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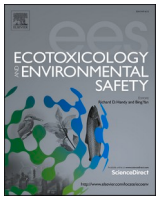

Response of *Chlorella vulgaris* to exposure to CuO NPs: Contributions of particulate and dissolved metal forms as modulated by tannic acid and pH

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ABSTRACT

A suspension of copper oxide nanoparticles (CuO NPs) is a mixture of dissolved and particulate Cu, the relative proportions of which highly depend on the water chemistry. However, the relationship between different proportions of particulate and dissolved Cu and the overall toxicity of CuO NPs is still unknown. This study investigated the response of *Chlorella vulgaris* to CuO NPs at varying solution pH and at different tannic acid (TA) additions, with a focus on exploring whether and how dissolved and particulate Cu contribute to the overall toxicity of CuO NPs. The results of the exposure experiments demonstrated the involvement of both dissolved and particulate Cu in inducing toxicity of CuO NPs, and the inhibition of CuO NPs on cell density of *Chlorella vulgaris* was found to be significantly ($p < 0.05$) alleviated with increased levels of TA and pH (< 8). Using the independent action model, the contribution to toxicity of particulate Cu was found to be enhanced with increasing pH values and TA concentrations. The toxic unit indicator better ($R^2 = 0.86$, $p < 0.001$) explained impacts of CuO NPs on micro-algae cells than commonly used mass concentrations ($R^2 = 0.27$–0.77, $p < 0.05$) across different levels of pH and TA. Overall, our study provides an additivity-based method to improve the accuracy of toxicity prediction through including contributions to toxicity of both dissolved and particulate Cu and through eliminating the uneven distribution of data due to large variations in total Cu, particulate Cu, dissolved Cu, Cu^{2+} activities, Cu-TA complexes and other Cu-complexes concentrations with varying water chemistry conditions.

1. Introduction

The use of nanoparticles (NPs) is still expanding and the global market of nanotechnology is estimated to reach $22.3 billion by 2024 (Wang et al., 2022). The growing presence of NPs in various products makes them more likely to enter the environment, while especially for metal-based NPs (MNPs) (Quigg et al., 2013) their fate and behavior are highly influenced by environmental factors such as pH and dissolved organic matter (DOM). The rationale of this study was that MNPs can dissolve in suspension, as shown in formulas (1) and (2) (Wang et al., 2016). Either lowering or increasing solution pH will affect the dissolution process. Our previous study further demonstrated that DOM can complex with metal ions in suspensions of MNPs, to subsequently promote the dissolution of MNPs at a lower concentration of DOM (Tan et al., 2021). Thereupon, MNPs suspensions constitute a mixture typically containing nano-structured particles and dissolved metals including free metal ions and metal (in)organic complexes. Their relative proportions in suspensions can be altered with varying levels of pH and DOM.

$$M_aO_b + (2b)H^+ \leftrightarrow aM^{(2b/a)+} + H_2O \quad (1)$$

$$M_aO_b + (2b)OH^- + bH_2O \leftrightarrow aM(OH)_{(4b/a)}^{(2b/a)-} \quad (2)$$

Where M_aO_b represents metal oxide NPs; $M^{(2b/a)+}$ represents the metal ions released from NPs; $M(OH)_{(4b/a)}^{(2b/a)-}$ represents the metal hydroxyl complexes.

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After a decade of research, both particulate and dissolved forms of MNPs were found to be harmful to living organisms. For instance, the cytotoxicity of Al_2O_3 NPs to algae was found to be directly driven by the ionic fraction (Pakrashi et al., 2013). Increasing numbers of studies showed the contribution of particles to suspension toxicity to be significant as well for organisms such as microbes (Zhai et al., 2016), duckweed (Song et al., 2015), zebrafish larvae (Hua et al., 2016) and lettuce (Liu et al., 2016). However, so far only very few studies investigated the relationship between the different proportions of particulate and dissolved metals in the suspension and the combined toxicity of suspensions of MNPs. Both dissolved and particulate forms of MNPs can induce oxidative stresses, impair genes or alter gene expression of organisms (Liu et al., 2020b). The exact contributions to toxicity of dissolved and particulate Cu are hard to distinguish by qualitative analysis, if only because their toxic strength may be different. If the particulate and dissolved forms act simultaneously to induce the overall toxicity of MNPs, the different toxicity of MNPs under varying environmental situations are very likely resulting from the different contributions of the two forms of MNPs (Hypothesis). Searching a method that can simultaneously reflect the toxicity contributions of particulate and dissolved forms will greatly improve the accuracy of toxicity prediction for MNPs.

Two additivity-based methods are fundamental and classic for mixture toxicity assessment: the concentration addition (CA) and independent action (IA) models. The combined effect of a mixture ($EC_{x\text{mix}}$ or $E(c_{\text{mix}})$) can be predicted by summing the scaled exposure levels causing effects of individual components (for CA, see formula (3)) or multiplying the responses induced by each component in a mixture (for IA, see formula (4)) without considering interactions (Liu et al., 2017). Based on our previous studies with plants (Liu et al., 2016), dissolved Cu did not interact with particulate Cu, which fits in the core assumption of CA and IA models. If the overall effect of a suspension of MNPs and the toxic effect of dissolved metals are known, the toxic effect of particulate forms in the MNPs suspension can be predicted using formula (4).

$$EC_{x\text{mix}}=\sum c_i/EC_{xi} \tag{3}$$

$$E(c_{\text{mix}})=1-\prod(1-E(c_i)) \tag{4}$$

Where EC_{xi} is the concentration of the i_{th} component provoking x % effect solely; c_i is the concentration of the i_{th} component in the mixture; $E(c_i)$ is the toxic effect on the test organism caused by component i in the mixture. The toxic unit (TU) is a well-known indicator for the toxic strength of a given chemical and was established on the basis of the CA concept (Liu et al., 2017). The sum of TUs (i.e., TU_{mix}, see formula (5)) can represent the toxic strength of a mixture, namely the toxicological effects of MNPs suspensions in the present study.

$$\begin{aligned}\text{TU}_{\text{mix}}&=\sum \text{TU}_i\\&=\sum C_i/EC_{50i}\\&=C_{\text{dissolved}}/EC_{50,\ \text{dissolved}}+C_{\text{particulate}}/EC_{50,\ \text{particulate}}\end{aligned} \tag{5}$$

Where $C_{\text{dissolved}}$ and $C_{\text{particulate}}$ respectively represent the dissolved metal and the particulate metal concentration in the MNPs suspension; $EC_{50,\ \text{dissolved}}$ represents the dissolved metal concentration at the 50% response level, which is a modeling parameter obtained based on dose-response curves; $EC_{50,\ \text{particulate}}$ represents the particulate metal concentration at the 50% response level, which is also a parameter obtained from fitting the dose-response curves.

As one of the most widely used additives in products including antimicrobial preparations, catalysts, semiconductors and heat transfer fluids (Wu et al., 2020), copper oxide nanoparticles (CuO NPs) were selected as an example of MNPs in this study. Owing to a short life cycle and fast metabolism (Saxena et al., 2021), *Chlorella vulgaris*, a globally present micro-green alga that plays an essential role in maintaining ecosystems health through producing organics and oxygen (Pakrashi et al., 2013), was used as an example of test organisms. Low molecular weight DOM is less prone to homo-aggregation (Pakrashi et al., 2013; Liu et al., 2020a) as compared with high molecular weight DOM such as humic substances. Tannic acid (TA) as a low-molecular weight organic acid is likely to interact with MNPs and was therefore selected as a DOM surrogate. To demonstrate the hypothesis that contributions of dissolved and particulate Cu to the toxicity of MNPs suspensions would be altered with varying environmental factors, this study was thus designed to quantify the changes in growth of *Chlorella vulgaris* as induced by CuO NPs across different levels of pH and TA by means of additivity-based methods. Specifically, the objectives of this study were: 1) to quantify the relative contributions of dissolved and particulate forms in suspensions to the overall toxicity of CuO NPs on micro-algae using the IA model; 2) to predict the combined toxicity of CuO NPs to micro-algae using the TU_{mix} indicator, and to compare its performance with the performance of commonly used mass concentrations of Cu species such as dissolved Cu, particulate Cu, total Cu, Cu^{2+} activities, Cu-TA complexes, and other Cu-complexes.

2. Materials and methods

2.1. Algal culture

The micro-algae *Chlorella vulgaris*, a single celled organism with advantages of quick reproduction and easy culturable (Shin et al., 2020) was selected as the test organism in the present study. *Chlorella vulgaris* was originally obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Stock cultures were kept in sterilized BG-11 medium (pH=7.1 ± 0.1, Table S1) and placed in a climate chamber at 24 ± 1 ℃ under a 12: 12 h light-dark photoperiod with a light intensity of 2000 lx provided by cool-white light fluorescent lamps. The algae suspension was gently shaken manually 6 times a day (US EPA, 2012). After that, 3 mL of algae suspension was inoculated into a 250 mL conical flask with 150 mL BG-11 medium to determine the micro-algae growth curve. Cell count was conducted every 4 or 12 h using a hemocytometer (Ratomski and Hawrot-Paw, 2021). under a UB200i optical microscope (Chongqing Optoelectronics Technology Co., China). The *Chlorella vulgaris* cells obtained a logarithmic growth phase after 85 h (duration: 85 h~148 h, Fig. S1). The 96 h read out was selected as the inoculation time point with an initial cell concentration of 8.5×10^5 cells mL^{-1} for the follow-up CuO NPs exposure tests. The morphology of the algal cells before and after exposure was observed using a Regulus 8100 Scanning Electron Microscopy (SEM, Hitachi, Japan).

2.2. Test chemicals and characterization

Uncoated CuO NPs (nominal size 40 nm, purity > 99.5%) were purchased from the Aladdin Industrial Corporation (Shanghai, China). Tannic acid (TA, purity > 99.8%) is a natural polyphenolic compound commonly found in natural environmental media, and was purchased from Tianjin Fengchuan Chemical Reagent Technology Corporation (Tianjin, China). The size and morphology of the CuO NPs in the culture medium were determined using a Tecnai G2 TF30 Transmission Electron Microscope (TEM, FEI Company, Netherlands). The particle size of the CuO NPs was analyzed using a Nano Measurer 1.2 (Fudan University, China). The hydrodynamic diameter and zeta potential of the CuO NPs in the culture medium were characterized using Dynamic Light Scattering (DLS) on a ZetaPALS Instrument (Brookhaven, US). The actual total concentrations of Cu in the CuO NPs suspension or in the solution of $Cu(NO_3)_2$ under different treatments were quantified using a Z2000 Flame Atomic Absorption Spectrometer (Hitachi, Japan). To quantify the percentage of dissolution of the CuO NPs at various levels of pH and TA, the suspension was centrifuged at 3000 *g* for 30 min to obtain the supernatant. The supernatant was then filtered through a 0.45 μm membrane and acidified with 65% HNO_3. The concentration of Cu in the supernatant was then determined by FAAS and regarded as the dissolved CuO NPs. The amount of particulate Cu was equal to the actual total

amount of Cu in the suspension minus the amount of Cu in the supernatant.

2.3. Algal bioassays

To study the influence of solution pH and the presence of TA on the toxicity of CuO NPs to *Chlorella vulgaris* and to quantify the contributions of dissolved and particulate forms to the overall toxicity of CuO NPs, three experiments were set up as follows:

(1) Assessment of the effects of solution pH on the toxicity of CuO NPs (pH-CuO NPs). Based on a preliminary range finding test, different amounts of CuO NPs were weighted and then added in the algal suspensions to reach nominal concentrations of 0, 25, 50, 100 and 150 mg L^{-1}. Because *Chlorella vulgaris* cells can tolerate the pH range of 3–11.5 (Mayo and Noike, 1994), and the pH value of freshwater lakes ranges from 4 to 8.5 (Alvo, 2009). To identify the influence of a pH change on toxicity of CuO NPs to *Chlorella vulgaris*, the pH values of algal suspensions at each nominal concentration of CuO NPs were adjusted to 4, 5, 6, 7.1 or 8 ± 0.2 respectively using either NaOH or HCl (0.1 M) every 24 h.
(2) Assessment of the effects of TA on the toxicity of CuO NPs at fixed pH (TA-CuO NPs). TA was first dissolved in the culture medium and diluted to concentrations of 0, 5, 10, 25 and 50 mg L^{-1} in the algal suspensions. Different amounts of CuO NPs were then added in the spiked TA suspensions to reach the same nominal concentrations as shown in experiment (1). The suspension pH values were fixed at 7.1 ± 0.2 using either NaOH or HCl (0.1 M) as well.
(3) Assessment of the effects of solution pH and TA on the toxicity of $Cu(NO_3)_2$ (pH-$Cu(NO_3)_2$ and TA- $Cu(NO_3)_2$). To mimic the influence of dissolved Cu in CuO NPs suspensions on micro-algae, different amounts of $Cu(NO_3)_2$ were weighted and then added in the algal suspensions or in the spiked TA suspensions to reach the nominal concentrations of 0, 0.5, 1, 5 and 10 mg L^{-1}. The adjustment of pH and TA in the system was the same as the above two experiments.

The matching of chemical stress and environmental stress in each treatment was completed using an orthogonal experimental design (see Table S2). All treatments were conducted in triplicate and under the culture conditions shown in Section 2.1. After 2, 24, 48, 72 and 96 h of exposure, the cell density was determined using a hemocytometer. Considering the potential impacts of varying pHs and TAs on *Chlorella vulgaris* growth, the control groups without CuO NPs were also tested by only adjusting pH value or TA concentration of the algal suspensions. As shown in Fig. S1 B and C, the growth of *Chlorella vulgaris* was the best at pH= 4, whereas the increase in TA concentration inhibited the growth of the micro-algae cells. A higher growth rate at pH= 4 may indicate a stress-induced proliferation or hormesis (Liu et al., 2020b), while a slower growth rate at higher concentrations of TA may be attributed to the detrimental effects of TA at the cellular level (Aguilera et al., 2016).

2.4. Metal speciation analysis

It is well known that Cu^{2+} can complex with not only different types of DOM such as TA, ferric ammonium citrate, and EDTA but also with different inorganic ligands such as OH^- and HCO_3^- (Tan et al., 2021). Varying pH may change the number of binding sites or the magnitude of binding affinities through affecting the degree of ionization of the above ligands in the solution (Zhao et al., 2017), while the added TA may compete with other ligands for binding with Cu^{2+} (Wang et al., 2015). Thereupon, in addition to CuO NPs dissolution, the relative proportions of Cu^{2+}, Cu-TA complexes and other Cu-complexes in suspensions may also vary a lot across different levels of pH and TA (Yadav et al., 2022). The concentrations of Cu-TA complex, other Cu-complex and total Cu-complex were all calculated based on the binding curves at various levels of pH and TA (Fig. S3) which were generated using the complex titration method shown in our previous study (Zhao et al., 2017). The Cu^{2+} activities in the system were measured using a CUO1503 Cu-ion selective electrode at equilibrium (Van London-pHoenix, US). The Cu concentrations in the supernatant and in the suspension measured using FAAS were regarded as dissolved Cu and total Cu respectively. To identify a better indicator for describing effects of CuO NPs across various levels of pH and TA, the micro-algae responses versus the commonly used mass concentrations of Cu species (the measured dissolved Cu, the calculated particulate Cu, the measured total Cu, the measured Cu^{2+} activity, the calculated Cu-TA complexes, the measured other Cu-complexes) and the TU_{mix} parameter were all fitted using a logistic non-linear regression model.

2.5. Data analysis

As shown in Section 2.3, the solution pH and presence of TA did affect the growth of *Chlorella vulgaris*. To investigate the influences of pH and TA on the toxicity of CuO NPs or $Cu(NO_3)_2$ towards *Chlorella vulgaris*, the following formula (6) (Zhao et al., 2016) was used to remove the effect of pH or TA itself on the growth of *Chlorella vulgaris* cells.

$$Inhibition\ rate = (D_c\text{-}D_t)/D_c \times 100\% \quad (6)$$

Where D_c and D_t respectively represent the cell density of control groups without CuO NPs (only adjusting pH value or TA concentration of the algal suspensions) and of treatment groups with CuO NPs at different sampling time i.e., 2, 24, 48, 72 and 96 h, cell mL^{-1}.

The logistic non-linear regression model was used to fit the effects of CuO NPs or $Cu(NO_3)_2$ on *Chlorella vulgaris*. Based on the established dose-response curves for $Cu(NO_3)_2$ (Figs. S4A and B), the biological effects induced by dissolved Cu in the CuO NPs suspension can be predicted when the dissolved concentration of Cu is known. The Cu concentrations in the CuO NPs suspension or in the $Cu(NO_3)_2$ solution, which inhibited 50% of the cell density (EC_{50}) or induced 50% of inhibition rate can be calculated as well. The actual total concentrations of Cu in the system were used in the above calculations. With the known effects of CuO NPs and dissolved Cu, the IA model (formula (4)) was used to quantify the contribution of particulate Cu to the overall toxicity of CuO NPs. The dose-response curve can be then established for particulate Cu in the CuO NPs suspension and the $EC_{50,\ particulate}$ value was calculated (Figs. S4C and D). The Origin 8.0 software (Origin Lab, USA) was used to process the data and for drawing. Statistical analysis was conducted using the one-way analysis of variance to compare the influence from various levels of pH or TA on the dissolution and on the effects of CuO NPs.

3. Results and discussion

3.1. Fate of CuO NPs in the culture medium

The TEM images revealed that the pristine CuO NPs (nominal size 40 nm) were spherical and present as aggregates in the culture medium for the micro-algae (Fig. 1). This is likely caused by Brownian motion, Van Der Waals forces and hydrogen bond formation among particles in the solution (Ghosh et al., 2011). The higher the solution pH, the darker the particle aggregates (Fig. 1A-E). At pH= 8, the averaged particle diameter equaled 481 ± 18 nm ($n = 10$) which was almost 9 times bigger than the average particle diameter measured at pH = 4 (54 ± 8 nm, $n = 10$), indicating a greater degree of aggregation of CuO NPs at a higher pH value. Similarly, the aggregates of CuO NPs were observed to be enlarged by the added TA (Figs. 1F to 1H). Especially at TA= 50 mg L^{-1} (Fig. 1I), the crystal morphology of CuO NPs could not be distinguished.

DLS analysis was conducted to further demonstrate the influence of

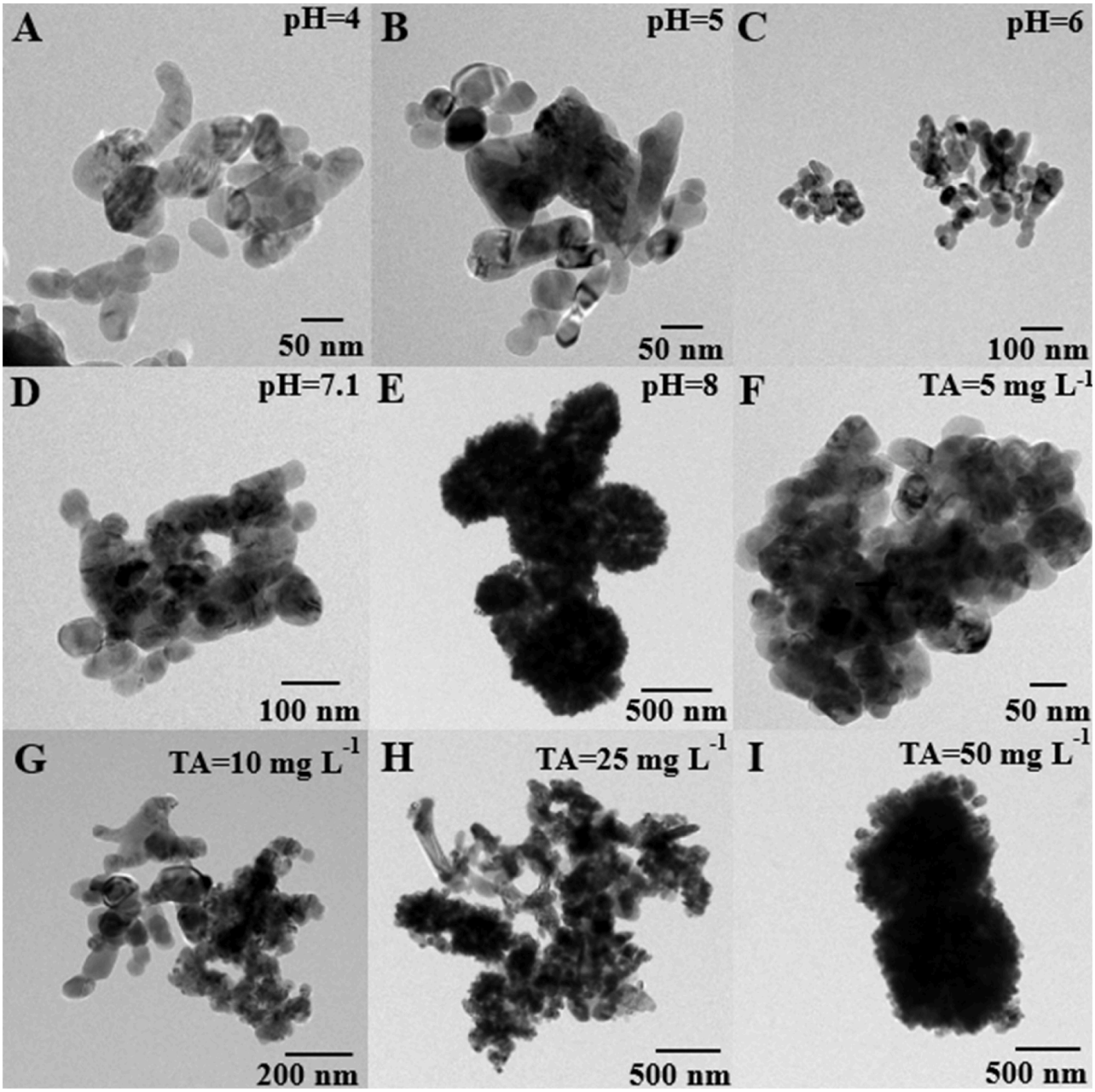

Fig. 1. The transmission electron microscope (TEM) images of CuO NPs (18.85 mg L^{-1}) prepared in micro-algae culture medium at various levels of pH and TA. The scale bars indicate the size (nm).

pH and TA on the dispersion or aggregation of CuO NPs. As pH of the suspension increased, the averaged hydrodynamic diameter obviously increased (Fig. 2A) and the absolute value of the zeta potential of the CuO NPs suspension greatly declined (Fig. 2C). At pH = 4, the zeta potentials ranged from − 11 to − 29 ± 1 mV and these values were the highest amongst all the pH-adjusted treatments. The greater the electronegativity of the CuO NPs surface, the greater the electrostatic repulsion between particles (Liu et al., 2020a), making them difficult to aggregate or form larger hydrodynamic diameter. For TA-spiked treatments, although the addition of TA (50 mg L^{-1}) significantly decreased the zeta potential of the suspended CuO NPs to even − 33 ± 2 mV (Fig. 2D), the particle hydrodynamic diameter (3452 ± 169 nm) was still much bigger than the ones without TA (1296 ± 73 nm) or with less amounts of TA (Fig. 2B). Only for total Cu= 18.85 mg L^{-1}, both hydrodynamic diameter and zeta potential were reduced by adding TA. These findings suggested that TA indeed increased the charge density of suspended CuO NPs. However, the increased electrostatic repulsion between particles due to TA adsorption cannot suppress the homo-aggregation of CuO NPs (Holsapple et al., 2005) when more particles were added in the suspension. Thereupon, lowering pH values or enhancing TA concentrations in suspensions with total Cu≤ 18.85 mg L^{-1} inhibits the aggregation of CuO NPs.

Our findings also confirmed that the proportion of dissolved Cu in the system was significantly altered by the varying conditions including solution pH, TA concentration, particle concentration, and exposure time. The lower the solution pH, the higher the percentage of dissolved Cu present in the CuO NPs suspension after 96 h of exposure (Fig. 3A). This finding confirmed that H^+ is able to significantly promote the dissolution of CuO NPs. As a comparison, the lowest percentage of dissolved Cu to total Cu in the suspension was at pH = 8. Bian et al. (2011) explained that copper hydroxide formed on the surface of CuO NPs at higher pH can inhibit further dissolution of CuO NPs, which resulted in

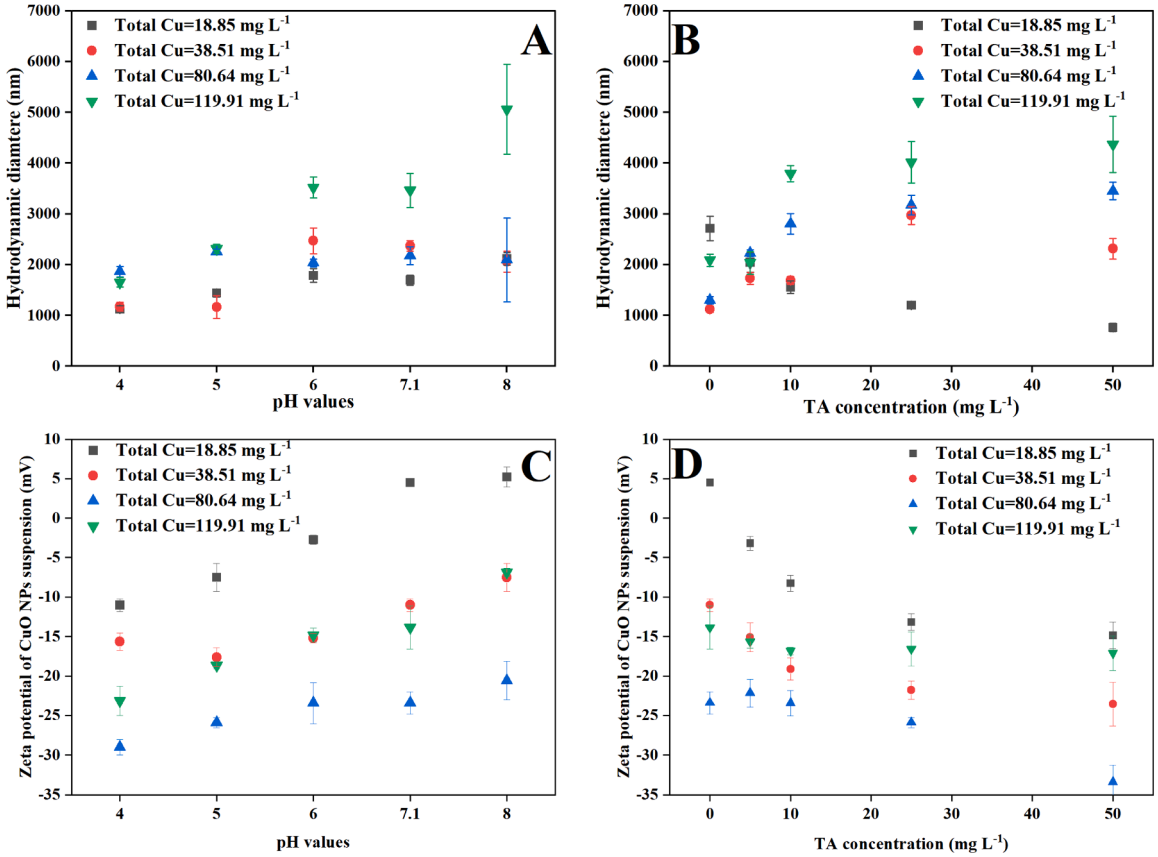

Fig. 2. The hydrodynamic diameter (A and B) and zeta potential (C and D) of CuO NPs suspensions without micro-algae cells at various pH values and TA concentrations, as measured using the dynamic light scatting (DLS) method. Data are presented as mean ± standard error of the mean ($n = 3$).

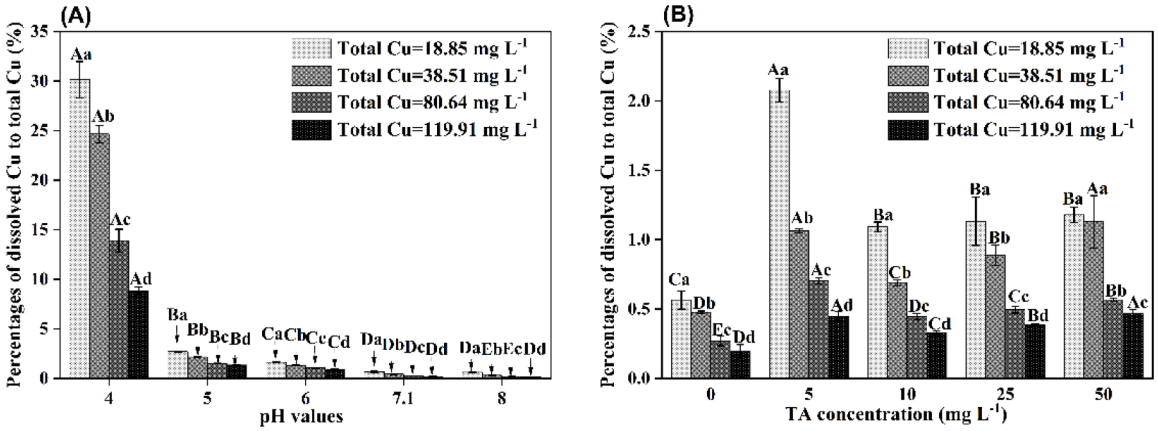

Fig. 3. Percentages of dissolved Cuin CuO NPs suspensions after 96 h exposure at various levels of pH (A) and TA (B, pH≈7.1). Capital letters represent the comparison between averaged dissolution percentages at the same concentration of total Cu namely intergroup comparison. Lower letters represent the comparison between averaged dissolution percentages at the same value of pH or TA namely intragroup comparison. Different letters indicate significant differences found using the One-way ANOVA test ($p < 0.05$). Total Cu indicates the measured total concentrations of Cu in CuO NPs suspension under different treatments. Data are presented as mean ± standard error of the mean ($n = 3$).

less dissolved Cu measured in the supernatant. For TA treatments, the presence of TA indeed increased the extent of dissolution of the CuO NPs, while this promoting effect did not monotonically increase with the increase of TA concentration. Although the percentages of dissolved Cu in the presence of TA were all higher than in case of absence of TA, the highest dissolution percentage of CuO NPs occurred at TA = 5 mg L^{-1} (Fig. 3B). This may be because as the TA concentration increased, the adsorption of TA on the surface of CuO NPs also increased significantly (Tan et al., 2021). The increased adsorption of TA on the particle surface would reduce the contact area between solute and solution which is inversely proportional to the dissolution rate according to the Noyes-Whitney equation (Borm et al., 2006), thereby inhibiting further dissolution of CuO NPs. When inspecting Figs. 1A and 1B, we also found that a low pH had a greater impact on the dissolution of CuO NPs as compared to increasing TA concentration. In addition, the total amount of Cu dissolved from CuO NPs generally increased with increasing

contact time and particle concentration (Fig. S2), while the percentages of dissolved Cu became less when adding more CuO NPs in the system across different levels of pH and TA (Fig. 3). Xiao et al. (2016) explained that particles were more likely to collide or aggregate at higher concentrations of NPs, which was also observed in the present study as shown above. Aggregation of NPs in solution was very likely to reduce the effective area of CuO NPs exposed to the culture medium and lead to a thicker diffusion layer on the CuO NPs surface, thus preventing further dissolution of CuO NPs.

3.2. Response of *Chlorella vulgaris* to CuO NPs suspension or to $Cu(NO_3)_2$ solution at different pH or TA concentrations

In general, the 96 h EC_{50} values of CuO NPs suspensions were all significantly lower than the 2 h or 24 h EC_{50} values (Fig. 4A and B). This indicates that the toxicity of CuO NPs suspensions increased gradually during 96 h of exposure. Xiao et al. (2016) ascribed the increased toxicity over time to the increased amount of Cu ions in the exposure medium. However, a significant increase in dissolution of CuO NPs over time was only observed at pH = 4, 5, or 6 in our study (Fig. S2). Baas et al. (2010) proposed that toxicity is a process in time, and an organism dies or the probability to die increases when its internal concentration exceeds a certain threshold. The increasing intake and accumulation of dissolved Cu and particulate Cu in CuO NPs suspensions may be the reason for a strict decrease in EC50 over time in the present study.

For metals or MNPs, pH is always the most important environmental factor affecting their fate and toxicity in the environment (Waalewijn-Kool et al., 2013). Fig. 4A and Table S3 showed that the 2 h and 72 h EC_{50} values of CuO NPs significantly increased with increasing solution pH. As a comparison, the 2 h, 24 h, 72 h and 96 h EC_{50} values of $Cu(NO_3)_2$ were all significantly enhanced by the increasing pH values, indicating that the toxicity of dissolved Cu towards *Chlorella vulgaris* decreased with increasing pH values in the exposure medium. An increase in pH undoubtedly increases the concentration of hydroxyl complexes in the solution. This finding was consistent with the previous study that the toxicity of $Cu(OH)^+$ is lower than that of Cu^{2+} (de Schamphelaere and Janssen, 2002). However, the above findings concerning dissolved Cu seemed to be not suitable to explain why the 24 h, 48 h and 96 h EC_{50} values of CuO NPs were not proportional to the solution pH value. Although the amount of Cu dissolved at pH = 8 was the smallest as compared to other pH values (Fig. S2), the 24 h, 48 h and 96 h EC_{50} values of CuO NPs decreased at pH = 8 (Fig. 4A). This phenomenon suggested that the toxicity of CuO NPs to *Chlorella vulgaris* can be statistically significantly alleviated by the increased pH values when pH < 8. Comparing Fig. 5B or C with 5D and 5E, the phenomenon of cell rupture caused by strong lipid peroxidation (Wu et al., 2023) disappeared as pH increased. At pH = 8, the absolute values of zeta potential of CuO NPs were obviously smaller than those at pH = 4–6 (Fig. 2C). This leads to a smaller electrostatic repulsion between negatively charged cells (Lin et al., 2012) and CuO NPs, and an increased surface coverage of CuO NPs that is likely to inhibit cell photosynthesis (Liu et al., 2020b). This may be also the reason for a relatively rough surface of the micro-algae cells at pH = 8 (Fig. 5F) compared to the cell surface of control group (Fig. 5A).

For TA-spiked treatments, the 2 h, 24 h, 48 h, 72 h and 96 h EC_{50} values of CuO NPs all significantly increased with increasing concentrations of TA (Fig. 4B and Table S3), indicating the toxicity of CuO NPs towards *Chlorella vulgaris* decreased with increasing TA concentration in

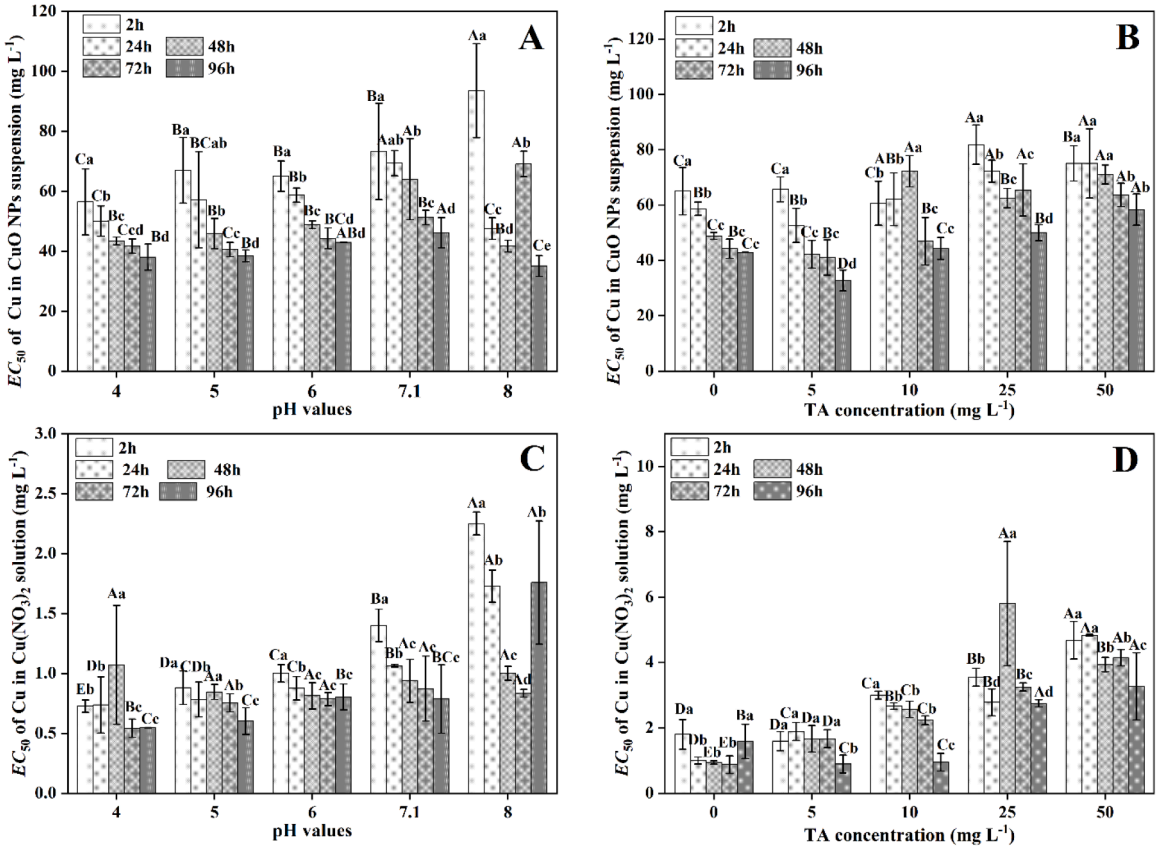

Fig. 4. The EC_{50} values of Cu in the CuO NPs suspension (A and B) or in the $Cu(NO_3)_2$ solution (C and D) at different levels of pH (4, 5, 6, 7.1, and 8) or TA (0, 5, 10, 25 and 50 mg L^{-1}, pH = 7.1). The EC_{50} value indicates the total Cu concentration in the CuO NPs suspension or in the $Cu(NO_3)_2$ solution which inhibited 50% of the cell density. Capital letters represent the comparison of averaged EC_{50} values during the same exposure period across different values of pH and TA. Lower letters represent the comparison of averaged EC_{50} values at the same value of pH or TA across different exposure periods. Different letters indicate significant differences found using the One-way ANOVA test ($p < 0.05$). Data are presented as mean ± standard error of the mean ($n = 3$).

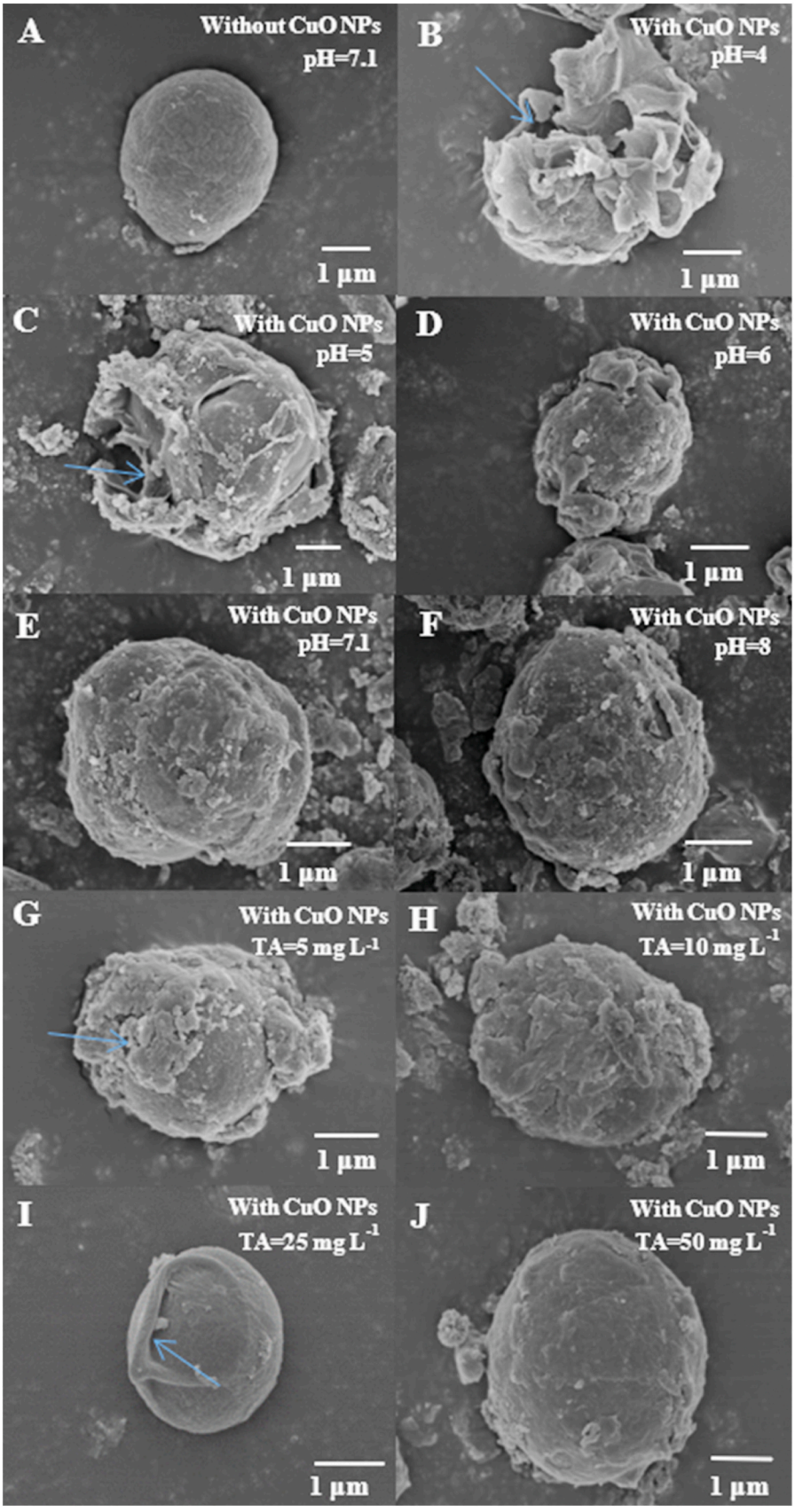

Fig. 5. Scanning electron microscope (SEM) images of *Chlorella vulgaris* cells exposed to CuO NPs (18.85 mg L^{-1}) after 96 h at various levels of pH or TA. Blue arrows indicate that the cells have been damaged to different degrees. Scale bars indicate size (μm).

the suspension. The TEM images also showed that the surface of algal cells became smoother when the TA concentration was high (Figs. 5I and 5J). This trend was observed for $Cu(NO_3)_2$ (Fig. 4C) as well, which may be because the complexation of Cu^{2+} with TA decreased the overall toxicity of $Cu(NO_3)_2$ through reducing the proportion of highly toxic Cu^{2+} in suspension. However, the above similar trends did not necessarily mean that the overall toxicity of CuO NPs was all attributed to the dissolved Cu in the presence of TA. Li et al. (2022) proposed that natural organic matter adsorbed on NPs can increase the electrostatic repulsion and decrease the direct contact of NPs with micro-algae cells. In addition, the 96 h EC_{50} value of Cu in CuO NPs suspensions at TA= 10 mg L^{-1} (44 ± 4 mg L^{-1}) was found to be more than 40 times bigger than the 96 h EC_{50} value in $Cu(NO_3)_2$ solution (1 ± 0.3 mg L^{-1}) when comparing Figs. 4B and 4D. According to Fig. 3B, when the mass concentration of Cu in CuO NPs suspensions > 38.51 mg L^{-1}, the percentage of dissolved Cu was smaller than 0.33 ± 0.01%. This meant that the concentration of dissolved Cu was only around 0.15 mg L^{-1} in CuO NPs suspensions with 44 ± 4 mg L^{-1} of total Cu which was too low to induce 50% inhibition of cell density. Thereupon, the particulate Cu should participate in inducing the toxicity of CuO NPs to *Chlorella vulgaris* at different levels of pH and TA.

3.3. Relationship between Cu species and responses of Chlorella vulgaris at different pH or TA levels

As discussed in Section 3.2, in addition to the dissolved form, the particulate form in CuO NPs suspensions also took part in inducing toxic effects of CuO NPs on *Chlorella vulgaris* cells. The IA model was thus used to further quantify the relative contributions of particulate Cu and dissolved Cu to the toxicity of CuO NPs at various levels of TA and pH. For pH-adjusted treatments, the contributions of particles and dissolved Cu were similar at pH = 4 and 5, while the contribution of dissolved Cu significantly decreased by 9–16% when solution pH started to rise from 6 to 8 (Fig. 6A). This finding can be mechanistically attributed to the strong reaction between H^+ and CuO NPs which intensified the dissolution of CuO NPs at pH = 4 and 5, while the promoting effect on particle dissolution decreased as the solution pH increased (Fig. 3A) and the $E_{dissolved}$ value decreased accordingly. Consequently, less than 5% of the percentage of dissolved Cu to total Cu (Fig. 3A) accounted for 62% of the overall toxicity of CuO NPs at pH = 5 (Fig. 6A). The observation that the toxicity of dissolved Cu is greater than the toxicity of particulate Cu was consistent with previous findings (Zhai et al., 2016). For TA-spiked treatments, the contribution to toxicity of dissolved Cu was significantly reduced by 34% when the TA concentration was 10 times enlarged (Fig. 6B). According to formula (4), there may be two reasons to explain why TA at a high concentration significantly weakened the contributions to toxicity of dissolved Cu. 1) the promoting effect of TA = 50 mg L^{-1} on the dissolution of CuO NPs was weaker as compared to the effect of TA = 5 mg L^{-1} (see Section 3.1), which reduced the content proportion and $E_{dissolved}$ of dissolved Cu in suspension. 2) the addition of TA significantly reduced the overall toxicity of CuO NPs suspensions (see Section 3.2) namely E_{mix} in formula (4). Thereupon, our hypothesis can be accepted that contributions of dissolved and particulate Cu to the toxicity of CuO NPs suspensions would be altered with varying solution pHs and TA concentrations.

The above discussion proved that both particulate and dissolved Cu in CuO NPs suspensions contributed to the toxicity of CuO NPs to *Chlorella vulgaris*. Therefore, we attempted to use TU, a mixture indicator that can include the toxic strengths of both particulate Cu and dissolved Cu, to optimize the prediction of CuO NPs toxicity. The inhibition rates of cell density in the CuO NPs suspensions were plotted as a function of different Cu species and the TU_{mix} parameter at varying levels of pH and TA (Fig. 7). Their predictive performance results followed the order that TU_{mix} (R^2 = 0.86, $p < 0.05$) > total Cu = particulate Cu (R^2 = 0.77, $p < 0.05$) > dissolved Cu (R^2 = 0.72, $p < 0.05$) > other-Cu complexes = free Cu^{2+} activities (R^2 = 0.62, $p < 0.05$) > Cu-TA complexes (R^2 = 0.27, $p < 0.05$). This order indicated that the simple application of mass concentrations such as Cu-TA complexes (Fig. 7E) and free Cu^{2+} activities (Fig. 7D) did not give reasonable predictions in estimating the impacts of CuO NPs on microalgae. This may be because the concentrations of these Cu species in suspensions are highly dependent on the water chemistry, which caused significant non-uniformity in the data distribution. For example, the lower the pH value of the solution, the lower the complexation ability of Cu^{2+} with ligands in solution (Fig. S3A), thus increasing the free Cu^{2+} activities in solution. Especially at pH = 4, the free Cu^{2+} activities were much higher than at other pHs (Fig. 7D), resulting in a big deviation of the actual *inhibition rate* from the values predicted using the logistic nonlinear regression model. Similarly, the complexation stability constants at TA = 25 and 50 mg L^{-1} (log K = 2.43 and 2.58, Fig. S3C) were also much bigger than those at lower TA concentrations (log K = 0.54 and 1.23). This led to much higher concentrations of Cu-TA complexes at TA = 25 and 50 mg L^{-1} as compared to those at lower concentrations of TA, and also big deviations of modeling results shown in Fig. 7E. Moreover, according to formula (1), the increased Cu-complexes would reduce the activities of Cu^{2+} in suspensions, driving further dissolution of CuO NPs. Because of the above chain of reactions, it is not possible to predict the toxicity of CuO NPs using only one Cu species. By including the concentrations of both particulate and dissolved Cu, the prediction was improved using total Cu concentration as an indicator of toxicity

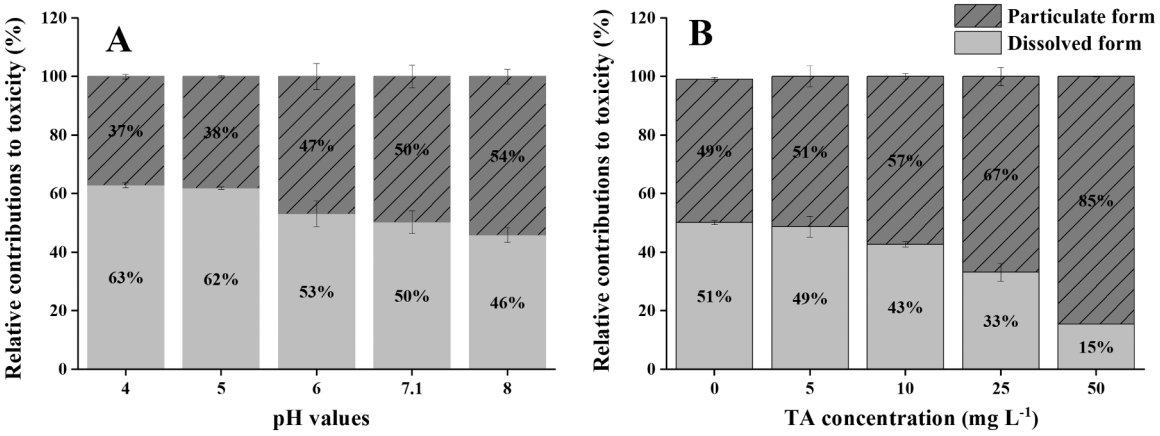

Fig. 6. The relative contributions of particulate Cu and dissolved Cu to toxicity (i.e., inhibition rates) caused by CuO NPs across various levels of pH (A) and TA (B) at the EC_{50} values towards *Chlorella vulgaris*. The effects caused by particulate forms of CuO NPs were calculated using the independent action (IA) model (Formula (4)). The dose-response curves were shown in Fig. S4. Data are presented as mean ± standard error of the mean (n = 3).

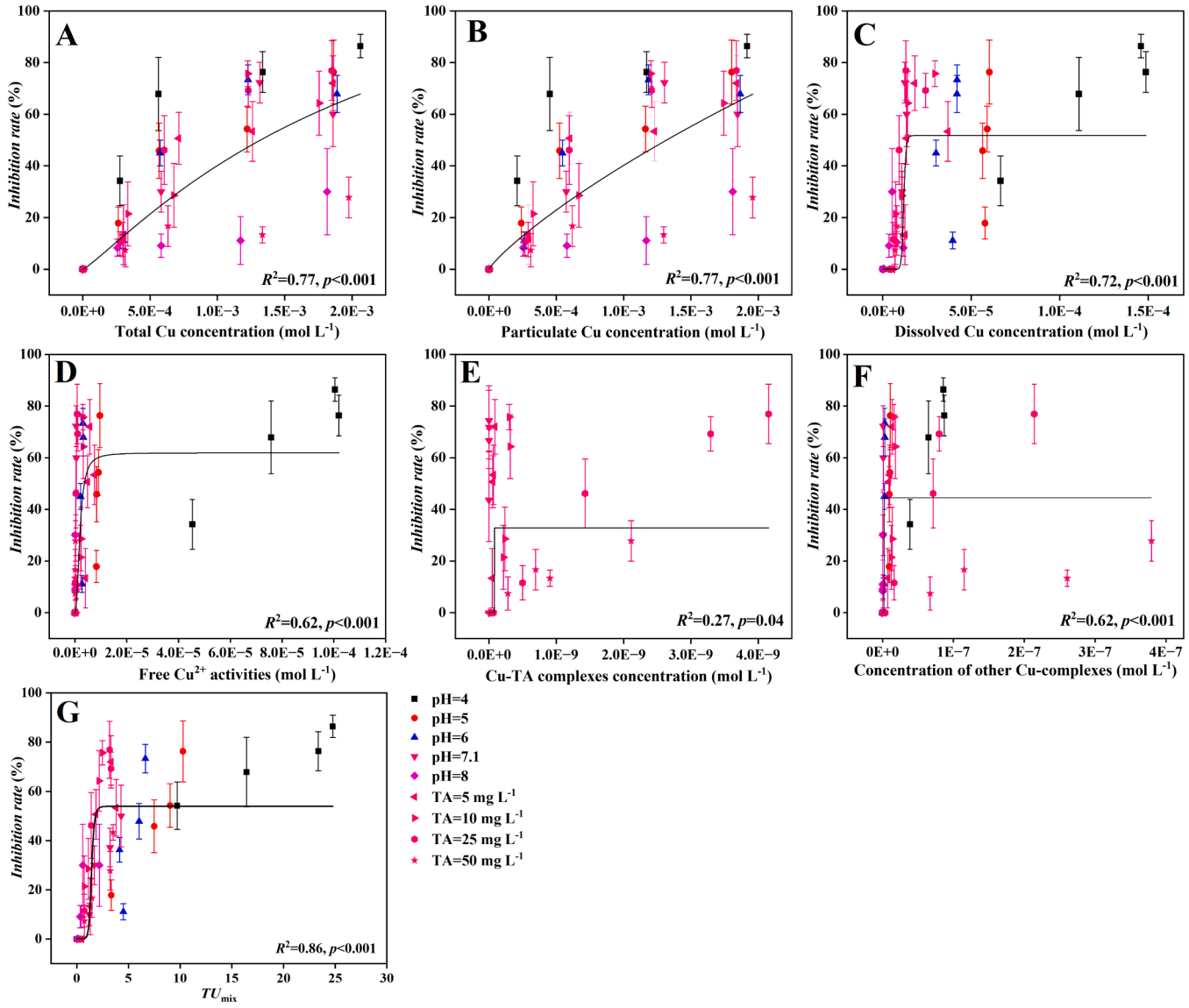

Fig. 7. Relationships between the concentrations of various Cu species or TU_{mix} and the inhibition rate of CuO NPs on cell density across various levels of pH and TA after 96 h exposure. Solid lines represent the statistically significant fits at the level of $p < 0.05$ using the logistic non-linear regression model. The concentration of other Cu-complexes indicates the amount of Cu^{2+} complexed with anionic ligands in solution other than TA. R^2 indicates the determination coefficient adjusted for the degrees of freedom. Data are presented as mean ± standard error of the mean ($n = 3$).

(Fig. 7A) but was still lower than the TU_{mix} prediction (Fig. 7G). As mentioned above, the toxicity of dissolved Cu was different from that of particulate Cu, and was influenced by environmental factors. By selecting TU_{mix} as the toxicity indicator, the problem of non-uniformity in data distribution can be well solved.

4. Conclusions

This study investigated whether and how the dissolved and particulate Cu in suspensions contribute to the toxic effects of CuO NPs on *Chlorella vulgaris* at various pH values and TA concentrations. By comparing the EC_{50} values of CuO NPs suspensions and of $Cu(NO_3)_2$ at different levels of pH and TA, it was confirmed that both dissolved forms and particulate forms of CuO NPs contribute to the overall toxicity to micro-algae. The inhibition of the growth of *Chlorella vulgaris* by CuO NPs was observed to be significantly reduced by increased levels of TA and by enhanced pH values at pH < 8. Using the IA model, the contribution to toxicity of particulate Cu in CuO NPs suspensions was quantified and found to be enhanced with increasing levels of pH and TA, while the contribution to toxicity of dissolved Cu changed in an opposite way. Therefore, the TU_{mix} indicator that can include the toxic strengths of both particulate and dissolved Cu was selected to assess the toxicity of CuO NPs. The predictive performance of TU_{mix} ($R^2 = 0.86$, $p < 0.001$) was superior than commonly used mass concentrations ($R^2 = 0.27$–0.77, $p < 0.05$) in assessing the overall toxicity of CuO NPs to *Chlorella vulgaris* cells. The above findings suggest that additivity-based methods can not only serve as a means to quantify the relative contributions of dissolved and particulate metals in MNPs suspensions, but also correct non-uniformity in the data distribution which is caused by varying water chemistry parameters and thus improve the toxicity prediction performance.

CRediT authorship contribution statement

Wei Zhuo: Writing – review & editing. **Pan Bo:** Writing – review & editing. **Qiu Hao:** Writing – review & editing. **Zhao Jing:** Writing – review & editing. **Wang Xia:** Data curation. **Liu Yang:** Writing – original draft, Conceptualization. **Peijnenburg Willie J.G.M.:** Writing – review

& editing. **Steinberg Christian E.W.:** Writing – review & editing. **Vijver Martina G.:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116035.

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