Method Article

Methods of determining titanium dioxide nanoparticles enhance inorganic arsenic bioavailability and methylation in two freshwater algae species

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**Abstract**

We developed the effect of titanium dioxide nanoparticles (nano-TiO\textsubscript{2}) on the bioaccumulation and biotransformation of arsenic (As), which remain largely unknown. We thus exposed two freshwater algae (\textit{Microcystis aeruginosa} and \textit{Scenedesmus obliquus}) to inorganic As with the aim of increasing our understanding on As bioaccumulation and methylation in the presence of nano-TiO\textsubscript{2}. Direct evidence of TEM and EDX image showed that nano-TiO\textsubscript{2} (anatase) entered the exposed algae. Thus, nano-TiO\textsubscript{2} as carriers boosted arsenic accumulation and methylation in these two algae species, which varied with both inorganic As speciation and algae species. Specifically, nano-TiO\textsubscript{2} could enhance markedly arsenate accumulation in \textit{M. aeruginosa} and arsenite accumulation in \textit{S. obliquus}. Similarly, we found higher content of As methylation in \textit{M. aeruginosa} of arsenite with 2 mg L\textsuperscript{-1} of nano-TiO\textsubscript{2} treatment and in \textit{S. obliquus} of arsenate treatment. Additionally, \textit{S. obliquus} exhibited higher As methylation compared to \textit{M. aeruginosa}, being more sensitive to As associated with nano-TiO\textsubscript{2} than \textit{M. aeruginosa}. Due to changes in pH levels inside these exposed algae, the As dissociation from nano-TiO\textsubscript{2} inside algal cell enhanced As methylation. Accordingly, the potential influence of nanoparticles on the bioaccumulation and biotransformation of their co-contaminants deserves more attention.

- Nano-TiO\textsubscript{2} entry is assumed to promote As accumulation into exposed algae.
- Nano-TiO\textsubscript{2} had different carrying capacities for different forms of As and algae.
- As dissociation from nano-TiO\textsubscript{2} is assumed to enhance As methylation in algae.

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Method

Nano-TiO₂ and arsenic preparation

We used the anatase form of nano-TiO₂ purchased from the Sigma-Aldrich Corporation with a particle size of less than 25 nm and a purity of > 99.7%. A nano-TiO₂ stock suspension (1 g L⁻¹) was prepared by first suspending nanoparticles in ultrapure water. We then sonicated the solution at 33 W for 30 min. The average hydrodynamic size of nano-TiO₂ was 193 ± 10 nm, as measured by the dynamic light scattering technique (DLS, Malvern Instruments, UK) at automatic attenuator mode. Experimental nano-TiO₂ concentrations of 100 μg L⁻¹ and 2 mg L⁻¹ were diluted from the stock suspension. The aggregate morphology of nano-TiO₂ was observed by a scanning electron microscope (SEM, S-4800, Hitachi, Japan). We used Na₃AsO₄·12H₂O and Na₂AsO₃ to prepare As stock solutions at 1 mM, which were stored at 4 °C in the dark until further use. Additionally, we measured the average hydrodynamic diameter (dₜₜ) and zeta potential (ζ) using DLS, and pH levels of nano-TiO₂ in BG-11 culture media with As(III) and As(V) of 10 μM at 0.1 mg L⁻¹ and 2 mg L⁻¹ TiO₂ concentrations.

Exposed algae species

The two freshwater alga species (S. Obliquus and M. Aeruginosa) used in our experiments were inoculated under sterile conditions in BG-11 media in Erlenmeyer flasks at 25 °C. The light-dark cycle used was 16:8 with a light intensity of 115 μmol photons m⁻² s⁻¹. For the following experiments, exposed algae were shaken at 100 rpm using a shaker to avoid settling.

Algae toxicity and stress

Toxicity of As(III) and As(V) was determined using 96 h growth rate bioassays. Algal cell at the exponential growth phase were added separately into a final concentration of As(III) (10 μM) and As (V) (10 μM) under increasing nano-TiO₂ levels (from 0 to 200 mg L⁻¹). The initial cell density of exposed algae was 10⁶ cells mL⁻¹. We used a hemocytometer and a microscope to measure algal cell density every 24 h until the end of the exposure experiment. The specific growth rate (µ) of cells was thus calculated according to the method reported by Zeng et al. [1]. Afterwards, the 96 h EC50 was calculated based on µ values of tested algal cells using a probability unit graphical method [2].
We examined alga stress from nano-TiO₂ associated with 10 μM of As(III) or As(V). Final concentrations of nano-TiO₂ were 0 mg L⁻¹ (control), 0.1 mg L⁻¹, and 2 mg L⁻¹. Cells at the exponential growth phase were added individually to As(V) and As(III) under the three separate aforementioned nano-TiO₂ concentrations. The initial cell density was 1 × 10⁶ cells mL⁻¹. At the same time, we conducted parallel experiments with final nano-TiO₂ concentrations of 0 mg L⁻¹, 0.1 mg L⁻¹, and 2 mg L⁻¹ (without the addition of As). We conducted Chl-a quantification after 96 h exposure. Additionally, we measured methane dicarboxylic aldehyde (MDA) to indicate the degree of lipid peroxidation (LPO) in this experiment, representing alga stress from As associated with nano-TiO₂. We detected MDA content using the thiobarbituric acid reactive substances (TBARS) method by applying a reagent kit (the Nanjing Jiancheng Biotechnology Institute, China) according to the manufacturer’s instructions [3].

Furthermore, we characterized nano-TiO₂ in algae and the culture media after both 0.1 and 2 mg/L of nano-TiO₂ exposure associated with 10 μM of As(III) and As(V) using TEM as well as energy-dispersive X-ray spectroscopy (TEM, H-7650, Hitachi, Japan; EDX, Genesis XM2) [4]. In brief, we fixed exposed algal cells using 2.5% glutaraldehyde and then refrigerated them for 12 h. Afterwards, the treated algal cells were washed thrice in a 0.1 M phosphate buffer (pH 7.0), then postfixed in 1% osmium tetroxide for 1 h, dehydrated through a graded series of ethanol (30%, 50%, 70%, 90%, 95%, and 100%) and embedded for 12 h. The resultant algal cells were incised using a diamond blade in an ultramicrotome (Leica UC7, Germany) to obtain cell ultrathin sections (approximately 70 nm in thickness) for TEM and EDX observations without staining. Direct evidence of TEM image and EDX observations was provided in Fig. 1.

![Fig. 1. Morphology of titanium dioxide nanoparticles in algae and culture media. Note: Arrows point to nano-TiO₂.](image-url)
Dissociation of inorganic As from nano-TiO₂ in algae cell homogenates

Inorganic arsenic dissociation in algal cell homogenates

In order to identify whether As dissociated from nano-TiO₂ inside algal cells, we conducted desorption experiments to detect the potential dissociation of inorganic As from nano-TiO₂ in algal cell homogenates, which followed the flowchart provided in Fig. 2. This information could provide insight into oxidative stress of As entry into algal cells facilitated by the presence of nano-TiO₂. In this experiment, the two algal species were selected and cultured to a cell density of 3 × 10⁷ cells mL⁻¹. We collected an algal pellet in a 50 mL centrifuge tube using a high-speed centrifuge (9000g) for 5 min. BG-11 was then added into the tube containing the alga pellet, and the tube was shaken to re-suspend the species. After 3 min of disruption using an ultrasonic cell disruption system, the algal cell homogenate was separated at a centrifugal force of 9000g for 5 min to remove algae residue, such as cell wall material. Finally, the algal cell homogenate was diluted with BG-11 to 100 mL for further usage. At the same time, we measured algal cell homogenate pH levels and total organic carbon (TOC) to understand the potential mechanisms of the dissociation.

Our preliminary results showed that As adsorption on nano-TiO₂ rapidly reached equilibrium (within 1 h) in the BG-11 medium. In turn, the solution of As associated nano-TiO₂ was prepared 1 h before use to make equilibrated adsorption of As(Ⅴ) and As(Ⅲ) onto nano-TiO₂. The final concentration of nano-TiO₂ and As was 2 mg L⁻¹ and 10 μM, respectively, diluted from their stock solutions with BG-11 for As adsorption onto nano-TiO₂ experiment. After mixing As and nano-TiO₂ for 1 h, the solution was centrifuged for 10 min at 12 000g to obtain the sediment of nano-TiO₂ associated with As by removing the supernatant. We then added 40 mL of the algal cell homogenate and BG-11 (used as the control) into the abovementioned sediment, respectively, to conduct the As desorption test in the algal cell homogenate. After shaking for 2 h at 180 rpm min⁻¹ in a shaker, we obtained the supernatant using a high-speed centrifuge (12 000g). We used 1 mL of the supernatant to measure As concentrations using inductively coupled plasma mass spectrometry (ICP-MS 7500a, Agilent). The difference in the final As

![Flowchart](image-url)

Fig. 2. Flowchart of desorption of inorganic As from nano-TiO₂ in algal cell homogenates.
concentration between BG-11 and the algal cell homogenate was the apparent As desorbed from nano-TiO$_2$, caused by the algal cell homogenate. Three replicates were prepared for this test.

**Inorganic arsenic deprecation from algae**

On the other hand, we examined the inorganic As deprecation from the algae species to further identify whether the dissociation of As from nano-TiO$_2$ inside algal cells occurred. The two selected alga species, with an initial cell density of 10$^6$ cells mL$^{-1}$, were pre-exposed to As separately for 96 h in the presence of nano-TiO$_2$, from which the final exposure concentrations were 1 µM As(V) and 0.8 µM As(III) in the presence of 20 mg L$^{-1}$ nano-TiO$_2$ for *M. aeruginosa* and 0.1 µM As(V) and 0.08 µM As(III) in the presence of 2 mg L$^{-1}$ nano-TiO$_2$ for *S. obliquus*. Under these exposure concentrations, algae did not exhibit any toxic effects. Moreover, As(III) and As(V) can absorb onto nano-TiO$_2$ by 98.91% ± 0.91% and 98.23% ± 0.87%, respectively. Thus, the As accumulated in exposed algae was largely associated with nano-TiO$_2$. The As solution was prepared by the same method described above, that is, it was prepared 1 h before use to equilibrate As(V) and As(III) adsorption on nano-TiO$_2$. We then individually centrifuged each pre-exposed algae species into a pellet at 3800g for 10 min. Afterwards, we used sterile ultrapure water (18.2 mΩ cm$^{-2}$) to twice wash each algal pellet before immersing each in an ice-cold phosphate buffer (1 mM K$_2$HPO$_4$, 5 mM MES, and 0.5 mM Ca(NO$_3$)$_2$) for 15 min to completely remove apoplastic As [5]. The collected pellets were then individually re-suspended in BG-11 media. After 12 h, 5 mL of supernatant was collected at 3800 for 10 min to measure total As and titanium (Ti) in media using ICP-MS [6,7]. At the same time, the desorption of As from nanoparticles in the absence of algae was comparably performed under the same treated conditions with the exception of using 12 000g centrifugation for 10 min, which further confirmed the deprecation of As from algal pellets. Moreover, we did not find any As desorbed from nano-TiO$_2$. Thus, the amount of total As in the supernatant was the As deprecation from the algae.

**Inorganic arsenic accumulation and methylation in the exposed algae**

An initial cell density of 10$^6$ cells mL$^{-1}$ was diluted from cell inoculum during the exponential growth phase. After being inoculated with *M. aeruginosa* or *S. obliquus*, each culture was treated immediately and separately with As(V) and As(III) at 10 µM and the three different concentrations of nano-TiO$_2$ (0 mg L$^{-1}$, 0.1 mg L$^{-1}$, and 2 mg L$^{-1}$, respectively) throughout a 4 d uptake period to evaluate the effects of As bioaccumulation and methylation affected by the presence of nano-TiO$_2$. Cultures devoid of nano-TiO$_2$ (0 mg L$^{-1}$) were used as controls. Each As(III) treatment was then monitored every 24 h by sampling 5 mL of the aliquot test solution until the end of experiment to detect whether As(III) oxidized into As(V). Finally, we individually collected two algal solutions of 25 mL each from separate flasks for all treatments, after which we centrifuged them into a pellet at 3800g for 10 min. Afterwards, two 5 mL supernatants were individually collected to measure total As and As speciation in the media, respectively, to calculate the bioconcentration factor (BCF) and to quantify As(III) oxidation in tested suspensions. Furthermore, before being immersed in the aforementioned ice-cold phosphate buffer for 15 min to completely remove apoplastic As, both algal pellets were washed twice using sterile ultrapure water (18.2 mΩ/cm$^2$) [5]. Afterwards, the two algal pellets were respectively digested after one pellet was dried under warm conditions at 60 °C in an oven while the other was freeze-dried in a vacuum freeze dryer. At this point, total intracellular As was measured to determine As bioaccumulation using the oven-dried algae samples, while As speciation was used to determine As methylation using the freeze-dried algae samples, including inorganic As, As(V), monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) [8]. The methylated amount of As inside algae was calculated as the sum of MMA and DMA in the exposed algae.

**Arsenic determination in algae and medium**

We determined total As and As speciation according to the previous methods used for algal sample preparation and As analysis [9]. In brief, approximately 0.02 g of oven-dried algal samples were treated overnight by means of microwave assisted digestion. Afterwards, samples were further diluted to measure As using ICP-MS. We had a good recovery rate (92.3% ± 5.6%) using a standard reference sample (GBW08521, the National Research Center for Standard Materials of China). Additionally, approximately 0.02 g of the freeze-dried algal samples was treated overnight. After microwave assisted digestion,
samples were filtered using 0.45 μm filters. We then used HPLC-ICP-MS (Agilent LC1100 series coupled with the Agilent ICP-MS 7500a) to measure As speciation in algal extracts and media.

**Calculation and statistics**

The measured As bioaccumulation in algae included free As in algal cells and its association with nanoparticles on cell surface and internalized cells. We then used the dry weight of BCF to estimate As bioavailability affected by the presence of nano-TiO₂, which was calculated using the following equation:

\[
\text{BCF} = \frac{\text{the } \text{As concentration in algae (µg/g dry weight)}}{\text{the As concentration in media (which includes the As adsorbed on nanoparticles; } \mu\text{g }\text{L}^{-1})} \times 1000 \ (1)
\]

Moreover, SPSS 12.0 was used to perform statistical analysis on the data. The data are shown as means with standard deviations (SD). The differences within treated groups were evaluated by two-way analysis of variance (ANOVA) with a least significant difference (LSD) range test at \(P<0.05\) significant levels.

**Conclusion**

Nano-TiO₂ promoted the accumulation and methylation of inorganic As in the two selected freshwater algae species investigated in this study. Evidence showed that nano-TiO₂ promotes As accumulation into exposed algae species. The dissociation of inorganic As from nano-TiO₂ contributes to its increased toxic effects on exposed algae, which be demonstrated by Chl-a and MDA. Subsequently, stress levels increased as a result of this As and nano-TiO₂ dissociation, thus potentially leading to greater As methylation in algae species. It is clear that As metabolism is variable between As forms and algae species as well as nanoparticles and environmental factors. Furthermore, As contamination is globally recognized as being high risk.

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