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Effect of TiO₂-nanoparticles on copper toxicity to bacteria: role of bacterial surface

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The impact of titanium dioxide nanoparticles (nano-TiO₂) on the aquatic environment is an important issue due to their increasing application. Although nano-TiO₂ was reported to show an effect on heavy metal toxicity to aquatic organisms, the underlying mechanism is not well understood. In this study, two bacterial species (*Bacillus thuringiensis* (*B. thuringiensis*) and *Bacillus megaterium* (*B. megaterium*)) from sediment were selected to study the effects of nano-TiO₂ on copper toxicity. Nano-TiO₂ was found to inhibit the growth of *B. thuringiensis* and enhance the oxidative stress damage caused by copper, whereas these effects were not observed for *B. megaterium*. Transmission electron microscopy and flow cytometry showed that *B. thuringiensis* has stronger association ability to nano-TiO₂ than *B. megaterium*. The existence of the S-layer on the surface of *B. thuringiensis* might be the possible reason, leading to the difference in copper toxicity. This indicates that the characteristics of bacterial surfaces might be important to the toxicity responses of nanoparticles.

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1. Introduction

Titanium dioxide nanoparticles (nano-TiO₂) are widely used in commercial and industrial fields because of their unique physical and chemical properties.^{1–3} It has been predicted that the annual production of nano-TiO₂ will reach 2.5 million tons by 2025.⁴ Therefore, the release of nano-TiO₂ into the aquatic environment will be inevitable, triggering potential risks to the aquatic ecosystem. Although the concentration of nano-TiO₂ in the natural aquatic environment is low (3–6 ng L⁻¹),⁵ studies have demonstrated that nano-TiO₂ could be toxic to aquatic organisms.^{6–11} Furthermore, nano-TiO₂ could interact with other contaminants already present in the environment, thus affecting the behavior and toxicity of these contaminants.^{12–14} Several studies have focused on the effect of nano-TiO₂ on heavy metal toxicity to aquatic organisms, such as *Daphnia magna*, algae and *Tetrahymena thermophile*.^{14–18} Only limited studies have reported the toxic influence of nano-TiO₂ on heavy metal toxicity to bacteria.

The number of bacteria in freshwater can reach 10⁸ ml⁻¹. Bacteria not only play an important role in nutrient cycling (*e.g.*, carbon, nitrogen and phosphorus), biomass decomposition and bioremediation of pollutants in the aquatic environment,¹¹ but also serve as the primary foundation nutrient source for organisms in higher trophic levels.¹⁹ The stability of the bacterial community is important for a healthy aquatic

ecosystem.^{20–22} Therefore, it is essential to study the effect of nano-TiO₂ on bacteria in this environment.

Several studies have found that nano-TiO₂ could inhibit bacterial growth²¹ and change microbial communities,²³ and that the toxicity of nano-TiO₂ to bacteria may be impacted by several factors. Firstly, the physical properties of nano-TiO₂, such as size and shape, were studied.^{24,25} Tong *et al.*²⁶ found that different shapes of nano-TiO₂ (nanotubes, nanorods, nanosheets and nanospheres) showed varying degrees of phototoxicity to *Escherichia coli* and *Aeromonas hydrophila* due to different alignment of nano-TiO₂ at the bacterial surface. In addition, different aqueous media conditions, such as pH, ionic strength and organic matter, could also affect the toxicity of nano-TiO₂ to bacteria by changing the agglomeration and sedimentation characteristics of nanoparticles and thus inhibiting the interaction of nanoparticles with bacteria.^{27–30} These differing results could be due to the different interactions between nano-TiO₂ and the bacterial surface. For example, Kumari *et al.*³¹ showed that the cytotoxicity of TiO₂-NPs to three different bacteria (*Bacillus alitudinis*, *Bacillus subtilis* and *Pseudomonas aeruginosa*) could be due to the adsorption of nano-TiO₂ to the bacterial surface, resulting in membrane damage and reactive oxygen species (ROS), which induced cytotoxicity. The interaction between nanoparticles and bacterial surface is, therefore, of great significance for evaluating the toxicity of nanoparticles.^{26,32}

The degree of interaction between nanoparticles and bacteria is related to the surface characteristics of the bacteria. Suresh *et al.*³³ found that Gram-positive bacteria (*B. subtilis*) were more sensitive than Gram-negative bacteria (*E. coli*) to nano-Ag due to different cell wall structures. Li *et al.*³⁴ reported that nano-TiO₂ showed higher toxicity to *B. subtilis* than

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Pseudomonas putida, because Gram-negative *P. putida* have a lipopolysaccharide membrane. Therefore, the difference in the microbial cell wall structures may be an important factor in the toxicity of nanoparticles to the bacteria. Moreover, bacterial surfaces are heterogeneous, consisting of complex substances (such as phospholipids, proteins and polysaccharides)³⁵ and are coated with different soluble microbial products (e.g., exopolysaccharides) secreted by the bacteria during metabolic processes.³⁶ There are numerous species of bacteria in the aquatic environment, and their surface characteristics are often diverse.³⁷ Therefore, exploring the interactions between different bacteria and nanoparticles will help to assess the risk of nanoparticles to the aquatic environment.

In this study, two individual bacteria with different surface properties were selected to investigate the effects of nano-TiO₂ on the biotoxicity of copper (Cu). Bacterial growth, ROS content, superoxide dismutase (SOD) and malondialdehyde (MDA) were measured to assess the toxic effects. Finally, the interaction of nanoparticles and two bacteria was investigated to elucidate the impacts of different surface characteristics on effect of nanoparticle on copper toxicity to bacteria.

2. Materials and methods

2.1. Nanoparticles preparation and characterization

Aeroxide P25 nano-TiO₂ particles were purchased from Acros Organics (Belgium). Stock suspensions of nano-TiO₂ (1 g L⁻¹) were prepared by adding 1 g of P25 nano-TiO₂ particles to 1000 mL of sterile Milli-Q water. The mixture was placed in an ultrasonic bath (100 W, 40 kHz) for at least 30 min to break up large agglomerates and homogenize the dispersion. The nano-TiO₂ stock solution was sonicated for a further 30 min before use. For the prepared suspension, surface areas were calculated using the BET method, with a Nova 2200e BET surface area analyzer (Quantachrome, FL, USA). Size (hydrodynamic diameter) and zeta potential (ζ -potential) of nanoparticles were measured using Zetasizer (Zetasizer Nano Series, Malvern Instruments, UK).

2.2. Bacterial cultivation

The two bacterial species, isolated from sediment, used in this study were identified as *Bacillus megaterium* and *Bacillus thuringiensis* by the Institute of Microbiology, Chinese Academy of Sciences, according to the morphology, physiological and biochemical characteristics and 16S rRNA gene sequence and *ilvD* gene sequence data.

Bacteria were incubated for 24 h at 37 °C, with shaking (SHP-150, Shanghai Jinghong). The nutrient agar medium composition included 5 g L⁻¹ NaCl, 3 g L⁻¹ beef extract, 10 g L⁻¹ peptone and agar 20 g L⁻¹ (NB solid medium), sterilized for 30 min at 121 °C. After incubation, the culture was washed twice with sterilized physiological saline (0.9%, w/v) following centrifugation at 3500g for 10 min. Finally, we resuspended the cells in sterilized physiological saline for exposure experiment use. The number of bacterial suspensions was found to be about 10⁹ CFU mL⁻¹. Sterilized physiological saline (9 g NaCl with 1 L ultra-pure water after sterilization for 30 min at 121 °C) was used as the experimental matrix throughout the study.

2.3. Exposure experiment

The toxicity exposure experiment was carried out in a 50 mL sterilized conical flask. Bacterial suspensions (1 mL; see details of bacterial cultivation) were added and diluted to 20 mL with sterilized physiological saline. The experiments were carried out in two systems (Cu-only and Cu + 1 mg L⁻¹ nano-TiO₂) in triplicate, and treatment without Cu (as control). The Cu concentration gradient was: 0, 50, 100, 200, 300 and 400 μ g L⁻¹. All flasks were sealed with sterilized film and cultured at 37 °C for 24 h, with shaking.

2.4. Bacterial growth

The viability of the bacteria was assessed by the flat colony counting method. To evaluate the toxic effect of nano-TiO₂ with Cu on bacterial viability after the exposure experiment, we diluted 1 mL of the experimental suspensions with typically ten-fold dilutions, three replicates of the dilution series, then spread 0.5 mL of different suspension concentrations onto nutrient agar medium plates (NB solid medium). These were cultured in the biochemical incubator at 37 °C. After 24 h, the flat colony counting method was used to calculate the number of CFU. Then, according to the corresponding dilution ratio, we calculated the quantity of bacteria, as CFU mL⁻¹.

2.5. Cu accumulation

After 24 h of exposure to nano-TiO₂ and Cu, 10 mL bacterial suspensions were collected and washed twice with sterilized physiological saline. The samples were then washed with 10 mM EDTA-Na₂ to remove the Cu²⁺ absorbed onto the cell wall. Subsequently, they were digested with 68% HNO₃ (Aristar grade) at 110 °C until the solution became completely transparent. The digested solution was transferred to a volumetric flask and adjusted to a constant volume of 10 mL with 2% HNO₃. The Cu concentration in the digested solution was analyzed by inductively coupled plasma mass spectroscopy (ICP MS, VG PQ2 TURBO). The Cu accumulation was expressed based on the CFU of bacteria.

2.6. Determination of ROS and enzyme activity

The test kits used to measure ROS, SOD and MDA were purchased from Nanjing Jiancheng Bioengineering Institute, China. ROS generation was tested with the fluorescence probe dichloro-dihydro-fluorescein diacetate (DCFH-DA). Cell suspensions (2 mL) were incubated with DCFH-DA at a concentration of 14 μ M at 37 °C for 60 min. The redundant DCFH-DA was removed by centrifugation and then suspended in 2 mL of physiological saline. To estimate the auto-fluorescence activity of nano-TiO₂ that may interfere with the DCFH dye, we tested a negative control of nanomaterials without bacterial cells. ROS generation of microorganisms was assessed by the DCF level using a fluorescence spectrometer.

Cells of 10 mL bacterial suspension were collected by centrifugation at 8000 rpm for 10 min, then washed twice with sterilized physiological saline, and transferred into 2 mL centrifugal tubes after centrifugation at 12 000g for 10 min. The supernatant



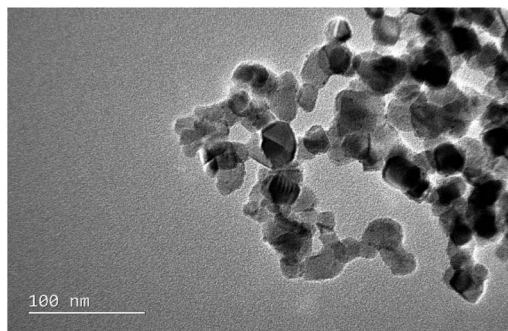


Fig. 1 TEM image of nano-TiO₂.

was removed and then disrupted with an ultrasonic cell crusher (Scientz-II, Ningbo Xinzhi Biological Company) in an ice bath following the addition of 1.5 mL sucrose buffer (0.25 mol L⁻¹ sucrose, 0.1 mol L⁻¹ Tris-HCl, pH 8.6). The homogenate was then centrifuged (4 °C, 16 000 g) for 20 min. The supernatant was used to determine SOD activities and MDA content, using the test kits (Nanjing Jiancheng Bioengineering Institute, China) according to the instruction manual.

2.7. Interaction of nanoparticles and bacteria

Transmission electron microscopy (TEM) and flow cytometry (FCM) were used to investigate the interaction of different bacterial surfaces with nano-TiO₂.

Bacterial cells were prepared according to bacterial cultivation, bacterial suspension was incubated with 1 mg L⁻¹ TiO₂ nanoparticles for 24 h and cultured in dark conditions at 37 °C. TEM samples were prepared by dropping a suspension of bacteria and nano-TiO₂ on copper grids (200 mesh, Zhongjingkeyi Technology Co., Ltd.). These were left to stand overnight to ensure adequate fixation, then the morphology of bacterial combined nano-TiO₂ was obtained by TEM (JEM-2100) at 100 kV.

The association of nanomaterials and bacterial cells can be evaluated by FCM.^{38,39} We followed the following procedure: 1 mL

bacterial suspension (as described in bacteria cultivation) was diluted to 20 mL with physiological saline, then the appropriate amount of nano-TiO₂ was added to obtain a concentration of 1 mg L⁻¹. We removed 300 μL samples from the bacterial suspension at 0, 0.5, 1, 2, 4, and 24 h and placed them into 2 mL centrifugal tubes. For time-dependent experiments, all 2 mL tubes were centrifuged at 1500 g for 15 min, then the bacterial cells were resuspended to 300 μL with phosphate buffer saline to remove the unadsorbed nano-TiO₂. In order to eliminate the interference of nano-TiO₂, we added 5 μL SYBR Green I nucleic acid dye (PCR) to the bacterial suspension, after mixing under dark conditions for staining on bacterial DNA about 30 min, as a marker of bacteria. Using CytoFLEX flow cytometry (Beckman Kurt) we measured all stained samples with light scattering, only the SYBR Green I dye positive signals were used to analyze, using the CytExpert 1.1.10.0 software for processing.

2.8. Statistical analysis

Results were expressed as mean value ± standard deviation ($n = 3$). Statistical significance was accepted if p value was < 0.05 . Statistical analyses (one-way analysis of variance (one-way ANOVA) and two-way analysis of variance (two-way ANOVA)) were conducted using IBM® SPSS® Statistics 20.0.

3. Results and discussion

3.1. Material characterization

The surface area of Aeroxide P25 (nano-TiO₂) was 55.29 m² g⁻¹. The average hydrodynamic diameter and ζ-potential were 827.1 ± 8.63 nm and 7.20 ± 0.17 mV, respectively. The nano-TiO₂ images were obtained by TEM (JEM-2100F, JEOL, Japan) operated at 100 keV. The morphology of nano-TiO₂ in aqueous solution was aggregated, as shown in Fig. 1.

3.2. Bacterial growth

As shown in Fig. 2, Cu exposure can significantly affect the growth of both bacterial species, compared to the control (Cu

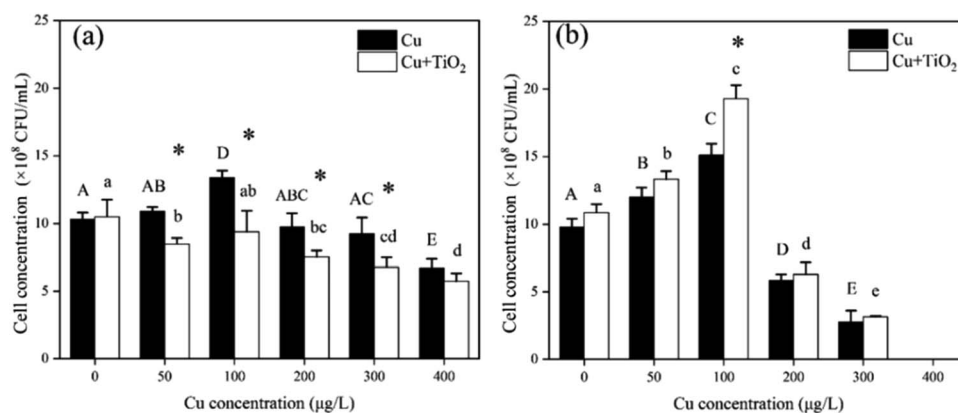


Fig. 2 *Bacillus thuringiensis* (a) and *Bacillus megaterium* (b) growth after exposure to different concentrations of Cu for 24 h in the absence and presence of 1 mg L⁻¹ nano-TiO₂; mean ± standard deviation, $n = 3$. The mean values sharing different letters are significantly different ($p < 0.05$, one-way ANOVA) among groups with different concentrations of Cu, *indicates that the number of bacteria have significant difference in the two systems (Cu-only or Cu + nano-TiO₂), T test $p < 0.05$.



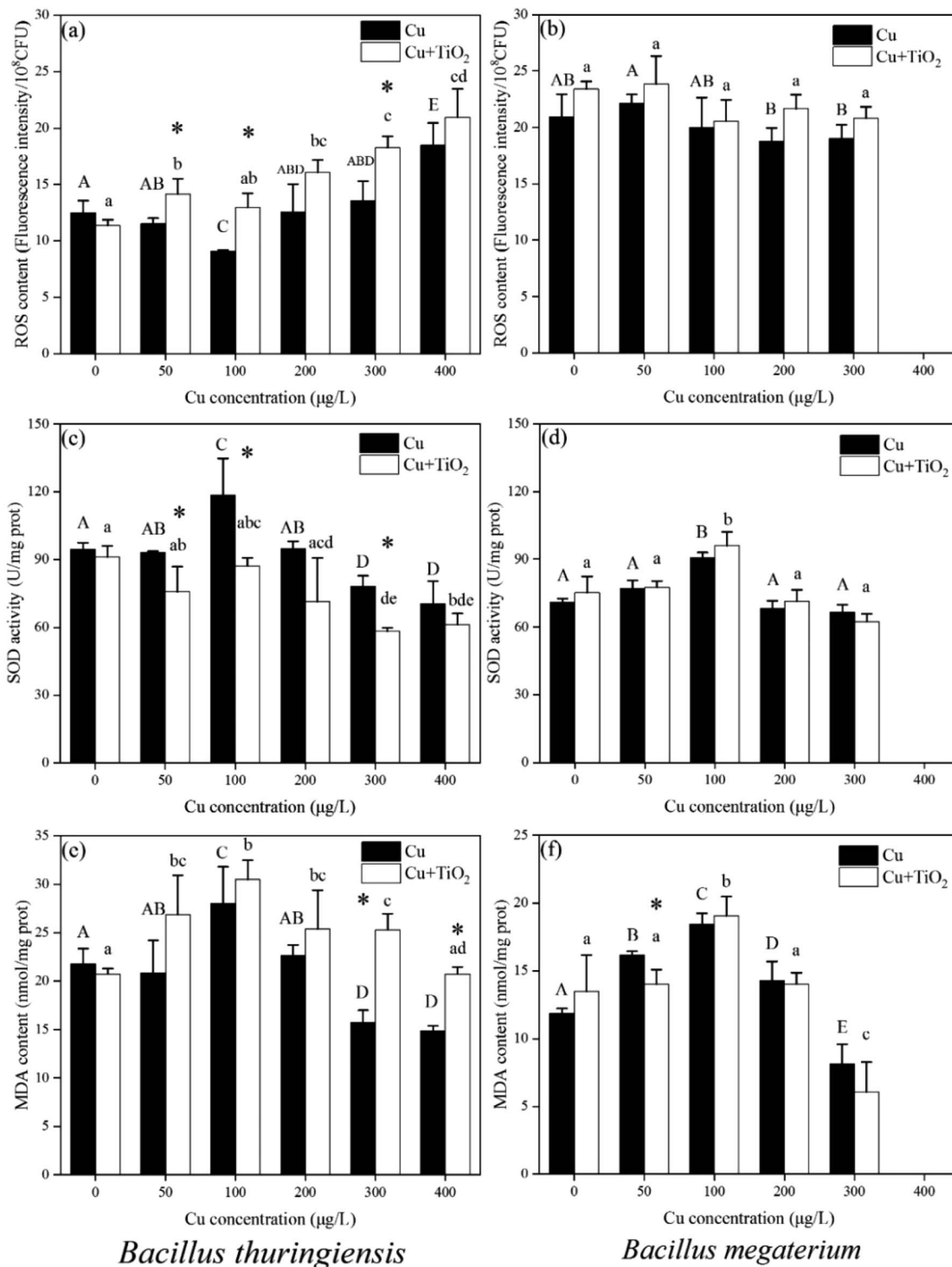


Fig. 3 ROS (a and b), SOD (c and d) and MDA (e and f) of *Bacillus thuringiensis* (left) and *Bacillus megaterium* (right) after exposure to different concentrations of Cu for 24 h in the absence and presence of 1 mg L⁻¹ nano-TiO₂; mean ± standard deviation, $n = 3$. The mean values sharing different letter are significantly different ($p < 0.05$, one-way ANOVA) among groups with different concentration of Cu, *indicates that the enzyme activity of bacteria have significant difference in the two systems (Cu-only or Cu + nano-TiO₂), T test $p < 0.05$.

concentration = 0 μg L⁻¹). In the Cu-only system, low Cu concentration promoted the growth of bacteria. As Cu concentration increased, the growth of the two bacterial species was

inhibited. The colony-forming units (CFU) of both bacteria reached a maximum at a Cu concentration of 100 μg L⁻¹. When Cu concentration reached 400 μg L⁻¹, the growth of *B.*



thuringiensis was lower than that of the control (T test $p < 0.05$), and all *B. megaterium* died. The exposure to 1 mg L^{-1} nano-TiO₂ (Cu concentration = $0 \text{ } \mu\text{g L}^{-1}$) did not distinctly affect the growth of bacteria compared to the control (T test $p > 0.05$). For the Cu + nano-TiO₂ system, the growth of *B. thuringiensis* was significantly lower than that in Cu-only system ($p < 0.05$, two-way ANOVA), indicating that nano-TiO₂ inhibited the growth of *B. thuringiensis*; *B. megaterium*, however, showed no significant difference compared with the Cu-only system ($p > 0.05$, two-way ANOVA).

3.3. ROS and enzyme activity

The ROS content of *B. thuringiensis* and *B. megaterium* after 24 h exposure is shown in Fig. 3(a) and (b), respectively. For *B. thuringiensis* in the Cu-only system, the ROS content at a Cu concentration of $100 \text{ } \mu\text{g L}^{-1}$ was significantly lower than that in the control (Cu concentration = $0 \text{ } \mu\text{g L}^{-1}$), while that at a Cu concentration of $400 \text{ } \mu\text{g L}^{-1}$ it was higher than that in the control.

The presence of nano-TiO₂ could significantly increase the ROS content of *B. thuringiensis* compared to that in the Cu-only system ($p < 0.01$, two-way ANOVA). However, for *B. megaterium*, the addition of nano-TiO₂ did not affect the ROS content in two exposure conditions.

As shown in Fig. 3(c) and (d), when the Cu concentration was $100 \text{ } \mu\text{g L}^{-1}$ in the Cu-only system, the SOD activities of both bacteria significantly increased compared to those of the control (Cu concentration = $0 \text{ } \mu\text{g L}^{-1}$). The SOD activity of *B. thuringiensis* decreased significantly when the Cu concentration was $300 \text{ } \mu\text{g L}^{-1}$ and $400 \text{ } \mu\text{g L}^{-1}$, while no significant changes were observed for *B. megaterium*. In the Cu + nano-TiO₂ system, the SOD activity of *B. thuringiensis* reduced significantly compared with that in the Cu-only system ($p < 0.05$, two-way ANOVA), indicating that nano-TiO₂ weakened the antioxidant enzyme activity. For *B. megaterium*, however, there was no significant difference in SOD activity in the absence or presence of nano-TiO₂ ($p > 0.05$, two-way ANOVA).

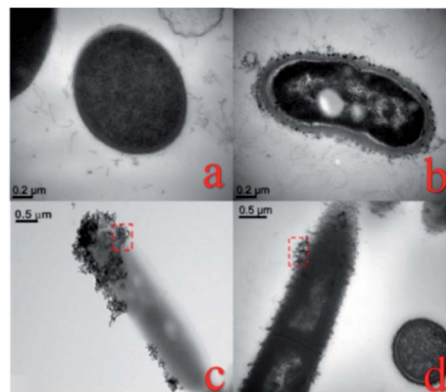


Fig. 5 TEM images of bacteria (a) *Bacillus thuringiensis* and (b) *Bacillus megaterium*, and (c) *Bacillus thuringiensis* and (d) *Bacillus megaterium* after 24 h exposure to 1 mg L^{-1} nano-TiO₂.

As the final product of lipid peroxidation, MDA could reflect the extent of intracellular lipid peroxidation.⁴⁰ As shown in Fig. 3(e) and (f), for *B. thuringiensis*, the MDA content in the Cu + nano-TiO₂ system was significantly higher than that in the Cu-only system when the Cu concentration exceeded $200 \text{ } \mu\text{g L}^{-1}$ (T test, $p < 0.05$). This result indicated that nano-TiO₂ might enhance the oxidative stress of Cu on *B. thuringiensis*. For *B. megaterium*, however, no difference was found in either the co-existence or the Cu-only system.

It was reported that Cu could induce oxidative stress in organisms. The ROS content reflects the oxidative stress, while SOD is an important antioxidant enzyme, and MDA is a lipid oxidation product mainly induced by ROS.^{41,42} In these experiments, it was found that the presence of nano-TiO₂ could significantly increase the ROS content, decrease SOD activity, and increase the MDA content in *B. thuringiensis*; however, the oxidative stress process did not significantly affect *B. megaterium* whether nano-TiO₂ was present or not.

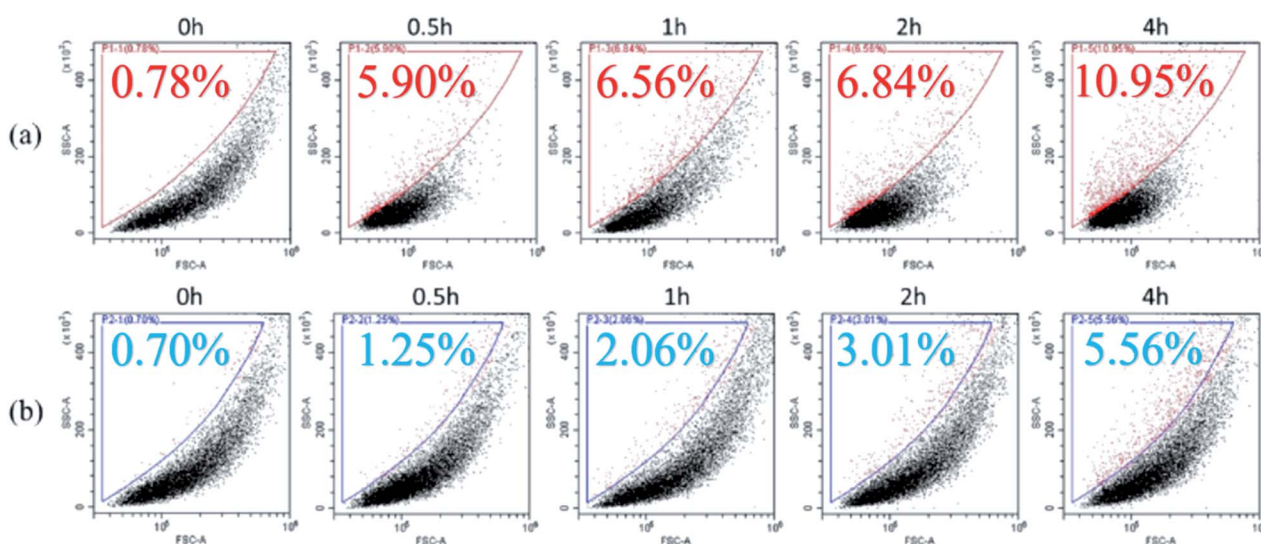


Fig. 4 Association of nano-TiO₂ with two bacteria (a) *Bacillus thuringiensis* and (b) *Bacillus megaterium* over time.



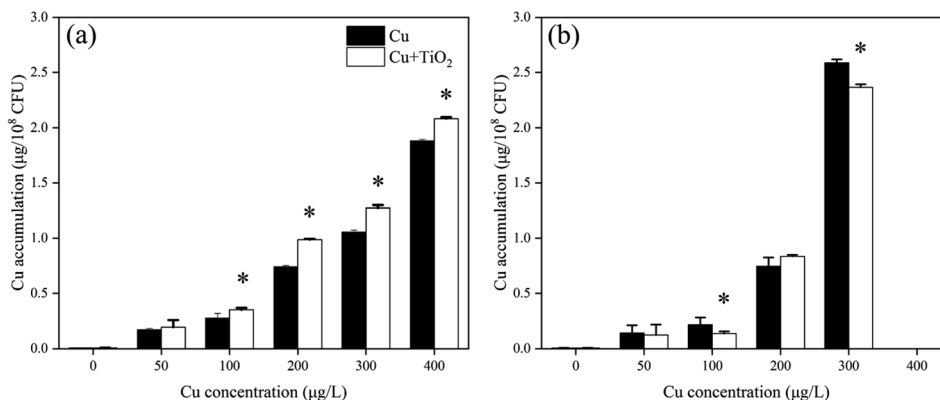


Fig. 6 *Bacillus thuringiensis* (a) and *Bacillus megaterium* (b) Cu accumulation after exposure to different concentrations of Cu for 24 h in the absence and presence of 1 mg L^{-1} nano-TiO₂; mean \pm standard deviation, $n = 3$, *indicates that the number of bacteria have significant difference in the two systems (Cu-only or Cu + nano-TiO₂), T test $p < 0.05$.

3.4. Interaction between bacteria surface and nano-TiO₂

It was found that nano-TiO₂ enhanced the toxicity of Cu to *B. thuringiensis*, but had no significant effect on *B. megaterium*. This difference in toxicity between the two bacterial species may be due to the differing interactions between nano-TiO₂ and their individual bacterial surfaces. It has been reported that the interaction between nanoparticles and bacteria may proceed in two steps: first, the nanoparticles adhere to the bacterial surface, leading to membrane damage; and then the intracellular components of the bacteria leak out, resulting in bacterial death.¹⁹ Simondeckers *et al.*²⁴ reported that the more TiO₂ nanoparticles adsorbed onto the bacterial surface, the higher the toxicity of TiO₂ nanoparticles to bacteria was found. The association between nanoparticles and bacteria may be a prerequisite in determining the toxicity of nanoparticles to bacteria.

To assess our hypothesis on the association of nano-TiO₂ with *B. thuringiensis* and *B. megaterium*, FCM analysis was carried out. FCM images showed the interaction between the nanoparticles and bacteria during contact. Data were showed as density plots. Gating sections, which were constructed manually using the supplied software of the FCM, were as control to compared with others to show percent cell having increase in side scatter but no increase in forward scatter. As shown in Fig. 4(a), after adding nano-TiO₂, the side scatter of *B. thuringiensis* gradually increased with time (exposure time of 0.5, 1, 2, 4 h corresponding to 5.90%, 6.84%, 6.56%, 11.95%, respectively). It showed that the nano-TiO₂ amalgamated with *B. thuringiensis* gradually. In contrast, the side scatter of *B. megaterium* increased less after the addition of nano-TiO₂ (Fig. 4(b)), indicating that the association between *B. megaterium* and nano-TiO₂ was lower. The results suggest that *B. thuringiensis* has a stronger association with nano-TiO₂ than *B. megaterium*, which could lead to increased toxicity of Cu to bacteria. Wang *et al.*⁴³ reported that the association of nanoparticles with bacteria might inhibit intracellular and extracellular substance transfer, cause cellular metabolism disturbance, and thus lead to cell death. The association of nanoparticles with the microbes would also facilitate the production of ROS.^{44,45} It is

certified by the increase in growth inhibition and ROS content in *B. thuringiensis* in current study.

The two bacterial species that we investigated are similar in shape and are both Gram-positive. Thus, it is believed that the difference in interaction behavior is due to the different surface characteristics. *B. thuringiensis* excretes crystalline proteins during metabolic processes, and its surface is covered by an S-layer, composed of proteins. *B. megaterium*, however, produces exopolysaccharides, which adhere to the bacterial surface.⁴⁶ We used TEM to determine the impact of the bacterial surface characteristics on the interactions between nano-TiO₂ and the bacteria. The results showed that the surface of *B. thuringiensis* was indeed enveloped by an S-layer (Fig. 5(a)), and exopolysaccharides aggregated around *B. megaterium* (Fig. 5(b)). Following exposure to 1 mg L^{-1} nano-TiO₂ for 24 h, more nano-TiO₂ attached to the surface of *B. thuringiensis* (Fig. 5(c)) than *B. megaterium*, *i.e.*, greater association formed between nano-TiO₂ and *B. thuringiensis* than between nano-TiO₂ and *B. megaterium*. Other researchers also found that proteins have a greater ability to aggregate with nanoparticles than polysaccharides.⁴⁷

Therefore, nano-TiO₂ adsorbed onto the surface of *B. thuringiensis* may compete with heavy metal ions for binding sites, thus influencing the regulation of heavy metals by the bacteria. When nano-TiO₂ and Cu co-existed, more Cu ions were able to enter into the *B. thuringiensis* cells subsequently, leading to increase of Cu accumulation (Fig. 6), growth inhibition and oxidative stress. For *B. megaterium*, however, the exopolysaccharides attached to the bacterial surface provided a protective barrier, thus obstructing contact with nano-TiO₂, and decreasing the effect of nano-TiO₂ on Cu toxicity to *B. megaterium*.^{48,49}

4. Conclusions

The results suggest that when nanoparticles co-exist with heavy metals, the characteristics of bacterial surface may influence the association of bacteria with nano-TiO₂, hence impacting the toxicity of heavy metals to bacteria. The study presented here demonstrates that the interaction between bacterial surface and



nanoparticles may be crucial to evaluate the toxicity of the nanoparticles.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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