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RESEARCH PAPER

Study of *Legionella pneumophila* treatment with copper in drinking water by single cell-ICP-MS

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Abstract

Legionella pneumophila is a persistent opportunistic pathogen that poses a significant threat to domestic water systems. Previous studies suggest that copper (Cu) is an effective antimicrobial in water systems. A rapid and sensitive quantification method is desired to optimize the conditions of *L. pneumophila* treatment by Cu and to better understand the interaction mechanisms between Cu and cells. In this study, we developed a highly sensitive single cell (SC)-ICP-MS method to monitor *L. pneumophila* cell concentration and track their uptake of Cu. The SC-ICP-MS method showed excellent sensitivity (with a cell concentration detection limit of 1000 cells/mL), accuracy (good agreement with conventional hemocytometry method), and precision (relative standard deviation < 5%) in drinking water matrix. The cupric ions (Cu^{2+}) treatment results indicated that the total *L. pneumophila* cell concentration, Cu mass per cell, colony-forming unit counting, and Cu concentration in supernatant all exhibited a dose-dependent trend, with 800–1200 µg/L reaching high disinfection rates in drinking water. The investigation of percentages of viable and culturable, viable but nonculturable (VBNC), and lysed cells suggested there always were VBNC present at any Cu concentration. Experimental results of different Cu^{2+} treatment times further suggested that *L. pneumophila* cells developed an antimicrobial resistant mechanism with the prolonged Cu exposure. This is the first quantification study on the interactions of Cu and *L. pneumophila* in drinking water using SC-ICP-MS.

Keywords Single cell-ICP-MS · *Legionella pneumophila* · Copper (Cu) · Antimicrobial resistance · Water treatment · Viable but nonculturable cell (VBNC)

Introduction

Legionella pneumophila is an opportunistic pathogen that is responsible for the most cases of Legionnaires' disease, a severe and potentially fatal pneumonia. The disease has been linked to the presence of *L. pneumophila* in drinking water distribution systems and premise plumbing drinking water systems. This high fatality rate (ranging from 5 to 30%, World Health Organization, 2019) disease outbreaks is rapidly increasing worldwide in recent years [1–4]. The presence of *L. pneumophila* in drinking water systems is a major public health concern, as it can cause disease outbreaks and result in widespread exposure to contaminated water. Therefore, it is critical to control the growth of *L. pneumophila* in the drinking water systems. The techniques commonly used to eliminate *Legionella* include thermal eradication, hyperchlorination, ozone, and ultraviolet (UV) treatments. However, these treatments have notable disadvantages, such as the risk of scalding and recolonization that occurs in a

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few months, the risk of pipe corrosion, and the formation of disinfection by-products. Copper (Cu) has been used to control *L. pneumophila* and other microorganisms in recent years [5–9]. However, the reported bactericidal activity of Cu on *L. pneumophila* is different from different studies and sometime conflicts [10]. Song et al. recently reported a comprehensive study of *L. pneumophila* treatment efficiency by Cu under different water chemistry conditions and the pathogen growth phases in drinking water system [9]. The results demonstrated that water chemistry and growth phase control the net effect of Cu on *Legionella*. Therefore, for practical application, the effective treatment conditions need to be evaluated case by case for different water systems. Cu has also been found to be an effective treatment for controlling other waterborne pathogens, such as *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecalis* [11]. However, evaluating the Cu dose effect in drinking water systems remains challenging because water chemistry can strongly affect it [9]. Up to now, the optimal conditions for using Cu to treat drinking water have not been well established and the interaction mechanisms between Cu and cells is still not fully understood. The efficacy of Cu treatment depends on several factors, including the type of Cu species used, the concentration of Cu in water, the contact time of Cu, the pH of water, and the presence of other chemicals, such as phosphate and chloride [9]. To better understand the impact of these factors and the mechanism of Cu-*L. pneumophila* interaction, it is essential to have a rapid and sensitive analytical method.

Another challenge of *L. pneumophila* control is its antimicrobial resistant nature. This opportunistic pathogen can convert into viable but nonculturable (VBNC) when treated with disinfectant and microcide, such as chlorine and Cu, and then revised back to the culturable stage under favorable condition [12–15]. This makes the complete disinfection of this pathogen very difficult. The mechanism of this conversion is not fully understood, due to the current technology that cannot quantitatively detect the different forms of cells, total intact cell (TC), viable and culturable cell (VC), and VBNC. New sensitive and rapid analytical methods are necessary.

The primary analytical techniques used for evaluating the efficiency of Cu treatment for *L. pneumophila* are inductively coupled plasma-optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), etc. [8, 9, 12]. Conventional ICP-MS analysis is limited to measurements of the average amount of metal per cell in a cell population after laborious acid digestion [16–18]. It cannot provide intracellular concentrations for individual cells needed to construct mass distributions across a heterogeneous population. Single cell (SC)-ICP-MS is a cutting-edge analytical technique that allows the quantification of elemental content in an individual cell and the distribution in the cell population. It has been increasingly used for studying interactions of metal-containing materials, including nanoparticles and bactericides, with algae, yeast, and other microorganisms [18–27], as well as human cancer cells [26, 28, 29]. SC-ICP-MS has key advantages: (1) it is very sensitive, capable of detecting intracellular metal concentration down to attogram (ag) per single cell and cell concentration down to a thousand cells per milliliter; (2) it can monitor Cu in cells directly without time-consuming acid digestion; (3) it provides the distribution of the metal content within a cell population rapidly, allowing for the discrimination of differences due to cell heterogeneity; (4) it can analyze a large number of individual cells in a short amount of time, allowing for high-throughput analysis. The SC-ICP-MS analysis employs time-resolved techniques utilizing a low flow rate high-efficiency nebulizer. The principle of the technique has been established [19, 30]. SC-ICP-MS is expected to have high potential for the investigation of *L. pneumophila* treatment and interaction mechanism with Cu and other metallic bactericides. However, a SC-ICP-MS method has not been developed for this type of analysis. In response, we developed a highly sensitive and rapid SC-ICP-MS method for quantifying Cu uptake by *L. pneumophila* at the single cell level. We have applied this SC-ICP-MS method to quantitatively evaluate the treatment efficiency by different Cu concentrations and contact times in a drinking water system. The technique is expected to lead applications for optimizing the opportunistic pathogen treatment conditions and studying the Cu and *L. pneumophila* interaction mechanism.

Materials and methods

Materials

Trace-metal-grade concentrated nitric acid (HNO_3, 67–70%), copper sulfate ($CuSO_4 \bullet 5H_2O$, > 99.9%), sodium hydroxide (NaOH, > 99.9%), sodium chloride (NaCl, > 99%), and isopropyl alcohol (> 99.9%) were all purchased from Fisher Scientific (Pittsburgh, PA, USA). Dissolved Cu (Cu^{2+}), magnesium (Mg), and bismuth (Bi) standard solutions (10 mg/L in 2% HNO_3) were purchased from High-Purity Standards (Charleston, SC, USA). Supplies for bacterial culture were purchased from Sigma-Aldrich, Inc., St. Louis, MO, in the USA (potassium hydroxide, activated charcoal, agar, L-cysteine) and Thermo Fisher Scientific Waltham, MA, in the USA (yeast extract, ACES buffer, alpha-ketoglutarate, thymidine). Ferric nitrate was purchased from MP Biomedicals (Solon, OH, USA). Ultrapure water (18.2 MΩ·cm) was prepared using a Millipore water purification system (Millipore, Billerica, MA, USA).

Bacterial strains and culture conditions

L. pneumophila strain Lp02 was used in this study as previously described [31]. The bacteria were grown on buffered charcoal yeast extract (BCYE) agar plates (supplemented with 0.4 mg/mL L-cysteine 0.1 mg/mL thymidine, 0.135 mg/mL ferric nitrate) for 2–4 days at 37 ℃. Several single colonies were transferred to a 16 mm × 150 mm test tube with 5 ml of BYE broth (N-(2-acetamido)-2-aminoethansulfonic acid-buffered yeast extract broth supplemented with 0.4 mg/mL L-cysteine, 0.1 mg/mL thymidine, and 0.135 mg/mL ferric nitrate). The initial optical density of the bacterial culture at a wavelength of 600 nm (OD_{600}) was 0.2. Cells were grown for 16 h (early stationary phase) at 37 °C with constant shaking at 200 rpm. After incubation, cells were pelleted by centrifuging at 5000 g for 5 min, and then the supernatant was discarded. Cells were washed twice with 5 mL of 0.9% saline solution. The cell pellet was resuspended in 5 mL of filter-sterilized ultrapure water and diluted to obtain $OD_{600} = 0.2$. The bacterial cell suspension was further diluted to obtain approximately $OD_{600} = 0.02$ to perform cell count using a hemocytometer for total *L. pneumophila* cell concentration. The cell count was determined according to standard protocol using a Leica model ICC50W microscope with phase contrast optics at 400 times magnification. Right after determining the cell concentration based on the hemocytometer calculations, bacterial cells were diluted with sterile water to a final concentration of cells of 3×10^6 cells/mL for the corresponding treatment experiment.

Drinking water sample collection and water parameters

The drinking water from a local municipal utility in Missouri was collected and used for these experiments. The drinking water was allowed to run from the faucet for about 5 min, then collected in pre-cleaned glass containers. The sample was left at room temperature for a few days until no disinfectant (chlorine) residue was left in the water. The water was then filtered through a 0.22-µm nylon membrane filter to remove any particulates and microorganisms, if present. The pH and dissolved organic carbon (DOC) of the water sample were measured and listed in Table 1. By following standard procedure, DOC was measured using a Shimadzu Model TOC-L TOC analyzer (Shimadzu, Overland Park, KS, USA). The water was also tested for native Mg and Cu concentrations by a conventional ICP-MS method using a PerkinElmer NexION 2000P ICP-MS (Shelton, CT, USA) after appropriate dilution using 1% HNO_3. The dissolved Mg and Cu standards in concentration range from 0.01 to 500 µg/L were applied to establish calibration curves. Bi (5 µg/L) was used as the internal standard added online during the ICP-MS analysis because of its superior performance and stability during the analyses of the drinking water samples. The reagent blank, duplicate, and spiked samples were also monitored for quality assurance. The concentrations are shown in Table 1.

Table 1 Drinking water sample important parameters

Sample parameter	Value
pH	7.10
Dissolved organic carbon (DOC)	3.81 mg/L
Total dissolved solids	243.0 mg/L
Cu concentration	120.0 µg/L
Mg concentration	32.2 mg/L

Cu treatment of *L. pneumophila* in drinking water

A Cu^{2+} stock solution of 100 mg/L was prepared using $CuSO_4 \bullet 5H_2O$ and adjusted $pH = 4.0 \pm 0.05$ to maintain Cu solubility. The intermediate/working solution of Cu^{2+} 10 mg/L was freshly diluted using ultrapure water right before each treatment experiment. The $pH = 7 \pm 0.05$ was adjusted using 0.1 N potassium hydroxide or 0.2 N nitric acid and the solution was sterilized using a 0.22-µm nylon membrane filter. Ultrapure water and 0.9% saline solution were tested by ICP-MS for blank background levels of Cu and Mg and then sterilized by using a 0.22-µm filter system.

The treatments were prepared by using 3×10^6 *L. pneumophila* cells/mL in 5 mL of water with the corresponding addition of Cu stock solution (10 mg/L) to achieve Cu concentrations at 0 (control), 100, 400, 800, and 1200 µg/L. Note that in native drinking water, 120 µg/L of Cu was present in addition to the spiked concentration. The bacterial cell suspensions in the water were incubated for 4 h at 37 °C with constant shaking at 200 rpm. The Cu concentration of 800 µg/L was selected for measuring the impact of contact time (0.08, 0.5, 2, 4, 8, and 24 h).

After treatment, cells were transferred to 15 mL of conical tubes and cells were pelleted by centrifugation for 5 min at 5000 g. The supernatant was removed by gentle pipetting to avoid loss of the cells. The supernatants of selected samples were collected for Cu analysis by conventional ICP-MS. The cell pellet was washed to remove residual Cu and then resuspended in 3 mL of ultrapure water. An aliquot of cell suspension was used for SC-ICP-MS analyses for Cu uptake by *L. pneumophila* through monitoring of ^{63}Cu and total cell concentration through monitoring of ^{24} Mg. The total cell (TC) concentration measured through ^{24}Mg SC-ICP-MS includes viable and culturable (VC), viable but nonculturable (VBNC), and nonviable (NV or dead) cells with intact cellular shape (NVBI). Only nonviable and lysed (NVL) cells cannot be detected. An aliquot of cell suspension was

used for counting colony-forming units (CFU) to determine the VC cell concentration after the Cu treatment.

Single cell-ICP-MS method

A PerkinElmer NexION 2000P ICP-MS equipped with a single cell sample introduction system and controlled by Syngistix for ICP-MS software (PerkinElmer, Shelton, CT, USA) was employed for SC-ICP-MS analysis. The detailed information about the PerkinElmer SC-ICP-MS sample introduction system (nebulizer, spray chamber, and injector) is shown in Table 2. Manual sample introduction was used for each sample at a flow rate of 14 µL/min following a 3-min rinse cycle with 0.1% nitric acid and ultrapure water. Nebulizer and make-up gas flows were optimized daily, but generally proceeded at 0.32 L/min and 0.70 L/min, respectively. The scan time was set to 100 s, with a dwell time at 50 µs. Prior to each experiment, the spray chamber was cleaned thoroughly with 99.9% isopropyl alcohol followed by ultrapure water.

CFU count for viable and culturable *L. pneumophila* cell concentration determination

An aliquot of 0.1 mL from each replicate of treated sample was transferred to a sterile microcentrifuge tube preloaded with 0.09% saline. Serial dilutions of (1:100–1:1000) were prepared and 0.1 mL was transferred onto a BCYE agar plate. After 72 h of incubation, colonies were enumerated manually.

Flow cytometry analysis to determine cell viability

L. pneumophila cell viability was assessed by a BD Accuri™ C6 Plus Flow cytometer (BD Biosciences, Becton-Dickinson, Franklin Lakes, NJ) with BD Accuri C6 Plus software and Live/Dead BacLight Bacterial Viability kit (SYTO® 9 green fluorescent nucleic acid stain and propidium iodide red fluorescent nucleic acid stain) following the manufacturer's instruction. *L. pneumophila* cells were treated with different concentrations of Cu (0, 100, 400, 800, and 1200 µg/L) for 4 h in drinking water at 37 °C, pH 7.10. After the treatment, cells were spin down and resuspended in ultrapure water for the flow cytometry analysis.

Table 2 Optimized SC-ICP-MS parameters

Parameter	Optimized value
Manual sampling flow rate	14 µL/min
Nebulizer	Meinhard TRP-90-A0.05
Spray chamber	Asperon single-cell
Injector	Quartz. 2.0 mm id
RF power	1600 W
Nebulizer gas flow rate*	0.32 L/min
AMS makeup gas flow rate	0.70 L/min
Analytes	^{24}Mg and ^{63}Cu
Transport efficiency (TE)*	44–56%
Sample analysis time	100 s
Dwell time	50 µs

*Parameters were optimized daily

Statistical analysis

Data analysis was performed using the statistical software Microsoft Excel 365 (Microsoft Corp.). Data are representative of triplicate and expressed as mean ± SD. Figures were drawn by OriginPro 2021 (Origin Lab, USA). The statistical analysis was performed by IBM SPSS Statistics 27.0.1 using one-way ANOVA with Duncan's post hoc test. An outcome was considered statistically significant if a p-value of < 0.05 was obtained.

Results and discussion

SC-ICP-MS method development and performance

Transport efficiency (TE) was measured by using the *L. pneumophila* cells by monitoring the intrinsic metal ^{24}Mg due to its high concentration in cells and relatively low background, using the following equation as previously reported [16]:

$$\mathrm{TE} = \frac{F}{Qs \cdot Nc \cdot t}$$

where Qs is the sample uptake rate (mL/s), Nc is the *L. pneumophila* cell concentration (number of cells/mL), t is the acquisition time (s), and F is the number of cells detected during acquisition time. A representative histogram of intracellular Mg mass distribution in *L. pneumophila* cell is shown in Fig. 1. The histograms of the suspensions were Gaussian shape except for some noise in the very low signal intensities, which were eliminated from the calculations. The TE ranged from 44 to 56% from day to day (measured daily for each experiment). This noise is to be expected since natural cell death happens and leads to generation of cell pieces during the log phase of cell growth.

The cell concentrations in cell suspension were measured by SC-ICP-MS and calculated by the Syngistix software using the measured TE and the measured counts per second for intrinsic Mg in each cell suspension. All the method conditions were optimized to the highest TE and reproducibility while ensuring cell integrity through the sample introduction system.

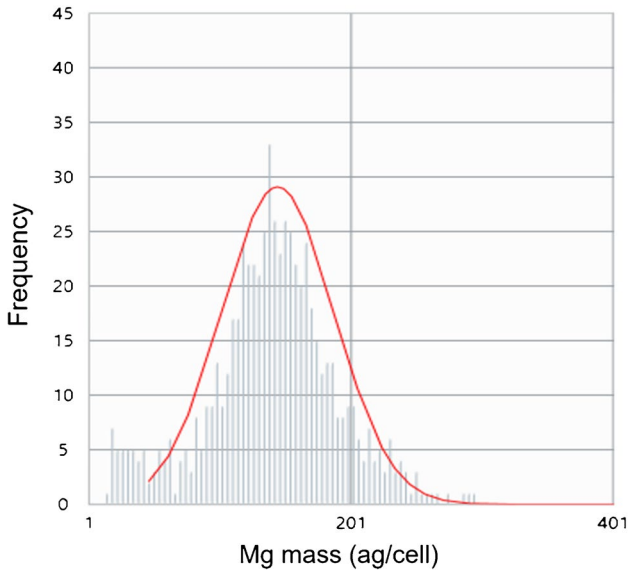

Fig. 1 A representative histogram of ^{24}Mg SC-ICP-MS from a *L. pneumophila* cell suspension

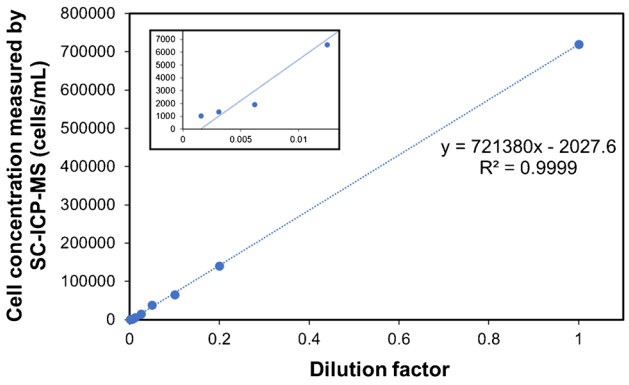

Fig. 2 SC-ICP-MS results from different dilutions of a stock cell suspension by measuring ^{24}Mg. The cell concentration in stock suspension is 7.2×10^5 cell/mL counted by a hemocytometer

The optimized method parameters are tabulated in Table 2.

To evaluate the Cu mass in each cell, external calibration curve with dissolved Cu standard solutions (0, 0.1, 1, 5, and 10 µg/L) was established. The detection limit was calculated to be 38 ag Cu/cell following the equation below [19]:

$$\mathrm{DL} = \frac{3\sigma}{\text{slope of calibration curve}}$$

where σ is the standard deviation of the blank, and DL is the mass detection limit (ag Cu/cell).

Due to the nature of *L. pneumophila* cells which tend to aggregate and attach to the wall or bottom of the container [32], verifying the linearity of cell suspension dilutions was necessary. As such, different dilutions of a suspension were made from 7.2×10^5 to 1000 cells/mL and the cell concentrations measured exhibited excellent linearity (Fig. 2).

Finally, to confirm the agreement between SC-ICP-MS and conventional hemocytometry counting, cell concentrations were determined using both the hemocytometer and SC-ICP-MS in three replicates of cell suspensions. The cell concentrations from both the hemocytometer and SC-ICP-MS gave good consistency. The SC-ICP-MS measured cell concentration as 2.7×10^5, 2.9×10^5, and 2.7×10^5 cells/mL, with a RSD of 4.2%, within 2 min during one run. The cell concentrations obtained by hemocytometer count were 2.8×10^5, 2.7×10^5, and 2.3×10^5 cells/mL, with a RSD of 10.2%, and it required more than 10 min for each sample count. The SC-ICP-MS measurement for cell count is not only much faster than the conventional cell count, but also has better reproducibility.

Cu concentration impact on *L. pneumophila* treatment efficiency

The histograms of Cu mass distribution in cell population after treatment (4 h, 37 ℃) with different Cu concentrations in drinking water are shown in Fig. 3. Compared with the control group (no Cu dose), the *L. pneumophila* cells exhibit obvious heterogenicity in Cu uptake, with the long tail observed after dosing Cu. The gradually right-shifted Gaussian fitting peak with the increase of Cu treatment concentration indicates the dose-dependent phenomenon of Cu uptake.

To further investigate the Cu concentration impact on *L. pneumophila* cell, the TC concentration and average Cu mass per cell were tested by SC-ICP-MS (Fig. 4a and b). During the SC-ICP-MS analysis, ^{24}Mg was monitored as an intrinsic metal in cells for TC concentration determination. As shown in Fig. 4a, the TC concentration detected by SC-ICP-MS decreases significantly ($p < 0.05$) with increased Cu treatment concentration. When dosing with 800 µg/L and 1200 µg/L of Cu, the Cu disinfection rate reached similarly high level, with lysed cell ratios of 41.3% and 46.9%, respectively, compared with the control group. As expected, the Cu mass in each cell increased with the increasing dosed Cu concentration (Fig. 4b), indicating that the uptake of Cu by *L. pneumophila* caused a significant decrease of cell concentration. Notably, there is no significant difference ($p > 0.05$) of the Cu mass per cell in the 800 µg/L Cu treatment group and the 1200 µg/L Cu treatment group, all close to ~ 300 ag Cu/cell. This should be the reason why TC concentration in the 1200 µg/L Cu treated group was only slightly decreased compare with that from the 800 µg/L Cu treated group.

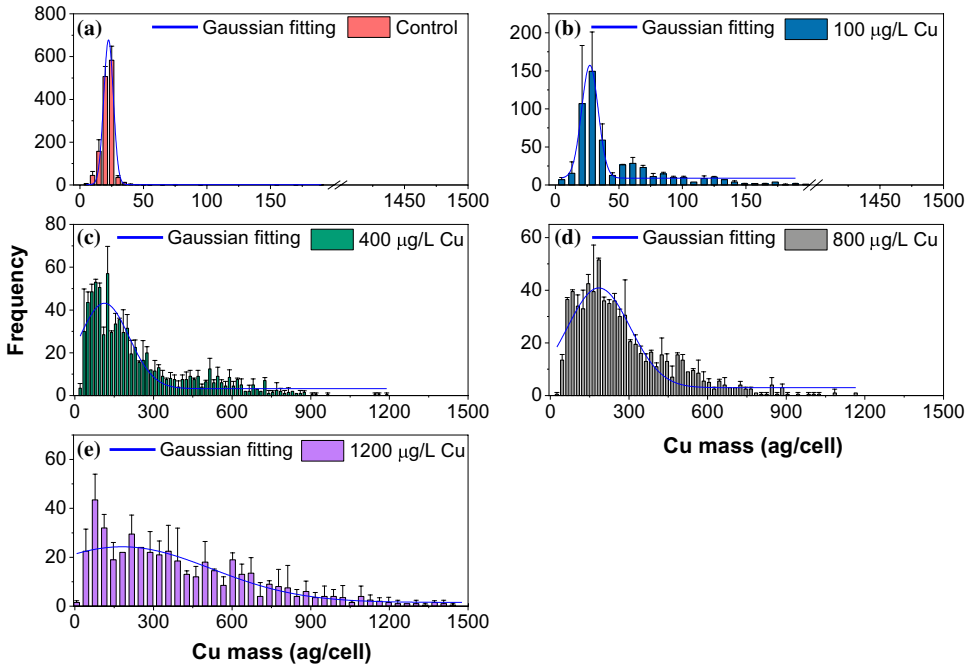

Fig. 3 Histograms of Cu mass distributions in *L. pneumophila* cell populations after 4-h treatment with different Cu concentrations (**a** for control; **b** for 100 μg/L Cu; **c** for 400 μg/L Cu; **d** for 800 μg/L Cu; **e** for 1200 μg/L Cu). The error bars in the SP-ICP-MS histograms represent standard deviation ($n = 3$)

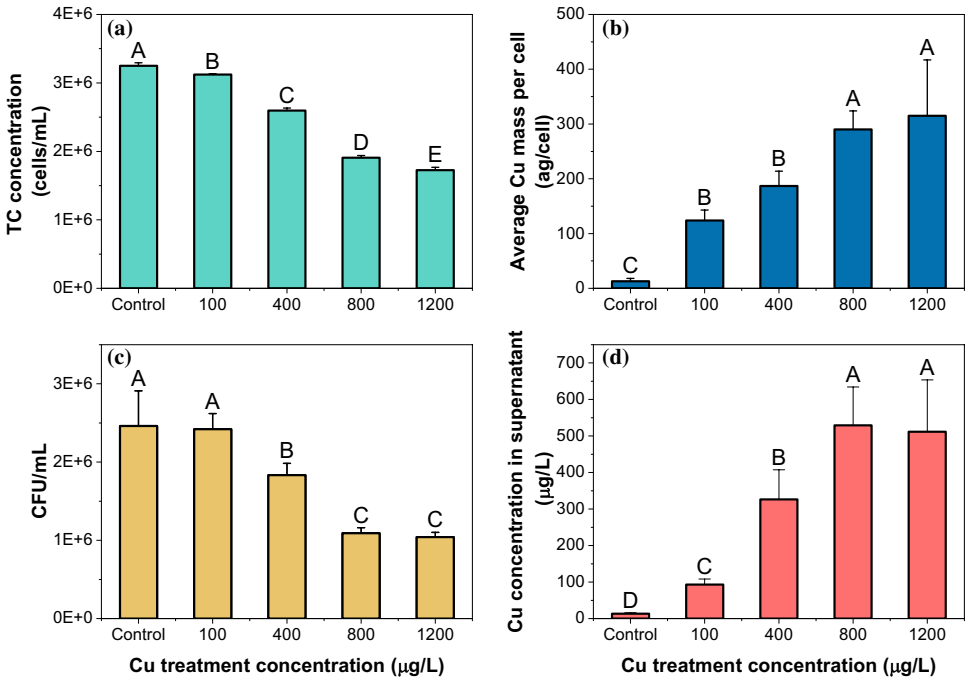

Fig. 4 Cu treatment concentration (0–1200 μg/L) effect on *L. pneumophila*. **a** *L. pneumophila* TC concentration detected by SC-ICP-MS and percentage of TC intact cells; **b** average Cu mass per *L. pneumophila* cell; **c** CFU/mL of *L. pneumophila* cell and percentage of VC; **d** Cu concentration in supernatant. Data are representative of triplicate and expressed as mean ± SD. The statistical analysis was performed by IBM SPSS Statistics 27.0.1 using one-way ANOVA with Duncan's post hoc test. Different letters (A, B, C, D, and E) on the bars indicate significant differences between different treatments (no same letter between 2 groups indicates significant difference, $p < 0.05$)

The SC-ICP-MS method can rapidly detect intact TC concentration, but it cannot distinguish the cell state. To examine culturable *L. pneumophila* cell, CFU count was determined after 72-h incubation and the results are shown in Fig. 4c. CFU counting results also display a dose-dependent trend, which agreed well with TC concentration results. The 800 μg/L and 1200 μg/L of Cu treatments also have similarly high disinfection level (56% and 58%, respectively). Interestingly, the VC cell concentrations detected by this plate count method were lower than the TC detected by SC-ICP-MS method in all groups, including control group at similar level (23% ± 2%). This is presumably due to the presence of VBNC *L. pneumophila* cells in the original cell culture or induced transformation to VBNC state during the 4-h experimental time, or possibly some died cells not lysed during this time.

In order to find out if the difference of TC detected by SC-ICP-MS and CFU count is due to VBNC cells or died but not

lysed cells, the cell viability was determined by flow cytometry analysis. The results in Fig. 5 indicate that most of the cells (93.9, 93.4, 92.1, 92.6, and 92.2% for control, 100, 400, 800, and 1200 µg/L Cu treatments, respectively) detected by SC-ICP-MS were viable cells. This confirmed that the more cells detected by SC-ICP-MS than the CFU count were VBNC *L. pneumophila* cells, i.e., TC ≈ VC + VBNC.

Putting all different experiments together, the changes of percentages of VC, VBNC, and NVL cells after different Cu concentration treatments are demonstrated in Fig. 6. As expected, the VC cell's percentage decreased and the NVL cell's percentage increased as the increase of Cu concentration. Interestingly, there is no obvious change about the ratios of VBNC cell after different Cu concentration treatments. Based on these percentage profiles, it could be speculated that after 4-h treatment, Cu is able to disinfect the *L. pneumophila* and change its state from VC to NVL cell, showing an obvious dose-dependent tendency, but did not affect the ratio of VBNC cell much. This seems indicating there are certain percentage

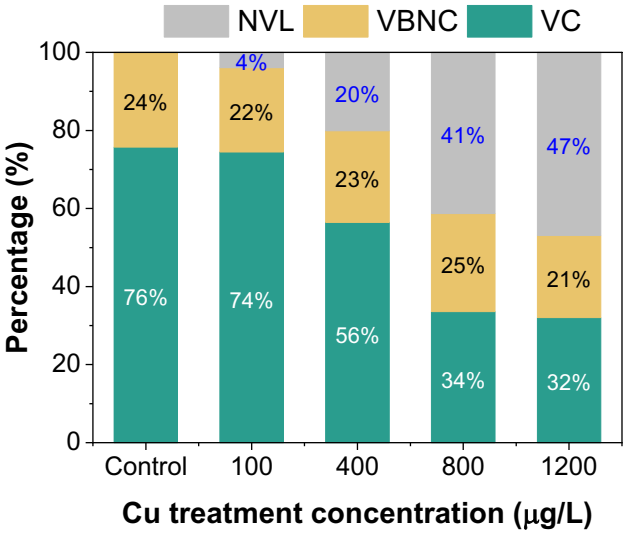

Fig. 6 Percentages of VC (viable and culturable) cells, VBNC (viable but nonculturable) cells, and NVL (nonviable and lysed) cells revealed by SC-ICP-MS and CFU count methods after different concentrations of Cu treatment

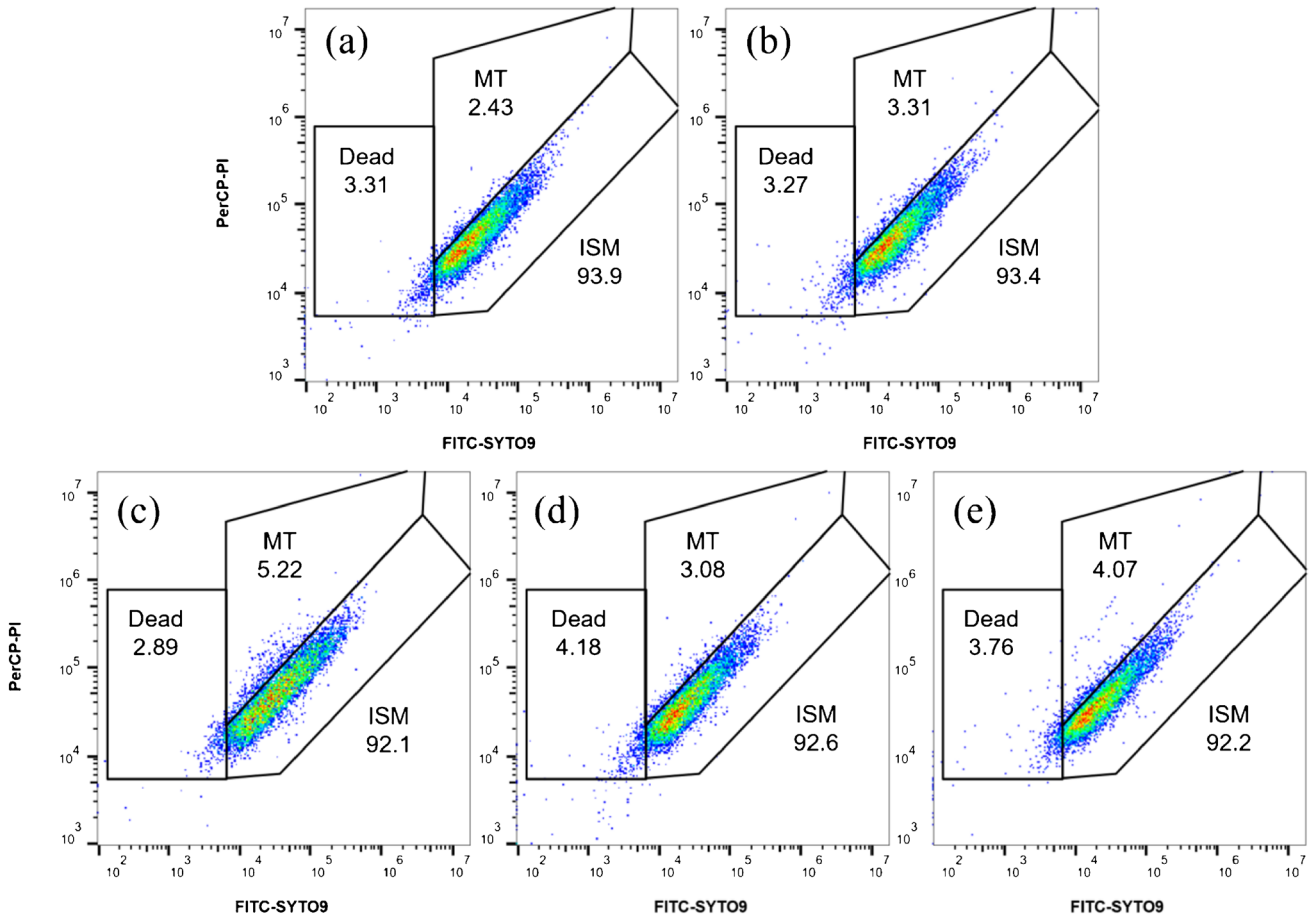

Fig. 5 Cell viability determined by flow cytometry analysis of *L. pneumophila* treated with different concentrations of Cu in drinking water at a contact time of 4 h. Representative 2D plots of **a** control (drinking water without Cu addition), **b** 100 µg/L Cu, **c** 400 µg/L Cu, **d** 800 µg/L Cu, **e** 1200 µg/L Cu. ISM: intact slightly damaged membranes (viable cell). MT, mixed type membranes (damaged cell)

L. pneumophila cells present as VBNC cells under this treatment condition.

To find out why there is no significant increase of disinfection efficiency as increasing Cu to 1200 µg/L from 800 µg/L, the Cu concentrations in the cell culture supernatants (media after removing the cells) were detected by conventional ICP-MS and the results are shown in Fig. 4d. It is clear that the soluble Cu concentrations in the high Cu treatment samples are much lower than the dosed Cu concentrations, especially for the 1200 µg/L Cu group, which is about the same as the 800 µg/L Cu dosed one. The difference of lost Cu between 800 and 1200 µg/L treatment groups is about 417 µg/L, indicating no available Cu increased when higher Cu dosed. The soluble Cu concentration after dosing 800 or 1200 µg/L Cu is around 500 µg/L as measured by the conventional ICP-MS method (Fig. 4d), which is similar with the previous reported [9]. It might be the maximum soluble Cu concentration in this drinking water chemistry situation. This could be the main reason why the efficiency of 1200 µg/L treatment is similar with the 800 µg/L Cu treatment group.

Other studies have also shown that *L. pneumophila* concentration in drinking water exhibits a dose-dependent trend with Cu concentration. A study conducted by Emilie et al. investigated the impact of Cu concentration on *L. pneumophila* concentration in a hospital hot water system [12]. The study found that as Cu concentration increased from 0.8 to 5 mg/L at pH 7.8, the log reduction in CFU counts of *L. pneumophila* cell increased from ~ 1 to ~ 5 after 672 h, demonstrating a clear dose–response relationship. Similarly, a study by Song et al. showed that with the increase of Cu concentration from 200 to 800 µg/L at pH 7, the log reduction of *L. pneumophila* increased from 1.5 to 4 after 6 h [9]. However, Cu can also react with other components in drinking water, such as chloride and natural organic matter (NOM). After entering drinking water, dosed Cu can react with chloride and NOM in drinking water forming different complexes, such as CuCl and $CuCl_2$, which have low solubility in water and are less available to organisms [9]. Another factor that can influence the relationship between Cu and *L. pneumophila* concentration in drinking water is the pH of the water. Recent studies found that the effectiveness of Cu in reducing *L. pneumophila* cell concentration was pH-dependent [9, 10], with the optimal pH being 7 when the dosed Cu concentration was 800 µg/L. Above this pH level, the antimicrobial effectiveness of Cu was reduced mainly due to the formation of Cu precipitation, as we observed in this study. For example, Song et al. treated tap water with 1000 µg/L Cu and monitored the soluble Cu content at different pH levels. After 20 min, the 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 [9].

Another important finding of our study is the alteration of the cell state during Cu treatment. The state transition from VC cell to NVL cell after Cu treatment should be associated with the "contact killing" process [33, 34]. Cu ions can bind to cell membrane surface, and then disrupt the lipid bilayer and cause membrane damage, leading to increased permeability and loss of essential ions and molecules from the cell. This can ultimately result in the lysis or rupture of the cell membrane, leading to the elevated NVL cell percentage [35]. Another toxicity mechanism to inactivate *L. pneumophila* is the formation of reactive oxygen species (ROS). Cu ions can generate ROS in the presence of water or oxygen. ROS can cause damage to cellular components such as proteins, lipids, and DNA, leading to cellular dysfunction and death [36]. Being unable to proliferate, *L. pneumophila* cells survive severe environmental conditions by entering the dormant state, i.e., VBNC cell state. A previous study reported that the Cu ion stressor can stimulate *L. pneumophila* to enter VBNC state [37]. Once induced into VBNC state by Cu treatment, *L. pneumophila* cells usually tend to shrink and form a spheric shape, which may reduce their metabolic activity by decreasing specific surface area and further reducing the exchange of Cu through their cell surface [13]. In addition, previous studies found that the bacterial physiology also changes significantly after entering the VBNC state. These changes include a decrease in the transmission efficiency of nutrients and substances, a decrease in metabolic and respiratory activity, alterations in the composition of the cell wall and membrane, and changes in gene expression levels [14, 15]. The decreased transmission efficiency, for example, hinders the Cu uptake and allows the *L. pneumophila* cells to survive with varying Cu concentration treatments. It should be noted that the VNBC *L. pneumophila* cells can be resuscitated by amoeba [38]; hence, further efforts are warranted to eradicate VNBC *L. pneumophila* in drinking water systems.

Cu treatment contact time impact on *L. pneumophila* treatment efficiency

From the above experiment results, it was found that 800 µg/L Cu treatment was effective in disinfecting *L. pneumophila* cells for a treatment contact time of 4 h. Further tests were performed to evaluate the impact of different contact times on the efficiency of treatment by using 800 µg/L Cu. The contact times of 0.08, 0.5, 2, 4, 8, and 24 h were tested. The histograms at different contact times are shown in Fig. 7. Even at ~ 0.08 h (5 min), unsymmetrical Cu mass histogram with a tail was observed, suggesting the rapid Cu uptake by *L. pneumophila* cells. Over time, the Gaussian fitting peak shifts to the right side and frequency of cell with higher Cu mass elevates gradually, leading to a histogram with more cells containing high Cu per cell. Interestingly, the Cu mass

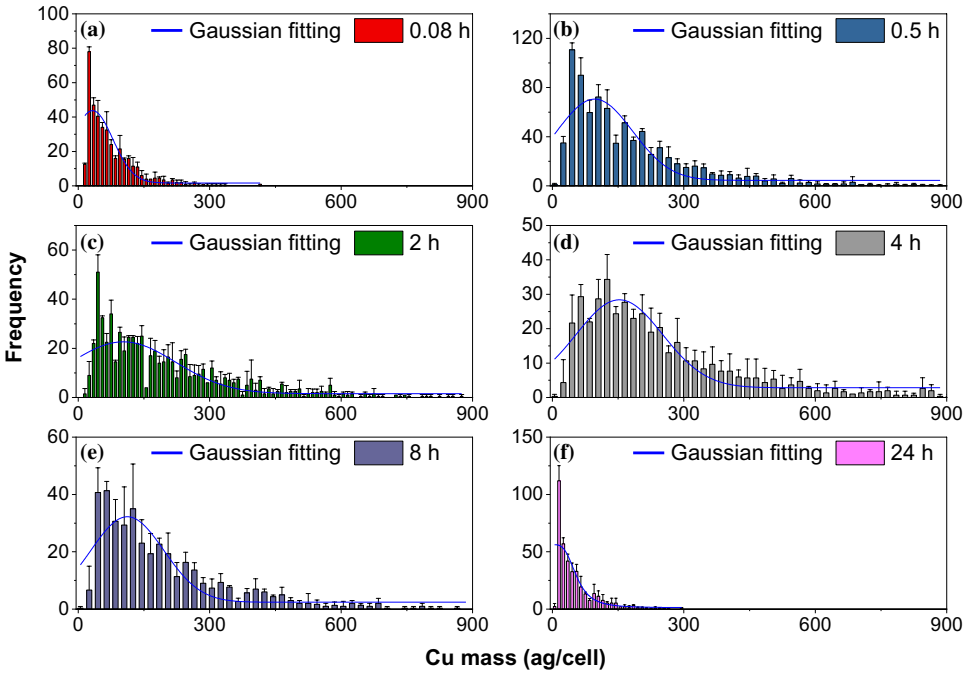

Fig. 7 Histograms of Cu mass distributions in *L. pneumophila* cell populations after treatment with 800 µg/L Cu at different contact times (**a** for 0.08 h; **b** for 0.5 h; **c** for 2 h; **d** for 4 h; **e** for 8 h; **f** for 24 h). The error bars in the SP-ICP-MS histograms represent standard deviation ($n = 3$)

distribution at 24 h is very similar with that at 0.08 h, which may be related to the potential resistance mechanism of *L. pneumophila* cells; i.e., some cells did not uptake Cu, or the cells actively expelled the Cu ions.

To investigate the Cu contact time impact on *L. pneumophila* cell, the TC concentration and average Cu mass per cell were tested by SC-ICP-MS (Fig. 8a and b). As shown in Fig. 8a, the TC concentration decreased first then increased ($p > 0.05$) slightly over time. In the first 2 h, TC concentration decreased rapidly and reached the highest disinfection rate of 62.9%. The Cu mass in individual *L. pneumophila* cell is shown in Fig. 8b. Unlike the experiments exploring the impact of different Cu concentrations where the Cu mass per cell showed a linear increase with increasing Cu dosage, herein the Cu mass per cell showed a volatile upward pattern in the first 8 h. For example, from 0.08 h to 0.5 h, the Cu mass increased by 133.3%, but it decreased by 8.6% from 0.5 h to 2 h. A similar phenomenon is also observed for the time period from 4 to 8 h. Afterwards, a sharp decline (71.7%) was observed at 24 h and Cu mass in a single cell is close to 0.08 h. This particular variation pattern, fluctuating increase then decrease over time, could be associated with the adjustment of *L. pneumophila* cells to adapt harsh environmental stressors and it may present the *L. pneumophila* cell's resistance to the Cu treatment quantitively.

CFU counting experiment was further conducted to determine the change of VC cell concentration over time and subsequently evaluate the percentage profiles of VC, VBNC, and NVL cells. The percentage of NVBI cell was ignored due to its insignificant level. The CFU counting displays a similar trend compared with the TC concentration detected by SC-ICP-MS, except at 24 h (Fig. 8c). The highest VC disinfection rate was found at 24 h. With the increasing Cu contact time, the ratio of NVL cell elevated first then decreased slightly, reaching the highest level at 2 h.

The Cu concentrations in the supernatants were measured by the conventional ICP-MS method after the treatments of cell samples (Fig. 8d). Cu concentration in the supernatant of Cu-treated water samples displayed an overall decreasing trend over time. Compared with dosed concentration of 800 µg/L, more than half of dosed Cu was lost from the supernatant after 24 h. SC-ICP-MS results also showed decreased cell concentrations due to cell death. As discussed above, the decreased Cu from the supernatant could result from the formation of Cu-associated precipitation due to the existence of NOM, chloride, etc. in real drinking water [9]. In addition, because of the electrostatic adsorption effect between Cu ion and cell membrane, part of dosed Cu might adsorb to the cell debris and then be removed during centrifugation.

The mechanism behind the relationship between Cu contact time and *L. pneumophila* cell concentration is likely multifactorial. Based on the present study, it is not true that the longer the treatment time, the more efficient the disinfection. On the contrary, longer contact times can not only stimulate the bacteria's adaptive mechanism, the VBNC state, which increases the risk of human infection, but lost more dissolved Cu due to the complicated drinking water matrix. Similar to our study, Song et al. found that Cu at a concentration of 800 µg/L inactivated *L. pneumophila* sharply after 4 h of exposure, but the antimicrobial effectiveness of Cu decreased after 6 h, with almost

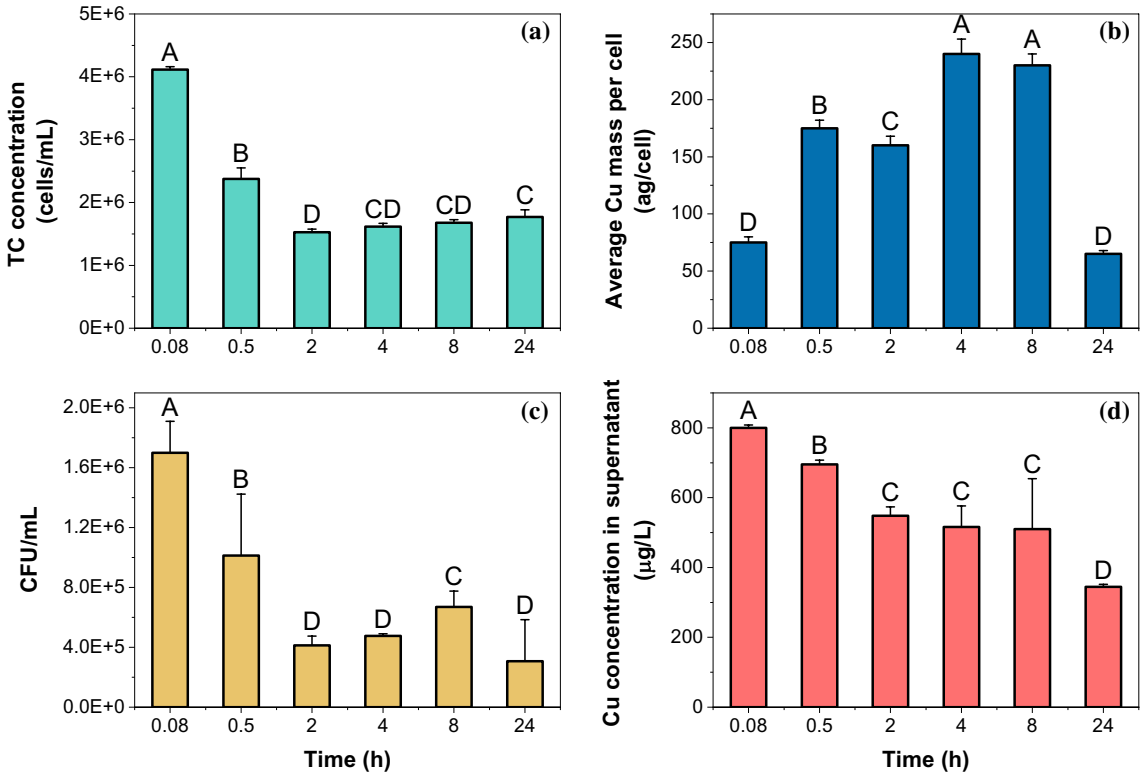

Fig. 8 Cu contact time (0.08–24 h) effect of 800 μg/L Cu treatment on *Legionella*. **a** The variation of *L. pneumophila* cell concentration; **b** Cu mass in individual *L. pneumophila* cell; **c** CFU count of *L. pneumophila* cell; **d** Cu concentration in supernatant. Data are representative of triplicate and expressed as mean ± SD. The statistical analysis was performed by IBM SPSS Statistics 27.0.1 using one-way ANOVA with Duncan's post hoc test. Different letters on the top of the bars indicate significant differences between different treatments (If no same letter between 2 groups, there is significant difference, $p < 0.05$)

the same log CFU reduction with 4 h [9]. The rebound effect of *L. pneumophila* cell concentration over time is likely due to selective pressure, whereby the surviving bacterial populations exhibit increasing resistance to Cu toxicity. Research has proposed that bacterial resistance to Cu may arise due to prolonged exposure to sublethal concentrations of Cu, which facilitate the organism's survival with minimal inhibition. Inhibition, rather than complete elimination, exerts selective pressure that promotes the emergence of a more robust and resistant bacterial population [39]. To a certain extent, *L. pneumophila* exhibits the capacity to neutralize Cu-generated ROS via the enzymatic action of superoxide dismutase. Superoxide dismutase catalyzes the conversion of superoxide radicals to hydrogen peroxide (H_2O_2), which is subsequently removed by the combined action of glutathione peroxidase-reductase and catalase, thereby effectively scavenging free radicals from the cellular environment [40, 41]. In addition, June et al. reported that there are heavy metal efflux islands located on the *L. pneumophila* genome. One specific efflux system—Cu(I)-translocating P-type ATPase CopA, encoded by the gene *lpg*1024—is suggested to be responsible for *L. pneumophila* Cu resistance [37]. The level of Cu mass in single cells in this study matches well with this report (Fig. 8b). It showed that *L. pneumophila* cells uptake Cu rapidly in the first half hour, then slow down the uptake, which may be due to the induction of this Cu efflux system. At 24 h, Cu mass per cell revised back to about the same as the 5-min contact time. This may be explained by the possibility that the cells actively expelled the Cu ions, or more likely, a portion of cells never take up the dosed Cu. This portion of cells is likely to be the VBNC *L. pneumophila* as evidenced by these experimental results at 24-h contact time.

Conclusions

The present study developed a rapid and highly sensitive SC-ICP-MS method for investigation of *L. pneumophila* treatment by Cu^{2+}. This SC-ICP-MS method can not only rapidly quantify the *L. pneumophila* cell concentration by monitoring ^{24}Mg, but also accurately detect the Cu concentration in individual cells as well as the Cu mass/cell distribution in a cell population to show the heterogeneity. The *L. pneumophila* cell concentration, average Cu mass per cell, CFU

counts, and Cu concentration in supernatant all exhibited a dose-dependent tendency, with 800 and 1200 µg/L reaching similarly high disinfection rates in drinking water. The investigation of percentages of VC, VBNC, NVBI, and NVL cells proved that Cu treatment can change VC cell to NVL cell effectively without forming NVBI cell, but Cu cannot affect the ratio of VBNC cell significantly. The contact time impact experiment demonstrated that there is no obvious proportional correlation between contact time and disinfection efficiency. The *L. pneumophila* cell possibly developed a bactericide resistant mechanism during treatment, or a portion of the *L. pneumophila* population, VBNC cells, never took up Cu during the treatment process. This novel methodology enables us to study the interaction of Cu and *L. pneumophila* quantitatively. This method is anticipated to be highly beneficial for understanding interactions between *L. pneumophila* and metal disinfectants.

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Author contribution All authors contributed to the study's conception and design. All authors commented on previous versions of the manuscript. XL conducted experiments with ICP-MS and SC-ICP-MS analyses and wrote the first draft of the manuscript, and edited the manuscript extensively. AS developed the SC-ICP-MS method and conducted SC-ICP-MS analyses, and edited the manuscript. AC performed the cell culture, CFU count, and Cu treatment experiments and edited the manuscript. LR developed the SC-ICP-MS method together with AS and edited the manuscript. JL conceived and designed research and edited the manuscript. SE supervised the project and edited the manuscript. DW supervised the cell culture and edited the manuscript. HY supervised the project and edited the manuscript. HS conceived and designed research experiments and prepared the paper together with LX. All authors read and approved the final version of the manuscript.

Data availability The data used in this study are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

This article does not contain any studies with human and/or animal participants.

Ethics approval Not applicable.

Consent to participate Not applicable.

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Disclaimer The research presented was not subject to EPA's quality system requirements. The views expressed in this article are those of the author(s) and do not necessarily represent the views or the policies of the US Environmental Protection Agency.

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