewed as being in a replicative state, which is simulated in pure culture experiments by the exponential growth phase. However, the stationary growth phase of *Legionella*, which can be induced by starvation and environmental stress, is thought to be more resistant to Cu.<sup>25</sup> The stationary growth phase, the stage entered after *Legionella* are released from the host organism, is also thought to be more relevant to *Legionella* transmissivity (i.e., virulence). However, previous work regarding the efficacy of Cu as a biocide focused on *Legionella* generated from nascent colonies grown on agar media (i.e., plate scrapes),<sup>16</sup> which contains a mixture of growth phases and, without other pretreatment steps, consists largely of exponential phase cells or an unknown mixture of exponential and stationary cells. More recent work has demonstrated that strain dependency results in a 4-fold difference in susceptibility,<sup>26</sup> but, to our knowledge, the impact of the physiological state of *Legionella* on the biocidal capacity of Cu has not been explicitly investigated.

Here, we provide an integrated and comprehensive examination of the impact of key water chemistry factors on Cu speciation and precipitation on the culturability of *L. pneumophila* in bench-scale experiments. We determine the impact of pH, levels of orthophosphate corrosion inhibitor, and NOM on the levels of total, soluble, and Cu<sup>2+</sup> ions in a simulated drinking water environment and investigate their resulting impact on exponential and stationary-phase *Legionella*. The results are contextualized with respect to trends in comparable field-data to improve guidance on the effective application of Cu as a *Legionella* control measure.

## MATERIALS AND METHODS

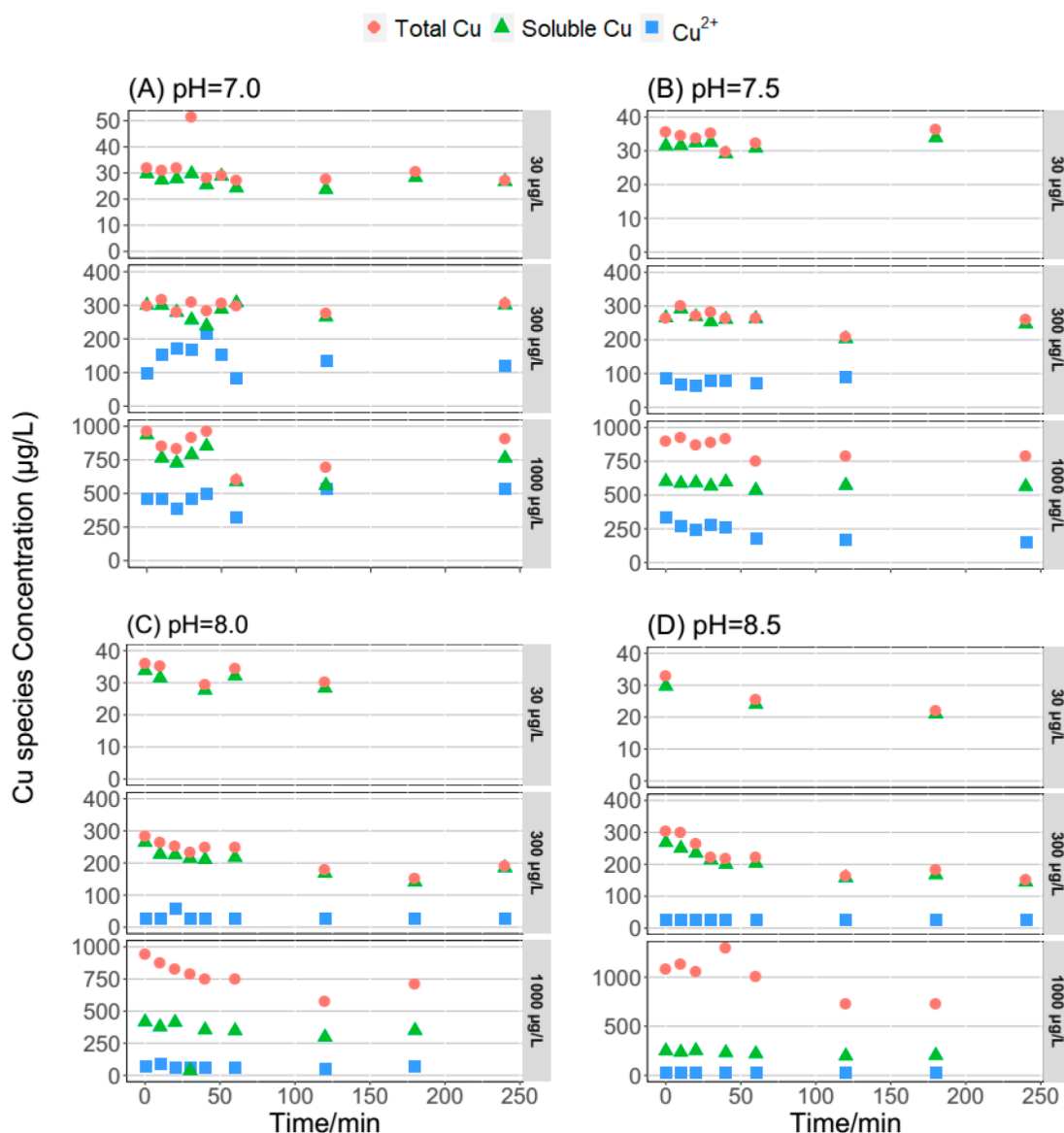
**Test Water, Reagents, and Glassware.** To represent a realistic background drinking water matrix for this and future studies, Blacksburg, VA tap water was used as the base water for all experiments. Blacksburg tap water was passed through three filters in sequence for use in all experiments: (1) ferric oxide column (Brightwell Aquatics), which removed municipally supplied phosphate-based corrosion inhibitor and some NOM; (2) granular activated carbon (GAC), which removed chlorine and additional NOM; and (3) a 0.22  $\mu\text{m}$  pore size mixed cellulose ester filter (MilliporeSigma) to remove particles and microorganisms. Afterward, the base water had <0.05 mg/L total chlorine (>98.3% removal), <10  $\mu\text{g/L}$  phosphorus (>97.5% removal), and <0.5 mg/L organic matter (Table S1). This water was autoclaved at 121  $^{\circ}\text{C}$  for 15 min. Glassware was used as a nonreactive, inert material to conduct all experiments. All glassware used was soaked in 10% nitric acid for 24 h, rinsed with reverse osmosis water, and autoclaved. All experiments were carried out at 37  $^{\circ}\text{C}$  in a walk-in incubator (Thermax, Warminster, PA) to mimic worst-case portions of building water plumbing systems (bottom of electric water heaters,<sup>27</sup> convectively mixed distal taps,<sup>28</sup> or tempered water after mixing valves) and eliminate temperature as a factor that may negatively impact *Legionella*

culturability. pH was adjusted with 0.2 N sulfuric acid or 0.1 N potassium hydroxide. Cu stock solutions of 100 mg/L Cu as CuSO<sub>4</sub> (Sigma-Aldrich, St. Louis, MO) were freshly prepared and adjusted to pH = 4.0  $\pm$  0.05 to maintain Cu solubility. Orthophosphate stock solutions were prepared by adding 9.52 g/L monobasic dihydrogen phosphate and 5.92 g/L dibasic monohydrogen phosphate targeting a solution with pH = 7.0. As per convention in water treatment, the summation of orthophosphate components (PO<sub>4</sub><sup>3−</sup>, HPO<sub>4</sub><sup>2−</sup>, and H<sub>2</sub>PO<sub>4</sub><sup>−</sup>) is noted in units “as PO<sub>4</sub><sup>3−</sup>” in the main text. Two NOM stock solutions were prepared from cleaned humic acid (HA) (Sigma-Aldrich) solutions after sequential alkalization and acidification to remove impurities<sup>29</sup> or Suwannee River II fulvic acid (FA) (Standard FA, International Humic Substance Society).

**Kinetics of Copper Precipitation.** Cu precipitation tests were performed in batch reactors (Figure S1). Base water was dosed with 0, 30, 300, and 1000  $\mu\text{g/L}$  Cu, and the pH was adjusted to 7.0, 7.5, 8.0, or 8.5 ( $\pm$ 0.05 pH units). Total and soluble Cu, Cu<sup>2+</sup> ions, and pH were measured initially in the base water, then immediately aliquoted to 50 mL glass reactors and capped without any headspace, which limited pH drift due to gas transfer. Reactors were sacrificed for sampling at  $t$  = 10, 20, 30, 40, 50, 60, 120, and 240 min. Control groups (with no Cu dosed) were confirmed to contain Cu < 10  $\mu\text{g/L}$  as background throughout the experiments.

**Copper Speciation.** The impact of pH and ligands on Cu speciation was evaluated in experiments set up in the same manner as the precipitation tests (Figure S2) in triplicate. When we analyzed the pH impact, Cu was added to achieve a final concentration of 1000  $\mu\text{g/L}$  Cu to base water at the target pH. When we analyzed the impact of Cu ligands, orthophosphate (at 0.5 and 1 mg/L orthophosphate as PO<sub>4</sub><sup>3−</sup>) or NOM (2 and 5 mg/L HA and FA as total organic carbon; TOC) was added to base water with pH = 7.0  $\pm$  0.02, followed by Cu dosing at 1000  $\mu\text{g/L}$  and pH readjustment to 7.0  $\pm$  0.02. This pH was selected to represent the lowest end of the typical pH range for water utilities and maximize the concentration of Cu<sup>2+</sup>. Biological replicates were obtained by repeating this process three times for every condition.

**Water Chemistry Measurements.** pH was measured with an Oakton (Vernon Hills, IL) portable pH meter with automatic temperature correction and was calibrated before each experiment and every 2 h during experiments. Total Cu samples were prepared by transferring 10 mL solutions from sacrificial reactors, after mixing by inverting, into sterile tubes (Evergreen, Buffalo, NY). Soluble Cu was operationally defined by filtration through a 0.45  $\mu\text{m}$  pore size nylon syringe filter (Whatman, GE Healthcare, Pittsburgh, PA). Both total Cu and soluble Cu samples were acidified with nitric acid (2% vol/vol) and initially measured by both inductively coupled plasma mass spectrometry (ICP-MS) and spectrophotometry with HACH Cu Ver1 reagents (method 8506, Hach, Loveland, CO) for a range of experimental conditions. Because the results of both methods were in good agreement (Figure S3), the colorimetric method was used to check concentrations during the experimental runs, and one of three replicates were cross-checked with the ICP-MS for QA/QC. Cu<sup>2+</sup> were measured by an ion selective electrode (Cole-Parmer, Vernon Hills, IL) following instructions provided by the manufacturer with 50 mL samples predosed with 1 mL of ionic strength adjustment (ISA) solution (5 N NaNO<sub>3</sub>). The ion selective electrode was checked every 2 h for calibration drift using



**Figure 1.** Cu species (total Cu, soluble Cu, and Cu<sup>2+</sup>) concentration as a function of time at pH values of 7.0, 7.5, 8.0, and 8.5. Cu<sup>2+</sup> levels at total Cu = 30 µg/L are not shown because they are below the probe quantification limit of 50 µg/L. For other conditions, Cu<sup>2+</sup> measurements <50 µg/L are plotted as 25 µg/L for visualization purposes.

methods recommended by the manufacturer (Cole-Parmer<sup>30</sup>), with a limit of quantification of 50 µg/L. Relative standard deviation (RSD) of the ion selective electrode was determined with 10 individual tests as <20% variation when total Cu > 100 µg/L and over 50% when total Cu < 50 µg/L (Figure S4).

***L. pneumophila* Cultivation Experiments. Bacterial Culture Preparation.** *L. pneumophila* cultures were freshly prepared for each experimental run. *L. pneumophila* serogroup 1 strain 130b was obtained from the U.S. Centers for Disease Control and stored at −80 °C in buffered yeast extract (BYE) broth (10 g of ACES, 10 g of yeast extract, 1 g of  $\alpha$ -ketoglutaric acid, 0.4 g of L-cysteine, and 0.25 g of ferric pyrophosphate per liter) with glycerol (20% vol/vol). Frozen stocks were isolated onto buffered charcoal-yeast extract (BCYE) agar plates (12 g of agar, 2 g of activated charcoal, 1 g of  $\alpha$ -ketoglutarate monopotassium, 10 g of ACES buffer, 2.8 g of potassium hydroxide, 0.25 g of iron pyrophosphate, 3 g of ammonium-free glycine, 80 000 IU polymyxin B sulfate, 0.001 g of vancomycin hydrochloride, 0.08 g of cycloheximide, and 0.4 g

of L-cysteine monohydrate per liter) and incubated for 72 h at 37 °C. BYE broth was inoculated with *L. pneumophila* at an initial optical density at wavelength of 600 nm (OD<sub>600</sub>) of 0.2 (Implen) by transferring 1 mL of sterile BYE broth onto t-streaked culture plates and harvesting colonies with a 3× flame-sterilized glass cell spreader. Inoculated BYE broth was incubated at 37 °C with agitation for either 9 h (exponential phase) or 14 h (early stationary phase), as determined by OD<sub>600</sub> growth curves (Figure S5). After broth culture, cells were pelleted by centrifuging at 5000g for 10 min, and the supernatant was decanted. Cell pellets were washed twice with 10 mL of base water, resuspended in 2 mL of base water, and diluted to  $\sim 3 \times 10^8$  colony forming units (CFU)/mL (McFarland Standard No. 1, OD<sub>600</sub> = 0.257).<sup>31</sup> Subsequently,  $\sim 3 \times 10^6$  CFU/mL dilutions were used for each experimental run.

**Copper Inactivation Tests.** Cu inactivation test procedures were similar to those of the Cu speciation tests (Figure S6). All Cu inactivation tests were performed at an initial total Cu of

800  $\mu\text{g/L}$ , unless otherwise stated, which represents the upper limit of recommended copper ion levels for copper inactivation.<sup>9,10,16</sup> After Cu dosing, ligand addition (at pH 7 only; none, 0.5, 3 mg/L phosphate as  $\text{PO}_4^{3-}$ , or NOM = 5 mg/L as TOC), pH adjustment (7.0, 7.5, 8.0, or 8.5), and mixing for 20 min to allow Cu speciation to stabilize (based on the solubility and speciation experiential results), glass bottles were moved to a biosafety cabinet to inoculate *L. pneumophila*. After being mixed, initial *L. pneumophila* levels were quantified as described below, and solutions were transferred to sterile 50 mL sacrificial glass reactors and incubated at 37 °C. Reactors were inverted to mix and sacrificed to quantify culturable *L. pneumophila*, targeting time points of  $t = 10, 20, 30, 40, 50, 60, 120, 240,$  and 360 min. All Cu inactivation data were fitted with Chick–Watson first-order inactivation curves, and the disinfection constants were estimated by best-fitted lines using the least-squares method. Biological replicates were obtained by repeating this process three times for every condition. At least one control representing background chemistry conditions and growth phases, without copper, was operated for each condition. For controls in Cu inactivation tests using early stationary phase *L. pneumophila*, in total 13 replicates (3 from pH = 7 and 8.5; 2 from  $\text{PO}_4^{3-} = 3$  mg/L and NOM = 5 mg/L; and 1 from pH = 7.5, 8, and  $\text{PO}_4^{3-} = 0.5$  mg/L) showed negligible reduction in *L. pneumophila* with time, and therefore controls were combined across experiments for statistical comparison.

***L. pneumophila* Enumeration.** One milliliter of solution was transferred to sterile tubes predosed with EDTA (molar ratio of EDTA:Cu = 5:1) to complex Cu. We confirmed that EDTA had no impact on the culturability of *L. pneumophila* in our experiments. Serial dilutions ( $1:10$ – $1:10^5$ ) were prepared with base water, and 10  $\mu\text{L}$  was transferred onto BCYE+GVCP agar plates using a multichannel pipet in triplicate (Figure S6). Only dilutions with 3–30 distinct *L. pneumophila* colonies were considered quantifiable. The lower limit of detection (LOD) (1 CFU counted) and limit of quantification (LOQ) (3 CFUs counted) of the serial dilution plating method were 100 and 300 CFU/mL, respectively, when 10  $\mu\text{L}$  was plated. Where the number of CFUs was expected to be few, 100  $\mu\text{L}$  was also directly spread plated to further lower the LOD and LOQ to 10 and 200 CFU/mL, respectively (i.e., 1 colony and 20 colonies observed). After being dried in the biosafety cabinet, plates were incubated at 37 °C and enumerated after 72 h.

**Drinking Water Utilities Field-Data Analyses.** Thirty-three large drinking water utilities were selected from a 1990 systematic survey of drinking water utilities<sup>32</sup> to be representative of their corrosion control methods (no corrosion control, pH control, inhibitor control, and both controls). Most recent (2018) customer confidence reports of the 33 utilities were utilized to extract data for 90% total Cu, pH, phosphate, and alkalinity. A subset of 11 utilities that contain all needed information were utilized to predict changes in Cu speciation in drinking water systems in the U.S. using equilibrium models.

**Statistical Analyses.** Statistical differences in Cu speciation and Cu inactivation data were determined using ANOVA with post hoc Tukey's Test or Kruskal–Wallis test with post hoc Dunn's Test (Bonferroni correction) in R (version 3.4.3). The summary statistics (mean, standard deviation) and statistical difference for left-censored (below LOQ or LOD) data sets were calculated using regression on order statistics

(ROS) and maximum likelihood estimation (MLE) regression test<sup>33</sup> in R (version 3.4.3). Significance threshold was set at  $P \leq 0.05$ . Calculations and graphs were generated using R (version 3.4.3) and Microsoft Excel 2016 (Microsoft). Thermodynamic equilibrium models were generated using MINEQL+ (version 4.6).

## RESULTS AND DISCUSSION

Changes in Cu speciation and precipitation in laboratory tests are reported first, followed by experiments quantifying the impact of these changes on the toxicity of Cu to *Legionella*. Finally, a systematic comparison of publicly available Cu monitoring data and predicted changes in the presence of toxic forms of Cu in drinking water systems in the U.S. using equilibrium models is reported.

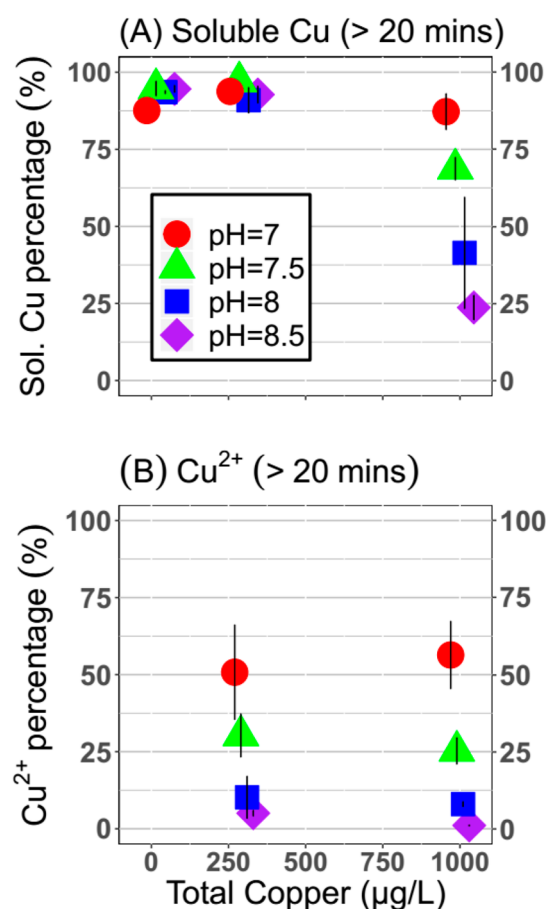
### Copper Speciation and Solubility Experiments.

**Copper Stability.** Water was dosed with Cu at 30, 300, or 1000  $\mu\text{g/L}$  at pH of 7.0, 7.5, 8.0, or 8.5 to examine precipitation and speciation kinetics (Figure S1). Cu species (total, soluble, and  $\text{Cu}^{2+}$ ) generally reached short-term equilibrium after 20 min of incubation (Figure 1). The mean pH value after incubation was  $7.02 \pm 0.08, 7.42 \pm 0.08, 7.82 \pm 0.10,$  and  $8.26 \pm 0.14$  for the targeted pH = 7.0, 7.5, 8.0, and 8.5 conditions, respectively, reflecting slight naturally occurring pH shifts. Cu did not significantly (<2%) precipitate at the lower doses of 30 and 300  $\mu\text{g/L}$  at any pH level. At 1000  $\mu\text{g/L}$ , more Cu precipitated (i.e., less soluble Cu was measured) at the higher pH, as would be predicted on the basis of solubility (Figure 2A). For instance, soluble Cu was 87.2% of total Cu at pH 7.0 and decreased to 68.7% at pH 7.5, 41.4% at pH 8.0, and 23.7% at pH 8.5. The level of  $\text{Cu}^{2+}$  ions decreased even more dramatically as the pH increased. At a dose of 300  $\mu\text{g/L}$  Cu at pH 7.0, 50.8% of the Cu was present in the form of  $\text{Cu}^{2+}$ , dropping to <11.7% at pH 8.5. A similar trend was observed at a Cu dose of 1000  $\mu\text{g/L}$  (Figure 2B).

**Copper Speciation.** The presence of Cu ligand-forming components reduced the solubility and/or level of  $\text{Cu}^{2+}$ . The addition of 2–5 mg/L humic acid or fulvic acid (as TOC) produced <50  $\mu\text{g/L}$  of  $\text{Cu}^{2+}$  and completely prevented precipitation (Figure 3). The condition with 5 mg/L HA was utilized for further Cu inactivation experiments. At 1000  $\mu\text{g/L}$ , the addition of just 0.5 mg/L  $\text{PO}_4^{3-}$  as orthophosphate at pH 7 reduced soluble Cu from 813.8  $\mu\text{g/L}$  (89.1% of total Cu) without  $\text{PO}_4^{3-}$  to 407.8  $\mu\text{g/L}$  (48.6% of total Cu) and reduced  $\text{Cu}^{2+}$  from 52.9% down to 6.1% (Figure 3). These observed soluble and  $\text{Cu}^{2+}$  levels were generally greater than those predicted by chemical equilibrium models, consistent with the short-term nature of the tests (hours) relative to the anticipated time to reach copper equilibrium conditions (days to weeks). This could be interpreted as a potential benefit to application of CSI systems, because the lower levels of copper predicted by complexation and precipitation did not occur.

### Impact of Cu on *L. pneumophila* Culturability.

**Exponential versus Stationary Phase.** Exponential phase *L. pneumophila* cultures were 2.5 times more susceptible to Cu relative to early stationary phase cultures (Figure 4). In 120 min, 800  $\mu\text{g/L}$  total Cu at pH = 7.0 caused complete loss of detectable CFUs ( $\sim 4.5$ -log reduction) relative to a 1.8-log reduction in CFUs for early stationary phase *L. pneumophila*. Control groups (no Cu dose) decreased <0.3 log CFU/mL in both the exponential phase (180 min test) and the early stationary phase (360 min test). Lin et al. showed that 800  $\mu\text{g/L}$  Cu was able to reduce *L. pneumophila* in DI water by over 6



**Figure 2.** Steady-state percentage of Cu measured at 20–240 min as (A) average soluble Cu and (B) average Cu<sup>2+</sup>. All data points represent the average of all data collected after 20 min of incubation; error bars represent standard deviation.  $n \geq 3$  (median = 6) for each data point. Cu<sup>2+</sup> levels at total Cu = 30 µg/L are not shown because it is below the Cu<sup>2+</sup> probe quantification limit of 50 µg/L. Because a substantial portion of the data was censored (Cu<sup>2+</sup> < 50 µg/L) in the Cu = 300 µg/L condition at pH = 8 and 8.5, as well as Cu = 1000 µg/L at pH = 8.5, means are not computed and are plotted at 25 µg/L for visualization purposes for these conditions.

log (presumed to be in the exponential phase due to using nascent colonies from plate scrapes) in 90 min.<sup>16</sup> Our data suggest that the transmissible form of *Legionella*, which is thought to exist outside of host organisms, can be more resistant to Cu than was previously understood.

**Impact of Cu Concentration.** To understand the Cu concentrations needed to reduce stationary phase *L. pneumophila*, three levels of Cu spanning biocidal level recommendations (200, 600, and 800 µg/L) were applied, with pH = 7.0 to maximize the concentration of Cu<sup>2+</sup> (Figure 2). At a dose of 200 µg/L total Cu, which yielded less than 10 µg/L Cu<sup>2+</sup> (Figures 1A and S6), there was only ~1.5 log reduction in *L. pneumophila* over 360 min as compared to 0.11 log reduction in a control without added Cu (Figure 5). At 600–800 µg/L total Cu, which were not significantly different ( $p > 0.05$  across all sampling points, ANOVA), where Cu<sup>2+</sup> was 317.4–423.2 µg/L in the Cu speciation tests, *L. pneumophila* was reduced by up to 4 log over 360 min.

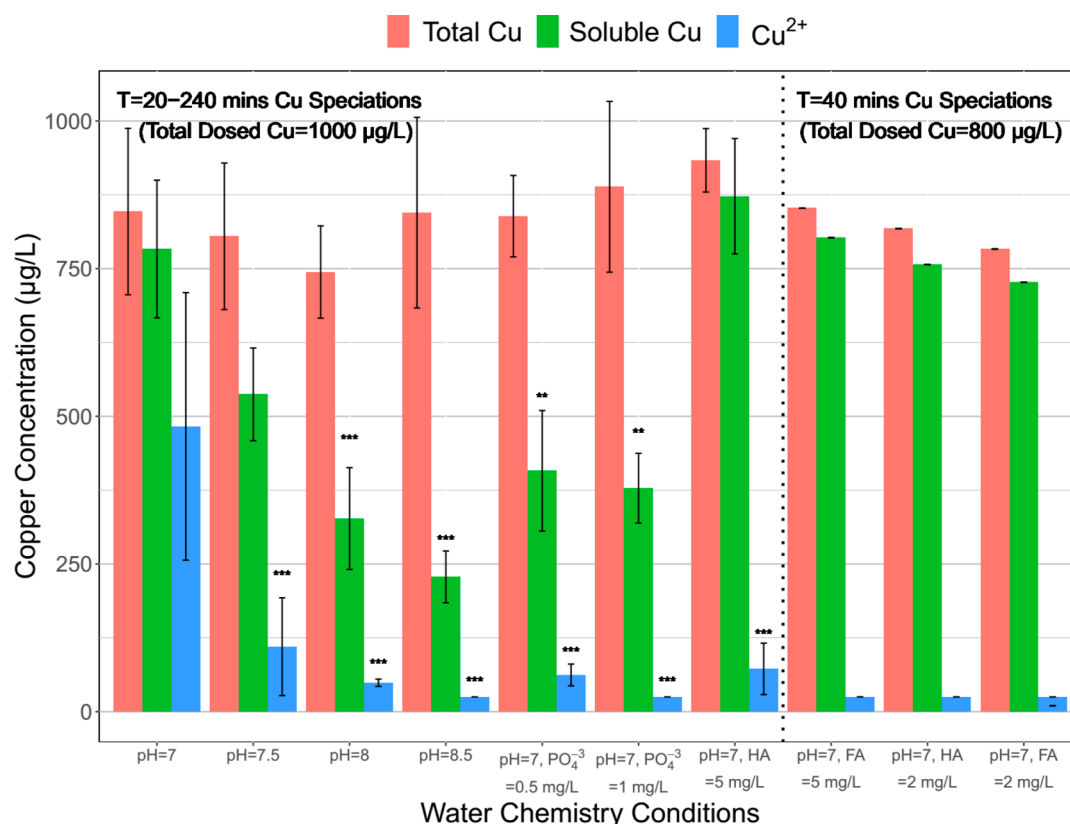
**Impact of Cu Speciation.** To analyze the effects of water chemistry on Cu inhibition of stationary *L. pneumophila*, 800 µg/L total Cu was dosed to reactors at pH = 7.0, 7.5, 8.0, and

8.5. At pH 7, parallel tests were conducted with 0.5 or 3.0 mg/L PO<sub>4</sub><sup>3-</sup> or 5 mg/L NOM as TOC. Control experiments without any Cu dosing indicated <0.7 log CFU reduction in all tested conditions over 360 min (Figure 6). As the pH increased from 7.0 to 8.5, the log-reduction in *Legionella* CFUs at  $t = 360$  min decreased from 3.65 to 1.34 (Figure 6). With the addition of 5 mg/L NOM, which is at the higher range of what is typical for drinking water,<sup>34</sup> the toxicity of Cu was virtually eliminated (Figure 6), consistent with undetectable Cu<sup>2+</sup> ions due to the formation of organic Cu ligands (Figure 3). With the addition of 0.5 mg/L PO<sub>4</sub><sup>3-</sup>, Cu was less biocidal than without phosphate, but it still reduced *L. pneumophila* by more than 2 log. At higher phosphate levels (3 mg/L PO<sub>4</sub><sup>3-</sup>), similar to the higher levels used in municipal drinking water systems for lead and Cu corrosion control, Cu only had a 1 log reduction in culturable *Legionella* and was not significantly different from the control ( $p$ -value = 0.092, ANOVA).

The negative effect of elevated pH on soluble Cu and toxicity toward *Legionella* has been demonstrated before with presumed exponential phase *Legionella*, but only at the two pH extremes of 7.0 and 9.0.<sup>10</sup> Here, we demonstrate Cu toxicity toward stationary phase *L. pneumophila*, with finer granularity over this key pH range, demonstrating there is a gradual drop off of Cu toxicity as Cu is precipitated. In addition, previous work reported that 20 mg/L dissolved organic carbon was needed to eliminate Cu toxicity, suggesting that only unrealistic levels of organic matter for drinking water would likely impact Cu toxicity.<sup>10</sup> However, here, we demonstrated that 5 mg/L organic carbon, at the higher range of what is typical for drinking water,<sup>34</sup> can also nearly eliminate Cu toxicity. On the basis of the copper speciation results, it is reasonable to expect that even doses as low as 2 mg/L HA or FA would have also reduced toxicity (Figure 3), in contrast to the prior study indicating no impact of 2 mg/L on Cu toxicity.<sup>10</sup> It is possible that the higher hardness in the earlier work (110 mg/L as CaCO<sub>3</sub>) versus this work (30 mg/L as CaCO<sub>3</sub>) maintained the antimicrobial effects of Cu<sup>2+</sup> in the presence of 2 mg/L. When we added 70 mg/L extra calcium hardness to our water with NOM, we observed a >50% increase in Cu<sup>2+</sup> levels in the water. Finally, we demonstrate for the first time that enhanced corrosion control, with the addition of phosphate-based corrosion inhibitors that complex Cu, also reduces Cu toxicity by 3.9× at concentrations relevant to drinking water.

**Estimating Cu Disinfection Coefficient.** The Chick–Watson model,  $\ln(N_t/N_0) = kCt$ , was used to calculate the Cu disinfection constant for the strain of *L. pneumophila* used in this study,  $k$  (L µg<sup>-1</sup> min<sup>-1</sup>), where  $N_t$  is the average of three replicates of culturable *L. pneumophila* at time  $t$ ,  $N_0$  is the average initial culturable *L. pneumophila* at time = 0 min, and  $C$  is the biocidal copper concentration. Four forms of copper were considered for modeling the reduction in *L. pneumophila*: measured total Cu, soluble Cu, Cu<sup>2+</sup>, and inorganic soluble Cu (inorganic ligands complexed portion of soluble Cu). The latter was estimated using thermodynamic equilibrium models based on the measured Cu<sup>2+</sup>, soluble Cu, pH, and published complexation constants for Cu(OH)<sup>+</sup> and Cu(OH)<sub>2</sub><sup>0</sup>. We assumed that chloride Cu complexes were negligible in the base water used in this experiment (Table S2).

The Cu disinfection constant for *L. pneumophila* ranged from  $4.1 \times 10^{-6}$  to  $8.9 \times 10^{-4}$  L µg<sup>-1</sup> min<sup>-1</sup> (Figure S7). Despite 2 orders of magnitude variation, all estimates fit the



**Figure 3.** Cu speciation (total, soluble, and  $\text{Cu}^{2+}$ ) in base water at various pH values (7–8.5) and with addition of ligand-forming constituents ( $\text{PO}_4^{3-}$ , NOM as humic acid (HA) or fulvic acid (FA)). For water chemistry conditions left of the dashed line, bars represent average and error bars represent standard deviation of triplicate independent reactors, including samples after 20–240 min incubation. For the pH = 8.5 and  $\text{PO}_4^{3-}$  dosing at 1 mg/L conditions,  $\text{Cu}^{2+}$  summary statistics were not computed due to the high amount of left-censored data (i.e., below the quantification limit of 50  $\mu\text{g/L}$ ); therefore, the means are displayed at 25  $\mu\text{g/L}$  for visualization purposes. Some loss of total Cu from target dose is due to attachment to the glass reactor at elevated pH. The average soluble Cu or  $\text{Cu}^{2+}$  levels that were significantly different from those at pH = 7 are indicated above the corresponding bars in the charts (Kruskal–Wallis test with post hoc Dunn’s test (Bonferroni correction) or regression by maximum likelihood estimation for left-censored data, “\*\*\*” for  $p < 0.001$  and “\*\*” for  $p < 0.01$ ). For water chemistry conditions to the right of the dashed line, the bars represent the results of one trial using total Cu = 800  $\mu\text{g/L}$  speciation with the indicated HA and FA doses to assess any potential differences between the two NOM sources after 40 min of incubation.

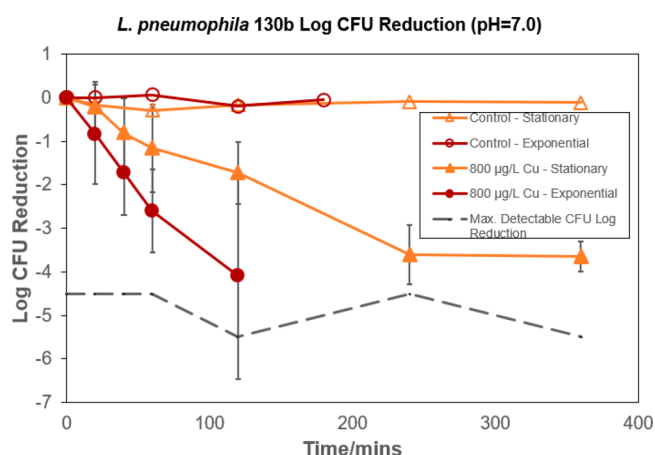
Chick–Watson model with  $R^2 > 0.8$  for all conditions, except the condition with 5 mg/L NOM-as TOC ( $R^2 > 0.5$ ). This was likely due to  $<0.6 \log_{10}$  reduction in Cu inactivation experiments and the large fraction of the soluble Cu existing as bioavailable organically bound Cu. Across this wide range of pH values, phosphate, and NOM, inorganic soluble Cu was the best measure of the biocidal concentration of Cu species in these experiments, as indicated by the lowest RSD across conditions (20.4%). The next best fit was soluble Cu (RSD = 44%), suggesting that in most drinking waters without significant amounts of organic matter, soluble Cu could be a good measure to predict the toxicity of Cu.

Only at pH = 8.5, where both soluble Cu and  $\text{Cu}^{2+}$  were reduced by 69.7% and >94.4%, respectively, as compared to pH 7.0, did the log CFU/mL reduction become significantly different relative to that at pH = 7 (Figure 6). The increase in pH from 7 to 7.5, which can occur within a plumbing system,<sup>35</sup> resulted in substantial reduction of  $\text{Cu}^{2+}$ , but the log CFU/mL reductions were not significantly changed. This is attributed to (1) inherent variation of microbial resistance to heavy metals in replicates observed in this study (e.g., Figure 6); (2) other forms of soluble Cu besides  $\text{Cu}^{2+}$  (assumed to be Cu hydroxide species in this study) still exerting toxicity toward *Legionella*; and (3) reduction in competition for complexation

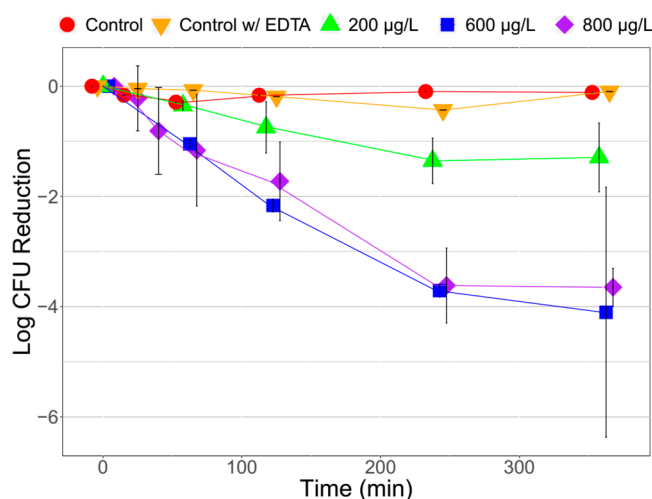
of toxic forms of Cu between hydroxide ions and biomass.<sup>23</sup> This clearly demonstrates that not all soluble Cu has the same biocidal capacity. In practical applications, only total and, in some instances (e.g., with high NOM), soluble Cu can be misleading in terms of quantifying biocidal action because water chemistry can strongly influence Cu solubility via precipitation and complexation.<sup>21,36,37</sup>

Estimating the Chick–Watson model disinfectant coefficient from Lin et al.’s work, using soluble copper measured in their experiment, yielded  $3.53 \times 10^{-4} \text{ L } \mu\text{g}^{-1} \text{ min}^{-1}$ , and this is approximately 100 times higher than that estimated herein, resulting in much faster Cu-induced *L. pneumophila* disinfection rates. Some key differences in experimental conditions likely contribute to this difference, including strains used<sup>26</sup> and life stage, as demonstrated herein. However, Lin et al.<sup>10</sup> defined soluble Cu as that which remained in suspension after pelleting cells, which would represent an underestimate of Cu due to complexation with cell debris relative to the 0.45  $\mu\text{m}$  filter definition herein. With these key differences, in combination with natural biological variability, it is likely these disinfectant constants are not substantially different.

**Total Cu and  $\text{Cu}^{2+}$  Trends among Drinking Water Utilities over the Last Three Decades.** We compared reported 90th percentile Cu levels at 33 major drinking water

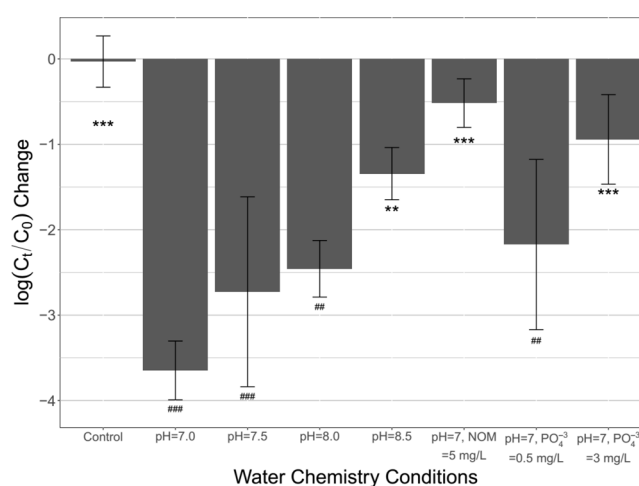


**Figure 4.** *L. pneumophila* log reduction in CFU for exponential phase and early stationary growth phase with and without 800 µg/L Cu at pH = 7. Data points and error bars represent the average and standard deviation of triplicate independent sacrificial inactivation reactors. Control shows one trial, measured over several time points to demonstrate stability during the experiment. Dashed line represents the maximum measurable log reduction based on the lower LOD, which varies at each time point according to the plate dilutions applied.

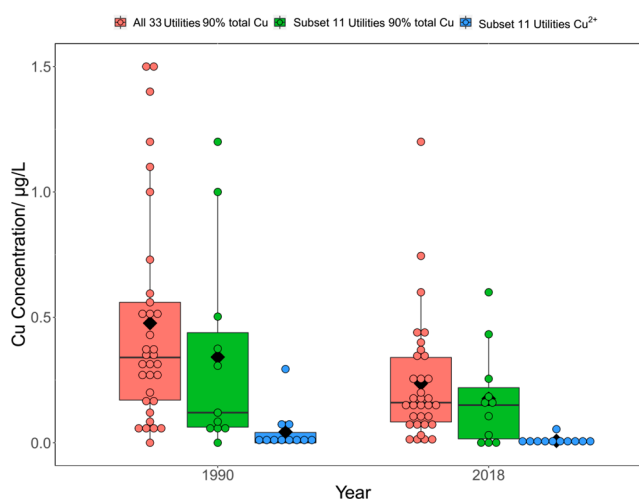


**Figure 5.** Early stationary *L. pneumophila* log reduction with different Cu doses as a function of time at pH = 7. Data points and error bars represent the average and standard deviation of triplicate independently sacrificed inactivation reactors. “Control” and “Control w/EDTA” were not dosed with Cu, with one trial for each, measured over several time points, to demonstrate stability in the control conditions.

utilities in 1990<sup>32</sup> to the most recent data for the same utilities in 2018 in publicly available consumer confidence reports. As expected, given that Cu pipes aged and corrosion control efficacy has improved, the average 90th percentile total Cu levels have decreased in more than 90% (30 of 33) of utilities surveyed, from an average of 477 µg/L in 1990 to 223 µg/L in 2018 (Figure 7). Out of 33 surveyed utilities, 11 had data on phosphate, pH, and alkalinity available to predict Cu<sup>2+</sup> levels in both 1990 and 2018 using MINEQL+ software with the assumption that all 90th percentile copper was soluble.<sup>37,38</sup> Overall, results from 1990 indicated a range of predicted Cu<sup>2+</sup>, from 0 to 293 µg/L, with a median level of 11 µg/L (Figure 7). The 2018 results indicated a range of Cu<sup>2+</sup> from 0 to 54 µg/L



**Figure 6.** Log reduction in early stationary phase *L. pneumophila* after 6 h of incubation under the indicated water chemistry conditions. Control group combines measurements without Cu at pH = 7, 7.5, 8, and 8.5, PO<sub>4</sub><sup>3−</sup> = 3 mg/L, and NOM = 5 mg/L as TOC (13 replicates total; 3 from pH = 7 and 8.5; 2 from PO<sub>4</sub><sup>3−</sup> = 3 mg/L and NOM = 5 mg/L; and 1 from pH = 7.5 and 8 and PO<sub>4</sub><sup>3−</sup> = 0.5 mg/L). Bars and error bars represent the average and standard deviation of 13 replicates (control) and triplicate (in Cu inactivation) samples. The average log(*C<sub>i</sub>/C<sub>0</sub>*) reduction values that were significantly different from those of the pH = 7 condition (ANOVA test, “\*\*\*” for *p* < 0.001; “\*\*” for *p* < 0.01) and the control condition (ANOVA test, “###” for *p* < 0.001; “##” for *p* < 0.01) are labeled below the bars.



**Figure 7.** Total Cu (90th percentile) extracted from Consumer Confidence Reports of 33 major drinking water utilities from 1990 and 2018. Total Cu (90th percentile) and estimated Cu<sup>2+</sup> of a subset of 11 utilities for thermodynamic equilibrium models. Boxplots represent the interquartile range (IQR), while whiskers represent a deviation of  $\pm 1.5 \times \text{IQR}$  from the median. Solid diamonds represent mean Cu concentrations.

and a median level of 1 µg/L. This analysis confirms that characteristic Cu<sup>2+</sup> levels likely decreased more dramatically (>10×) than did total or soluble Cu (2×). This is a potential contributing factor that has not previously been suggested related to increasing incidence of Legionnaires’ disease, even though Cu pipes are the primary material used in commercial buildings. Other potentially prominent factors thought to contribute to increasing rates of Legionnaires’ disease include

improved diagnosis and reporting, aging of the national water infrastructure, and an aging population.<sup>39</sup>

## ■ IMPLICATIONS

On the basis of the results presented herein, to achieve at least a 2 log reduction in bulk water *L. pneumophila* in 6 h, it would be necessary to maintain a level of copper inorganic complexes at about 600  $\mu\text{g/L}$ . While passive control of *L. pneumophila* has been documented, the increased use of phosphate-based corrosion control has decreased baseline levels of Cu in drinking water systems down to an average 90th percentile Cu of  $\sim 250 \mu\text{g/L}$ . The increased phosphate dose also likely disproportionately decreased the forms of soluble Cu that are most biocidal (Figures 6 and 7). For CSI systems, EPA and WHO recommend regular monitoring of total Cu levels,<sup>3,40</sup> and published studies suggest that 200–800  $\mu\text{g/L}$  for Cu and 10–80  $\mu\text{g/L}$  for silver will be effective.<sup>3,14–16</sup> Our results demonstrated that these levels would not be sufficient for a wide range of relevant conditions, including higher pH, high levels of NOM, or the presence of orthophosphate corrosion inhibitors, at least for copper alone.<sup>16</sup> This study also helps explain noted discrepancies in the observed effects of copper on *Legionella* reported in the literature.<sup>7,9–12</sup> In practice, to balance corrosion inhibition and counter other water chemistry effects on Cu biocidal efficacy, in-building water chemistry adjustment and other applicable *L. pneumophila* control technologies should be considered. At a minimum, those implementing CSI should measure soluble Cu and organic carbon. Organic carbon concentrations in the range of 2–5 mg/L are at higher risk of decreasing Cu biocidal efficacy.<sup>10</sup> Here, we focus only on the effect of copper ions. Follow-up research on silver ions, which have been demonstrated to have synergistic antimicrobial effects with copper, would also be of value.<sup>16</sup>

Other factors not examined in this study are also likely to impact Cu toxicity, such as *Legionella* strain. Recently, an up to 4-fold variation in the susceptibility to cupric chloride was reported among various environmental strains of *Legionella* tested.<sup>26</sup> In addition, exposure to Cu may induce viable but nonculturable (VBNC) *Legionella* that may regrow once the external stress (i.e., Cu) is removed or changes in conditions encourage their growth (e.g., amoeba host), as has been demonstrated with other biocides, but not with Cu.<sup>41–44</sup> Finally, *Legionella* residing within amoeba hosts and/or biofilms would likely require higher levels of Cu to be inactivated than indicated in this work, as the amoebae serve as an additional barrier to exposure to biocidal levels of Cu<sup>45</sup> and Cu may form organic complexes with biomass, reducing its biocidal capacity. Such factors require additional research.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.0c06804>.

Figure S1, Copper stability bench-scale testing workflow; Figure S2, Copper speciation bench-scale testing workflow; Figure S3, Measured copper concentration comparison between inductively coupled plasma mass spectrometry (ICP-MS) and HACH Method 8506; Figure S4, Cupric ion selective probe relative standard deviation changes as a function of total copper levels; Figure S5, *L. pneumophila* strain 130b growth curve;

Figure S6, Copper inactivation bench-scale tests workflow; Figure S7, Disinfection rate  $k$  comparison across different water chemistry conditions using disinfectants as  $\text{Cu}^{2+}$ , soluble Cu, inorganic soluble Cu, and total Cu; Table S1, Typical base water characters before and after three filter treatments; and Table S2, MINEQL+ model of representative base water conditions (pH = 7, alkalinity = 40 mg/L) predicting Cu (800  $\mu\text{g/L}$  or  $1.25 \times 10^{-5} \text{ M}$ ) species at equilibrium (assuming no solids' formation to represent short-term steady state) (PDF)

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### Author Contributions

M.A.E., W.J.R., and Y.S. designed the experiments and assisted in the analysis of results. Y.S. conducted the experiments and prepared the first draft of the manuscript. A.P. was a co-PI on the NSF grant and provided feedback on the experimental design and analysis of the results. All authors contributed to the writing of the manuscript and approved the final version.

### Notes

The authors declare no competing financial interest.

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