**Using sludge fermentation liquid to reduce the inhibitory effect of copper
oxide nanoparticles on municipal wastewater biological nutrient removal**

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ABSTRACT The deterioration of biological nutrient removal (BNR) can occur with the release of engineering nanomaterials into wastewater treatment plants (WWTPs). Also, large amounts of waste sludge are generated in WWTPs, which can be reutilized as a useful resource. In this study, the use of sludge fermentation liquid to reduce CuO nanoparticles (NPs) toxicity to municipal wastewater BNR was reported. In the BNR system supplemented with sodium acetate, which was widely used as additional carbon source of municipal wastewater in literatures, the appearance of 2.5 mg/L CuO NPs for 5.5 h decreased the total nitrogen (TN) removal efficiency from 81.4% to 59.0%, but the TN removal was recovered to 78.7% after sodium acetate was replaced by sludge fermentation liquid. It was found that CuO NPs induced excessive generation of reactive nitrogen species (RNS), which led to the disorder of redox status, low levels of energy and reduction equivalents generations, and deterioration of denitrification. Further investigation revealed that cysteine in fermentation liquid played a vital biological role in reducing nanotoxicity by facilitating the synthesis of glutathione, which reduced excessive RNS generation, increased key proteins expression, guaranteed the metabolisms of intracellular energy and substrate smoothly, and finally recovered the BNR performance.

Keywords: Sludge fermentation liquid; Nanotoxicity; Reactive nitrogen species; Redox balance; Protein expression

1. Introduction

Biological nutrient removal from wastewater is an effective approach for prevention of eutrophication of water bodies. Usually, in biological nutrient removal (BNR) process wastewater ammonium nitrogen ($\text{NH}_4^+\text{-N}$) is removed via nitrification-denitrification pathway, i.e, $\text{NH}_4^+\text{-N}$ is firstly oxidized to nitrite and nitrate under aerobic conditions, and then reduced to nitrite, nitric oxide, nitrous oxide, and finally to nitrogen gas. Phosphorus is eliminated by phosphorus accumulating organisms (PAO) via enhanced

biological phosphorus removal mechanism, which includes phosphorus anaerobic release and aerobic uptake. However, the efficiency of BNR can be affected by operational and environmental conditions, such as carbon source, sludge retention time, temperature, etc (Chen et al., 2004; Mulkerrins et al., 2004). Also, large amounts of waste sludge are produced in BNR process, which need to be treated before being discharged into the environment. One strategy for sludge management is moving towards reutilization of it as useful resources to produce valuable products, such as methane, hydrogen or short-chain fatty acids (Appels et al., 2008).

Over the past decades, engineering nanomaterials have been implemented in various applications related to biotechnology, material science and optical technology (Lee et al., 2010). The increased production and utilization of nanomaterials resulted in their releases into environments, which would cause the toxic effects on mammalian cells (Wang et al., 2012), plants (Atha et al., 2012), and model bacteria (Zhao et al., 2013). The released engineering nanomaterials can easily enter into wastewater treatment plants (WWTPs) via civil sewage systems (Kaegi et al., 2011). Thus, the influences of engineering nanomaterials on activated sludge, bacterial community structure, and nitrogen and phosphorus removals were studied recently (Lombi et al., 2012; Priester et al., 2014; Zheng et al., 2012). For example, it was found that the long-term exposure of CuO nanoparticles (CuO NPs) at low level (1.4 mg Cu/L) inhibited the methanogenesis process of wastewater in an anaerobic bioreactor (Otero-Gonzalez et al., 2014). Hou et al. (2015) confirmed that the presence of CuO NPs affected sludge properties such as flocculation, dewaterability and composition of extracellular polymeric substances, and some metal oxides nanoparticles (such as ZnO NPs and SiO₂ NPs) caused negative influence on nitrogen and phosphorus removals, (Zheng et al., 2012; Zheng et al., 2011).

For some nanomaterials their toxicity comes mainly from the released ions, and thus depends on

environment conditions, such as medium type, pH, ionic strength, which can change the stability and dissolution properties of nanomaterials (Li et al., 2013). Also, some chemicals (such as humic-like substances and those organic compounds with thiol functional groups) have been reported to combine with the released ions, which results in the decrease of nanotoxicity (Pokhrel et al., 2013). For example, Li et al. found that the presence of dissolved organic matter (DOM) lowered the toxicity of ZnO NPs to *E. coli* by reducing the concentration of free Zn^{2+} released from ZnO NPs (Li et al., 2013). Nevertheless, for other nanomaterials (such as CuO NPs), our study had shown that it was the particles instead of the released ions responsible for the toxicity (Su et al., 2015). It seems that the approaches reported in the literature for reducing the toxicity of nanomaterials by reacting with free ions may not be appropriate in the case of CuO NPs.

It is well-known that excess generation of reactive species can lead to the broken of intracellular redox balance, which affects various cellular processes, such as energy metabolism and protein synthesis (Patel et al., 1999). Nanomaterials have larger ratio of surface to volume, and thus have a high reactivity, which can easily lead to the excessive production of reactive molecular species. The exposure of nanomaterials caused the unbalance of cellular redox status, which attributed to their toxicities to various cells or microorganisms (Nel et al., 2006). For example, under aerobic conditions, the study on human cell lines suggested that the excessive generation of reactive oxygen species (ROS) induced by Ag NPs negatively affected cellular metabolism (Verano-Braga et al., 2014). In addition, both Ag NPs and ZnO NPs were observed to induce oxidative stress to bacteria and mammalian cells, which further disrupted the signal pathway (Setyawati et al., 2013b), caused apoptosis (Setyawati et al., 2014), led to plasma membrane leakage (George et al., 2009), or triggered inflammatory response (Chia et al., 2015). In denitrification process, although the generation of ROS is hardly triggered in cells due to the absence of molecular oxygen

(Apel and Hirt, 2004), the metabolic intermediate nitric oxide can be easily transformed to reactive nitrogen species (RNS), a diverse range of antimicrobial compounds (Patel et al., 1999). Apparently, the toxicity of nanomaterials to denitrification would be mitigated if the generation of RNS could be decreased.

CuO NPs are among the metal oxide nanoparticles with a wide application in wood preservation, antimicrobial textiles, and catalysts for monoxide oxidation (Gabbay et al., 2006), which, however, exhibit the most potent toxicity compared to other metal oxide nanoparticles (Karlsson et al., 2008). The global production of CuO NPs was 640 tons in 2015, and the estimated production would be 1600 tons in 2025 (Future Markets Inc., 2015). The increasing production and utilization of CuO NPs might result in their intentional or unintentional releases into the environment. It is therefore of great significance to evaluate the environmental risks of CuO NPs. It was reported in our previous study that CuO NPs could enter into denitrifiers cells and the intracellular CuO NPs interfered key protein expression, which was the major mechanism for their toxicity (Su et al., 2015). In the present study, the data showed that the presence of CuO NPs in municipal wastewater treatment system inhibited biological nutrient removal performance. The aim of this study was to find out an efficient way to reduce the inhibitory effect of CuO NPs to municipal wastewater BNR via mitigating the generation of reactive nitrogen species by the use of waste activated sludge fermentation liquid. Firstly, the influence of sludge fermentation liquid on the toxicity of CuO NPs to municipal wastewater BNR performance was studied. Then, the mechanisms for sludge fermentation liquid remarkably reducing the toxicity of CuO NPs were explored by investigating the composition of sludge fermentation liquid, the intracellular nitrosative and redox status, the metabolism of substrate and energy, and the expressions of key proteins.

2. Materials and methods

2.1. Municipal wastewater and sludge

The municipal wastewater and waste activated sludge were obtained from a WWTP in Shanghai, China. The main characteristics of wastewater are as follows: soluble chemical oxygen demand (SCOD) 170-215 mg/L, ammonia-nitrogen ($\text{NH}_4^+\text{-N}$) 19-29 mg/L, and soluble ortho-phosphorus (SOP) 2.6-3.7 mg/L. The sludge was withdrawn from the secondary sedimentation tank of the above WWTP, and then used for fermentation liquid preparation. After settling at 4 °C for 24 h the sludge had total suspended solids (TSS) 14365 ± 921 mg/L and mixed liquid volatile suspended solids (MLVSS) 10457 ± 618 mg/L.

2.2. Preparation and characterization of nanoparticles stock suspension

CuO NPs (mean diameter < 40 nm, purity > 99%) were purchased from Sigma-Aldrich (St. Louis, MO). The CuO NPs stock suspension (50 mg/L) was prepared in deionized (DI) water, and 30 min sonication (25 °C, 500 W, 40 kHz) in a bath sonicator was used to make stock suspensions homogeneous before use. For size characterization, the powder CuO NPs were ultrasonicated in alcohol and then dropped into Ni grids for transmission electron microscopy (TEM) analysis using JEM-2010 HT (JEOL, Japan) at an acceleration voltage of 200 kv. X-ray diffraction (XRD) analysis was conducted on a Bruker D8 Advance diffractometer equipped with a Cu K α radiation source. Size distribution and surface charge in DI water and wastewater were characterized using dynamic light scattering (DLS) technique on Zetasizer NanoZS (Malvern, UK), and the data were analyzed by Zetasizer Software v7.02 (Malvern, UK). The results of the characterizations were shown in Fig. S1 (Supplementary data).

2.3. Preparation and characterization of sludge fermentation liquid

Anaerobic fermentation of waste activated sludge was conducted at pH 10 and 37 °C for 8 d, which was detailed in our previous publication (Ji and Chen, 2010). The fermentation liquid was obtained by centrifugation at 4800 rpm for 30 min (see Table 1 for the main characteristics), and then stored at 4 °C for use.

The amino acid compositions in fermentation liquid were analyzed according to the reference (Nemzer et al., 2011). In brief, 5 mL of fermentation liquid was mixed with 10 mL of 10% trichloroacetic acid solution, and the mixture was settled for 1 h at 4 °C. After being centrifuged at 12000 rpm for 30 min, the supernatant was adjusted to pH 2.0 ± 0.2 , following by centrifugation again at 12000 rpm for 20 min. Finally, the supernatants were filtered through a 0.45 μ m Millipore filter and the contents of amino acids were determined by amino acid auto-analyzer model L-8900 (Hitachi High-Technologies Corporation, Japan).

2.4. Experiments of sludge fermentation liquid affecting CuO NPs toxicity to BNR

The experiments were conducted in four identical sequencing batch reactors (SBR-1, SBR-2, SBR-3 and SBR-4) with working volume of 500 mL each. Five minutes before the end of low dissolved oxygen phase, 2000 mL of biomass was taken from the parent SBR (see Supplementary data for its operation), centrifuged at 4500 rpm for 5 min to remove the supernatant, washed three times with 0.9% NaCl solution, and then resuspended in tap water with a final volume of 200 mL. The aliquot was divided equally into four SBRs, and then 450 ml of municipal wastewater supplemented respectively with sodium acetate and sludge fermentation liquid was added to SBR-1 and SBR-2, and SBR-3 and SBR-4. The influent SCOD in all reactors was 400 mg/L, and the influent NH_4^+ -N and SOP were adjusted to 35 ± 1 and 6 ± 0.5 mg/L by NH_4Cl and KH_2PO_4 . CuO NPs (stock suspension) were then added to reactors SBR-2 and SBR-4 with concentration of 2.5 mg/L in the influent. All other operations were the same as described in the section of “Operation of parent sequencing batch reactor” (Supplementary data). After the exposure for 5.5 h, the effluent was obtained at the decanting stage and the concentrations of NH_4^+ -N, SOP, NO_3^- -N, and NO_2^- -N were measured.

2.5. Experiments of main organic components in sludge fermentation liquid affecting CuO NPs

toxicity to BNR

Protein, polysaccharide, volatile fatty acids (VFAs) and amino acids were the main organic compositions in sludge fermentation liquid. To investigate the individual role on toxicity reduction, various components (including protein, polysaccharide, VFAs, and amino acids) were respectively prepared in different reactors based on the detected concentrations in wastewater containing fermentation liquid. Then, the influent SCOD was adjusted to 400 mg/L by sodium acetate. In this study dextran and bovine serum albumin (BSA) were used respectively as the model compounds of polysaccharide and protein. The synthetic wastewater was prepared according to previous publication with minor modification (Zheng et al., 2011), which contained: NH_4Cl (35 mg N/L), KH_2PO_4 (5 mg P/L), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (10 mg/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (8 mg/L) and trace element solution (1 mL/L). The composition of trace element is shown in Table S1 (Supplementary data). The experiments were conducted according to the procedure described above.

To describe the magnitude of toxicity reduction of different components, toxicity reduction rate (TRR) was introduced in this study. The TRR was defined as following:

$$TRR_x = \frac{R_{N,x} - R_{N,NPs}}{R_{N,Control} - R_{N,NPs}} \times 100\%$$

Where $R_{N,x}$ is the NO_x^- (including nitrate and nitrite) removal efficiency in the presence of CuO NPs and sludge fermentation liquid main composition x (VFAs, protein, polysaccharide, and amino acids), and $R_{N,Control}$ and $R_{N,NPs}$ are the NO_x^- removal efficiencies of the control and the CuO NPs exposure groups, respectively.

2.6. Experiments of cysteine alleviating CuO NPs toxicity towards denitrification

The strain of *Paracoccus denitrificans* (ATCC 19367), purchased from American Type Culture Collection (Manassas, VA, USA), was employed as the test denitrifying microbe. Prior to the exposure

experiment, *P. denitrificans* was cultivated aerobically in Difco nutrient broth at 30 °C in a shaker with constant agitation (200 rpm) for 24 h, and then harvested from broth media (see Supplementary data). To examine the possible effects of CuO NPs on denitrification in the presence of cysteine, *P. denitrificans* was exposed to 0.25 mg/L CuO NPs, and then 0 and 5 mg/L of cysteine were supplemented in the mineral medium (see Table S2, Supplementary data) modified from the literature (Błaszczuk, 1993). Bacterial cultures were inoculated at an initial OD₆₀₀ of 0.05, and then cultivated anaerobically in the dark at 30 °C and 200 rpm. After the exposure for 24 h, the concentrations of NO₃⁻-N and NO₂⁻-N and the growth and viability of bacteria were measured (Zheng et al., 2014).

2.7. Assays for ATP and reduced and oxide glutathione levels

To evaluate the energy level, the measurement of intracellular ATP level was performed according to the instruction of the ATP kit provided by Nanjing Jiancheng Institute of Biological Engineering (China). At the end of exposure experiment, the bacterial cells were collected and washed with 0.1 M PBS buffer for twice. Then, the ATP was extracted according to the literature (Yang et al., 2002). In detail, the suspended cells in a microcentrifuge tube were heated immediately in a boiling water bath for 10 min. After cooling in ice for 30 s, the mixture was centrifuged (12000 rpm, 4 °C for 5 min), and 30 µL of supernatant was taken and mixed with reagents in sequence. Finally, the data were obtained at 636 nm absorbance via a microplate reader (BioTek, USA). The reduced glutathione (GSH) and oxide glutathione (GSSG) levels were measured with the use of enzymatic cycling methods (Rahman et al., 2006). The rates of 2-nitro-5-thiobenzoic acid formation were calculated, and the total glutathione and GSSG concentrations in the samples were determined by using linear regression to calculate the values obtained from the standard curve. The GSH concentration was determined by subtracting the GSSG concentration from the total GSH concentration.

2.8. Determination of key proteins synthesis

The quantification of key proteins of denitrifying bacteria was conducted by multiple reaction monitoring (MRM) technique. After the exposure for 24 h, triplicated samples were obtained by centrifugation at 5000 rpm firstly and the total protein was extracted. Prepared STD buffer (containing 4% sodium dodecyl sulfate (SDS) and 150 mM Tris-HCl (pH 8.0)) were added in the bacteria pellets, and then the samples were treated by vortex mixing, heating in water bath for 5 min, and sonic disruption (80 W, 10 s operation and 15 s break for 10 cycles). Finally, the supernatant was collected by centrifugation (4 °C, 15000 rpm, 10 min) and protein concentration was determined.

The digestion of extracted proteins was performed as described in our previous publication (Su et al., 2015), and then 80 µg of peptide was used for the subsequent liquid chromatography-tandem mass spectrometry (LC-MS) analysis. The protein translation initiation factor (IF-2) was used as the internal standard. In brief, after the separation by LC, MS analysis of samples were conducted using AB SCIEX QTRAP 5500 system interfaced with the Nanospray III source and triplicate LC-MS/MS runs were performed for each sample. The parameters for MS analysis were as below: positive ion mode for 60 min, mass range of 100-1000 m/z, and mass tolerance 250 mDa. Raw quantitation data were confirmed by AB SCIEX ProteinPilot 4.5 software with the restriction of confidence > 0.95, and the peptide transition intensity was analyzed quantitatively by software Skyline 2.5.0 (University of Washington). Triplicated biological samples were used in the MRM quantification test, and the differential expressed proteins were considered to be significantly regulated compared to the control when the *p* was less than 0.05.

2.9. Other Analytical Methods

The measurements of NH_4^+ -N, SOP, NO_3^- -N, NO_2^- -N, TSS, MLVSS, SCOD, polysaccharide, soluble protein, volatile fatty acids (including acetic, propionic, isobutyric, n-butyric, isovaleric, and n-valeric acids)

were according to our previous publication (Ji and Chen, 2010). Details for the determinations of ROS, RNS, cell viability and NADH/NAD⁺ are presented in Supplementary data.

2.10. Statistical analysis

All samples were run in triplicate, and the results were expressed as mean $\pm$ standard deviation. An analysis of variance (ANOVA) was used to test the significance of results and $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Effect of sludge fermentation liquid on CuO NPs toxicity to BNR process

In the literature sodium acetate or acetic acid has been used widely as carbon source or additional carbon source of BNR (Park et al., 2010). In this study the reactor received municipal wastewater supplemented with sodium acetate in the absence of CuO NPs was set as the control. As shown in Fig. 1, the effluent concentrations of NH₄⁺-N and phosphorus were not significantly affected by CuO NPs ($p > 0.05$). In the reactor exposed to CuO NPs, the removal efficiencies of NH₄⁺-N and soluble ortho-phosphors were 98.7% and 88.7%, respectively, which were close to the data in the control test (99.2% and 89.3%). However, the effluent NO₃⁻-N and NO₂⁻-N in the CuO NPs reactor were increased respectively to 13.15 and 0.84 mg/L, which were much higher than those in the control (5.86 and 0.36 mg/L). In literatures, ammonia oxidation was also observed to be unaffected by other nanoparticles, such as SiO₂ and ZnO NPs (Zheng et al., 2012; Zheng et al., 2011). With the co-presence of CuO NPs and fermentation liquid, the effluent NO₃⁻-N and NO₂⁻-N levels declined to 6.62 and 0.48 mg/L, respectively. In this reactor, the corresponding removal efficiency of total nitrogen (NH₄⁺-N, NO₃⁻-N and NO₂⁻-N) reached 78.6%, which was much higher than that in the CuO NPs reactor (58.9%). Obviously, in wastewater biological nutrient removal process, denitrification was more susceptible to CuO NPs than

phosphorus removal and ammonia oxidation, and the use of sludge fermentation liquid significantly alleviated the inhibitory effect of CuO NPs. In the following text the mechanism for sludge fermentation decreasing the toxicity of CuO NPs to denitrification was explored.

3.2. The role of main organic compositions in fermentation liquid on toxicity reduction

It was reported that the increase of wastewater COD concentration was beneficial to recover the viability of activated sludge exposed to ZnO NPs when the influent COD was insufficient (Zheng et al., 2015). Usually, the removal of 1 mg N and P consumes respectively 6-8 mg and 7-10 mg COD (Sedlak, 1991). In the current study the concentrations of SCOD in all reactors were the same (400 mg/L), and ammonium nitrogen, and soluble phosphorus were respectively 35 and 6 mg/L, indicating that the amount of carbon source was sufficient for BNR. Therefore, it was not the COD concentration that causing the toxicity reduction in sludge fermentation liquid reactor.

The data in Fig. 2A showed that VFAs, polysaccharide, protein and amino acids were the main organic constitutes in fermentation liquid, which accounted for around 87% of the total SCOD. As depicted in Fig. 2B, the presence of polysaccharide did not significantly affect the denitrification, but VFAs and protein promoted the denitrification efficiencies in the reactors containing CuO NPs. Protein can form protein corona via biophysicochemical interactions and affect the nanotoxicity (Tenzer et al., 2013). The toxicity reduction was respectively 9.2%, 0.6% and 20.3% in the case of VFAs, polysaccharide and protein. As seen in Fig. 2B, however, even all these three organic compositions appeared in wastewater, the toxicity reduction was only 27.4%, which was still much lower than that observed in the test of using sludge fermentation liquid (88.5%). It seems that VFAs, protein and polysaccharide were not the main reason for the toxicity of CuO NPs decreased by sludge fermentation liquid.

In the current study sludge fermentation liquid was acquired by fermenting waste activated sludge at

pH 10. There are large amounts of protein in sludge, which can be easily hydrolyzed to amino acids under alkaline conditions. As seen in Figs. 2A and 2B, although the concentration of amino acids in sludge fermentation liquid was lower than that of VFAs, polysaccharide and protein, it remarkably alleviated the toxicity of CuO NPs to denitrifying microbes. When only amino acids were added to the CuO NPs system the toxicity reduction could reach 66.0%, and the simultaneous appearance of amino acids together with VFAs, protein and polysaccharide (i.e., the hybrid components) in the reactor of CuO NPs resulted in a toxicity reduction of 83.8%, which was almost the same as that achieved by the use of sludge fermentation liquid. Further analysis showed that the predominant amino acids in sludge fermentation liquid were alanine (Ala), citrulline (Cit), aminobutyric acid (α -ABA), cysteine (Cys), isoleucine (Ile), leucine (Leu) and phenylalanine (Phe) (Fig. 2C). After the fermentation liquid was added into wastewater, the corresponding concentrations of these predominant amino acids were 4.14, 0.52, 0.73, 5.01, 0.35, 0.41, and 0.19 mg/L, respectively. When these amino acids were added individually to the CuO NPs system according to the content, the data in Fig. 2D revealed that cysteine exhibited marvelous potential on toxicity reduction, which accounted for 85.9% of toxicity reduction function of the all amino acids. The presence of cysteine in fermentation liquid was therefore the main cause of toxicity reduction.

The physiochemical properties of CuO NPs in different experimental conditions were investigated to fate of nanoparticles in wastewater. According to the data of zeta potentials, CuO NPs in wastewater with the presence of protein and amino acids showed more negative charge (Figure S2A, Supplementary data). The size distributions of CuO NPs were shown in Figure S2B (Supplementary data), which indicated that the presence of VFAs and polysaccharide did not significantly affect the nanoparticle size in wastewater. However, the nanoparticle in wastewater including protein and amino acids exhibited smaller size than the one in wastewater. It seems that protein and amino acids can adsorb on the surface of nanoparticle,

leading to more negative surface charges and smaller aggregations. In addition, the transformations played a major role in the toxicity of nanoparticles. Wang et al. (2013) found that CuO NPs underwent sulfidation with the presence of sulfide, and the resulting CuS NPs were less cytotoxic than CuO NPs. In the present study, XRD was employed to identify the phase transformation of CuO NPs, and the results indicated that no significant characteristic peaks of nanoparticles in different conditions matched well with CuS reference spectra (Figure S3, Supplementary data). Therefore, there was no sulfidation in wastewater system with the presence of fermentation liquid or amino acids, and the toxicity reductions were not caused by phase transformations of CuO NPs.

3.3. Biological mechanism of cysteine decreasing the toxicity of CuO NPs towards denitrifier

P. denitrificans, was reported widely in activated sludge, water, soil, and sediments (Knowles, 1982), which was therefore used in this study as a model denitrifying microbe to explore the biological mechanism of cysteine. As seen in Fig. S4A (Supplementary data), the nitrate removal efficiency was decreased from 85.3% (in the absence of CuO NPs) to 58.6% (in the presence of 0.25 mg/L CuO NPs). However, the nitrate removal efficiency was increased to 74.0% after the addition of cysteine. In addition, CuO NPs caused excessive nitrite accumulation (Fig. S4B, Supplementary data), but cysteine reduced the final concentration of nitrite. In the literature, cysteine was observed to attenuate the toxicity of Ag NPs via scavenging the released Ag^+ , because the functional group (-SH) in cysteine showed high-affinity binding with Ag^+ (Pokhrel et al., 2013). However, our previous study revealed that the toxicity of CuO NPs came mainly from the nanoparticles rather than the released copper ions (Su et al., 2015). It seems that cysteine might play other roles in the reduction of CuO NPs toxicity in this study.

For most nanoparticles, the origin of nanotoxicity has been ascribed to the excessive generation of reactive oxygen species, which causes the damage to cellular metabolism and structure (George et al., 2009;

Setyawati et al., 2013a; Xia et al., 2008). The data in Figs. S5A and S5B (Supplementary data) indicated that the bacteria exposed to CuO NPs exhibited higher ROS generation than the control, and the addition of cysteine declined the ROS level. Nevertheless, the average intensity of ROS fluorescence was only decreased for 20%. ROS is usually generated in aerobic respiration process (Apel and Hirt, 2004). In the current study, denitrification occurred under anoxic condition, which suggests that there might be other reasons for the origin of CuO NPs toxicity. Nitric oxide (NO) is an important intermediate of denitrification, which can cause the formation of reactive nitrogen species (RNS). RNS, a diverse range of antimicrobial molecules, possess negative influence on biological process by arousing nitrosative stress (Patel et al., 1999). As seen in Fig. 3A the fluorescence intensity distribution of the generated RNS was altered by CuO NPs, which was also affected by the addition of cysteine. Further analysis indicated that the average fluorescence intensity was remarkably increased from 561 to 1761 by CuO NPs (Fig. 3B), and the RNS level was decreased to 757 after cysteine was added. By comparing the data of ROS (Fig. S5, Supplementary data) and RNS (Fig. 3), it is clear that the presence of CuO NPs in BNR system resulted in a much higher production of RNS than ROS (3.1-fold versus 1.5-fold). Apparently, one important reason for cysteine alleviating the toxicity of CuO NPs towards denitrification was attributed to its role on the reduction of RNS production.

Fig. 4A illustrated the relationship between the RNS generation from denitrification, the synthesis of GSH by cysteine, and the transformation of GSH. In denitrification, NO, the precursor of RNS, is generated as an intermediate. The excessive generation of RNS induced by CuO NPs would lead to the transformation from GSH to its oxidized form glutathione disulfide (GSSG). Glutathione (GSH) is a major antioxidant and redox regulator in cells (Penninckx and Elskens, 1993), which can be synthesized via γ -glutamyl cycle pathway with the involvement of cysteine. Cysteine has been reported to be the

rate-limiting factor in cellular GSH biosynthesis as the sulfhydryl group (-SH) in cysteine can be served as a proton donor, which is responsible for the biological activity (Meister, 1988). Intracellular glutathione usually exists in two forms, i.e., reduced as GSH and oxidized form GSSG, and the balance between GSH and GSSG (i.e., the redox couple GSH/GSSG) is important to cellular metabolism for its regulation of redox status (Masip et al., 2006). Generally, more than 90% of the total glutathione pool exists in the reduced form (GSH) (Zitka et al., 2012), and the ratio of GSH/GSSG is greater than 9:1, which is agreed with the observation made in the control test of this study (Fig. 4B). However, as seen in Fig. 4B a significant decrease in the ratio of GSH/GSSG (1.9:1) was observed when CuO NPs appeared in denitrification system. Then, the addition of cysteine dramatically increased the GSH/GSSG to 9.5, indicating that cysteine recovered the redox balance broken by CuO NPs.

In literature, N-acetyl-cystein (NAC), a derivative of cysteine, was observed to recover cell viability after being inhibited by nanomaterials via restoring the membrane potential and reducing the production of intracellular oxidants (Setyawati et al., 2015; Siddiqui et al., 2013). Besides, the presence of NAC also recovered the intracellular GSH to basal level, indicating that NAC was capable of stimulating the biosynthesis of GSH (Ruffmann and Wendel, 1991). Based on the biological roles as scavenging free radical, GSH was employed as an effective addition to reduce the production of oxidants (Setyawati et al., 2015). In addition, Tay et al. (2014) found that the GSH-ligand Au nanomaterials induced less cellular internalization and down-regulated oxidative state. In these studies, the anti-oxidative molecules (including NAC and glutathione) were able to recover the cell viability via reducing ROS generation. However, these studies focused on the toxicity reduction for cells living in aerobic condition. In the present study, the anaerobic microbe (denitrifier) was studied, and we found that cysteine, existing in fermentation liquid, possessed the potential for toxicity reduction. Also, it was found that the mechanism

for cysteine reducing the toxicity was different as cysteine could synthesize glutathione to reduce excessive reactive nitrogen species (RNS) generated in the denitrification process. It is well-known that cysteine is widely present in the environment, and thus it can be easily obtained, such as fermentation liquid of waste activated sludge, amino acid-containing wastewater, etc.

It was reported that the variation of intracellular redox state gave influences on gene expression and protein synthesis of living organisms, such as plant cells (Buchanan and Balmer, 2005). It is well known that cysteine is one of the basic components for protein synthesis (Fig. S6, Supplementary data), and large amounts of functional proteins are involved in denitrification (Nojiri et al., 2009). For example, as carbon source is necessary for denitrification and iron is the essential trace element acting as the active center for redox reactions in metalloproteins, the transporter membrane protein and periplasmic binding protein are responsible for the transport of carbon source and iron from the outside to the inside of cells (Braun and Braun, 2002; Schneider, 2001). Also, the transfer of electrons in denitrification process relies on the participations of NADH flavin oxidoreductase, electron transfer proteins and cytochrome. In addition, the denitrification reactions are catalyzed by denitrifying enzymes (such as nitrate reductase and nitrite reductase). From the data in Fig. 5A, it is clear that the expressions of all these proteins, which had been inhibited by CuO NPs, were up-regulated after the addition of cysteine.

Energy (ATP) is required for microbial activities, such as synthesis of biological molecules, membrane transport and cell proliferation (Elston et al., 1998). To denitrification, NADH is the direct electron donor for denitrification, which is then transformed to NAD^+ via electron transfer chain. The carbon source is firstly transported into cells via transporter protein, and then the intracellular metabolism of glucose generates NADH and ATP via a series of reactions (Schneider, 2001). Previous studies reported that the intracellular glucose concentration was significantly affected by glucose transport, which was the

rate-controlling step (Dresner et al., 1999). Then, the increased concentration of intracellular glucose improved the cellular ATP content and NADH level (Eto et al., 1999; Hayashi et al., 2000). Similarly, this phenomenon was verified in our previous study (Su et al., 2015), and the down-regulated glucose transporter proteins induced less intracellular glucose, which further caused less generations of NADH and ATP. Since the expression of transporter protein was restored by cysteine in the present study, more carbon source could be utilized by cells (Fig. S7, Supplementary data), which would result in more productions of ATP and NADH. As illustrated in Figs. 5B and 5C, the ATP and NADH level, compared to the control, was significantly decreased by CuO NPs, but they were recovered almost to the original ones after the addition of cysteine. The same observations were made with the growth and viability of microbes (Fig. S8, Supplementary data).

4. Conclusions

According to this study it can be seen that the release of CuO NPs into municipal wastewater biological nutrient removal process caused great magnitude of reactive nitrogen species generation, which negatively influenced the balance of microbial redox status, the metabolisms of intracellular energy and NADH, the expression of denitrification-related key proteins, and the growth of BNR microbes. The deterioration of denitrification was therefore observed. By using sludge fermentation liquid, the toxicity of CuO NPs to BNR could be significantly alleviated due to the existence of cysteine increasing the level of reduced glutathione, which scavenged the excessively produced RNS, maintained the redox status, mitigated the negative influences induced by CuO NPs, and finally recovered the BNR performance.

Acknowledgments

This work was financially supported by the National Science Foundation of China (51425802, 41301558 and 51278354), the Program of Shanghai Subject Chief Scientist (15XD1503400), the Fundamental

Research Funds for the Central Universities, and the Collaborative Innovation Center for Regional Environmental Quality.

Supplementary data

This file contains additional methods, Tables S1 and S2, and Figure S1-S8. This materials is available free of charge via the Internet at <http://www.journals.elsevier.com>.

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524

Table

Table 1. The compositions of sludge fermentation liquid.

Figure captions

Fig. 1. Effects of fermentation liquid (FL) on the toxicity of CuO NPs to BNR effluent concentrations of $\text{NH}_4^+\text{-N}$ and SOP (A) and $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ (B).

Fig. 2. The SCOD percentage of main organic compositions in fermentation liquid (A) and their influences on denitrification performance and toxicity reduction (B); the analytic results of detectable amino acids in fermentation liquid (C) and their effects on the denitrification performances and toxicity reduction (D). The mixture of VFAs, polysaccharide (Po), protein (Pr) and amino acids was expressed as “Hybrid components”. Error bars represent standard deviations of triplicate tests.

Fig. 3. Influence of CuO NPs and the addition of cysteine on the variation of RNS (A: distribution of fluorescence intensity; B: statistics analysis of fluorescence intensity). SS represented the side scatter of light from the measurement in flow cytometer.

Fig. 4. Schematic metabolic pathway of intracellular GSH synthesis and transfer (A), and the influences of cysteine addition to CuO NPs system on intracellular GSH/GSSG (B).

Fig. 5. Influence of cysteine addition to the CuO NPs system on the expressions of key denitrification proteins (A), intracellular NADH/NAD^+ (B) and ATP (C). Error bars represent standard deviations of triplicate tests.

Graphical Abstract

Table 1. The compositions of sludge fermentation liquid

SCOD ^a	VFAs ^{a,b}	protein ^c	polysaccharide ^c	NH ₄ ⁺ -N ^c	SOP ^c
5080 ± 187	2722 ± 148	983 ± 43	439 ± 67	16.4 ± 0.6	9.2 ± 0.4

a. The unit is mg COD/L.
b. VFAs contain acetic acid (1350 ± 67), propionic acid (433 ± 61), isobutyric acid (211 ± 38), n-butyric acid (237 ± 46), isovaleric acid (438 ± 51), n-valeric acid (53 ± 11).
c. The unit is mg /L.

Figure 1

Fig. 1. Effects of fermentation liquid (FL) on the toxicity of CuO NPs to BNR effluent concentrations of $\text{NH}_4^+\text{-N}$ and SOP (A) and $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ (B).

Figure 2

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