Biotoxicity of nickel oxide nanoparticles and bio-remediation by microalgae

Chlorella vulgaris

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ABSTRACT

Adverse effects of manufactured nickel oxide nanoparticles on the microalgae Chlorella vulgaris were determined by algal growth-inhibition test and morphological observation via transmission electron microscopy (TEM). Results showed that the NiO nanoparticles had severe impacts on the algae, with 72 h EC50 values of 32.28 mg NiO L−1. Under the stress of NiO nanoparticles, C. vulgaris cells showed plasmodysis, cytomembrane breakage and thylakoids disorder. NiO nanoparticles aggregated and deposited in algal culture media. The presence of algal cells accelerated aggregation of nanoparticles. Moreover, about 0.14% ionic Ni was released when NiO NPs were added into seawater. The attachment of aggregates to algal cell surface and the presence of released ionic Ni were likely responsible for the toxic effects. Interestingly, some NiO nanoparticles were reduced to zero valence nickel as determined by X-ray diffraction (XRD). The maximum ratios of nickel reduction was achieved at 72 h of exposure, in accordance with the time-course of changes in soluble protein content of treated C. vulgaris, implying that some proteins of algae are involved in the process. Our results indicate that the toxicity and bioavailability of NiO nanoparticles to marine algae are reduced by aggregation and reduction of NiO. Thus, marine algae have the potential for usage in nano-pollution bio-remediation in aquatic system.

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1. Introduction

Nanoparticles (NPs) have received much attention due to not only their unique properties in optic, physical, chemical and biological processes, but also their potential effects on the ecosystem and human health (Colvin, 2003; Sayes et al., 2005; Moore, 2006; Wiesner et al., 2006). As water resources are particularly vulnerable to direct and indirect contamination by nanomaterials (NMs), their potential toxicity to aquatic biota should be evaluated. Most work in this area has focused on widely used NMs, such as fullerences (Oberdörster, 2005; Lovern and Kapler, 2006), nTiO2 (Hund-Rinke and Simon, 2006; Lovern and Kapler, 2006; Wang et al., 2008; Arujoa et al., 2009), nZnO (Franklin et al., 2007; Arujoa et al., 2009; Wong et al, 2010), nCuO (Arujoa et al., 2009) and nAg (Navarro et al., 2008a). These NMs caused growth inhibition of algae, behavioral changes and acute mortality in water fleas (Daphnia species), cell damage in fish brain and the expression changes of some molecular biomarkers. Still, much less is known about the interaction between NMs and aquatic biota as well as the fate of NMs in water. This information is necessary for evaluating the risks of specific nanoparticles in aquatic environments.

Nanosized nickel oxide (nNiO) possesses many unique properties compared to its bulk counterpart and is extensively used as catalyst, battery electrode, electrochromic films, sensors magnetic materials and diesel-fuel additive (Salimi et al., 2007; Rao and Sunandana, 2008). Along with the rapid development of coastal areas, NiO NPs from welding process become an important source of nano-pollution in coastal seawaters (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans). NiO nanoparticles may be released by direct aerial emission of particles to surface waters, leakages and spills, and indirect storm-water runoff from land (Wiesner et al., 2006). Similarly to other NPs, NiO nanoparticles pose risks for the environment and human health. It has been reported that risks from inhalation exposure to NiO nanoparticles existed in mammal as determined with in vitro assays (Oya et al., 2007). However, data on the effects of nanosized NiO on aquatic organisms are not available.

Several reports have shown that dead algal powder and living organisms have the ability to reduce noble metal (gold, silver
and platinum) from their ionic solution, providing the possibility to remediate mining wastes or using organisms as the eco-friendly nanofactories for synthesis of nanoparticles (Gardea-Torresdey et al., 2002; Konishi et al., 2007; Singaravelu et al., 2007). However, review of literature revealed that the reduction of metal oxide nanoparticles to zero valence forms has been unexplored, which aroused our interest in the present investigation.

This study was designed to assess the toxicity of nickel oxide nanoparticles to algae. Batch cultured Chlorella vulgaris were employed as the testing organism to investigate their growth and morphological changes under NPs exposure. The fate of NPs in algal media with or without algae was also investigated, including their solubility and aggregation. To our knowledge, this is the first study on the toxicity and bio-remediation of NiO nanoparticles on algae.

2. Materials and methods

2.1. Preparation of NiO nanoparticles and structural characterization

NiO nanoparticles were prepared by a homogeneous precipitation method with an aqueous solution of nickel nitrate hexahydrate and urea. The purity and mean size of NiO nanoparticles were measured by TEM (Transmission electron microscopy, JEOL TEM-100SX electron microscope) and X-ray diffraction (XRD) analysis (see Fig. S1, Supplementary data). From TEM measurement, the preliminary average size of NiO NPs was 20 nm, agreed with the calculated results by Bragg equation. The stock suspension of NiO nanoparticles was prepared in algal medium and was ultrasonicated for 30 min before use.

2.2. Biotoxicity assays

2.2.1. Chlorella vulgaris culture

The algae were cultured in 250 mL erlenmeyer flasks containing 60 mL of sterilized f/2 medium which were capped with loose cotton, and these flasks were placed on a shaker, with the cycle of 30 min reciprocating shake and 15 min stop. The shaker system simulated the current of nature water body to keep the turbulence of culture medium. The cultures were kept at 23 ± 1 °C under illumination of approximately 73.6 μmol m⁻² s⁻¹ with daily cycles of 12-h light and 12-h night. The cell density of culture was monitored spectrophotometrically at 684 nm (OD₆₈₄, optical density at 684 nm) with a spectrophotometer (Beckman DU800) every 24 h. During the exponential growth phase, correlation between optical density (OD₆₈₄) and cell density was determined by counting with a hemocytometer. The regression equation between cell density (y = 10⁶ mL⁻¹) and OD₆₈₄ (x) was calculated as y = 62.79509x − 1.00981 (p < 0.01, R = 0.99628). Morphological observations were conducted with a Nikon AZ100 microscope.

2.2.2. Growth-inhibition test

In general, the Organization for Economic Co-operation and Development (OECD) 201 algal growth-inhibition test guidelines were followed, and cells in exponential growth phase were used for all experiments. Each growth-inhibition test consisted of seven NiO nanoparticles concentrations: 0, 10, 15, 20, 30, 40 and 50 mg L⁻¹ with three replicates for each concentration. Two separate trials were performed using the same batch of algae and NiO nanoparticles. In order to compare the contents of photosynthetic pigments, thirty milliliters of each algal culture were collected to analyze chlorophyll – a contents of treated groups. Chlorophyll – a concentrations were determined using 90% acetone extractions and measured by spectrophotometer (Beckman DU800).

Inhibitory rate of growth was obtained by the formula (1)

\[
\text{Inhibitory Rate (IR)}\% = (1 - N/N₀) \times 100\%
\]  

(1)

where N is the cell density (cell per milliliter) in the NiO nanoparticles treated cultures N₀ is the cell density (cell per milliliter) in the control culture.

The median effective concentration (EC₅₀) values for inhibition of cell growth were calculated using SPSS software.

2.2.3. TEM observation of algal cells

The ultrastructural alterations of C. vulgaris induced by NiO nanoparticles were observed with TEM (JEM-2000FX transmission electron microscopy) analysis. After 96 h exposure, C. vulgaris treated with 30 mg L⁻¹ NiO nanoparticles were collected by centrifugation for TEM analysis. Each sample was analyzed for at least five view fields at different magnifications.

2.2.4. The protein assays

Thirty milliliters of each algal culture treated by 15 mg L⁻¹ NiO nanoparticles were collected to analyze soluble protein contents of algae. Separated algal cells were ground using a tissue grinder in 1 mL of 20 mM phosphate buffer (pH 7.4) and 0.1 g white quartz sand in a mortar sitting in an ice bath. The protein content was measured using the Bio-Rad Bradford 595 nm kit.

2.3. Bioreduction analysis

2.3.1. X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS)

To determine the valence state of nickel as well as the amount of element Ni and NiO inside algal cells, XRD and XPS analysis was conducted with samples of treated algae. After 96 h exposure, algae treated with 15 mg L⁻¹ NiO NPs were collected, dried at 60 °C and then sintered at 650 °C for 4 h under nitrogen protection (10–15 mL min⁻¹).

2.3.2. Inductively coupled plasma-mass spectrometry (ICP-MS)

To assess solubility of Ni from nanoparticles in seawater, a series of NiO NPs stocks (10, 15, 20, 30, 40 and 50 mg L⁻¹ NiO) were prepared with sterilized seawater. The NiO suspensions were vacuum filtered with 0.45 μm Millipore filter and concentrations of Ni²⁺ of filtrates were analyzed by ICP-MS. The acidification process was omitted before measurement to avoid the NiO particles below 450 nm in filtrates were changed into ionic Ni.

When exposed to 15 mg L⁻¹ NiO nanoparticles, algal samples with the different NiO NPs exposure times were vacuum filtered with 0.45 μm Millipore filter to separate algae from culture medium. The filtrates were acidified with HNO₃ and measured with ICP-MS to determine Ni content. The absorbed NiO by algal cells could be calculated by the total NiO (T_NiO, also determined by ICP-MS) minus the NiO in filtrates (F_NiO). Therefore, the absorption% = (T_NiO – F_NiO)/T_NiO × 100%.

2.4. Statistics

Data are presented as means ± standard deviations and were tested for statistical significance using analysis of variance (one-way analysis of variance, ANOVA). Linear regressions were conducted using the Origin 7.5 software. Values were considered significantly different when the probability (p) was less than 0.01.

3. Results

3.1. Biototoxicity of nNiO to algae

3.1.1. Growth inhibition

The concentration-inhibition curves are shown in Fig. 1. Five concentrations were selected for exposure to NiO NPs. The
inhibitory rate (IR) increased with the dose increasing and represented statistically significant differences after 72 h exposure \( (p < 0.01) \). The IR exhibited negative figures before 48 h exposure, indicating so-called hormesis effects of poisoning. The EC\textsubscript{50} for 72 h was 32.28 (10.77–38.53) mg L\textsuperscript{-1}/C\textsubscript{0} while increased to 44.33 (16.35–48.56) mg L\textsuperscript{-1}/C\textsubscript{0} for 120 h exposure. It seemed that the growth inhibition was reversible. Changes in chlorophyll – a contents showed the similar tendency, the concentration of chlorophyll – a declined with the increase of NiO concentrations, especially under the 40 and 50 mg L\textsuperscript{-1}/C\textsubscript{0} NiO treatments (Fig. 2).

### 3.1.2. Ultrastructural alterations

The ultrastructure of \textit{C. vulgaris} was compared between control and treated cells (Fig. 3). In control cells, the entire cell was enclosed by a cell wall, and the plasma membrane was close to the cell wall. Chloroplasts, which are one of the most important organelles, contained numerous well-compartmentalized thylakoids. The content of cells was intact (Fig. 3A and C). In some of treated cells, however, the plasma membrane was detached from the cell wall (Fig. 3B and E). The cytosol was leaked due to the disruption of plasma membrane (Fig. 3B, D, and F) and the plasma membrane as well as cell wall were degraded completely in some cases (Fig. 3B, white triangle). The thylakoids showed disordered grana lamella which indicated that the photosynthesis could be affected (Fig. 3D). In treated groups, deposits of NiO nanoparticles were observed as black aggregates (Fig. 3B, D, and F, arrows).

### 3.2. The aggregation and deposition of NiO

As shown in Fig. 4, the NiO nanoparticles formed aggregates and deposited after being added to the aqueous medium. In Fig. 4E and F, NiO nanoparticles were dispersed into algal culture medium with or without algae. When the algae were absent, little nanoparticle settled (A2–F2); by contrast, NiO nanoparticles visibly aggregated and settled within minutes after the addition of algae. The amount of deposition was positively correlated to the NiO concentration. The NiO deposits appeared looser and bigger than that in the algae-absent groups (Fig. 4A, the inset picture). As observed with differential interference contrast (DIC) microscope, the nanoparticle aggregates were black and the algae were white embossed dots (Fig. 4C). When the same image was observed with fluorescence microscopy, the algal cells were visible by their red fluorescence, indicating the algal cells aggregated with NPs.

### 3.3. Bioreduction of NiO

#### 3.3.1. The XRD analysis

Fig. 5 shows a XRD spectrum of NiO nanoparticles before (a) and after (b) being added to \textit{C. vulgaris} culture medium. The curve “a” was the XRD spectrum of NiO nanoparticles prepared by homogeneous precipitation method. When nanosized NiO was added into the \textit{C. vulgaris} culture, new diffraction peaks (Fig. 5b) appeared, which indicates the existence of elemental nickel.

#### 3.3.2. The XPS analysis

The ratio of Ni\textsuperscript{0} and NiO at different exposure time was further investigated using XPS spectroscopy. As seen in Fig. S2. (Supplementary data), the binding energies (BE) of Ni 2p could be resolved into Ni 2p and Ni 2p A doublets, which suggested that there were two valence of Ni as Ni\textsuperscript{2+} and Ni\textsuperscript{0}. The ratios of two chemical states of nickel (Ni\textsuperscript{2+} and Ni\textsuperscript{0}), which provide information on bioreduction rate, were calculated from Area CPS (eV) of Nickel 2p. The ratios of bioreduction at different exposure time are presented in Table S1. The ratio of Ni\textsuperscript{0} reached the highest point at 72 h of exposure.

#### 3.3.3. ICP-MS analysis

#### 3.3.3.1. The release of Ni\textsuperscript{2+}

Ni ions were detected by ICP-MS in all seawater suspension samples (Table S2., supplementary data). In generally, Ni\textsuperscript{2+} increased as the concentration of NiO increased...
except at 30 mg L\(^{-1}\) NiO group. The mean solubility of NiO in seawater was 0.14%. In 50 mg L\(^{-1}\) NiO treated group, 0.1100 mg L\(^{-1}\) Ni\(^{2+}\) was detected.

### 3.3.3.2. Bio-absorption of Ni

Bio-absorption of Ni was measured by ICP-MS. Ni content of algal filtrates varied with exposure time. The absorption percentage increased after 48 h exposure, indicating that the absorption process occurred before the bioreduction of NPs (Fig. 6).

### 4. Discussion

#### 4.1. Biotoxicity of nNiO to algae

Our results indicated that exposure to NiO nanoparticles caused growth inhibition and morphological alteration, resulting in biotoxicity on green algae. And the shading effects caused by cell-NP aggregates as well as the released Ni ions led to the biotoxicity of nNiO to algae.
4.1.1. Shading effects

The noticeable algal cell/NiO aggregation implied the aggregates entrapped and shaded the algal cells (Fig. 4D), leading to a reduction in the light available to those cells and thus inhibiting their growth. Similar phenomenon was also observed by Aruoja et al. (2009) and Wang et al. (2008) who used additional two green algae to test the toxicity of TiO$_2$ NPs. It is clear from this and previous studies that the NPs aggregation behavior strongly affected the growth of photo-autotroph in aquatic systems due to shading effects.

4.1.2. Released ions

For other metallic NPs, such as ZnO, released ions have been reported as the main reason of toxicity (Franklin et al., 2007; Kahru et al., 2008; Aruoja et al., 2009). Bulk NiO is almost insoluble in water (International Chemical Safety Cards). While for nano-NiO, trace Ni$^{2+}$ was detected in solution which implied the characteristics of nanoparticles. In our previous study, the EC$_{50}$ of nickel ions was 1.73 mg L$^{-1}$, which showed that Ni$^{2+}$ was more toxic than nNiO (Li et al., 2009). The data were much more than 38 times than ionic Ni concentrations in nano-NiO suspension presented here. Results of this study suggest that released ions may contribute adverse effect on microalgae but not the only reason.

In general, the existed forms were complicated when NiO added into algal medium. Preliminary nano-size NiO aggregated and settled. Part of them changed into ionic Ni and transported into element Ni. It was dynamic process and ionic Ni maybe the transitional state from NiO to element Ni. Further studies are needed.

Fig. 4. Aggregates of NiO nanoparticles in algal culture medium with (A–E) and without (B, F) algal cells. The inset pictures of A and B illustrate the same sample but in bright field microscope (400×). Aggregates of NiO nanoparticles in culture medium, as visible to the naked eye (A, B), in DIC microscopy (C) and in fluorescence microscopy (D); Algal culture medium at different concentrations of NiO: A-0 mg L$^{-1}$, B-15 mg L$^{-1}$, C-30 mg L$^{-1}$, D-45 mg L$^{-1}$, E-60 mg L$^{-1}$ and F-75 mg L$^{-1}$ with (E) and without (F) algal cells, bar = 50 µm for the inset pictures of A and B, C, D and bar = 1 cm for E and F.
4.2. Bio-remediation of nNiO by algae

4.2.1. Flocculation and settlement

As Fig. 4 showed, algal cells accelerated NPs aggregation and might have reduced the toxicity to free cells in solution, which is in accordance with the decline of IR after 120 h high-concentration nNiO exposure in this study (Fig. 1). Actually, the phenomenon of aggregation has been reported elsewhere for uncoated, non-modified oxide nanoparticles, for instance, ZnO (Adams et al., 2006), TiO₂ (Lovern and Kapler, 2006) and CeO₂ (Limbach et al., 2005), which is considered to be their inherent property. Generally, the ionic strength, the solution pH and organic matter as well as the surface charge of NPs play key roles in the aggregation behavior of nanoparticles. In this study, the presence of algae as well as their secretion increased the organic matter of aquatic systems. In addition, relative higher pH (varied from 8.2 to 8.5) of seawater also accelerated the aggregation of NPs. Navarro et al. (2008b) thought organic matter might influence the surface speciation and charge of NPs and thus affect their aggregation/deposition properties. Therefore, the interactions between NPs and organic matter may determine the NPs’s fate in aquatic systems, which will undoubtedly influence their bioavailability in solution. In this regard, algae may promote sedimentation of NPs into the environment and are likely candidates for bio-remediation of nano-pollution. The ability of algae to detoxify the NPs should be considered when evaluating nanomaterial’s potential risks in aquatic ecosystem. By controlling parameters of the suspension, such as temperature, ionic strength, pH, as well as the surface charge of NPs, the deposition/aggregation process of NPs may be regulated, which may provide the potential guidance to remediation of nano-pollutants in aquatic systems.

4.2.2. Bioreduction of nNiO

Bioreduction of nNiO to nNi may be the other reason for the decrease in IR on algal growth after 120 h high-concentration nNiO exposure. According to the classification of The International Agency for Research on Cancer (IARC, http://www.iarc.fr), nickel compounds (including NiO) are listed as one of 95 carcinogens to humans (group 1) (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans) and are harmful to aquatic organisms (International Chemical Safety Cards), while metallic nickel is considered as possible carcinogen to humans (group 2B) (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans) and it is less toxic than NiO. Therefore, the reduction from nNiO to nNi may lead to weakened toxicity. This opens up new research areas for bio-remediation of nano-pollution. This is the first study demonstrating that the zero valence metal is reduced from its metal oxide nanoparticles in algal cultures. It is believed that the enzymes of the organisms play an important role in the reduction (Wiesner et al., 2006). The coincidence of the maximum soluble protein content and the maximum ratio of reduction of Ni presented here (at 72 h), suggest that proteins may be the important factor in that process. Further studies are needed.

5. Conclusion

In summary, through the use of algal growth inhibition assay, physiological and morphological observations as well as XRD and XPS measurements, we have shown that NiO nanoparticles have adverse effects on growth of algal cells. At the same time, living algae have the ability to accelerate the aggregation of NPs as well as to reduce NiO nanoparticles to zero valence nickel. These results indicate that the green algae may be promising organisms for bio-remediating nano-pollution.

![Fig. 5. The XRD spectra of NiO nanoparticles before (a) and after (b) interact with algal cells. The red circles represent the diffraction peaks of Ni.](image)

![Fig. 6. Time course of changes in soluble protein content of C. vulgaris, the bioreduction rate and absorption rate when exposed to 15 mg L⁻¹ NiO nanoparticles. The bioreduction rate (%) was calculated by the XPS data of area CPS (eV) of Nickel 2p. The absorption rate (%) was calculated by the data of ICP-MS. Percent variation of protein content and the ratio of Ni⁰/Ni were expressed as mean ± standard deviation (SD) of two replicated cultures.](image)
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Appendix A. Supplementary data


References


