

Impacts of Sediment Organic Matter Content and pH on Ecotoxicity of Coexposure of TiO₂ Nanoparticles and Cadmium to Freshwater Snails *Bellamya aeruginosa*

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Abstract The environmental factors are expected to affect the ecotoxicity of heavy metals in the presence of engineered nanoparticles (NPs) in aquatic ecosystems. However, in sediment scenario, little is known regarding their impacts on the ecotoxicity of co-exposure of sediment-associated heavy metals and NPs. This study evaluated the impacts of different levels of organic matter (OM) (4.8–11.6%) and pH (6–9) on the ecotoxicological effects of co-exposure of sediment-associated titanium dioxide nanoparticles (TiO₂-NPs) and cadmium (Cd) to a freshwater gastropod *Bellamya aeruginosa*. The burdens of Ti and Cd and biomarkers of DNA damage, Na⁺/K⁺-ATPase, lipid peroxidation (LPO), and protein carbonylation (PC) in the hepatopancreas were determined following 21 days of exposure. At background level of OM (4.8%) in sediments, TiO₂-NPs significantly promoted Cd accumulation in low-Cd treatments (5 mg/kg) but did not promote Cd accumulation in high-Cd treatments (25 mg/kg). At the relatively higher OM levels (7.1 and 11.6%), TiO₂-NPs significantly enhanced Cd accumulation and toxicity as evidenced by aggravated DNA damage, decreased Na⁺/K⁺-ATPase activities, and increased LPO and PC levels. Moreover, Cd burdens in both low-Cd and high-Cd treatment were positively correlated with corresponding Ti burdens, indicating TiO₂-NPs partially acted as carrier of Cd. At all pH levels, in low-Cd treatments, TiO₂-NPs did not affect Cd accumulation, LPO, and PC levels but significantly enhanced DNA damage and slightly facilitated the inhibition of Na⁺/K⁺-ATPase activities. In high-Cd

treatments, only at pH 9, TiO₂-NPs significantly enhanced Cd accumulation and toxicity. Our results implied that interaction between TiO₂-NPs and OM or pH significantly affected the accumulation and toxicity of Cd in *B. aeruginosa*, but the underlying mechanisms need further investigation. Additionally, it should be noted that the potential ecological risk of co-exposure of NPs and coexisting pollutants might be closely species-specific and related to environmental media.

Due to their unique reactivity and optical and electromagnetic properties, engineered nanoparticles (NPs) have been extensively used in scientific research, industrial applications, consumer products, health care technology, and environmental protection (Stone et al. 2010) and thus increasingly find their way into aquatic environments (Petersen et al. 2011), posing a potential risk to aquatic ecosystem (Scown et al. 2014). Research of potential ecological risk of NPs is increasingly prevalent in the ecotoxicological field (Savolainen et al. 2010). Although the majority of NPs have been recognized as emerging toxic contaminants to aquatic organisms, the potential ecological risks of NPs also may be related to the complicated interactions between NPs and coexistent pollutants (Vale et al. 2014), and environmental factors might play an important role in these interactions (Christian et al. 2008).

Among all kinds of NPs, titanium dioxide nanoparticles (TiO₂-NPs) are one of the most extensively used nanomaterials (Vance et al. 2015). They are generally utilized as common additives to a variety of commercial products and are routinely found in sunscreens, coatings, cosmetics, pharmaceuticals, environmental catalysts, food colorants, plastics, soaps, nanofibers and nanowires, bandages, alloys, contact lenses, and textiles (Menard et al. 2011). TiO₂-NPs

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have been expected to accumulate rapidly in the aquatic environment during the next decade (Farre et al. 2009). Given their excellent affinity and sorption capacity to chemicals, once released into the aquatic environment, TiO₂-NPs may act as a carrier of many persistent toxic substances (PTS) (Hartmann et al. 2012). Some studies have shown that the presence of TiO₂-NPs may markedly alter the bioavailability and ecotoxicity of PTS to aquatic organisms (Sun et al. 2007; Tan et al. 2011; Zhang et al. 2007; Yang et al. 2014). It has been demonstrated that the sorption capacity of TiO₂-NPs to PTS in water is governed by nanoparticle characteristics and surrounding environmental factors, such as pH, natural organic matter (NOM) content, ionic strength, and electrolyte valence (Domingos et al. 2009; Praetorius et al. 2014). Therefore, environmental factors might significantly affect ecotoxicity of coexisting PTS via governing interactions between NPs and PTS.

So far, studies on the effects of environmental factors on the ecotoxicity of coexposure to NPs and PTS are scarce. Only Rosenfeldt et al. (2015) recently indicated that toxicity of copper in the water to *Daphnia magna* was reduced in the presence of TiO₂-NPs and organic material. However, little is known regarding such impact in the case of sediments. Aquatic organisms in the water may only be affected if the loading of NPs and PTS is frequent or continuous. It is well-known that sediments are an ultimate depository of NPs and PTS, thus benthic organisms may be at higher risk of exposure. *Bellamya aeruginosa* is a freshwater benthic gastropod and is quite common and abundant throughout Chinese freshwater ecosystems. Its wide distribution in aquatic ecosystems, convenient culturing in the laboratory (Ma et al. 2010), and relatively high sensitivity to different kinds of pollutants make *B. aeruginosa* an ideal test species for sediment toxicity assessment (Ma et al. 2009; Zheng et al. 2013). Therefore, the application of *B. aeruginosa* to assess the ecotoxicity of coexposure to NPs and PTS will be a practical choice.

Generally, molecular biomarkers are widely used to test the subacute toxicity of vast quantities of pollutants and might provide insights into the potentially toxic mechanism. In previous studies, a battery of biomarkers was frequently used to assess NPs toxicity on invertebrate species. For example, Hu et al. (2010) used a set of oxidative stress biomarkers, such as superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA), as well as DNA damage to evaluate toxicities of TiO₂ and ZnO nanoparticles to the earthworm *Eisenia fetida*. Likewise, Tsyusko et al. (2012) investigated the toxicity of silver nanoparticle on *E. fetida* by determination of a series of biomarkers of biochemical and genetic alterations, such as stress response genes expression, heat shock protein 70 (HSP70), CAT, glutathione *S*-transferase (GST), and

protein carbonylation (PC). It is well-known that cadmium (Cd) is a nondegradable cumulative pollutant in aquatic ecosystems. Many studies showed that Cd stress could cause oxidative damage (Maria et al. 2014), DNA damage (Nigro et al. 2015), and the inhibition of Na⁺/K⁺-ATPase activities (Lionetto et al. 1998) in aquatic animals.

In the present study, we examined biomarkers of genotoxic effects (DNA damage), Na⁺/K⁺-ATPase activities, and oxidative stress (MDA, and PC) of sediment-associated TiO₂-NPs and Cd, in single and combined 21-day exposure under different levels of organic matter (OM) and pH in the hepatopancreas of *B. aeruginosa*. The burdens of Ti and Cd in the hepatopancreas were correspondingly determined. The purpose was to investigate the effects of sediment OM and pH levels on ecotoxicity of Cd in the presence of nontoxic concentration of TiO₂-NPs.

Materials and Methods

Test Species

B. aeruginosa from stock cultures in our laboratory was used as test organism. They were originally collected from natural populations in Wuhan botanic garden (Wuhan, China) and successfully reared under standard laboratory conditions for several generations (Ma et al. 2010). Snails were fed ad libitum with commercial fish food (Sanyuan[®], Beijing, China) and kept at a water temperature of 24 ± 1 °C and a light:dark photoperiod regime of 16:8 h. Toxicity tests were initiated with adult snails (1 year old, average shell length 20.82 ± 1.32 mm, average body weight 2.24 ± 0.39 g) from an age-synchronized culture. Only apparently healthy snails were used. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from National Institutes Health and approved by the Ethical Committee of Jishou University.

Chemicals and Characterization

The pristine TiO₂-NP powder (advertised particle size, 10 nm; 99.9% purity) was purchased from Boyu Gaoke New Materials Co., Ltd. (Beijing, China) and characterized prior to the toxicity test. The average particle size was found by TEM (H-7500, HITACHI, Japan) to be 11.6 ± 2.4 nm; a Brunauer–Emmett–Teller (BET) surface area is 140.3 m²/g, and the crystal phase is anatase (>99%). Cd(CH₃COO)₂·2H₂O (99.99% purity) and humic acid (90% purity) were obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). The commercial reagent kits for determining the activities or contents of Na⁺/K⁺-ATPase, MDA, PC, and protein were supplied by Nanjing

Jiancheng Bioengineering Institute (Nanjing, China). Unless otherwise specified, all other reagents and chemicals were of analytical grade and also were purchased from Aladdin Reagent Co., Ltd.

Preparation of Test Sediments

Sediments were collected from Dehang Nature Reserve (28°10'35.89"N, 109°38'23.69", western Hunan Province, China). Wet sediments were passed through 80-mesh nylon sieve to remove coarse particles and allowed to settle thoroughly. Finally, the overlying water was decanted. The resulting sediment samples were oven-dried at 80 °C overnight to eliminate undesired fauna and then homogenized. These sediment samples were stored at 4 °C for subsequent experiments. The properties of this sediment can be summarized as follows: grain size composition of 72% sand, 11% silt, and 17% clay (on a weight basis), pH of 8.0, total organic carbon of 4.8% (w/w, on dry weight basis), total nitrogen of 1064 mg/kg, total phosphorus of 1215 mg/kg, and a relatively low background concentrations of heavy metals (with 58.62, 32.13, 16.22, 111.43, 0.31, and 25.32 mg/kg of Cr, Ni, Cu, Zn, Cd, and Pb, respectively), which were below the threshold effect concentrations of sediment quality guidelines for freshwater ecosystems (MacDonald et al. 2000).

Experimental Set-Up

The present study consists of two independent experiments which involved the effects of different levels of OM (experiment 1) and pH conditions (experiment 2) on co-exposure of TiO₂-NPs and Cd to *B. aeruginosa*. Experiment 1 contained OM background level (4.8%) and two actual amended OM levels (7.1 and 11.6% on dry weight basis). Experiment 2 contained pH background level (8.0) and two given pH levels (6.0 and 9.0, adjusted using 2 mol/L HCl or NaOH). Based on the environmentally relevant levels of Cd in Chinese freshwater sediments (Zhu et al. 2010) and our previous studies for *B. aeruginosa* (Ma et al. 2009), two nominal concentrations of Cd (5 and 25 mg/kg, added as Cd(CH₃COO)₂·2H₂O) were tested. Actual measured concentrations of Cd in the sediments by ICP-OES were 5.26 ± 0.3 and 25.87 ± 0.6 mg/kg (the background level of Cd is 0.31 g/kg), respectively. Only one nontoxic concentration (1 g/kg dry sediment) of TiO₂-NPs was tested based on a previous study under similar exposure scenarios in freshwater snail *Physa acuta* (Musee et al. 2010). Actual measured concentrations of Ti in sediments by ICP-OES was 3.22 ± 0.12 g/kg (the background level of Ti is 2.69 g/kg). The exposure concentrations of Cd and TiO₂-NPs were reported as nominal concentrations. According to

our experiment design, there were a total of 18 treatments in each experiment. The experiments were conducted in triplicate per treatment.

Sediment Spiking

For experiment 1, based on 4.8% of background OM level in the dry sediments, humic acids were introduced into batches of dry sediments to make two final amended OM concentrations of 7.1 and 11.6%, which were determined with potassium dichromate oxidation method. Briefly, a total of 500 g of dry sediments were used in each treatment. Humic acids were fully mixed with dry sediments in an agitator for at least 1 h. Then, TiO₂-NPs (1 g/kg dry sediment) powders for each treatment were added and continuously mixed for at least 1 h. These sediments were stirred by adding Milli-Q water (1: 1, v/v) in the 4-L test chamber to obtain wet slurry sediments and allowed to settle for 72 h at 20 °C. A series of Cd spiking solutions were prepared from dilutions of Cd stock solution (2 g/L, as pure Cd) and diluted to the volume of resulting wet sediments with Milli-Q water, such solutions were added to the sediments followed by manually stirring with a wooden spoon for at least 24 h to achieve a uniform distribution of spiked toxicants throughout sediments, and then kept in closed chamber at 20 °C for 14 days to ensure chemical equilibrium between the sediments and water (Simpson et al. 2004). For experiment 2, according to the above-mentioned method, TiO₂-NPs were first added, and then a series of Cd spiking solutions were spiked into the sediments. The pH of sediments was adjusted using 0.1 mmol/L of HCl or NaOH until a steady state. The sediments also were subjected to chemically equilibrate at 20 °C for 14 days. The control groups were treated in the same way.

Chronic Sediment Toxicity Tests

Two experiments were conducted simultaneously. Based on our previous studies with *B. aeruginosa* (Ma et al. 2010), 21-day sediment toxicity bioassays were performed to assess the chronic effects and metals accumulation in the hepatopancreas. The overlying water (Milli-Q water) was gently added overtop the sediment of each test chamber. Chambers were then placed into a water-bath tank and allowed to settle for 72 h before introduction of test organisms. Twelve selected snails were added to each test chamber in a size-stratified random order. Each treatment consisted of three chambers (36 organisms). The exposures were conducted under static conditions with aeration. The test chambers were kept at 24 ± 1 °C and a light/dark regime of 16/8 h. Each test chamber was covered with nylon mesh with a small hole. Test organisms were fed throughout the experiment with commercial fish food

(5 mg/snail/day, Sanyuan[®], Beijing, China) (Ma et al. 2010) every 2 days. Water quality, including dissolved oxygen, temperature, ammonia, and pH, was checked every 2 days. The survival rate of test organisms in each chamber was above 90% during the exposure period. After 21-day exposure, test organisms were recovered from sediments and rinsed with Milli-Q water. Three randomly selected organisms per chamber were immediately used for the determination of DNA damage. The remaining organisms were snap-frozen in liquid nitrogen, and then the hepatopancreas were carefully dissected from visceral mass, and stored at $-80\text{ }^{\circ}\text{C}$ until further assay.

Quantification of Cd and Ti in the Hepatopancreas

The hepatopancreas samples were digested with HNO_3 – HClO_4 method. The hepatopancreas samples were dried at $80\text{ }^{\circ}\text{C}$ to constant weight and then digested with 3 mL of concentrated HNO_3 plus 2 mL of HClO_4 in acid-washed Teflon tubes for 5 min, followed by heating on the electric heating plate at $200\text{ }^{\circ}\text{C}$ until completion of digestion. For Cd determination, the contents of digestion tubes were diluted to 10 mL of 1% HNO_3 and filtered through a syringe-driven filter ($0.45\text{ }\mu\text{m}$). Similar weight samples of the certified reference material GBW08571 (mussel, Beijing Century Aoke Biotech Co., Ltd., China) also were analyzed followed by the same digestion procedure to ensure the accuracy of analytical procedure and the measured values were found within in the certified range. For Ti determination, the above-digested solutions were further digested with 5 mL of the sulphuric acid–ammonium sulphate. Subsequent quality control (QC) measures were performed as described by Sun et al. (2007). Cd and Ti contents were in the same way determined with ICP-OES. Cd and Ti contents in different samples were calculated based on the dry weight.

Biochemical Analysis

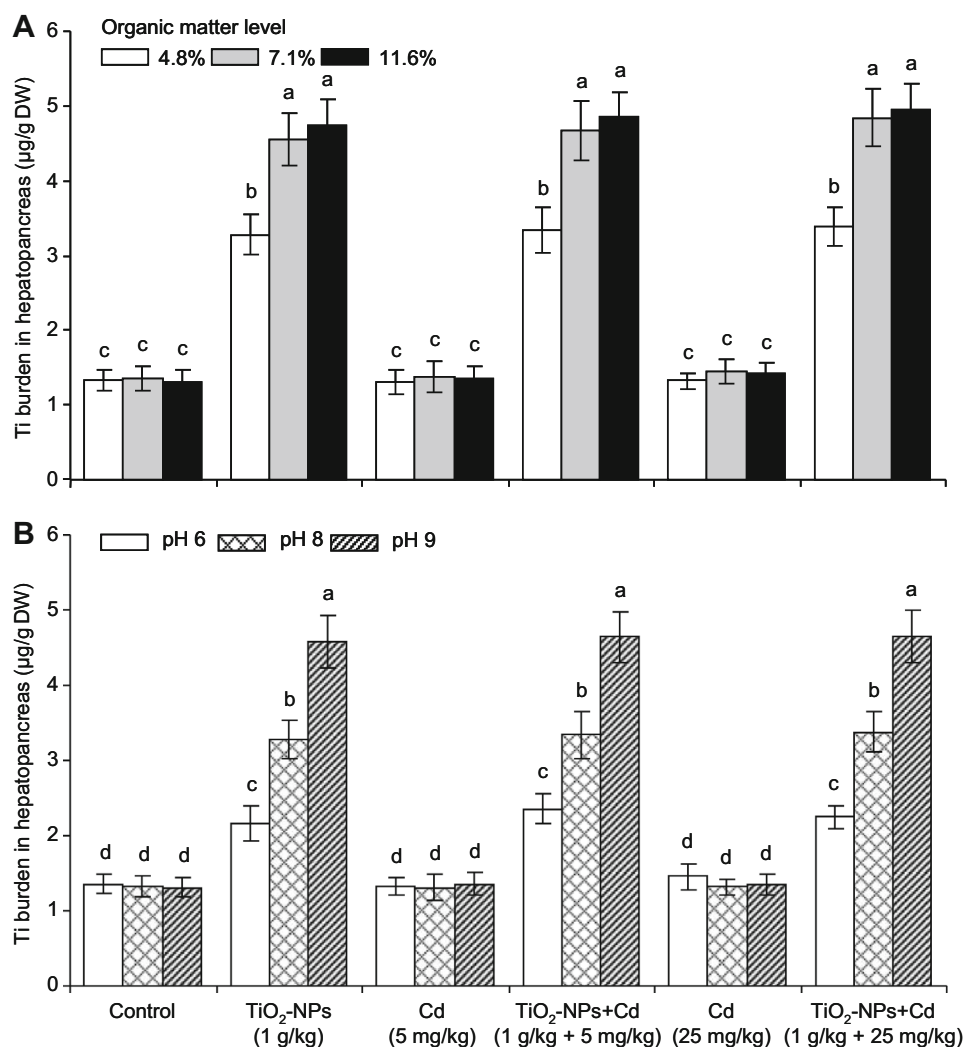
The frozen hepatopancreas samples were homogenized in glass homogenizer under ice-cold conditions by adding Tris–HCl buffer (1: 9, w/v, 0.01 mol/L, pH 7.4, containing 0.0001 mol/L of EDTA-2Na, 0.01 mol/L of sucrose, and 0.8% of NaCl) and a small amount of protease inhibitor (0.001 mmol/L of phenylmethylsulfonyl fluoride). The homogenates were centrifuged at $1000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. Half of the supernatant was collected for determination of Na^+/K^+ -ATPase activities and protein carbonyl levels. The remaining content was continuously centrifuged at $9000\times g$ for 20 min at $4\text{ }^{\circ}\text{C}$, and the resulting supernatant was collected to measure MDA levels. The supernatant samples were stored at $-80\text{ }^{\circ}\text{C}$ before analysis. Na^+/K^+ -ATPase activities were measured using

phosphorus molybdenum blue colorimetric methods at 636 nm according to the instructions of the commercial assay kit (Jiangsu, China). One unit (U) of Na^+/K^+ -ATPase activity was defined as 1 μmol phosphorus generated from ATP catalyzed by Na^+/K^+ -ATPase in 1 mg protein per hour. The Na^+/K^+ -ATPase activity was expressed as U/mg protein. Lipid peroxidation (LPO) products measured as malondialdehyde (MDA) contents were determined at 532 nm using the thiobarbituric acid (TBA) method followed the instructions of the commercial assay kit (Jiangsu, China), and MDA contents were expressed as nmol/mg protein. Protein carbonyl (PC) levels were measured at 370 nm using 2, 4-dinitrophenylhydrazine (2, 4-DNPH) method followed the instructions of the commercial assay kit (Jiangsu, China), and expressed as nmol carbonyl groups per mg protein. The protein contents in the supernatant from hepatopancreas homogenates were quantified using protein assay kit (Jiangsu, China) with the Bradford protein assay method (Bradford 1976).

Determination of DNA Damage

The DNA damage of hepatopancreas cells was evaluated by alkaline comet assay based on the methods of Singh et al. (1988) and Xu et al. (2013) with some modifications. Briefly, freshly isolated hepatopancreas was rinsed with 500 μL of ice-cold phosphate buffered saline (PBS), and then cut into pieces thoroughly in a 1.5-mL centrifugal tube, 1 mL of PBS was added, and blew with micropipette to suspend cells, kept at temperature for 3 min. The resulting cell suspension was centrifuged at $500\times g$ for 2 min and the supernatant was discarded. The cells were washed and resuspended in ice-cold PBS at 1×10^6 cells/mL. Because DNA damage is associated with cell death (Tice et al. 2000), before comet assay the cell viability was evaluated by trypan blue (0.4% w/v) exclusion techniques. In this study, cell viabilities all the treatments were more than 90% of that in the controls. After that, 30 μL of preliquified (at $75\text{ }^{\circ}\text{C}$) 0.5% normal melting agarose (NMA) was pipette onto the frosted side of a preheated (at $60\text{ }^{\circ}\text{C}$) slide and was spread evenly. After drying, the slide was slightly heated, and then 70 μL of preliquified NMA was pipette onto the slide and rapidly covered with a coverslip, kept at $4\text{ }^{\circ}\text{C}$ for 10 min, and afterwards, the coverslip was gently removed. The cell suspension (10 μL) was mixed with 90 μL of preliquified (at $37\text{ }^{\circ}\text{C}$) 0.7% low melting agarose (LMA). The mixture (75 μL) was pipette immediately onto above precoated slide and covered with a coverslip, kept at $4\text{ }^{\circ}\text{C}$ for gelation for 10 min, and then the coverslip was gently removed. The slide was immersed into ice-cold lysis buffer (2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA (pH 10.0), 1% Na-sarcosinate,

Fig. 1 Ti burden in the hepatopancreas of *Bellamya aeruginosa* following single and combined 21-day exposure to TiO₂ nanoparticles and cadmium under different levels of organic matter (a) and pH (b). Data are mean \pm standard deviation ($n = 3$). Different letters indicate significant differences between the values as obtained by ANOVA and post hoc LSD test ($p < 0.05$), where the letter “a” is assigned to the groups with highest values. Ti burden is expressed in a dry weight tissue basis



supplemented with 10% dimethylsulfoxide (DMSO), and 1% Triton X-100 for 90 min.

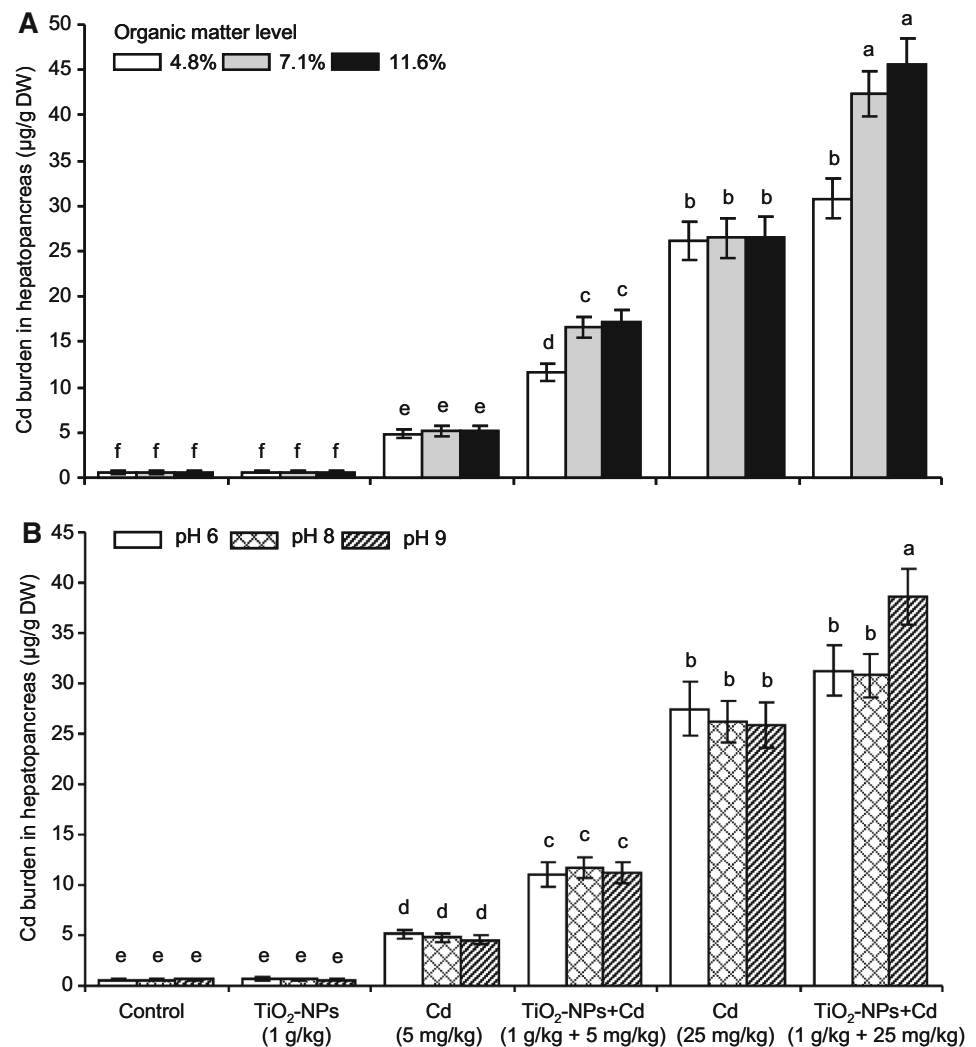
After being rinsed with PBS, the slide was placed in electrophoresis tank containing freshly prepared electrophoresis buffer (300 mM NaOH with 1 mM Na₂EDTA, pH >13) to unwind DNA for 30 min at 4 °C before electrophoresis for 25 min at 25 V (300 mA). The slide was then neutralized thrice with 0.4 mM Tris-HCl (pH 7.4) at 10-min intervals and stained with 20 µL of propidium iodide (2 µg/mL) for 10 min. All operations were conducted under red light to avoid light-induced damage. For each slide, 50 randomly selected cells were viewed using a fluorescence microscope (Nikon, ECLIPSE 80i, Japan) with an excitation filter of 515–560 nm, nonoverlapping cells were captured with a CCD camera at $\times 200$ magnification. Images were analyzed using the Comet Assay Software Project (CASP). The measurement of tail DNA content percentage (%TD) and olive tail moment (OTM) was used to assess DNA damage. OTM was defined as the product of the tail length and the fraction of total DNA in the tail and calculated as

$[(\text{tail mean} - \text{head mean}) \times (\text{tail DNA \%}/100)]$. Three slides were evaluated per treatment, and the averaged median values for OTM from each slide were calculated for each treatment group (Pourrut et al. 2011).

Statistical Analysis

Statistical analyses were accomplished using the IBM SPSS 20.0 statistical software package for windows (SPSS Inc., Chicago, IL). All values were expressed as means with the corresponding standard deviation (SD). Based on normality of data (Kolmogorov–Smirnov and Levene’s test), one-way analysis of variance (ANOVA) with the post hoc least significant difference (LSD) multiple comparison tests were conducted to assess differences between the treatments. Pearson product-moment correlation analysis was performed to examine the correlations between Ti burdens and Cd burdens in the hepatopancreas under co-exposure of TiO₂-NPs and Cd. Differences were considered statistically significant when $p < 0.05$.

Fig. 2 Cd burden in the hepatopancreas of *Bellamya aeruginosa* following single and combined 21-day exposure to TiO₂ nanoparticles and cadmium under different levels of organic matter (a) and pH (b). Data are mean \pm standard deviation ($n = 3$). Different letters indicate significant differences between the values as obtained by ANOVA and post hoc LSD test ($p < 0.05$), where the letter “a” is assigned to the groups with highest values. Cd burden is expressed in a dry weight tissue basis



Results

Ti and Cd Burden in Hepatopancreas

The addition of TiO₂-NPs into sediments significantly increased Ti burdens in the hepatopancreas of *B. aeruginosa* compared with the controls (Fig. 1). In the case of different levels of OM, similar change pattern of Ti burdens was found in different TiO₂-NPs treatments. Ti burdens under higher OM levels (7.1 and 11.6%) were significantly higher than that under low OM levels (4.8%) but not in OM level-dependent manner. In the case of different pH treatments, Ti burdens showed a distinct pH-dependent increase.

The Cd burden profiles in the hepatopancreas of *B. aeruginosa* are shown in Fig. 2. In the case of different levels of OM, in both low-Cd and high-Cd alone treatments, overall Cd burdens were significantly higher than that of the controls, but OM levels did not affect Cd burdens. In TiO₂-NPs and Cd co-exposure treatments, at low-

Cd concentration (5 mg/kg), Cd burdens were significantly promoted by TiO₂-NPs, resulting in more than three times as that of corresponding Cd alone treatments, and higher OM levels (7.1 and 11.6%) significantly facilitated Cd accumulation. At high-Cd concentration (25 mg/kg), in contrast with Cd alone treatments, under low OM level (4.8%), Cd burdens were not affected by TiO₂-NPs, but under high OM levels (7.1 and 11.6%), Cd burdens were significantly promoted by TiO₂-NPs, resulting in more than 1.5 times as that of corresponding Cd alone treatments. In the case of different pH treatments, in both low-Cd and high-Cd alone treatments, likewise, overall Cd burdens also were significantly higher than that of controls, and Cd burdens were not affected by pH levels. In TiO₂-NPs and Cd co-exposure treatments, at the low Cd concentration, Cd burdens were significantly elevated by TiO₂-NPs but were not in pH-dependent manner. At high Cd concentration, Cd burdens were not affected by pH of 6 and 8, but at pH of 9, Cd burdens were significantly elevated by TiO₂-NPs.

Pearson product-moment correlation analysis showed that, in the case of different levels of OM, Cd burdens in the hepatopancreas were positively correlated with corresponding Ti burdens ($r = 0.99$ with $p < 0.05$ for low-Cd treatment, $r = 0.97$ with $p = 0.046$ for high-Cd treatment). However, in case of different pH values, no significant correlation between Cd burdens and Ti burdens was found ($r = 0.307$ with $p = 0.401$ for low-Cd treatment, $r = 0.756$ with $p = 0.173$ for high-Cd treatment).

DNA Damage

The DNA damages of the hepatopancreas in *B. aeruginosa* by measuring comet %TD and OTM are illustrated in Figs. 3 and 4, respectively. It can clearly be seen that both %TD and OTM showed a similar change pattern following single and combined 21-day exposure to TiO₂ nanoparticles and cadmium at different levels of OM and pH, implying they have same indicating efficiency for DNA

damage. In the case of different levels of OM, low-Cd alone exposure (5 mg/kg) did not induce DNA damage, high-Cd alone exposure (25 mg/kg) significantly induced DNA damage by more than 30-fold increase of %TD and 20-fold increase of OTM compared with the control, respectively. In TiO₂-NPs and Cd co-exposure treatments, as OM levels increased, DNA damage by low-Cd were significantly enhanced in the presence of TiO₂-NPs compared with corresponding low-Cd alone exposure and reflected a OM-dependent manner. At high-Cd concentration and low OM level (4.8%), no significant increases of DNA damage were observed relative to corresponding high-Cd alone exposure; however, at high-Cd concentration and higher OM levels (7.1 and 11.6%), DNA damages were significantly enhanced in the presence of TiO₂-NPs, but there was no significant difference between these two OM level treatments. In the case of different pH, regardless of controls, TiO₂-NPs and Cd alone exposure or co-exposure, DNA damages at pH of 9 were significantly greater

Fig. 3 Tail DNA percentage (%TD) from comet assay of the hepatopancreas in *Bellamya aeruginosa* following single and combined 21-day exposure to TiO₂ nanoparticles and cadmium under different levels of organic matter (a) and pH (b). Data are mean \pm standard deviation ($n = 3$). Different letters indicate significant differences between the values as obtained by ANOVA and post hoc LSD test ($p < 0.05$), where the letter “a” is assigned to the groups with highest values

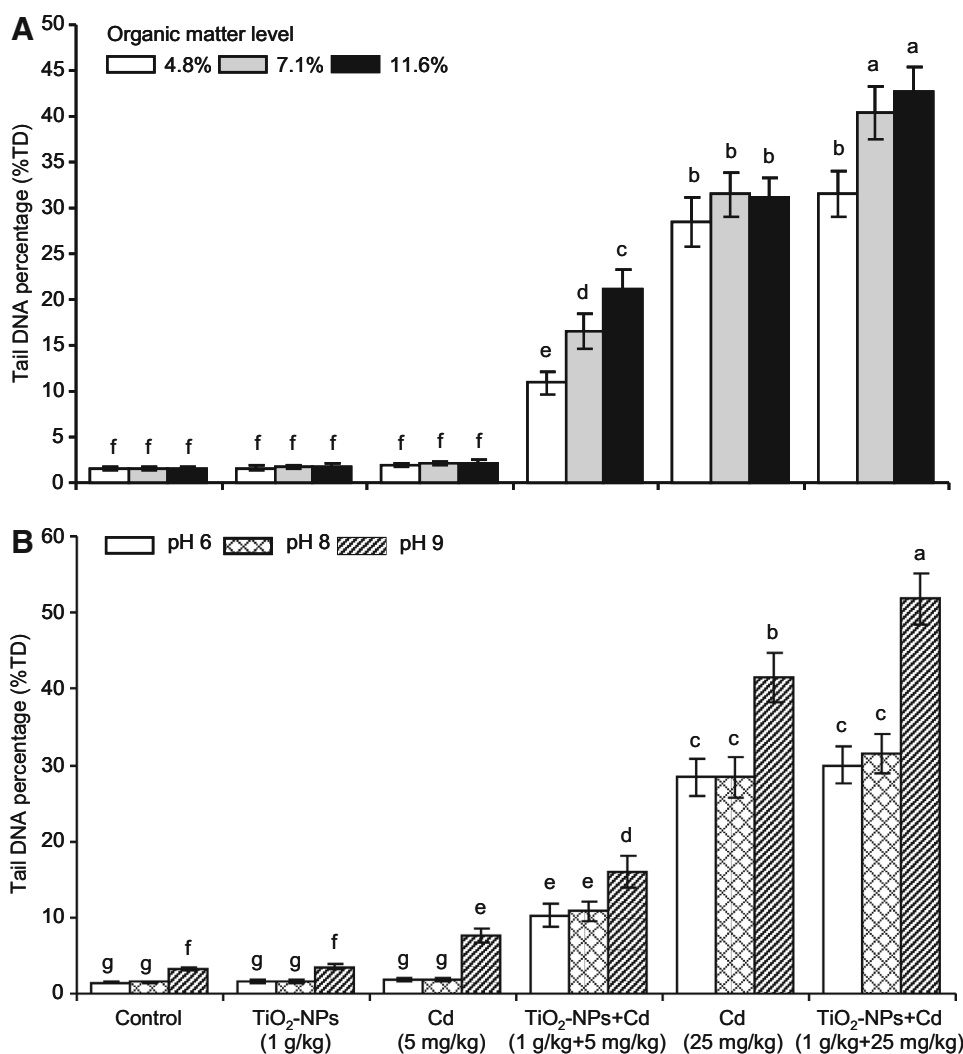
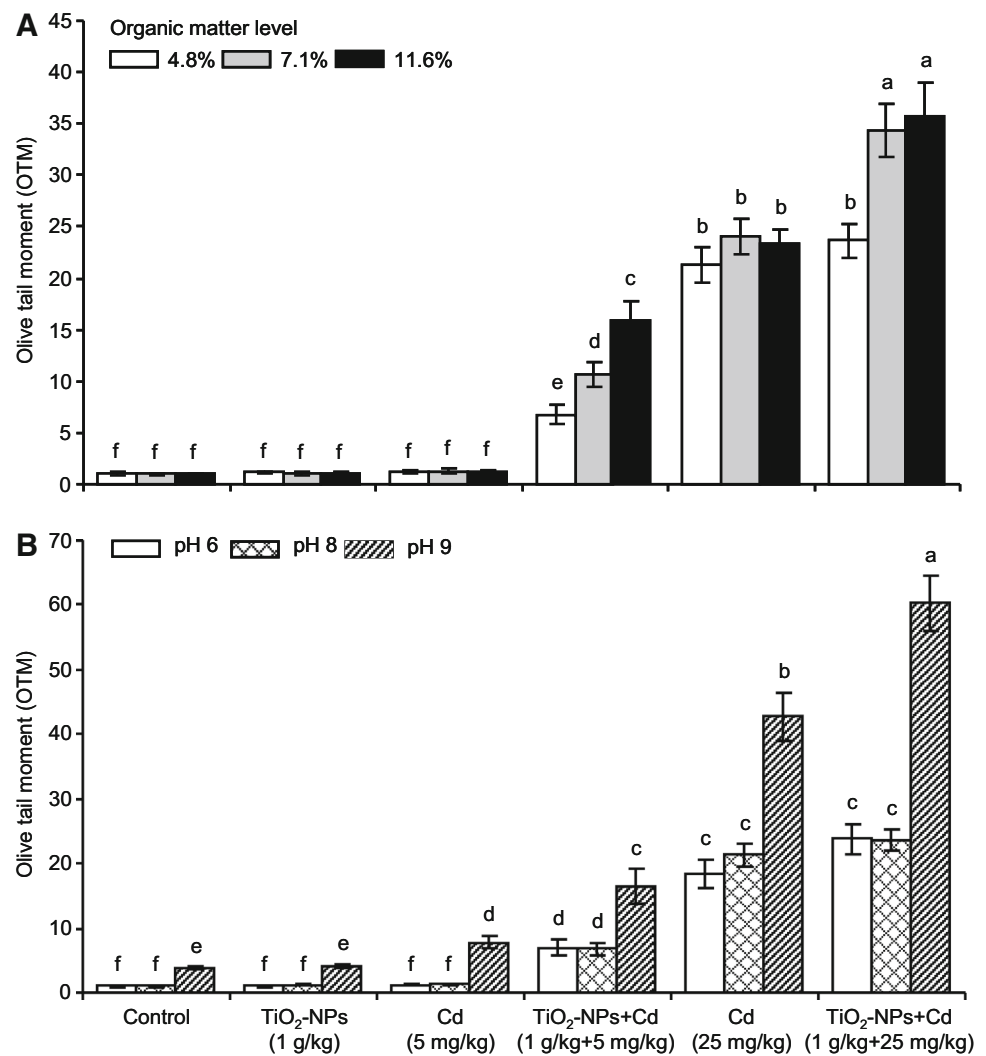


Fig. 4 Olive tail moment (OTM) from comet assay of the hepatopancreas in *Bellamya aeruginosa* following single and combined 21-day exposure to TiO₂ nanoparticles and cadmium under different levels of organic matter (a) and pH (b). Data are mean ± standard deviation (*n* = 3). Different letters indicate significant differences between the values as obtained by ANOVA and post hoc LSD test (*p* < 0.05), where the letter “a” is assigned to the groups with highest values



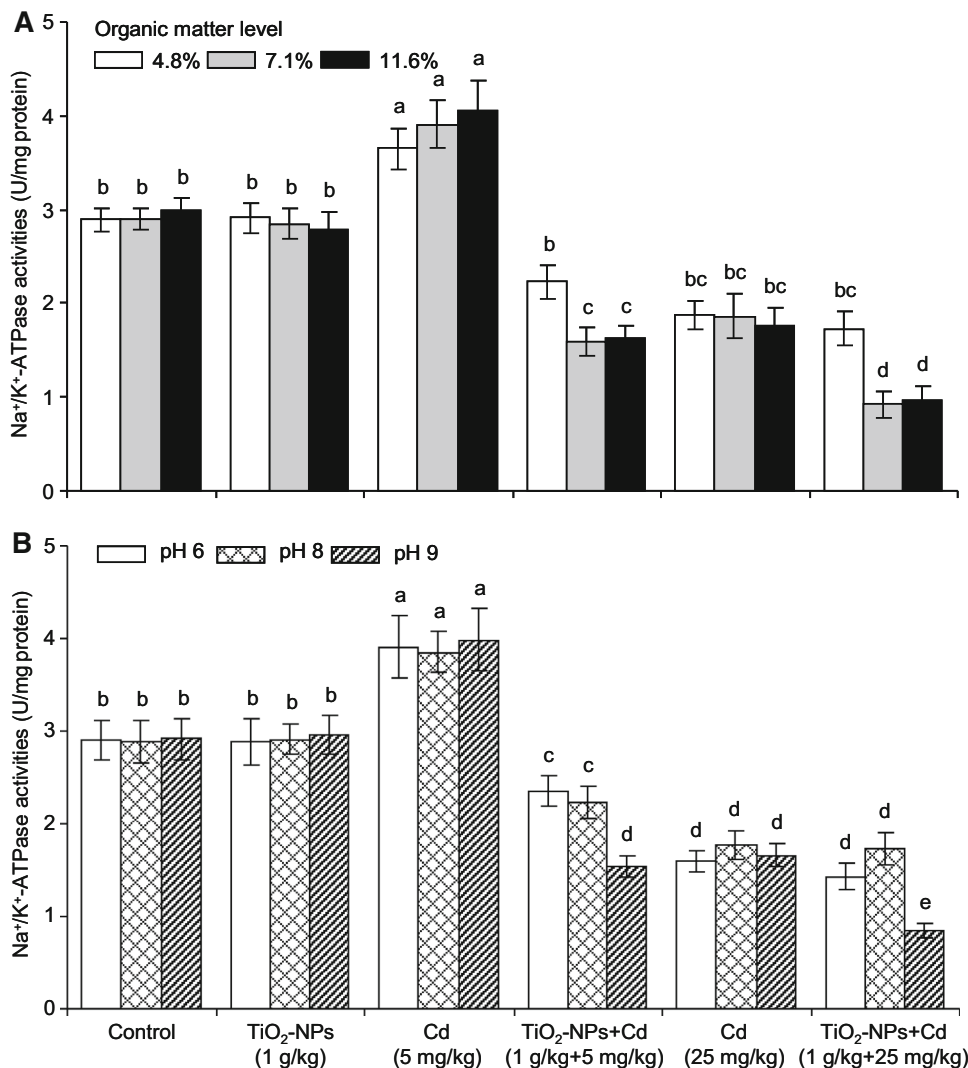
than that at pH of 6 and 8. In the TiO₂-NPs and Cd co-exposure treatments, DNA damage by low-Cd were significantly elevated in the presence of TiO₂-NPs compared with corresponding low-Cd alone exposure. At high-Cd concentration and pH 6 and 8, no significant DNA damage increases were observed when compared to corresponding high-Cd alone exposure, at high-Cd concentration and pH of 9, DNA damages were significantly elevated in the presence of TiO₂-NPs compared with corresponding high-Cd alone exposure.

Na⁺/K⁺-ATPase Activity

The Na⁺/K⁺-ATPase activities of hepatopancreas in *B. aeruginosa* are shown in Fig. 5. Regardless of OM or pH levels, in both low-Cd and high-Cd alone treatments compared with the controls, ATPase activities followed a changing trend of being significantly activated at low-Cd treatments (5 mg/kg) and significantly inhibited by ~50%

at high-Cd treatments (25 mg/kg) but not in an OM-/pH-dependent manner. Basically, under TiO₂-NPs and Cd co-exposure, the changes of ATPase activities were contrary to that of DNA damage (Figs. 3, 4). In the case of different levels of OM, ATPase activities were significantly inhibited by ~50% by low-Cd stress in the presence of TiO₂-NPs compared with the controls or corresponding low-Cd alone exposure. The higher OM levels (7.1 and 11.6%) facilitated this inhibition but not in OM-dependent manner. At high-Cd concentration and low OM level (4.8%), no significant decreases of ATPase activities were observed relative to corresponding high-Cd alone exposure. However, at high-Cd concentration and higher OM levels (7.1 and 11.6%), ATPase activities significantly decreased by up to 50% compared with corresponding high-Cd alone exposure. In the case of different pH treatments, likewise, ATPase activities were significantly inhibited (<50%) by low-Cd stress in the presence of TiO₂-NPs compared with the control and corresponding low-Cd alone exposure, the

Fig. 5 Na^+/K^+ -ATPase activities of the hepatopancreas in *Bellamyia aeruginosa* following single and combined 21-day exposure to TiO_2 nanoparticles and cadmium under different levels of organic matter (a) and pH (b). Data are mean \pm standard deviation ($n = 5$). Different letters indicate significant differences between the values as obtained by ANOVA and post hoc LSD test ($p < 0.05$), where the letter “a” is assigned to the groups with highest values



highest pH level facilitated this inhibition. Compared with corresponding high-Cd alone exposure, only at pH of 9, ATPase activities significantly decreased by more than 50%.

Oxidative Damage

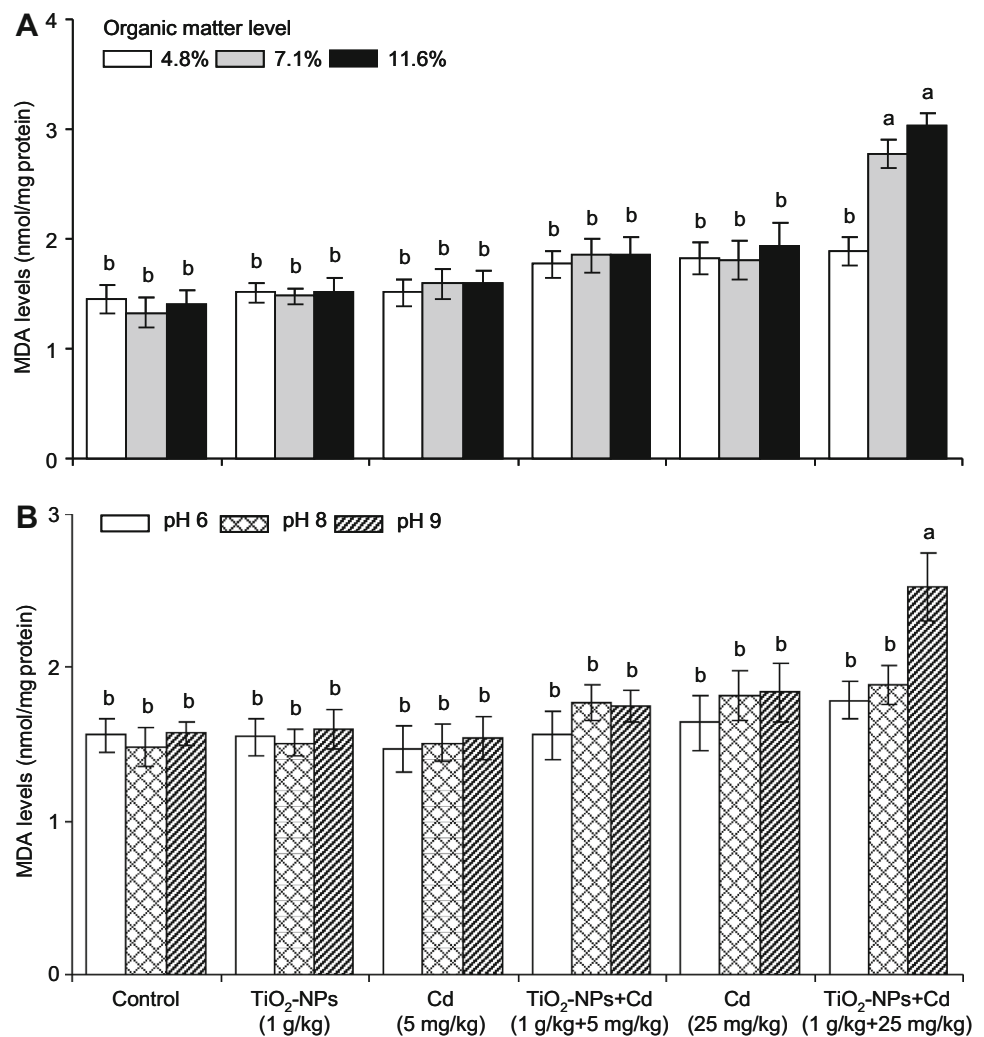
LPO of the hepatopancreas in *B. aeruginosa* by measuring MDA contents are displayed in Fig. 6. Obviously, only at higher OM levels (7.1 and 11.6%) and at the highest pH level, the MDA contents increased remarkably compared to that of any other treatments. In both cases of different OM and pH levels, the PC levels of hepatopancreas in *B. aeruginosa* showed a similar trend (Fig. 7). In both low-Cd and high-Cd alone treatments, the PC levels were significantly elevated compared with the controls by more than 40 and 130%, respectively, but not in OM-/pH-dependent manner. In TiO_2 -NPs and Cd co-exposure treatments, at higher OM levels (7.1 and 11.6%), the PC levels

were significantly elevated by Cd stress in the presence of TiO_2 -NPs compared with corresponding Cd alone exposure. Similarly, only at pH of 9, the PC levels significantly elevated compared with corresponding Cd alone exposure.

Discussion

To date, limited studies regarding the effects of organic matter (Wang et al. 2008; Yang and Xing 2009; Li et al. 2014) and pH (Chen et al. 2014) on the interaction between NPs and coexisting pollutants were based on water column. However, no data are available for the effects of organic matter and pH on the ecotoxicity from co-exposure of NPs and pollutants in sediments. In our study, the DNA damage, Na^+/K^+ -ATPase activities, the levels of MDA and PC, and Cd burdens in the hepatopancreas of *B. aeruginosa* were determined for the assessment of ecotoxicity.

Fig. 6 MDA levels of the hepatopancreas in *Bellamya aeruginosa* following single and combined 21-day exposure to TiO₂ nanoparticles and cadmium under different levels of organic matter (a) and pH (b). Data are mean \pm standard deviation ($n = 5$). Different letters indicate significant differences between the values as obtained by ANOVA and post hoc LSD test ($p < 0.05$), where the letter “a” is assigned to the groups with highest values



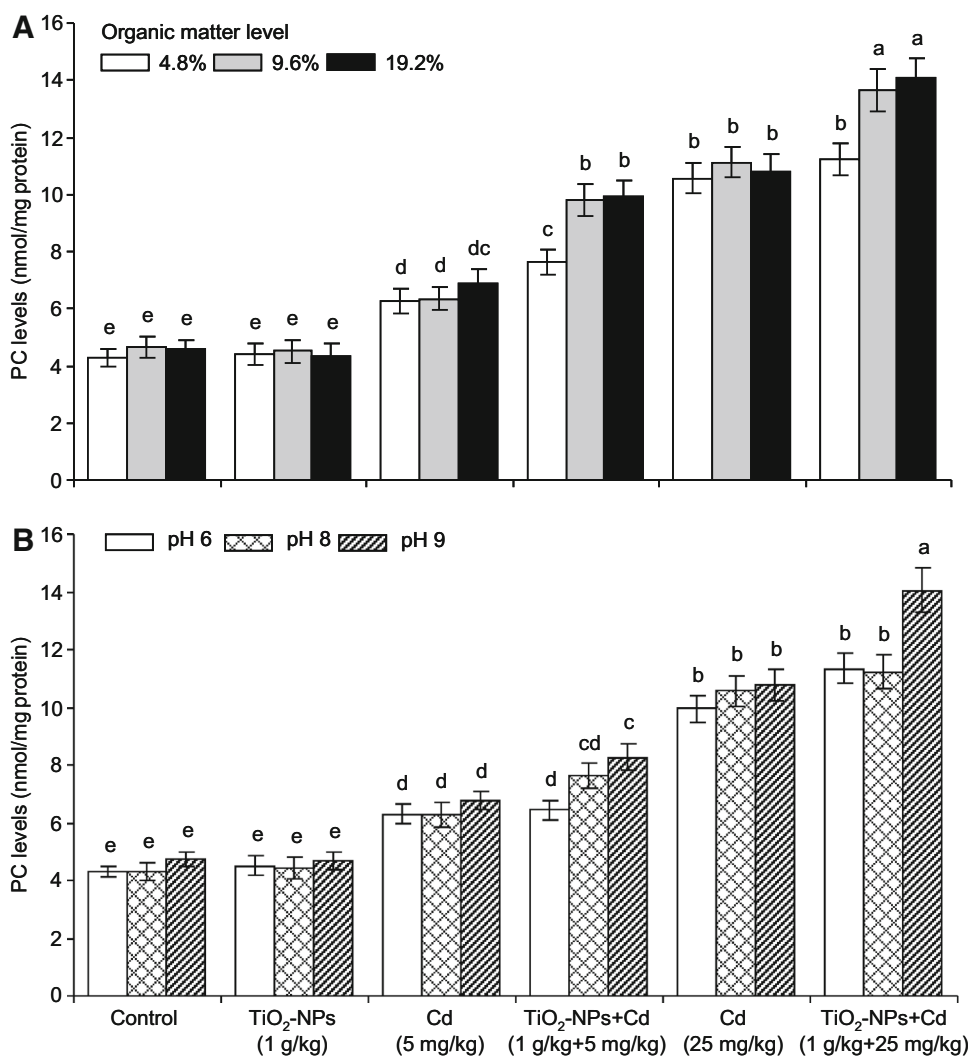
Effect of Organic Matter on Ecotoxicity from Co-exposure of TiO₂-NPs and Cd

In this study, under Cd alone exposure, different levels of OM did not affect Cd accumulation, Na⁺/K⁺-ATPase activities, and the levels of MDA and PC in the hepatopancreas of *B. aeruginosa*. Under TiO₂-NPs and Cd co-exposure, at the background level of OM (4.8%), TiO₂-NPs significantly promoted Cd accumulation in low-Cd treatments (5 mg/kg), which was in accordance with previous studies in terms of waterborne bioaccumulation testing with *Cyprinus carpio* (Zhang et al. 2007), *Daphnia magna* (Tan et al. 2011, 2014), and *Tetrahymena thermophila* (Yang et al. 2014). However, at the background level of OM, TiO₂-NPs did not promote Cd accumulation in high-Cd treatments (25 mg/kg). We speculated relatively high-Cd concentration in the sediments might mask the role of limited concentrations of TiO₂-NPs. At the higher OM levels (7.1 and 11.6%), in both low- and high-Cd treatments, TiO₂-NPs significantly increased Cd accumulation, and thus aggravated DNA

damage, declined the Na⁺/K⁺-ATPase activities, and enhanced LPO and PC, moreover, DNA damage coincided well with Cd accumulation.

Our results also suggested that under TiO₂-NPs and Cd co-exposure, Cd burdens were always positively correlated with Ti burdens, indicating TiO₂-NPs partially acted as the carrier of Cd. The relatively higher OM levels may enhance the toxicity of Cd to *B. aeruginosa* via increasing Cd accumulation. The interaction between TiO₂-NPs and OM levels in the sediments potentially governed the accumulation and toxicity of Cd to *B. aeruginosa*. Previous studies have demonstrated that NPs can interact with abundant organic ligands, including natural organic matter (NOM), which can result in the formation of surface coating on the NPs (Thio et al. 2011) and in reduced aggregation through charge stabilization and steric stabilization mechanisms (Domingos et al. 2009; Petosa et al. 2010; Praetorius et al. 2014; Grillo et al. 2015) and potentially increased sorption of coexisting pollutants onto NPs. For example, Wang et al. (2008) indicated that OM

Fig. 7 Protein carbonyl (PC) levels of the hepatopancreas in *Bellamyia aeruginosa* following single and combined 21-day exposure to TiO₂ nanoparticles and cadmium under different levels of organic matter (a) and pH (b). Data are mean ± standard deviation (n = 5). Different letters indicate significant differences between the values as obtained by ANOVA and post hoc LSD test (p < 0.05), where the letter “a” is assigned to the groups with highest values



coating greatly enhanced pyrene sorption by TiO₂-NPs. Yang and Xing (2009) showed that phenanthrene sorption by TiO₂-NPs was enhanced significantly by coated humic acids. Li et al. (2014) also indicated that HA coating enhanced the sorption of phenanthrene onto TiO₂-NPs. As a benthic organism, *B. aeruginosa* mainly feed on particulate matters and organic detritus from sediments (Chen and Song 1975). Hence, the increased Cd burdens in the hepatopancreas might result from the direct ingestion of complexes of TiO₂-NPs and Cd enhanced by OM. Contrary to our findings, Rosenfeldt et al. (2015) reported in the presence of organic matter and TiO₂-NPs in the water column, the toxicity of Cu to *Daphnia magna* was further reduced, and this decrease of toxicity coincided with a lowered Cu concentration in water. Their results implied that the toxicity of Cu to *Daphnia magna* mainly resulted from the concentration of free Cu rather than complexes of TiO₂-NPs and Cu, because Cu in the complexes could not be ingested by *D. magna*. Therefore, although OM can enhance the adsorption of pollutants onto TiO₂-NPs, the

toxicity of coexisting pollutants largely depend on the feeding pattern of test species and test media (sediments or water column).

Effect of pH on Ecotoxicity from Co-exposure of TiO₂-NPs and Cd

Under Cd alone exposure, the Cd burdens, Na⁺/K⁺-ATPase activities, and levels of MDA and PC in the hepatopancreas of *B. aeruginosa* were not affected by pH levels, but DNA damages were significantly increased at pH of 9. Under TiO₂-NPs and Cd co-exposure, in low-Cd treatments (5 mg/kg), regardless of pH levels, TiO₂-NPs did not affect Cd accumulation, the levels of LPO and PC, but significantly enhanced the DNA damage, slightly facilitated the inhibition of the Na⁺/K⁺-ATPase activities (by less than 50%). In high-Cd treatments (25 mg/kg) and at pH 9, TiO₂-NPs significantly promoted Cd accumulation, enhanced DNA damage, declined the Na⁺/K⁺-ATPase activities, and enhanced LPO and PC. Our study

indicated that high pH of sediments not only caused DNA damage to *B. aeruginosa* but also resulted in increased toxicity of Cd via increasing Cd accumulation. It had been demonstrated that ambient pH exerts a significant effect on NPs in terms of particle charge and the size of aggregates, which depend on the point of zero charge (pHpzc, when the surface of NPs does not possess electrical charge) of NPs, generally, when ambient pH value closes to pHpzc, the electrostatic repulsion between NPs will reduce, leading to aggregation of NPs (Dunphy Guzman et al. 2006; Pettibone et al. 2008). The pHpzc of anatase TiO₂-NPs is 6.3 (Finnegan et al. 2007). Therefore, it is easy to aggregate in natural water bodies at pH 6–8. It was confirmed that the stability and mobility of TiO₂-NPs were markedly enhanced when pH moved far from its pHpzc, especially at alkaline conditions (Jiang et al. 2008; Domingos et al. 2009). It is expected that increased TiO₂-NPs stability may enhance adsorption of pollutants onto TiO₂-NPs. In our study, no obvious increased Cd accumulation and toxicity in hepatopancreas at pH 6 and 8 (closing to pHpzc) may possibly be explained by decreased ingestion of complexes of TiO₂-NPs and Cd, which resulted from substantial aggregation of TiO₂-NPs. This also was partially evidenced by poor correlation between Ti burdens and Cd burdens. At pH 9, relatively more complexes of TiO₂-NPs and Cd potentially increased their opportunity being ingested by *B. aeruginosa*.

Conclusions

Our findings clearly indicated that in the case of coexistence of non-toxic concentration of TiO₂-NPs and Cd in sediments, the interaction between TiO₂-NPs and important environmental factors OM and pH significantly affected the accumulation and toxicity of Cd in *B. aeruginosa*. The relatively higher levels of OM increased Cd accumulation and ecotoxicity of Cd to *B. aeruginosa* but not in OM-dependent manner. High sediment pH (9) not only caused pronounced DNA damage to *B. aeruginosa* but also led to overall increased toxicity of Cd. Therefore, investigations regarding ecological risk of NPs should be addressed not only on their potential impacts on coexisting pollutants but also on the effects of relevant environmental conditions. Additionally, our results were different from the findings obtained in waterborne exposure test (Rosenfeldt et al. 2015), although OM and pH may significantly affect the interaction between NPs and pollutants, the potential ecological risk of co-exposure of NPs and pollutants might be closely related to the feeding pattern of aquatic species and environmental media. Our study only

addressed the effects of sediment OM and pH levels on ecotoxicity of Cd in the presence of nontoxic concentration of TiO₂-NPs. The underlying mechanisms require further investigation.

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Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

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