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Algae (*Raphidocelis*) reduce combined toxicity of nano-TiO_2 and lead on *C. dubia*

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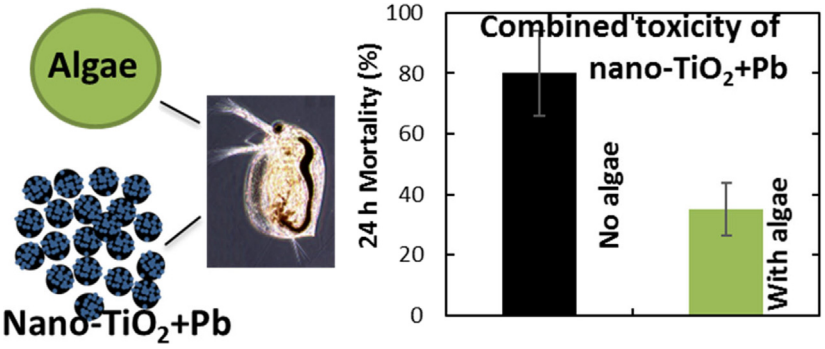
HIGHLIGHTS

- Algae reduce the combined toxicity of nano-TiO_2 and lead on *C. dubia*.
- Algae do not significantly impact Pb accumulation, depuration or distribution processes.
- Food energy may enhance ROS neutralization or Pb immobilization in *C. dubia*.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanoparticles (NPs) often serve as carriers of background toxins and enhance their toxicity on aquatic organisms such as *Ceriodaphnia dubia* (*C. dubia*). However, foods, especially algae, are also present in natural water and impacts this type of toxicity. This study investigated the effect of algae on the combined toxicity of nano-TiO_2 and lead (Pb). A mixture of yeast-trout chow-cereal leaves (YTC) was also used as another model food. Results indicated that, both algae and YTC significantly reduce the combined toxicity of nano-TiO_2 and Pb. Further investigation indicated that the ingestion of algae had minimal impacts on Pb uptake by, Pb depuration from, and Pb distribution within the *C. dubia*. Therefore, the toxicity reduction from algae ingestion should come from mechanisms other than the change in Pb mass and speciation in *C. dubia*, which will need future investigation. Nevertheless, the effect of food on the mitigation of combined toxicity of NPs and heavy metals must be considered when assessing the toxicity of nanoparticles in the natural environment because food always exists in natural waterbodies where aquatic organisms grow.

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1. Introduction

The 2012 worldwide production of common nanoparticles (NPs), such as titanium dioxide, iron oxide, zinc oxide, carbon nanotubes, etc., varied from 10,000 tons to >20,000 tons (Piccinno et al., 2012). Nano-TiO_2 has been widely used in paints, plastics, sunscreens, solar

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cells, and as food additives (O'regan and Grätzel, 1991; Lademann et al., 1999; Liang et al., 2000; Macwan et al., 2011; Bachler et al., 2015). The 2012 worldwide production of nano-TiO_2 surpassed 10,000 tons, and much of it was released into the environment through wastewater discharge or waste disposal pathways (Kiser et al., 2009; Piccinno et al., 2012). Although nano-TiO_2 is considered less toxic than most other nano-sized transition metal oxides (Heinlaan et al., 2008), its interactions with other environmental compounds (toxic and non-toxic) may alter the degree of its toxicity on aquatic life as well as its fate and transport in the environment. For instance, algae can enhance the uptake of NPs by *C. dubia* (Dalai et al., 2014a); the natural organic matter in natural water can reduce the toxicity of NPs and heavy metals (Fan et al., 2016); calcium or hardness can influence the uptake pathway of NPs (Tan et al., 2016).

Heavy metals may be present in surface water due to discharges from industrial, agricultural, and mining processes (Duruibe et al., 2007). Lead (Pb) is one of the most concerned heavy metals (Lewis, 1985). The U.S. National Primary Drinking Water Regulations (NPDWR) limit Pb in drinking water with an action level of 15 μg/L (EPA, 2016). Recent studies have shown that NPs, including nano-TiO_2, enhance the toxicity of heavy metals such as Pb in many organisms (Wang et al., 2011a; Wang et al., 2011b; Hu et al., 2012a; Dalai et al., 2014b;). Hu et al. (2012a) also reported that nano-TiO_2 and nano-CeO_2 significantly increase Pb toxicity on *C. dubia*. Wang et al. (2011a, 2011b) showed that nano-Al_2O_3 and nano-TiO_2 significantly increase arsenic (As) toxicity. Nano-TiO_2 significantly increases the accumulation of As, cadmium (Cd), copper (Cu), and Pb in freshwater bivalves and zebrafish (Hu et al., 2011; Fan et al., 2018). Collectively, NPs act as carriers of heavy metals to facilitate their transport to aquatic species. This phenomenon is characterized as the "Trojan-horse effect" (Hu et al., 2012a). Consequently, non-toxic or low toxic NPs significantly enhance the toxicity of heavy metals in the environment.

Algae are essential food sources for fish, water fleas, snails, and other aquatic life in freshwater and seawater. Previous research has indicated that algae can reduce the toxicity of heavy metals for water fleas or mussels through a depuration process (Svensson, 2003; Petersen et al., 2009) or adsorption/removal mechanisms (Roy et al., 1993). In contrast, algae increase the bioavailability and adverse effects of NPs on water fleas and snails (Dalai et al., 2014a; Su et al., 2018). These findings not only demonstrated that algae and other food types may play complex roles in the toxicity of NPs and heavy metals, but they also raised more questions: How do algae or other foods that commonly seen in the surface water, such as yeast, cereal, and fish food, influence the combined toxicity of NPs and heavy metals? Do algae serve as carriers of NPs and heavy metals to enhance the toxicity, or as energy sources to reduce the toxicity? The objective of this research was to investigate the effects of foods on the combined toxicity of nano-TiO_2 and Pb on aquatic organisms, using algae and the yeast-trout chow-cereal leaves (YTC) mixture as two model food types and *C. dubia* as the model organism recommended by EPA (EPA, 2002).

2. Materials and methods

2.1. Chemicals

All chemicals used for this research were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA) unless otherwise specified. $CaSO_4{\cdot}2H_2O$ (98%), Na_2SeO_4 (99%), $NaHCO_3$ (100.2%, Pb < 5 mg/kg), $MgSO_4$ (Pb < 0.001%), and KCl (99%) were used to prepare a culture medium. $Pb(NO_3)_2$ was used to prepare the Pb stock solution, and trace metal grade nitric acid was used for sample acidification and digestion. A certified Pb standard solution at 1000 ppm was used to develop standard calibration curves for the Pb analysis. The TiO_2 NPs (5–10 nm, anatase, 99%) purchased from Skyspring Nanomaterials Inc. (Houston, TX, USA) was used to make relevant testing solutions. All test solutions were prepared using Milli-Q water, which had a resistivity of 18.2 MΩ·cm.

2.2. Culturing of C. dubia and acute toxicity test

The starter *C. dubia* was purchased from MBL Aquaculture (Sarasota, FL, USA), and the algae (*Raphidocelis*) and YTC mixture (Pb < 1 μg/L) were purchased from ABS Inc. (Fort Collins, CO, USA). The mass culture, individual culture, and toxicity tests were conducted in a laminar flow hood (SVC-6AX, Streamline® laboratory products, Fort Myers, FL, USA) that was located in a temperature-controlled chamber with a constant temperature of 25 °C. Lighting within the chamber was set at a light-to-dark cycle of 16 h: 8 h using an illuminati light. The culture medium was prepared with synthetic water of moderate hardness (pH = 7.8 ± 0.2, hardness = 85 ± 5 mg/L as $CaCO_3$) which consisted of appropriate amounts of $CaSO_4{\cdot}2H_2O$, Na_2SeO_4, $NaHCO_3$, $MgSO_4$, and KCl, following the EPA standard method EPA-821-R-02-012 (EPA, 2002).

The acute toxicity test was conducted according to the EPA standard method, EPA-821-R-02-012 (EPA, 2002). In brief, 20 healthy neonates at an age of <24 h (which were brood from 7 to 14 day old individually cultured *C. dubia* adults) were used for each test concentration. Each test concentration used four 30-mL medicine cups as test reactors. Each test reactor contained 15 mL test solution that contained NP, Pb, and/or food prepared using the culture medium and five neonates. As a quality control (QC) check, the neonates were washed with a fresh culture medium three times before being transferred to the test reactor in order to eliminate any food residual adsorbed from the culture reactors. A plastic dropper with a 3-mm diameter opening tip was used to transfer neonates to prevent any damage of the neonates. After 24 h, the neonates were visually inspected for mortality. The survival rate of all controls in acute toxicity tests exceeded 90%.

2.3. Pb adsorption onto different particles

The Pb(II) stock solution (1000 mg/L) was prepared by dissolving an appropriate amount of $Pb(NO_3)_2$ in MQ water, and then acidifying it with nitric acid to reduce the pH to <4. A total of three parallel series of Pb(II) solutions, with different selected concentrations of Pb (up to 2500 μg/L for each series), were prepared from the Pb(II) stock solution with the culture medium in HDPE bottles. A selected amount of dry nano-TiO_2 was added to each of the bottles of one series, making a nano-TiO_2 concentration of 200 mg/L in each bottle. For the next series of bottles, the same volume of algae stock solution was added to each of the bottles, making a cell concentration of 1.8×10^5 cells/mL in each bottle. The algae cell concentration (1.8×10^5 cells/mL) was consistent with that in the individual culture of *C. dubia*, which can provide sufficient nutrients for the testing organism (EPA, 2002). The volume increase caused by the algae stock solution was negligible (0.6%). The third series of bottles served as a control series, without adding any particles. All bottles were mixed using a mechanical shaker for 24 h. After mixing, the test solutions were collected and centrifuged, and the supernatants were collected for residual Pb(II) analysis. The final pH of all test solutions after 24 h of mixing ranged between 7.6 and 7.8.

2.4. Preparation for toxicity experiments

To test nano-TiO_2 toxicity, a series of 70 mL test solutions of various nano-TiO_2 concentrations (from 0 to 1200 mg/L) were prepared by adding appropriate amounts of dry nano-TiO_2 particles into a series of HDPE bottles that contained 70 mL of culture medium. To test the algae impact on nano-TiO_2 toxicity, another group of bottles that contained different concentrations of nano-TiO_2 but the same algae concentration of 1.8×10^5 cell/mL was also prepared with culture medium. To test Pb toxicity, a series of 70 mL test solutions with various Pb(II) concentrations (up to 2500 μg/L) was prepared by diluting the Pb(II) stock solution with the culture medium in HDPE bottles. All test

solutions were mixed for 24 h with a mechanical shaker before use. Control experiments that used only culture medium and culture medium algae (without NPs) were also conducted. A total of 60 mL of each test solution was divided into four test reactors for toxicity tests.

To test the combined toxicity of Pb and different particles (algae, YCT, nano-TiO_2), a series of 70 mL test solutions with different Pb(II) concentrations (up to 2500 μg/L) were prepared. Different particles or particle combinations, including algae, YTC, nano-TiO_2, algae nano-TiO_2, and YTC nano-TiO_2, were added to these test solutions, and then mixed for 24 h with a shaker. The nano-TiO_2 was added as dry particles. The algae and YTC were added as stock solutions of algae (3.0×10^7 cell/mL) and YTC (1700 mg/L as total solids), respectively. The concentrations of algae, YTC, and nano-TiO_2 in the test solutions were 1.8×10^5 cell/mL, 6.8 mg/L (as total solids), and 200 mg/L, respectively. The increases in volume caused by algae and YTC additions were 0.6% and 0.4%, respectively, which were considered negligible. Control groups that contained only particles, without Pb, were also included. The final pH values in test solutions after 24 h of mixing were in a range of 7.6 to 7.8. A total of 60 mL of each test solution was used for a toxicity test using four replicate reactors. The remaining solutions were used for soluble Pb(II) analysis.

2.5. Pb uptake by C. dubia

In principle, the neonates used in toxicity tests should be used in Pb uptake tests. However, because the neonates were extremely fragile and could be easily damaged during the multiple manual steps of the test, they were not feasible for the Pb uptake study. Consequently, adult *C. dubia* were used to investigate Pb uptake (Wang et al., 2011a; Hu et al., 2012b).

Approximately 100 adult *C. dubia* were washed three times with a clean culture medium. They were placed in test solutions that contained Pb, Pb algae, Pb nano-TiO_2, and Pb nano-TiO_2 algae, respectively. The concentrations of Pb, nano-TiO_2, and algae in solutions were 2500 μg/L, 200 mg/L, and 1.8×10^5 cell/mL, respectively. After designated time periods, *C. dubia* were picked and washed three times again with a clean culture medium to remove any nano-TiO_2 and algae that were loosely attached to the surface of *C. dubia*. This was followed by filtering through a 0.297 mm standard sieve. The washed *C. dubia* were then manually counted and transferred to a digestion vessel. Ten mL of trace metal grade nitric acid were added for digestion at 95 °C for 12 h using a hot-block digester (Watlow mini-0R10-000G). After digestion, the sample volume was reduced due to evaporation. It was then increased to 10 mL using MQ water, and prepared for soluble Pb analysis. A control experiment was also conducted using a clean culture medium. The uptake experiments were conducted in duplicate.

2.6. Pb depuration from adult C. dubia in a clean medium and a medium with algae

The depuration experiment procedure was similar to that used by Gillis et al. (2005). In brief, the adult *C. dubia* were initially placed in test solutions that contained Pb nano-TiO_2, and Pb nano-TiO_2 algae, respectively, to uptake Pb. The experimental procedures of Pb uptake, and the preparation of various test solutions that contain Pb, nano-TiO_2, and algae were the same as the Pb uptake test, except that uptake duration of Pb was fixed at 4 h for this test. The *C. dubia* were then picked and washed three times with the culture medium and transferred to reactors that contained a fresh culture medium and a fresh culture medium algae (1.8×10^5 cell/mL), respectively, for depuration tests. During the depuration period, no food or particles were added to the reactors. After a selected depuration time, *C. dubia* were picked, washed, filtered, counted, and digested. The Pb content in the digestion solution was analyzed to calculate the residual Pb in *C. dubia*. The depuration experiments were conducted in duplicate.

2.7. Mortality of pre-exposed C. dubia neonates in clean medium and medium with algae

The pre-exposure solution was prepared using a culture medium that contained 2500 μg/L of Pb and 200 mg/L of nano-TiO_2. The pre-exposure procedure of *C. dubia* was similar to the toxicity test procedure. After a selected period of exposure time, the live neonates were picked, washed, and transferred to a clean culture medium and a culture medium that contained 1.8×10^5 cell/mL of algae, respectively. The survival or mortality of the *C. dubia* was monitored for 24 h.

2.8. Pb analysis

The residual solutions from different experiments were first centrifuged at 2000 G for 10 min. The supernatants were then acidified with trace metal grade nitric acid to reach a pH < 2, and preserved in a refrigerator if the Pb was not immediately analyzed. The soluble Pb(II) was determined using a graphite furnace atomic adsorption spectrometer (GFAA) (Perking Elmer AAnalyst 600), which has a detection limit of 0.5 μg/L for Pb.

2.9. Statistical analysis

All toxicity tests were conducted with four replicates. For the toxicity of Pb alone, one-way analysis of variance (ANOVA) with "Pb concentration" as the factor was conducted to determine any statistical significance of mortality ($p < 0.05$). Two-way ANOVA analysis with "Pb concentration" and "type of particle" as factors was conducted to determine the statistical significance ($p < 0.05$) of mortality between different treatment groups.

3. Results and discussion

3.1. Nano-TiO_2 toxicity

We initially tested the toxicity of nano-TiO_2, with or without the presence of algae. Results indicated that nano-TiO_2 up to 1200 mg/L did not result in the death of *C. dubia* in both scenarios (data not shown). This observation was in agreement with others who also reported a low toxicity of nano-TiO_2 on *D. magna* and *C. dubia* (Heinlaan et al., 2008; Hu et al., 2012a).

The average size of algae, *Raphidocelis*, used in this research had a length of 8–14 μm and a width of 2–3 μm (OECD, 2011). Dalai et al. (2014a) reported that algae facilitate the uptake of nano-TiO_2 by *D. magna*. Hypothetically, the increased uptake of nano-TiO_2 may increase the toxicity on *C. dubia*. However, in the NP concentration range that we tested, algae did not impact the toxicity of nano-TiO_2 on *C. dubia*.

3.2. Pb adsorption onto nano-TiO_2 and algae

Fig. 1 shows the impact of nano-TiO_2 and algae on the soluble Pb concentration in the culture medium at pH 7.8. Pb could form precipitation with hydroxide, carbonate, and sulfate in the culture medium. Therefore, only a small fraction of the total added Pb was soluble. For example, the soluble Pb concentration was only 350 μg/L when the total Pb concentration was 2500 μg/L in the culture medium, and this soluble Pb concentration curve can be considered as a solubility curve. While algae could reduce the soluble Pb concentration due to adsorption, nano-TiO_2 had a much greater reduction in the soluble Pb concentration, to <10 μg/L for all Pb concentrations tested (Fig. 1). Thus, nano-TiO_2 is an excellent sorbent of Pb.

C. dubia use appendages as a filter to select particulate foods of certain sizes for ingestion (Ebert, 2005). The particle sizes that could pass through the appendage for digestion by *C. dubia* ranged from 100 nm to 5000 nm (Geller and Müller, 1981). Hu et al. (2012a) reported that,

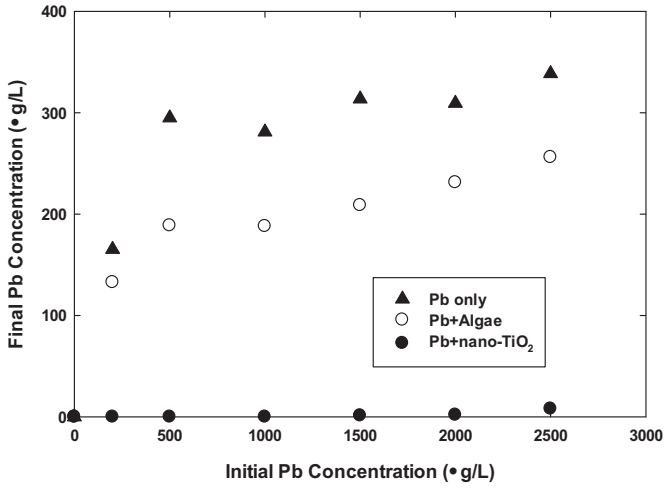

Fig. 1. Soluble Pb(II) concentration in a culture medium at pH 7.8 with and without nano-TiO_2 (200 mg/L) or algae (1.8×10^5 cells/mL).

in the same culture medium, the nano-TiO_2 particles used in this study had hydrodynamic sizes that ranged from 30 nm to 600 nm. As a result, most of the nano-TiO_2 particles, along with the adsorbed Pb, could enter the *C. dubia* body.

3.3. Toxicity of Pb with or without nano-TiO_2 and/or algae

Fig. 2 shows the effect of nano-TiO_2 and/or algae on the toxicity of Pb. The concentration of Pb alone (up to 2500 μg/L) did not significantly impact the toxicity of Pb ($p = 0.82$, one-way ANOVA). This result was consistent with previous research that showed that Pb toxicity was insignificant in *D. magna* (Gale et al., 1992). Erten-Unal et al. (1998) reported that the toxicity of Pb was a function of its solubility in water; the LC_{50} of Pb in the form of $PbSO_4$ in very hard water was 3166 mg/L for *D. magna*. Our culture medium had a moderate hardness with a pH of 7.8, which contained hydroxide, carbonate, and sulfate ions that could form precipitation with Pb. The major Pb species in a solution similar to our culture medium was $Pb_3(CO_3)_2(OH)_2$ precipitate (Escudero-García et al., 2013), which has much less toxicity than soluble Pb ions. As a result, when there were no other particles present in the culture medium, Pb was mostly in the form of precipitates, resulting in low toxicity on *C. dubia*.

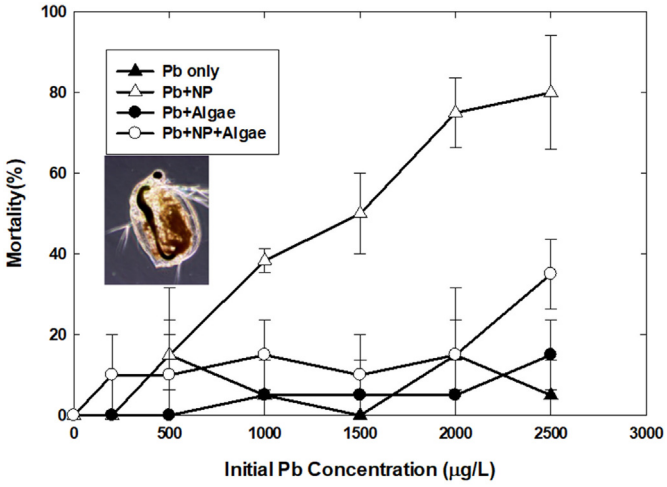

Fig. 2. The 24 h mortality of *C. dubia* in the presence of Pb, with or without other particles (nano-TiO_2 and algae). Concentrations of particles: NPs = 200 mg/L; Algae = 1.8×10^5 cells/mL. Photo was *C. dubia* from 1 h uptake with Pb (2500 μg/L) NPs (200 mg/L). Standard deviation was represented by the error bar attached to each point; the number of data for each point were 4 ($N = 4$); $p < 0.05$ indicated statistical significant.

Importantly, Fig. 2 shows that, with the addition of nano-TiO_2 particles, Pb toxicity increased significantly ($p < 0.05$). When the initial Pb concentration was 2500 μg/L, the presence of nano-TiO_2 increased the *C. dubia* mortality to 80%. Table 1 shows that, with the addition of nano-TiO_2, Pb became a significant factor impacting toxicity ($p < 0.05$). As indicated earlier, Pb could form precipitation in moderate hardness water. Therefore, even high Pb concentration would not change the toxicity if there is no particles in the solution. However, nano-TiO_2 could carry Pb to *C. dubia* therefore alter the toxicity pattern of Pb alone. Because the toxicity from soluble Pb ions alone was negligible due to the low soluble Pb concentration, and the toxicity from nano-TiO_2 alone was minimal at the concentration we used (data not shown), the significant increase in toxicity when both Pb and nano-TiO_2 were present came from Pb being accumulated on the nano-TiO_2 surface. The photo in Fig. 2 was the *C. dubia* after exposure to both Pb and nano-TiO_2, indicating that the gut was filled with nano-TiO_2. Apparently, nano-TiO_2 particles served as a carrier and delivered high amounts of Pb to *C. dubia* (Wang et al., 2011a; Wang et al., 2011b; Hu et al., 2012a). Note that the pH in the mid-gut of water fleas (*Daphnia*) is typically in a range of 6.0 to 6.8 (Ebert, 2005), lower than that of the culture medium (pH = 7.8), which results in Pb desorption from nano-TiO_2 (Hu and Shipley, 2012), making more free Pb ions available with the gut for tissue uptake. This could increase ROS production in the tissue and result in a greater toxicity (Ercal et al., 2001). The soluble Pb ions could also replace essential trace elements like Zn in the antioxidant enzyme and damage the enzyme (Bray and Bettger, 1990). Both effects could cause the death of *C. dubia*. In addition, the hydroxyl surface group of nano-TiO_2 could form surface complex with Pb(II) as Ti-O-Pb (Vohra and Davis, 1997). It could interact with the surface of the biological tissue or membrane that is composed of negatively charged glycosaminoglycans (GAGs) (Huang et al., 2010), resulting in toxicity.

High NP concentration could reduce the density of sorbed heavy metals. In the meantime, it increases NP uptake by *C. dubia*. If the NPs concentration exceeds the bioaccumulation limit of *C. dubia*, the increase in NP concentration could reduce heavy metal uptake therefore the toxicity (Wang et al., 2011a; Wang et al., 2011b). It was also reported that the maximum nano-TiO_2 concentration that could enhance As(V) toxicity was 50 mg/L (Wang et al., 2011a). The toxicity of Pb from 200 mg/L of nano-TiO_2 in this study might be increased if a lower nano-TiO_2 concentration was used. Therefore, the concentration effect of nano-TiO_2 on Pb toxicity must be considered when evaluating its ecological safety.

3.4. Effect of algae on Pb toxicity with or without nano-TiO_2

Algae are natural food for *C. dubia*. It could adsorb toxic metals (Roy et al., 1993), and carry them to *C. dubia*. Therefore, it is expected that algae increase Pb uptake by *C. dubia* and enhances Pb toxicity. However, Fig. 2 shows that algae have a very minimal impact on Pb toxicity of *C. dubia* ($p = 0.59$). Furthermore, in the presence of algae, increasing Pb concentration did not impact the toxicity ($p = 0.18$). Similar findings were also reported for Ti[4] (Dalai et al., 2014a). It is also possible that

Table 1
Two-way ANOVA P-value Between Treatment Groups.

Treatment group	Pb concentration	Type of particle	Interaction[a]
	P-value	P-value	P-value
Pb only – Algae Pb	0.18	0.59	0.19
Pb only – NP Pb	<0.05	<0.05	<0.05
Pb only – YTC Pb	<0.05	0.20	<0.05
NP Pb – AlgaeNP Pb	<0.05	<0.05	<0.05
YTC Pb – AlgaePb	<0.05	<0.05	0.13
NP Pb – YTC NP Pb	<0.05	<0.05	<0.05

[a] Interaction = Pb concentration × type of particle.

algae provided energy for *C. dubia* to mitigate the toxicity imposed by Pb ions.

Fig. 2 also shows that the presence of algae significantly reduced the combined toxicity of nano-TiO_2 Pb on *C. dubia* ($p < 0.05$). For example, at the initial Pb concentration of 2500 μg/L, *C. dubia* mortality decreased from 80% to 35% as a result of 1.8×10^5 cells/mL of algae. We reasoned that, algae could provide metabolic energy for *C. dubia* to enhance its anti-oxidation defense against Pb-mediated oxidative stress. Furthermore, algae may impact the distribution of Pb on nano-TiO_2, thus impacting the toxicity.

3.5. Effect of algae on the uptake of Pb by C. dubia

Algae could adsorb heavy metals or NPs (Roy et al., 1993; Nolte et al., 2017) and increase their uptake by *C. dubia*. However, if a medium contains both heavy metals and NPs, the role of algae on their uptake is not clear. It may enhance the uptake of both heavy metals and NPs, or occupy some spaces within the gut therefore reduce their uptake. Fig. 3 shows the impact of algae on the Pb content in adult *C. dubia*, with and without nano-TiO_2. The Pb content in *C. dubia*, in the absence of algae or nano-TiO_2 particles, was 1.15 ng per *C. dubia* after 6 h of culturing in a medium that contained only Pb. Algae increased the Pb content in *C. dubia* to 3.79 ng per *C. dubia*, which is three times its value without algae. We also determined that the background Pb content was 0.46 ng per *C. dubia*. Therefore, the accumulated Pb from the test solutions that contained Pb-only and Pb algae was 0.69 and 3.33 ng per *C. dubia*, respectively. Apparently algae served as a carrier to significantly increase Pb uptake by *C. dubia* by approximately five fold. Nevertheless, compared to the test solutions that contained NPs, this additional accumulation of Pb caused by algae was very low.

Fig. 3 points out that nano-TiO_2 significantly enhanced Pb uptake by *C. dubia*, with a total Pb content of 14.37 ng per *C. dubia* after 6 h of culturing. Thus, the additional Pb uptake from the culture medium was 13.91 ng per *C. dubia*, which was 20 times the value of that from the Pb-only solution. Furthermore, the presence of algae slightly reduced the Pb content to 11.56 ng per *C. dubia*. Tan et al. (2011) also reported that the algae reduced Cd and Zn assimilation efficiency in *D. magna* when mixed with nano-TiO_2. Both algae and NPs could adsorb and carry Pb to *C. dubia*, but algae had lower Pb adsorption capacity than nano-TiO_2 (Fig. 1). Therefore, the amount of Pb carried to *C. dubia* by algae was much less than that carried by nano-TiO_2. Wang et al. (2011a) reported the gut of *C. dubia* had limited space to hold NPs. In the presence of two carriers, it is possible that algae which adsorbed less Pb occupied part of the gut space and reduced NP uptake. Wang et al. (2011a, 2011b) reported that the increase in NP concentration could reduce arsenic accumulation on the NP surface through dilution, thereby reducing the toxicity of arsenic on *C. dubia*. By the same token, the reduced uptake of Pb resulting from algae might contribute to the reduced *C. dubia* mortality. One discrepancy that remains to be investigated is that the reduction in 2.81 ng Pb per *C. dubia* caused by algae may not be significant enough to reduce *C. dubia* mortality from 80% to 35%.

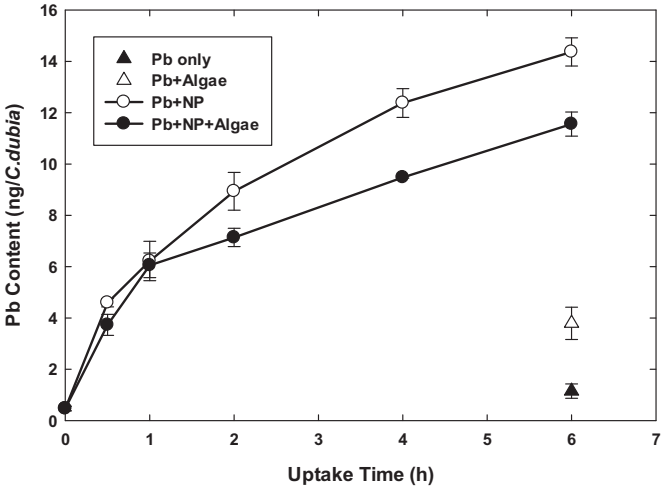

Fig. 3. The Pb content in adult *C. dubia* bodies in the presence of NPs and/or algae. Condition of the exposure medium: [Pb] = 2500 μg/L; NPs = 200 mg/L; Algae = 1.8×10^5 cells/mL. Standard deviation was represented by the error bar attached to each point; the number of data for each point were 2 ($N = 2$).

3.6. Effect of ingested algae on Pb depuration from C. dubia

Petersen et al. (2009) reported that algae could help remove carbon nanotubes from *D. magna*. A similar process might occur if *C. dubia* has both heavy metals and NPs in their bodies. Other than that, the ATP based protein transporter could also remove heavy metals (Nies, 1999). There was also a suspicion that algae might provide energy to *C. dubia* to transport Pb out of the tissue. In order to examine the hypothesis that algae helped remove Pb after uptake, the *C. dubia* was initially exposed in a medium that contained Pb nano-TiO_2 and Pb nano-TiO_2 algae, respectively, for 4 h to allow for Pb uptake. They were then placed in a clean medium (without algae and nano-TiO_2) for depuration. Fig. 4 shows that the Pb in *C. dubia* in both treatment groups had similar depuration rates, which indicated that the algae originally accumulated in the guts of *C. dubia* did not facilitate the removal of Pb from *C. dubia*. In particular, the Pb content in *C. dubia* was nearly identical for both treatment groups after a short period (2 h) of depuration. The photos for the *C. dubia* exposed to Pb nano-TiO_2 algae show that, after 6 h of depuration, there was still a significant amount of nano-TiO_2 in the digestive tract of *C. dubia*. The ingested algae did not help the removal of Pb through removing nano-TiO_2 carrier from digestive tract. Tan and Wang (2017) reported that using nano-TiO_2 as fake food in the depuration of Cd and Zn from *D. magna* resulted in a similar assimilation efficiency compared with algae at a similar concentration. Apparently, nano-TiO_2 could not provide energy for *D. magna* to transport heavy metals out. By the same token, algae did not help with Pb removal from *C. dubia* through energy related pathway. Because the Pb content after 2 h of depuration was the same for both treatment groups, the presence of algae within the gut did not reduce the mortality of *C. dubia* through Pb removal.

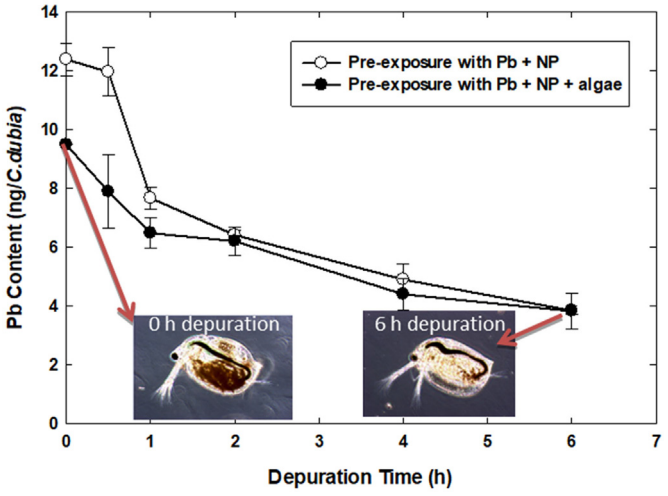

Fig. 4. The Pb depuration from *C. dubia* in a clean culture medium after exposure in a culture medium that contained Pb NPs and Pb NPs Algae, respectively. Conditions of the exposure medium: [Pb] = 2500 μg/L; NPs = 200 mg/L; Algae = 1.8×10^5 cells/mL; exposure time before depuration: 4 h. Photo was *C. dubia* from 0 and 6 h depuration after pre-exposure with Pb NPs Algae. Standard deviation was represented by the error bar attached to each point; the number of data for each point were 2 (N = 2).

3.7. Effect of ingested algae on Pb distribution in C. dubia

The body of a *C. dubia* is comprised of two compartments: a gut and body tissue excluding gut (Gillis et al., 2005). There was a thought that, when co-ingested with Pb nano-TiO_2, algae could immobilize Pb within the gut, resulting in Pb being less available for tissue uptake, hence reducing Pb toxicity. To validate this hypothesis, we conducted an experiment to determine if the depuration of nano-TiO_2 could change the Pb content in *C. dubia* that had been pre-treated with algae. Algae were used to accelerate nano-TiO_2 depuration (Gillis et al., 2005). Initially, the *C. dubia* were exposed in medium that contained Pb nano-TiO_2 and Pb nano-TiO_2 algae, respectively, for 4 h to load Pb. Subsequently, the *C. dubia* were placed in a culture medium that contained algae for Pb depuration. Fig. 5 shows that Pb depuration rates for these two treatment groups were similar. The typical guts passage time (GPT) of foods in *C. dubia* was 2–55 min, depending on the type and concentration of the food (Cauchie et al., 2000). The photos in Fig. 5 show that, for both types of *C. dubia*, 1 h of depuration time removed most of the nano-TiO_2 from the guts.

As indicated in Fig. 5, the uptake of algae quickly removed nano-TiO_2 from the guts, so the sorbed Pb on nano-TiO_2 at the beginning of the depuration experiment was also removed. The residual Pb in the *C. dubia* was associated with other body tissues, and the uptake of algae from depuration solution did not help its removal. Therefore, when compared to the depuration in clean medium (Fig. 4), newly ingested algae only accelerated the removed Pb associated with the nano-TiO_2 within the guts. The additional Pb removal that resulted from new algae ingestion was about the same, regardless of the presence or absence of algae in the gut at the beginning of the depuration experiment. Therefore, the pre-ingested algae should not reduce the mortality of *C. dubia* through Pb immobilization.

3.8. Effect of algae on the survival of C. dubia that were pre-exposed with Pb and NPs

The *C. dubia* neonates were initially exposed in a culture medium that contained both Pb (2500 μg/L) and nano-TiO_2 (200 mg/L) for 2, 4, and 6 h to accumulate Pb and nano-TiO_2. They were then placed in a clean medium and a medium that contained algae, respectively, to examine the 24-h mortality. Fig. 6 shows that algae significantly reduced the 24-h mortality of *C. dubia* that was pre-exposed to Pb NPs for 4 and 6 h. Because most Pb was assimilated into body tissues during the pre-exposure, algae in the medium in this survival experiment only served as a food source, without significantly changing the Pb content in *C. dubia*. The algae must have played a significantly different role in reducing the *C. dubia* mortality.

Fig. 3 indicates that algae only slightly reduced Pb content when mixed with Pb nano-TiO_2. However, this small Pb difference could not fully explain the significant decrease in *C. dubia* mortality from 80% to 35% (Fig. 2). In addition, the algae did not change the Pb depuration rate or Pb distribution in *C. dubia* when co-ingested with Pb nano-TiO_2 (Figs. 4 and 5). We conjectured that algae provided metabolic energy for *C. dubia* to reduce Pb toxicity. The main toxicity mechanism of heavy metals was the production of ROS (Ercal et al., 2001), and the algae could provide metabolic energy to boost antioxidation defense through elevating antioxidants such as glutathione (GSH) and superoxide dismutase (SOD) that neutralize the ROS (Poljsak et al., 2013). In addition, natural antioxidants were found in many algae (Kelman et al., 2012). Romay et al. (1998) also reported that a pigment from blue-green algae had antioxidant properties in vitro. Therefore, the algae may also directly serve as antioxidant when ingested by *C. dubia*. In addition, metallothionein (MT) is a type of protein that can bind with heavy metals to reduce toxicity, and the MT level is dependent upon heavy metal exposure and nutrient (Moltedo et al., 2000). Algae, as a food source, are possible to provide the energy to increase MT production which immobilizes the heavy metal in tissue. Because algae did not significantly reduce the total amount of Pb in *C. dubia*, some Pb could be immobilized by protein and become less available for tissue utilization and ROS production. Thus, algae could significantly reduce Pb-mediated mortality in *C. dubia*.

3.9. Effect of food type on the combined toxicity of Pb and nano-TiO_2

Fig. 7 shows the toxicity of Pb when YTC was used as the food. YTC is another food type recommended for *C. dubia* in the EPA standard method (EPA, 2002). The yeast and cereal are commonly present in the environment, and trout chow represents fish food that is available in the environment. The concentration of YTC (6.8 mg/L as total solid) used in the toxicity test was consistent with the concentration range recommended by EPA (EPA, 2002). Statistical analysis indicated that YTC did not significantly impact Pb toxicity ($p = 0.20$). However, the

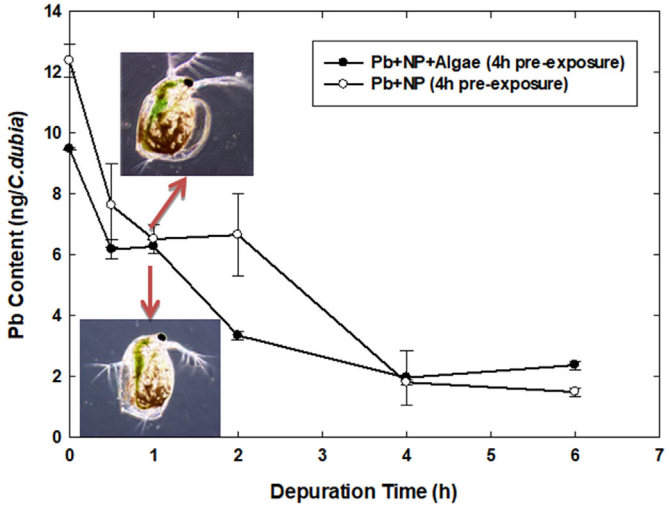

Fig. 5. The Pb depuration from *C. dubia* in a culture medium that contained algae after exposure in a culture medium that contained Pb NPs and Pb NPs Algae, respectively. Conditions of the exposure medium: [Pb] = 2500 μg/L; NPs = 200 mg/L; Algae = 1.8×10^5 cells/mL; pre-exposure time before depuration: 4 h. Photos are *C. dubia* from 1 h depuration after pre-exposure with Pb NPs (top) and Pb NPs Algae (bottom). Standard deviation was represented by the error bar attached to each point; the number of data for each point were 2 (N = 2).

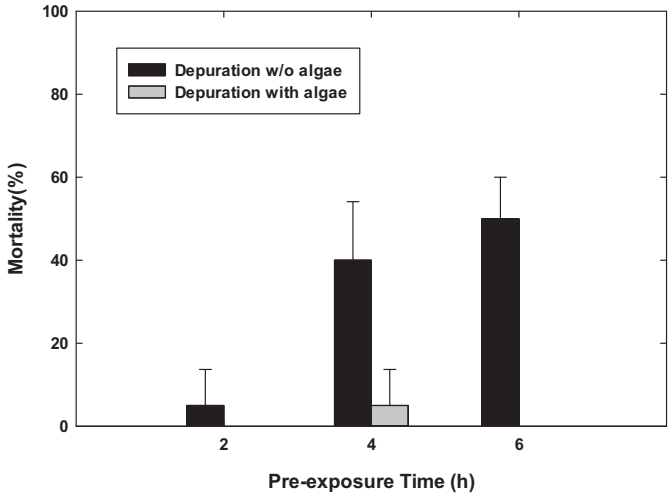

Fig. 6. The 24 h mortality of *C. dubia* in a clean medium and a medium containing algae, after exposure to a culture medium containing only NP Pb. Conditions of the exposure medium: [Pb] = 2500 μg/L; NPs = 200 mg/L; pre-exposure time before depuration: 2, 4, and 6 h, respectively. Concentrations of algae in depuration reactor: Algae = 1.8×10^5 cells/mL. Standard deviation was represented by the error bar attached to each point; the number of data for each point were 4 (N = 4).

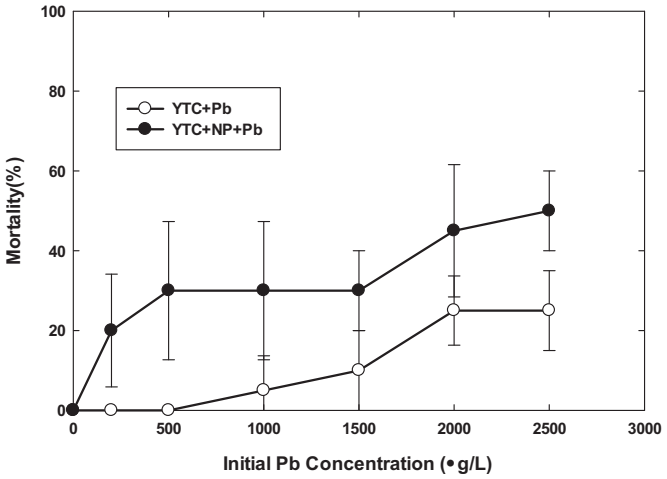

Fig. 7. Effect of yeast-trout chow-cereal leaves (YTC) on the 24-h mortality of *C. dubia* in the presence of Pb and Pb nano-TiO_2, respectively. Concentrations of particles: NPs = 200 mg/L; YTC = 6.8 mg/L as total solid. Standard deviation was represented by the error bar attached to each point; the number of data for each point were 4 (N = 4); $p < 0.05$ indicated statistical significant.

Pb concentration became a significant factor to impact the toxicity ($p < 0.05$). It is possible that YTC could provide energy to mitigate the Pb toxicity, but it also carried a higher amount of Pb to *C. dubia*. Compared to Pb algae (Fig. 2), YTC Pb also exhibited a slightly greater toxicity ($p < 0.05$). Importantly, YTC significantly reduced the combined toxicity of Pb nano-TiO_2 ($p < 0.05$) from 80% (Fig. 2) to 50% (Fig. 7) at a Pb concentration of 2500 μg/L. Compared to the results in Fig. 2, algae were more promising in reducing Pb toxicity than YTC. The effect of food type on the Pb-mediated toxicity in *C. dubia* may be attributed to their physical or biological properties, such as surface characteristics, adsorption capacity of NPs and Pb, the binding between food and NPs or Pb, food uptake or digestion efficiency, food concentration, natural antioxidant in food, and energy provided by the food.

4. Conclusions

Nano-TiO_2 can significantly elevate Pb toxicity via enhanced transport to *C. dubia*. Importantly, our data showed that algae and YTC can reduce the combined toxicity of Pb and nano-TiO_2. Algae, at a concentration of 1.8×10^5 cells/mL, significantly reduced the mortality of *C. dubia* from 80% to 35% in the presence of both Pb (2500 μg/L) and nano-TiO_2 (200 mg/L). From the view of Pb accumulation, the presence of algae with both Pb and nano-TiO_2 slightly reduced Pb uptake, but did not change the depuration rate of Pb. Further, algae did not immobilize Pb in the digestive tract, nor change the Pb distribution in the body. YTC exhibited a lower reduction in the combined toxicity of Pb and nano-TiO_2 than algae. The present finding suggests that food needs to be considered when assessing the toxicity of nanoparticles in the realistic environment, and further investigation on the mechanisms of toxicity mitigation from food will be needed.

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