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The combined toxic effect of nanoparticles and lead in the presence of algae (Raphidocelis)

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THE COMBINED TOXIC EFFECT OF NANOPARTICLES AND LEAD IN THE PRESENCE OF ALGAE (*RAPHIDOCELIS*)

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This dissertation consists of the following four articles:

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Paper II, found on pages 42–74, is intended for submission to *Environmental Science and Technology*.

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ABSTRACT

The elevated toxicity of toxic metals in the presence of nanoparticles (NPs) has raised significant concerns on the NP environmental safety. This research focuses on the impact of environmental conditions, specifically algae, on this type of toxicity. An indicator aquatic organism, *Ceriodaphnia dubia* (*C. dubia*), was used to examine the combined toxicity and toxicity mitigation from algae by monitoring its 24-h mortality. Four papers are included in this research, which focus on four aspects of the combined toxicity and the corresponding algae (*Raphidocelis*) impact. The first paper examines the algae impact on the combined toxicity of lead (Pb) and nano-TiO_2. The effect of algae was determined through Pb accumulation by, depuration from, and distribution within the *C. dubia*. The second paper develops a two-compartment kinetic model to quantify the iron (Fe) and Pb accumulation in *C. dubia* in the presence of nano-TiO_2. The model was used to investigate the major uptake, depuration, and distribution pathways of Fe and Pb in *C. dubia*. The third paper presents a toxicokinetic-toxicodynamic (TK-TD) model that was used to develop fundamental understanding of the combined toxicity of Pb and nano-TiO_2, more specifically, the role of nano-TiO_2 in the combined toxicity. The fourth paper focuses on the algae impact on the combined toxicity of Pb and an emerging containment, micro-plastic (MP). The combined toxicity mechanisms and the effect of algae were investigated through Pb and MP interaction, Pb accumulation, and MP accumulation. This research provides a fundamental understanding on the role of algae, a natural food source for aquatic organisms, on the toxicity of NPs in the presence of other background toxins.

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1. INTRODUCTION

1.1. COMBINED TOXIC EFFECT

In the natural environment, different kinds of toxic elements can pose toxic effects on organisms. A combined toxic effect is one kind of toxic effect caused by multiple toxic elements exposed together.[1] For combined toxicity, the adverse effects may increase more than simply adding the effect of an individual toxic element,[2] and challenge the existing toxicity evaluation methods.[3-6] Over the past several decades, anthropogenic activities have increased not only the level of containments, but also their variety in the environment. Numerous emerging containments and their interaction with existing environmental toxic elements have raised new risks, and seriously threaten the environmental safety throughout the entire food web. Liu et al.[7] found the mixture of TiO_2, SiO_2, and ZrO_2 nanoparticles significantly increased cellular oxidative damage in algae. Cedergreen et al.[8] reported that the co-exposure of fungicide and insecticide resulted in higher toxicity on planktonic crustaceans. Fan et al.[9] demonstrated that nano-TiO_2 increased heavy metal bioavailability on benthic organisms. Lee et al.[10] illustrated the combined effect of gold (Au) and polystyrene nanoplastics on fish. Therefore, as a public health concern, this complex combined toxic effect has increasingly attracted public attention.

1.1.1. Heavy Metal Toxicity. Heavy metals are one of the traditional environmental hazards. The extensive application of heavy metals in industry, agriculture, medicine, and technology have inevitably led to a wide heavy metal distribution in the environment.[11] Heavy metals can lead to serious health issues, including organ and nerve damage.[12] Pb exposure can result in severe damage to the brain and kidney.[13] Mercury (Hg)

can damage the brain and peripheral nervous system.[14] Cadmium (Cd) can damage bone and the liver.[15] Moreover, heavy metal toxicity is also on the basis of concentration, speciation, solubility, and environmental conditions.[12] For example, arsenic (As) toxicity is highly dependent on As speciation;[16] copper (Cu) exhibits higher toxicity at acidic conditions;[17] zinc (Zn) toxicity is affected by humic substance;[18] and Pb toxicity is a function of its solubility.[19] Collectively, heavy metals exhibit broad and critical impacts on the environment and health safety. Aquatic organisms can selectively or nonselectively uptake soluble and solid components from the environment. Apparently, soluble heavy metals could be directly ingested by organisms. Other than soluble component uptake, heavy metals also interact (e.g., adsorption) with particles in the environment.[20, 21] Consequently, heavy metals could also be ingested by organisms with these solid components. Although the U.S. EPA restricts the heavy metal level in aquatic systems,[22-24] in this scenario, the combined toxic effect of solid components and heavy metals would alter their toxicity pattern and raise the risk in the environmental safety.

1.1.2. Nanoparticle Toxicity. NPs are particles between 1 and 100 nm. Commonly used NPs include TiO_2, ZnO, SiO_2, CeO_2, Ag/AgO, FeO_x, AlO_x, and carbon nanotube,[25] and the production of these NPs was estimated in 2012 to range from 10,000 tons to more than 20,000 tons.[25] These NPs are extensively used in numerous commercial products (e.g., electronics,[26] agriculture,[27] healthcare,[28] additives,[29] paint,[30] and solar cell).[31] In the past decade, the rapid nanotechnology development and its broad application lead NPs to become an emerging environmental contaminant. The negative environmental impacts of NPs have attracted more and more attention. Xia et al.[32] found nano-ZnO induced oxidant injury, inflammation, and resulted in cell death; Asharani et al.[33] revealed silver

nanoparticles caused the death of zebrafish; Aruoja et al.[34] illustrated that nano-CuO was more toxic than bulk Cu to aquatic organisms; Li and Huang[35] reported that carbon nanotube showed toxicity to planktonic crustaceans in short-term mortality and long-term growth. In addition to the type of NP, one of the NPs toxicity mechanisms is related to contact with the biological membrane; therefore, NPs toxicity also depend upon their surface properties, e.g. functional group,[35] and surface charge.[36] These toxicity studies not only bring public attention to the toxicity of NPs themselves, but also raise new questions: As emerging contaminants, how do NPs interact with existing contaminants? Do NPs enhance the toxicity of existing contaminants, or mitigate the toxic effect? Will this interaction raise new challenges in environmental safety evaluation?

1.1.3. The Combined Toxic Effect of NPs and Heavy Metals. As indicated earlier, heavy metals are present in all aquatic systems. As a result, NPs inevitably mix with heavy metals when released into the environment. The combination of NPs and heavy metals can alter their toxicity pattern and raise new challenges in environmental safety.[5, 6, 10, 37-39] In this dissertation, the study of the combined toxicity will mainly focus on heavy metals and NPs.

Among all the NPs, metal and metal oxides based NPs represent significant portion of the market.[25] The toxicity of these NPs are depends on their physical and chemical properties. Some NPs are extremely toxic to aquatic organisms, e.g., nano-ZnO,[32] nano-CuO,[34] which has raised important concerns. Meanwhile, some NPs are considered as environmental friendly, e.g. nano-TiO_2.[40] However, researchers also found that nano-TiO_2 may interact with many environmental compounds and alter their fate and transport as well as their toxicity in the environment.[3, 4, 41] Heavy metal toxicity is also significantly affected

by nano-TiO_2. Hu et al.[6] reported that Pb toxicity is enhanced by nano-TiO_2; Wang et al.[39] illustrated that nano-TiO_2 elevates the As toxicity; Fan et al.[42] found Cu toxicity is increased in the presence of nano-TiO_2. This phenomenon is characterized as the "Trojan horse effect".[6] Briefly, NPs concentrates heavy metals on its surface and serves as a carrier to facilitate their transport to aquatic organisms. As a result, the heavy metal accumulation is significantly increased in organisms, thereby increasing the toxicity. Although nano-TiO_2 itself is less toxic, the combined toxic effect of nano-TiO_2 and heavy metals can also alter their toxicity pattern. Moreover, because nano-TiO_2 is considered environmentally friendly or less toxic, the public does not tend to attach great importance to the combined toxic effect of nano-TiO_2 and heavy metals in the environment.

1.2. ENVIRONMENTAL CONDITIONS

In aquatic systems, various environmental conditions, e.g., pH, hardness, organic matter, temperature, exist with heavy metals and NPs. These conditions not only maintain the ecological system, but also significantly affect the toxicity. For example, pH can affect As speciation to alter the toxicity;[43] dissolved organic matter can reduce the toxicity of Cu;[44] and high hardness can increase the LC_{50} of Cd, Cu, Zn, and Pb on water flea.[45] Other than heavy metals, the fate and toxicity of NPs are also affected by environmental conditions. Van Koetsem et al.[46] found the amount of suspended matter in aquatic systems affected the aggregation of CeO_2 NPs, thereby changing the toxicity. Majedi et al.[47] reported low water temperature increased the solubility of nano-ZnO, which is one of the toxicity mechanisms of Zn-based NPs. Furthermore, environmental conditions can mitigate or aggravate the toxicity of NPs and exhibit a different toxicity pattern from the

theoretical study. For example, nano-ZnO is considered a toxic NP to organisms, but Yung et al.[48] demonstrated that salinity reduced the toxicity of nano-ZnO. In contrast, nano-TiO_2 is a typical environmentally friendly material with negligible biological effects,[40] but Mitrano et al.[49] illustrated that under sunlight, UV radiation caused nano-TiO_2 surface transformation and resulted in reactive oxidative species (ROS) production to increase toxicity. Collectively, environmental conditions complicate the fate and toxicity of heavy metals or NPs in a real environment. Therefore, to reveal the realistic environmental impacts of heavy metals or NPs, environmental conditions must be considered in the evaluation of toxic elements.

In the natural environment, the coexistence of NPs and heavy metals may result in complex interactions and toxicity changes, and further blur the boundary of environmental safety. Environmental conditions not only affect the fate and toxicity of heavy metals or NPs individually, but also change the interaction between heavy metals and NPs. Therefore, in the combined toxicity evaluation system, environmental conditions have a more complex impact than individual toxic elements. For example, pH is the most important parameter that affects all aspects of the aquatic system, including the combined toxicity of NPs and heavy metals, by altering the solubility and speciation of the heavy metals, the activity of organisms, and the properties of NPs. In addition to the individual component, the interaction between NPs and heavy metals is dominated by the pH.[50] Other than the effect of pH, Fan et al.[41] found organic matter, commonly found in all aquatic systems, can alter Cu speciation and further influence the combined effect of nano-TiO_2 and Cu in the environment. These studies demonstrated the great complexity of environmental conditions, impact on combined toxicity.

Among all the environmental conditions, algae is a ubiquitous factor. Algae are present in all aquatic environments, and serves as a food source for the aquatic life in both freshwater and seawater. Therefore, algae will inevitably participate in the toxic process when it is ingested with NPs and/or heavy metals. It was found that algae can reduce the toxicity of heavy metal through a depuration process,[51, 52] or adsorption/removal mechanisms.[20] In addition, NPs or heavy metal can increase ROS production and induce toxicity in organisms.[53, 54] However, algae as a food source can provide energy for organisms to boost antioxidant synthesis or directly serve as an antioxidant to mitigate this type of toxicity.[55, 56] Other than these toxicity mitigations, some researchers found algae may increase the bioavailability and adverse effects of NPs on aquatic lives.[4, 57] These studies revealed algae may play a complex role, which must be evaluated in regard to the combined toxicity of NPs and heavy metals under realistic conditions.

1.3. KINETIC AND DYNAMIC MODEL

In the combined toxicity of heavy metals and less toxic NPs, e.g. nano-TiO_2, the toxicity mainly derives from the excessive heavy metal uptake.[6, 58] Model prediction is an effective approach to achieve better understanding of the toxicity mechanisms. The toxicokinetic- toxicodynamic (TK-TD) model is based on a series of processes to predict the toxic element accumulation and corresponding toxicity in organisms. The TK-TD model has been used in various toxicity studies, such as single toxic element, toxic element mixture, and synergistic toxicity.[8, 59, 60] Therefore, the TK-TD model can be used to simulate and quantify the combined toxicity of heavy metals and NPs. The TK model is used to predict toxic element accumulation in organisms in the presence of NPs. The

corresponding toxic element accumulation is then applied in the TD model to reveal the toxicity mechanisms.

1.3.1. Toxicokinetic Model. As a type of commonly used toxicity test organisms, planktonic crustaceans can be characterized as two compartments: gut and body tissue (exclude gut), and each compartment has an independent pathway to uptake and depurate toxic elements.[61] The gut is a major tissue of organisms that extends from the mouth to the anus. Toxic elements accumulated in the gut from active uptake through the mouth are eventually depurated from the anus. For other body tissues, one of the toxic element accumulation pathways is diffusion from environment. Other than diffusion, the gut, as the digestive system, is responsible for transporting nutrients to the tissue.[62] Therefore, toxic elements accumulated in the gut of organisms can also transfer to body tissue. To eliminate

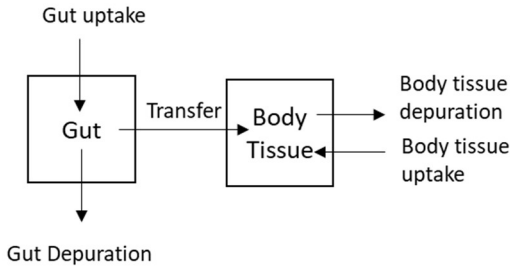

Figure 1.1. Scheme of toxicokinetic model in *C. dubia*.

toxic elements from body, the gut and body tissue also have independent pathways. Figure 1.1 briefly shows the toxic element uptake, elimination, and transfer pathways in organisms. The corresponding TK model equations in the presence of NPs are described in Paper II. In this two-compartment assumption, toxic elements in the gut could be easily removed from body, and toxic elements accumulated in body tissue are metabolically

available and respond to the toxicity.[61] Therefore, the TK model will predict the toxic elements in both gut and body tissue, and the predicted toxic element accumulation in the body tissue will then be applied in the TD model to conduct the toxicity mechanism study.

1.3.2. Toxicodynamic Model. The TD model is used with the TK model to predict the toxicity in terms of survivorship. The basic theory of the TD model is that if the toxic element accumulation in body tissue exceeds a threshold concentration that test organisms can tolerate, hazards arising from the exceeded toxic element accumulation start to accumulate and the probability of death increases.[59] Figure 1.2 summarizes this toxicodynamic process, and the corresponding TD model equations are described in Paper II and III. Two parameters in the TD model, threshold and killing rate, could reflect the toxicity of certain elements. The changing of any or both parameters could reflect the toxicity change with the addition of other compounds.[59] In the presence of NPs, especially less toxic nano-TiO_2, if heavy metals are the sole contributor to the toxicity, the threshold and killing rate should be consistent at different toxic element concentrations.[59] Although this TD model does not incorporate the NPs, the threshold and killing rate of the heavy metal determined from different NP concentrations can be used to reflect the impact of NPs on the combined toxicity, and reveal the toxicity mechanisms.

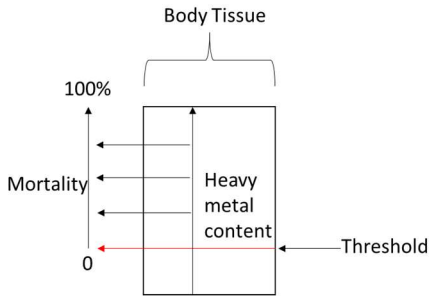

Figure 1.2. Scheme of the toxic process in *C. dubia*.

1.4. RESEARCH SIGNIFICANCE

The evaluation of the combined toxicity of heavy metal and NPs becomes important due to the increasing NPs release and toxicity pattern change. Currently, there are two major approaches to investigate the toxic element toxicity. One approach is generally focused on the characterization of different biological indicators in test organisms, such as ROS, metallothionein (MT), and superoxide dismutase (SOD).[42, 63, 64] Different biological tissues have different responses to the toxic element, and the damage of these tissues can elevate or suppress the release of certain proteins. By monitoring the level of these specific biological indicators, one can quantify the damage induced by the toxic element, and also specify the damaged tissue and the toxicity mechanism. However, the biological indicator approach involves excessive amounts of reagent testing, and the increased or decreased biological indicator level cannot differentiate if the damage comes from heavy metals or NPs. In addition, the initial condition of the individual test organism also significantly affects the level of the biological indicators. These weaknesses challenge the use of this approach in the combined toxicity study.

The other approach emphasizes the fate and transport mechanisms of the toxic elements,[3, 37, 59] which are related to the NP properties and other environmental conditions. These conditions can significantly affect the assimilation efficiency of toxic elements in test organisms. The transport of toxic elements within different tissues is also part of the research focus under this approach, and it can help to find the target tissue for specific elements. Based on these mechanisms, one can quantify the impact of different conditions on the toxic element accumulation, and reveal the assimilation pathway. There are also some limitations for this approach. For example, the activity of test organisms can

significantly affect the toxic element assimilation. To ensure the conditions do not affect the activity of test organisms, low concentrations of toxic elements and NPs that do not kill the test organisms have to be used. Therefore, this approach lacks the connection between toxic element accumulation and toxicity.

In addition to the theoretical combined toxicity studies, environmental conditions can induce great complexity on the combined toxicity in a realistic environment. Their toxicity pattern can significantly differ from theoretical studies. Currently, few studies have addressed the environmental impact on the combined toxicity. Lacking this information might result in overestimation or underestimation of the combined toxicity when evaluating their environmental impact. Among all the environmental conditions, algae may play a complex role in the combined toxicity. On one hand, algae are a food source for organisms that may mitigate the toxicity. On the other hand, algae are also carriers of heavy metals and NPs, which may elevate the toxicity. Due to the multifaceted performance, the evaluation of algae in the study of combined toxicity has become of great interest.

In this research, we quantified the heavy metal accumulation in organisms in the presence of NPs, and connected the accumulation with mortality to reveal the combined toxicity mechanisms. A realistic environmental condition, algae, was also considered in this toxicity study to evaluate the corresponding environmental impact. In addition, a first-order kinetic model will be established to quantify accumulation of heavy metals under different conditions, therefore revealing the toxicity mechanisms. This research will provide a reliable approach to determine effects of different components/conditions on the combined toxicity of heavy metals and NPs.

1.5. DISSERTATION SUMMARY

In this dissertation, four papers are used to systematically illustrate the impact of environmental conditions, specifically algae, on the combined toxicity of heavy metals and NPs. The test aquatic organism was *C. dubia*, based on the EPA method.[65] The contents of the four papers are described as follows.

In Paper I, the algae impact on the combined toxicity of Pb and nano-TiO_2 was investigated. The algae impact on the combined toxicity was examined by using 24 h mortality of *C. dubia*. The mechanism study was conducted through the physical aspect; more specifically, it investigated the effect of algae on the Pb accumulation, depuration, and distribution in *C. dubia*.

In Paper II, a two-compartment kinetic model was established to predict the Pb and Fe accumulation in *C. dubia* in the presence of nano-TiO_2. To validate the model results, different nano-TiO_2 concentrations were used, and the distribution of Pb and Fe in *C. dubia* was also examined. To extend the model application, the condition of algae impact on Pb accumulation in the presence of nano-TiO_2 was also examined. Furthermore, the predicted Pb accumulation results were linked with a toxicodynamic model to examine the feasibility of applying this model in toxicity prediction.

In Paper III, the combined toxicity mechanism of Pb and nano-TiO_2 was investigated through a TK-TD model. The TK model developed from Paper II was used to predict the Pb accumulation in the presence of different concentrations of nano-TiO_2. The predicted Pb accumulation was then applied in the TD model to reveal the toxicity mechanisms, and more specifically, the role of nano-TiO_2 in the combined toxicity. Finally,

the effect of algae on the combined toxicity of Pb and nano-TiO_2 was also examined through the TK-TD model.

In Paper IV, the combined toxicity of an emerging contaminant, MP, and Pb was investigated. The effect of algae on this combined toxicity mitigation was also examined. The toxicity mechanisms with and without algae were investigated through three aspects: 1) MP and Pb interaction, specifically soluble Pb concentration and Pb adsorption on MP; 2) Pb accumulation, and 3) MP accumulation. Finally, the contribution of soluble Pb and MP adsorbed Pb on the combined toxicity was quantified.

PAPER

I. ALGAE (*RAPHIDOCELIS*) REDUCE COMBINED TOXICITY OF NANO-TiO_2 AND LEAD ON *C. DUBIA*

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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, food, especially algae, is 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.

Keywords: Toxicity, *C. dubia*, Nano-TiO_2, Lead, Algae, YTC

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 more than 20,000 tons (Piccinno et al., 2012). Nano-TiO_2 has been widely used in paints, plastics, sunscreens, solar cells, and as food additives (O'regan et al., 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. Pervious 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., 2017). 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. MATERIAL 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 less than 24 h (which were brood from 7-14 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 less than 4. A total of three parallel series of Pb(II) solutions, with different selected

concentrations of Pb (up to 2,500 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 1,200 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 2,500 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 2,500 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 2,500 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 2,500 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 2,000 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 1,200 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

Figure 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 2,500 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 less than 10 μg/L for all Pb concentrations tested (Figure 1). Thus, nano-TiO_2 is an excellent sorbent of Pb.

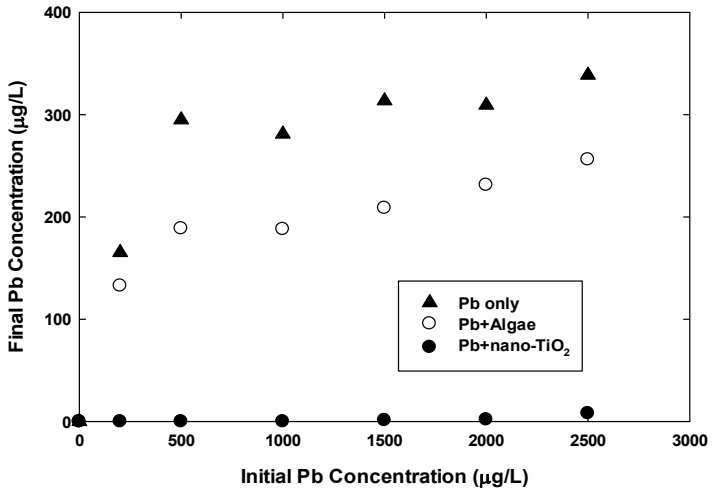

Figure 1. Soluble Pb(II) concentration in a culture medium at pH 7.8 with and without nano-TiO_2 (200 mg/L) or algae ($1.8x10^5$ cells/mL).

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 5,000 nm (Geller and Müller, 1981). Hu *et*

al. (2012a) reported that, 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

Figure 2 shows the effect of nano-TiO_2 and/or algae on the toxicity of Pb. The concentration of Pb alone (up to 2,500 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 3,166 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 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*.

Importantly, Figure 2 shows that, with the addition of nano-TiO_2 particles, Pb toxicity increased significantly ($p<0.05$). When the initial Pb concentration was 2,500 µg/L, the presence of nano-TiO_2 increased the *C. dubia* mortality to 80%. Table 1 shows

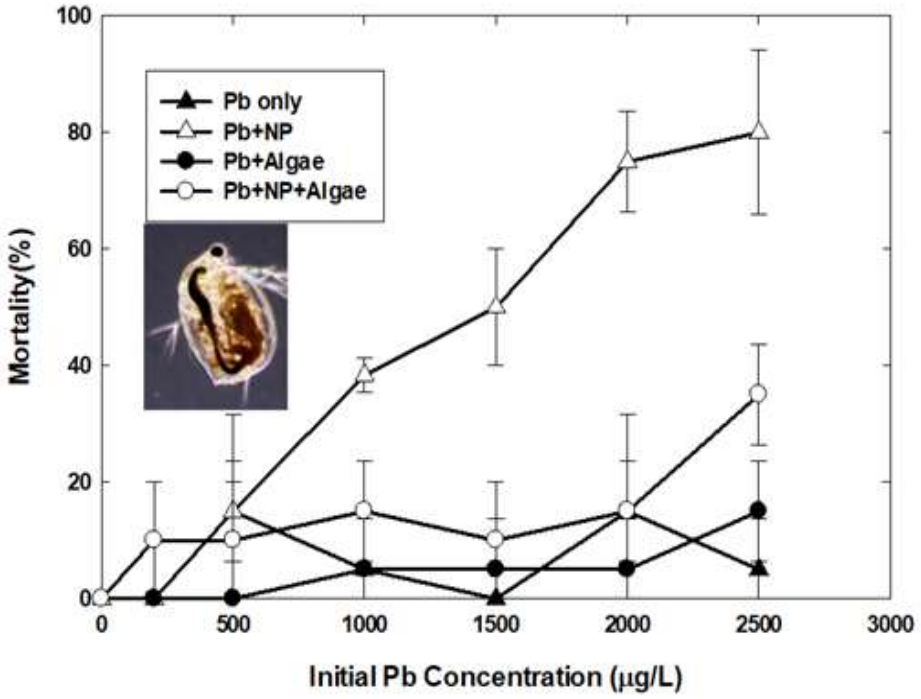

Figure 2. The 24 h mortality of *C. dubia* in the presence of Pb, with or without other particles (nano-TiO_2 and algae). Concentrations of particles: NPs = 200 mg/L; Algae = $1.8x10^5$ cells/mL. Photo was *C. dubia* from 1 h uptake with Pb (2500 μg/L) + NPs (200 mg/L). Standard deviation was represented 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.

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 Figure 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.

Table 1. Two-way ANOVA P-value between treatment groups.

Treatment Group	Pb concentration P-value	Type of particle P-value	Interaction* 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 – Algae+NP+Pb	<0.05	<0.05	<0.05
YTC+Pb – Algae+Pb	<0.05	<0.05	0.13
NP+Pb – YTC+NP+Pb	<0.05	<0.05	<0.05

*Interaction = Pb concentration × Type of particle

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

Figure 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 2,500 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

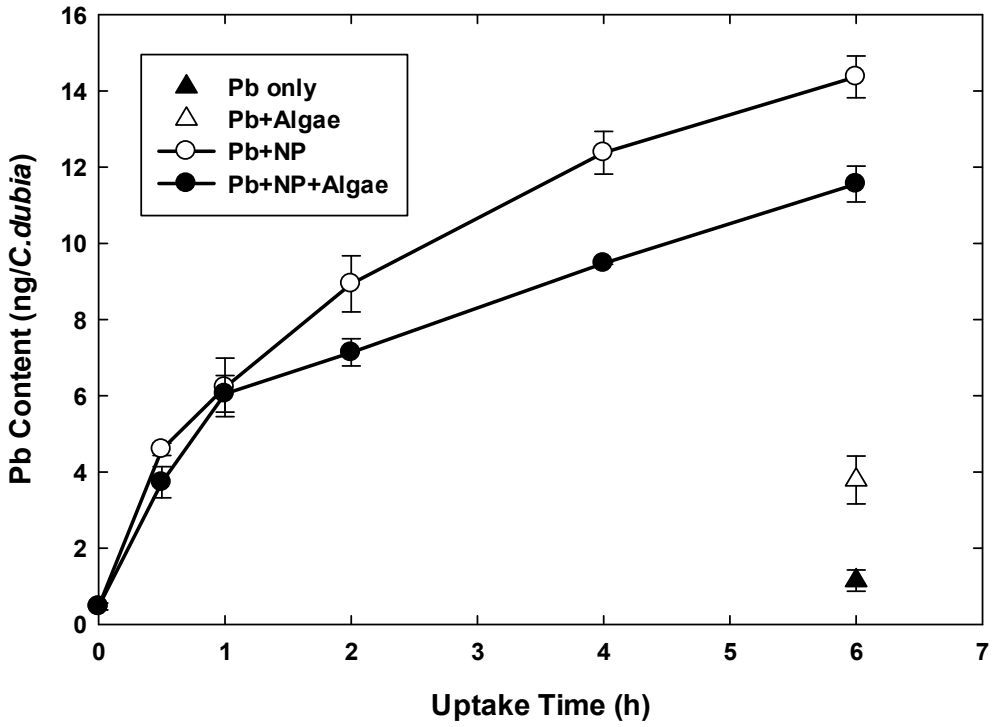

Figure 3. The Pb content in adult *C. dubia* bodies in the presence of NPs and/or algae. Condition of the exposure medium: [Pb] = 2500 μg/L; NPs = 200 mg/L; Algae = 1.8×10^5 cells/mL. Standard deviation was represented by the error bar attached to each point; the number of data for each point were 2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

metals and NPs, or occupy some spaces within the gut therefore reduce their uptake. Figure 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.

Figure 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 (Figure 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%.

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. Figure 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.

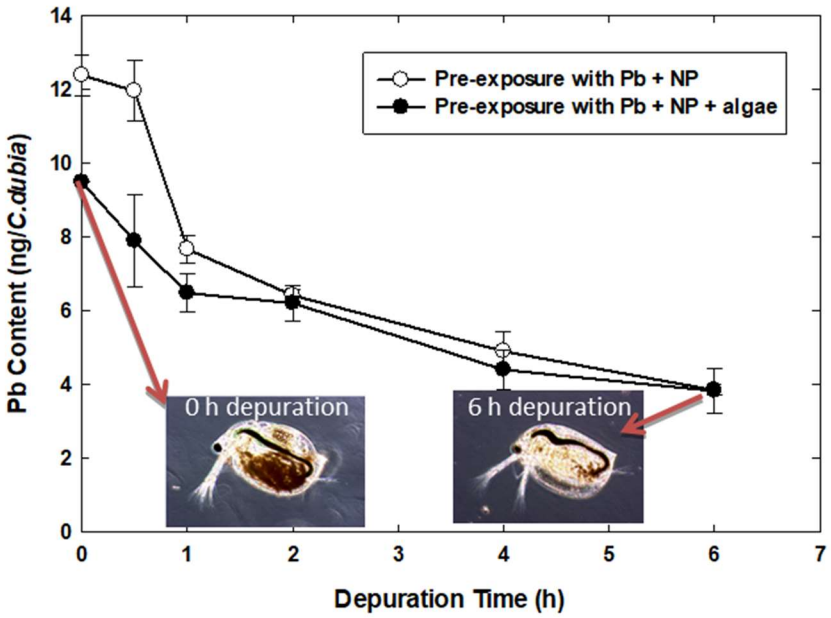

Figure 4. The Pb depuration from *C. dubia* in a clean culture medium after exposure in a culture medium that contained Pb + NPs and Pb+NPs+Algae, respectively. Conditions of the exposure medium: [Pb] = 2500 µg/L; NPs = 200 mg/L; Algae = $1.8x10^5$ cells/mL; exposure time before depuration: 4 h. Photo 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. Figure 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 Figure 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 Figure 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 (Figure 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 (2,500 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. Figure 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.

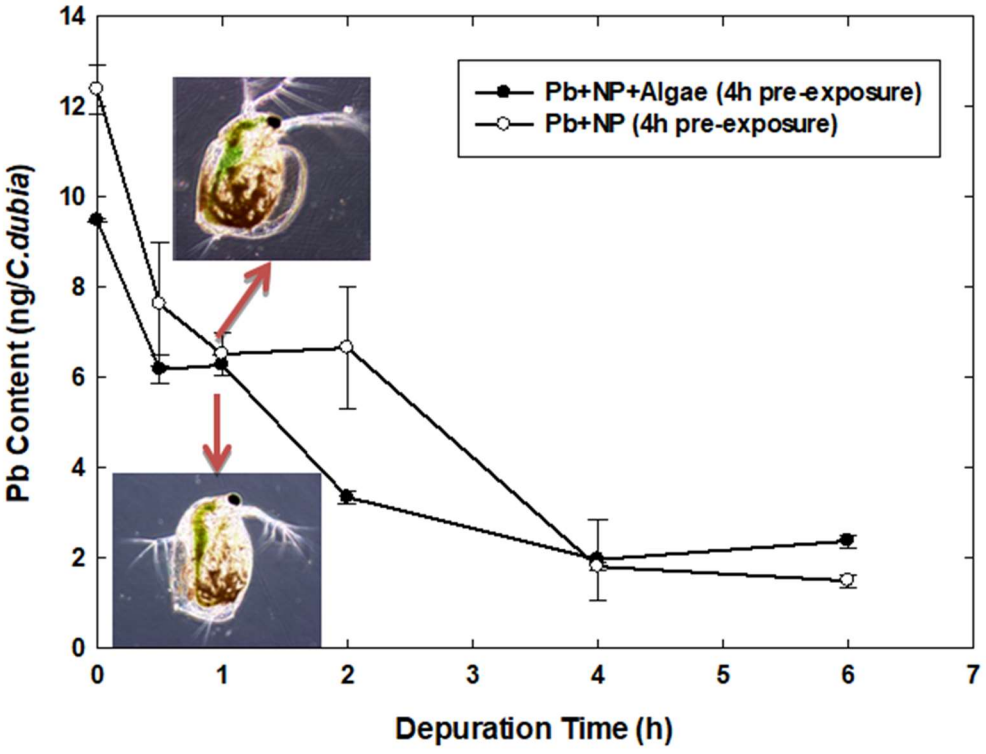

Figure 5. The Pb depuration from *C. dubia* in a culture medium that contained algae after exposure in a culture medium that contained Pb + NPs and Pb+NPs+Algae, respectively. Conditions of the exposure medium: [Pb] = 2500 μg/L; NPs = 200 mg/L; Algae = $1.8x10^5$ cells/mL; pre-exposure time before depuration: 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).

Figure 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% (Figure 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 (Figures 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

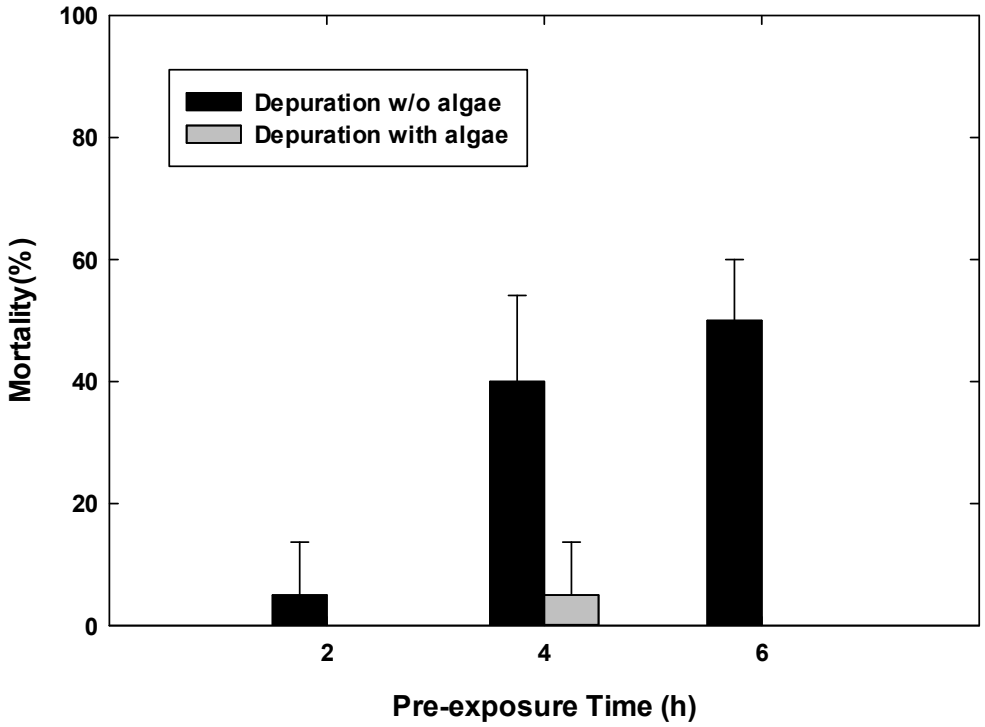

Figure 6. The 24 h mortality of *C. dubia* in a clean medium and a medium containing algae, after exposure to a culture medium containing only NP+Pb. Conditions of the exposure medium: [Pb] = 2500 μg/L; NPs = 200 mg/L; pre-exposure time before depuration: 2, 4, and 6 h, respectively. Concentrations of algae in depuration reactor: Algae = $1.8x10^5$ 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).

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

Figure 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 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 (Figure 2),

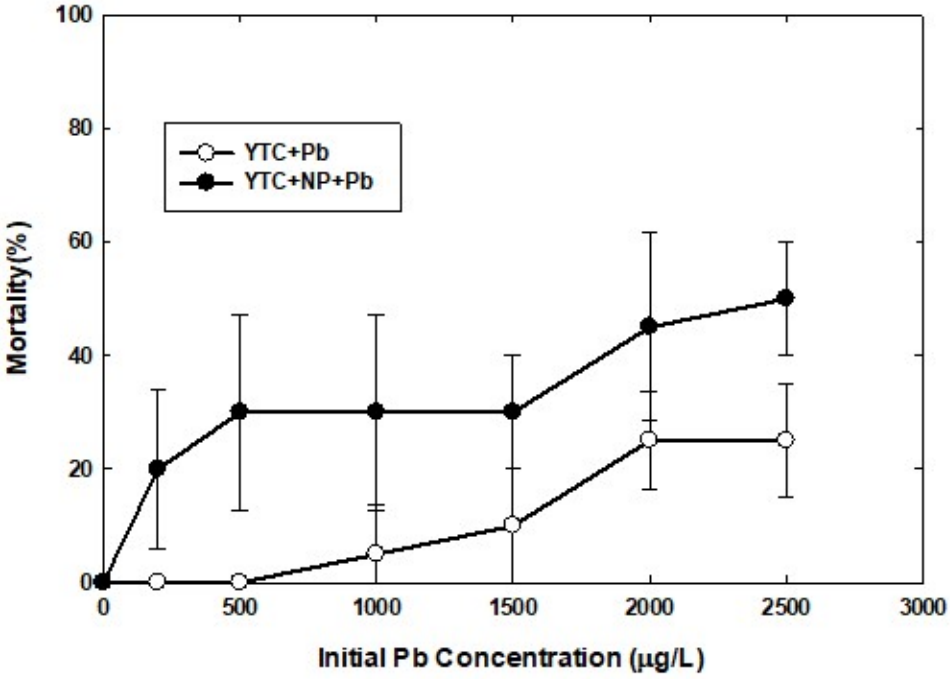

Figure 7. Effect of yeast-trout chow-cereal leaves (YTC) on the 24-h mortality of *C. dubia* in the presence of Pb and Pb + nano-TiO_2, respectively. Concentrations of particles: NPs = 200 mg/L; YTC = 6.8 mg/L as total solid. Standard deviation was represented by the error bar attached to each point;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

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% (Figure 2) to 50% (Figure 7) at a Pb concentration of 2,500 µg/L. Compared to the results in Figure 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 (2,500 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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II. QUANTIFYING THE EFFECT OF NANO-TiO_2 ON THE TOXICITY OF LEAD ON *C. DUBIA* USING A TWO COMPARTMENT MODELING APPROACH

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ABSTRACT

Nanoparticles (NPs) can significantly influence toxicity imposed by metals. However, this impact has not been well quantified. In this research, we investigated the effect of nano-TiO_2 on lead (Pb) accumulation and the resultant toxicity using *Ceriodaphnia dubia* (*C. dubia*) as the testing organism. We used a two compartment modeling approach, which included a two compartment accumulation model and a toxicodynamic model on the basis of Pb tissue accumulation, to quantify this impact. The effect of algae, a natural food of *C. dubia*, on the combined toxicity of Pb and nano-TiO_2 was also quantified. The two compartment accumulation model could well quantify Pb accumulation kinetics in two compartments of *C. dubia*, the gut and the tissue (the rest of the body parts) in the presence of nano-TiO_2. Modeling results suggested that the gut

quickly accumulates Pb through active uptake from the mouth, reaching the maximal level within 2 h. However, the tissue slowly accumulates Pb from the gut at a steady rate. The predicted Pb distribution within *C. dubia* was verified by depuration modeling results from an independent depuration test. The toxicity test data indicated that the survivorship of *C. dubia* as a function of accumulated Pb in the tissue can be well described by a toxicodynamic model. Effects of algae on Pb accumulation in different compartments of *C. dubia* and the toxicity in the presence of nano-TiO_2 were also quantified using the two compartment modeling approach. The two compartment modeling approach provides a useful tool in assessing the effect of NPs on aquatic ecosystems where toxic metals are present.

Keywords: Nano-TiO_2, Lead, Toxicity, Algae, Two compartment model

1. INTRODUCTION

Recent anthropogenic activities have led to a significant release of nanoparticles (NPs) into the environment.[1] In addition to causing adverse environmental effects alone, NPs could also adsorb toxic metals and carry them into the body of aquatic organisms, enhancing the toxicity of the toxic metals in the environment. This effect challenges the existing toxicity evaluation paradigm for toxic metals.[2-6] Understanding the accumulation and distribution of toxic metals in aquatic organisms is essential in assessing metal toxicity in the presence of NPs.

Previous reports attributed the NP-enhanced toxicity to the excessive accumulation of toxic metals in the whole body.[2,7] For example, lead (Pb) accumulation in *Ceriodaphnia*

dubia (*C. dubia*) was increased by approximately 20 times in the presence of nano-TiO_2, leading to toxicity enhancement.[8] In the absence of NPs, researchers have used process-based kinetic modeling approach to quantify toxic metal accumulation in the entire body and the corresponding toxicity, based on the metal uptake, transfer, and depuration pathways.[9-11] However, because NPs alter the metal distribution pattern in different parts of the body, this whole body approach is unable to predict metal toxicity in the presence of NPs.

Gillis et al.[12] proposed that the whole body of a *Daphnia* can be divided to two compartments: the gut and the body tissue (tissue) excluding the gut. Heavy metals in the gut have less toxicity and can also be easily removed, but those in the tissue are metabolically available to induce toxicity. As a result, when assessing the effect of NPs on metal toxicity, models are needed to quantify metal accumulation in different compartments of *C. dubia*. Moreover, under realistic environmental conditions, other factors may also affect metal accumulation and distribution in the organism and therefore the overall toxicity.[13,14] For example, algae inevitably participate in the heavy metal accumulation process due to their extensive distribution in the environment, and the heavy metal distribution within the organism due to their physiological function. Therefore, the effect of algae on metal bioavailability, distribution, and toxicity is also of great interest when assessing the metal toxicity in the presence of NPs in the environment.

A toxicodynamic model has been used to link toxic metal accumulation with toxicity.[9-11] The model describes a time-dependent hazard based on toxic metal accumulation in the entire body.[9,15] However, because heavy metal accumulation in the tissue is more relevant to toxicity[12] and NPs alter the metal distribution pattern within the

body of the aquatic organism by disproportionally carrying significantly more heavy metals to the gut, toxicity prediction based on whole body accumulation is no longer appropriate. Heavy metal accumulation in the tissue must be considered in a toxicodynamic model in order to better quantify the effect of NPs on metal toxicity.

In this study, we developed a two compartment accumulation model to predict the accumulation kinetics of Pb in the gut and the tissue of *C. dubia* in the presence of nano-TiO_2. Accumulated Pb in the tissue was applied to the toxicodynamic model to predict toxicity. We used *C. dubia*, an EPA recommended toxicity test organisms, as the indicator organism for toxicity.[16] We also quantified the effect of *Raphidocelis,* a common freshwater algae, on Pb accumulation and toxicity in the presence of nano-TiO_2, to shade light on the toxicity of Pb and NPs under more realistic environmental conditions.

2. THEORETICAL ASPECTS

2.1. TWO COMPARTMENT ACCUMULATION MODEL

The whole body of *C. dubia* can be characterized as two compartments: the gut and the body tissue excluding gut.[12] Toxic metals can accumulate in both compartments, with each compartment having independent uptake pathways. The toxic metal accumulated in the gut is from active uptake through the mouth, and that accumulated in the tissue is in part from the surrounding environment through diffusion.[17] In addition, the gut, as the digestive system, is responsible for nutrient transport via both passive and active mechanisms.[18] Therefore, toxic metal accumulated in the gut can also transfer into the tissue. The gut and the tissue also have independent pathways to depurate the toxic metals

from *C. dubia*. Figure 1 schematically illustrates the two compartment model of uptake, depuration, and transfer of toxic metals in *C. dubia*. We propose the mathematical model below to delineate the accumulation kinetics of toxic metals:

$$C_T = C_{gut} + C_{body} \quad (1)$$

$$\frac{dC_{gut}(t)}{dt} = k_{in} - (k_{12} + k_{1e}) \times C_{gut}(t) \quad (2)$$

$$\frac{dC_{tissue}(t)}{dt} = jC_D(t) + k_{12}C_{gut}(t) - k_{2e}C_{tissue}(t) \quad (3)$$

where $C_T(t)$ = total toxic metal accumulated in whole body (ng/flea) at time t; $C_{gut}(t)$ = toxic metal accumulated in the gut (ng/flea) at time t; $C_{tissue}(t)$ = toxic metal accumulated in the tissue (ng/flea) at time t; $C_D(t)$ = dissolved metal concentration (ng/L) at time t; k_{in} = toxic metal gut influx rate (ng/flea/h); k_{12} = toxic metal transfer rate constant from the gut to the tissue (h^{-1}); k_{1e} = toxic metal depuration rate constant from the gut (h^{-1}); j = the tissue accumulation constant that reflects the diffusion from the surrounding test solution (L/flea/h); k_{2e} = toxic metal depuration rate constant from tissue to the surrounding solution through diffusion (h^{-1}); t = exposure time (h).

Toxic metals in water are either associated with particles or in the soluble form. The particulate forms of the toxic metals, including the metal precipitates and metals adsorbed by NPs, are accumulated in the gut through active uptake by *C. dubia*. Settled particles can also be re-suspended and ingested by *C. dubia*.[19] Because the gut of *C. dubia* has a fixed space to maintain NPs, the gut uptake rate of heavy metals is proportional to the adsorbed metal concentration on the NP surface. In a system that contains Pb and nano-TiO_2, nearly 100% of Pb is adsorbed by nano-TiO_2.[8] Thus, the total Pb concentration can

be used to calculate the Pb concentration absorbed by nano-TiO_2. Therefore, the influx rate of Pb to the gut through active ingestion from the mouth can be expressed as:

$$k_{in} = \frac{C_T}{C_{NP}} \times k \quad (4)$$

where C_{NP} = the NP concentration (mg/L); k = the gut uptake constant through active mouth uptake (mg/flea/h).

The above different differential equations were solved by using the WolframAlpha to find the analytical solutions.[20]

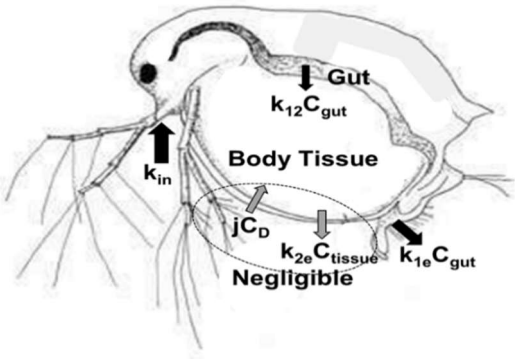

Figure 1. The schematic of the two compartment accumulation model. The gut accumulation considers active uptake through the mouth (k_{in}), losses to the body tissue ($k_{12}C_{gut}(t)$), and losses to the outside ($k_{1e}C_{gut}(t)$). The body tissue accumulation considers influx from the outside ($jC_D(t)$) and influx from the gut ($k_{12}C_{gut}(t)$), as well as losses to the outside ($k_{2e}C_{tissue}(t)$). The Pb exchange between the body tissue and the surrounding solution through diffusion is negligible.

2.2. TWO COMPARTMENT DEPURATION MODEL

A two compartment depuration kinetic model was used to fit the experimental data from a depuration test, to determine the metal distribution at the beginning and during the depuration process.[12] This model assumes that depurations from both the gut and the tissue follow first-order kinetics:

$$M_T(t) = M_{gut}(0)e^{(-k_g t)} + M_{tissue}(0)e^{(-k_b t)} \quad (5)$$

where $M_T(t)$ = mass of heavy metal in the whole body (ng/flea) at time t during depuration; $M_{gut}(0)$ = mass of heavy metal in the gut at the beginning of depuration (ng/flea); $M_{tissue}(0)$ = mass of heavy metal in the tissue at the beginning of depuration (ng/flea); k_g = the gut depuration rate constant (h^{-1}); k_b = the body depuration rate constant (h^{-1}); and t = the depuration time (h). Note that $M_{gut}(0)$ and $M_{tissue}(0)$ for the depuration equation are equal to $C_{gut}(t)$ and $C_{tissue}(t)$ for the accumulation equation, where t is the time used to accumulate Pb before depuration tests.

The kinetic parameters were determined by using the least-squares algorithm during the nonlinear regression analysis of the experimental data. The goodness of the data fitting was evaluated by the coefficient of determination (R^2).

2.3. TOXICODYNAMIC MODEL

A toxicodynamic model can be used with the two compartment accumulation model to predict the toxicity in terms of survivorship. The principle of this toxicodynamic model is that if toxic metal accumulation in the tissue (metabolically available) exceeds a threshold value, hazard starts to accumulate, resulting in an increase in the probability of death.[9-11] For this research, we hypothesize that the maximum exceedance of the toxin tissue accumulation contributes equally with the exposure time to the hazard. This hypothesis is similar to the C*t (disinfectant concentration × time) concept used for water disinfection process – a certain C*t value is correlated to a certain level of disinfection. Because the Pb tissue accumulation increases with the increase in the exposure time, the

terminal Pb tissue accumulation at the end of the exposure, $C_{tissue}(t)$, is the maximal Pb tissue accumulation. As a result, the hazard can be expressed as:

$$H(t) = k_k \times (C_{tissue}(t) - C_{TH})t \quad (6)$$

where H(t) = hazard (dimensionless) at time t; k_k = killing rate (flea/ng/h); C_{TH} = toxic metal threshold concentration (ng/flea).

For this research, the negative control group did not show any toxicity. Therefore, the survival probability can be expressed as:[9]

$$S(t) = \begin{cases} 1 & if\ C_{tissue}(t) \le C_{TH} \\ e^{-H(t)} & if\ C_{tissue}(t) > C_{TH} \end{cases} \quad (7)$$

where S(t) = survival probability of the test organism at time t.

By combining Equations (6) and (7) we can develop a toxicodynamic model for this application:

$$S(t) = \begin{cases} 1 & if\ C_{tissue}(t) \le C_{TH} \\ e^{-k_k \times (C_{tissue}(t) - C_{TH})t} & if\ C_{tissue}(t) > C_{TH} \end{cases} \quad (8)$$

The kinetic parameters were determined by using the least-squares algorithm during the nonlinear regression analysis of the experimental data. The goodness of the data fitting was evaluated by the coefficient of determination (R^2).

3. MATERIALS AND METHODS

3.1. CHEMICALS, NPS, AND ORGANISMS

All the chemicals used in this research were acquired 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 culture medium buffer. $Pb(NO_3)_2$ was used to prepare the Pb stock solution. Trace metal grade nitric acid was used for sample digestion. A certified Pb standard solution at 1000 mg/L was used to develop standard calibration curves for Pb analyses. Nano-TiO_2 (5-10 nm, anatase, 99%) purchased from Skyspring Nanomaterials Inc. (Houston, TX, USA) was used to prepare testing solutions. Algae (*Raphidocelis*) and a mixture of yeast, trout chow, and cereal leaves (YTC) (Pb < 1 μg/L) acquired from ABS Inc. (Fort Collins, CO, USA) were used as food for *C. dubia* mass culture. The same algae (*Raphidocelis*) were also used in the accumulation, depuration, and toxicity test. The *C. dubia* starter was purchased from MBL Aquaculture (Sarasota, FL, USA), and cultured in our lab using culture medium that contained 1.8×10^5 cell/mL of algae and 6.8 mg/L of YTC (as total solid) based on the EPA standard method, EPA-821-R-02-012.[16]

All test solutions were prepared using Milli-Q (MQ) water (resistivity = 18.2 MΩ·cm). The culture medium buffer, moderate hardness synthetic water (pH = 7.8 ± 0.2, hardness = 85± 5 mg/L as $CaCO_3$), was prepared by dissolving appropriate amount of $CaSO_4{\cdot}2H_2O$, Na_2SeO_4, $NaHCO_3$, $MgSO_4$, and KCl into MQ water following the EPA standard method.[16] The stock solution of 1000 mg/L Pb(II) was prepared by dissolving an appropriate amount of $Pb(NO_3)_2$ into MQ water, and acidified with the trace metal grade nitric acid to a pH of less than 4.

All mass culture and toxicity tests were conducted in a laminar flow hood (SVC-6AX, Streamline® laboratory products, Fort Myers, FL, USA) within a temperature-controlled chamber (25 °C). The chamber was set at a light to dark cycle of 16 h to 8 h to mimic the natural day and night light cycle.

3.2. ACCUMULATION TEST

For the accumulation test, test solution containing 2,500 μg/L of Pb(II) was prepared by diluting the Pb stock solution with 1 L of culture medium buffer in 1 L HDPE bottle. An appropriate mass of dry nano-TiO_2 particles was added to the bottle to obtain a 50 mg/L nano-TiO_2 concentration. The test solution was then mixed using a mechanical shaker for 24 h and the final pH was in the range of 7.4-7.8. After mixing, 100 mL of the test solution was transferred to each 125 mL HDPE bottle reactors to conduct the accumulation test.

Adult *C. dubia* (2 week old) were used to conduct the accumulation test. Four reactors, representing 4 accumulation time points, 1, 2, 4, and 6 h, respectively, were used. For each reactor, approximately 50 adult *C. dubia* were used. Before adding to the reactor, *C. dubia* were washed three times with clean culture medium buffer to eliminate any food adsorbed on the surface of *C. dubia* during culturing. After designated exposure time periods (1, 2, 4, or 6 h), *C. dubia* were collected and washed with clean culture medium buffer three times to remove particles on the surface. The *C. dubia* were the filtered through a 0.297 mm sieve, counted, and transferred to a digestion vessel that contained 5 mL nitric acid. They were digested at 95°C for 12 h in a hotblock digester with Watlow mini controller (Watlow mini-0R10-000G). The digested sample was then diluted and analyzed for soluble Pb. The total Pb accumulation in each *C. dubia* was then calculated based on the soluble Pb concentration of the digestion solution. A control group that did not contact the test solution was also was also included, and used to determine the background Pb content, which was 0.12 ng/flea. The net accumulation of Pb during the accumulation

process was calculated by subtracting the background Pb content from the total accumulations. All accumulation experiments were conducted in duplicate.

The impact of algae on Pb accumulation was also tested by following a similar procedure as above, except that the test solution contained not only 2,500 μg/L of Pb and 50 mg/L of nano-TiO_2, but also algae. Two algae concentrations, 1.8×10^5 and 5.4×10^5 cell/mL, were used. The algae concentrations were achieved by transferring appropriate volumes of the algae stock solution (3×10^7 cell/mL) to the test solution containing Pb and nano-TiO_2.

3.3. DEPURATION TEST

The Pb depuration test was conducted by following the procedure used by Gillis, et al.[12] In brief, different depuration times, 0, 1, 2, 4, 6, and 8 h, were used. For each depuration time, approximately 50 adult *C. dubia* were initially placed in a 100 mL test solution that contained 2,500 μg/L of Pb and 50 mg/L of nano-TiO_2 for 2 h to uptake Pb. The *C. dubia* were then collected and washed with clean culture medium buffer three times. The group that had 0 h depuration time was directly digested without going through the depuration process. The rest 5 groups of *C. dubia* were transferred to 5 reactors that contained 100 mL depuration solution (clean culture medium buffer + 1.8×10^5 cell/mL of algae), respectively, for depuration. After pre-selected depuration times (1, 2, 4, 6, and 8 h), *C. dubia* were collected, washed, filtered, counted, and digested in nitric acid. The Pb concertation in the digestion solution was measured and was used to calculate the total Pb content in the *C. dubia*. The background Pb content was determined using a group of 50 *C. dubia* that did not go through the Pb uptake and depuration procedures and was the same

as that measured for the accumulation control group (0.12 ng/flea), which was expected as the control groups were cultured under identical conditions. The final accumulated Pb in *C. dubia* was calculated by subtracting the background Pb content from the total Pb content.

3.4. TOXICITY TEST

The toxicity tests were conducted by following an EPA method.[16] Two types of toxicity tests were conducted. The first type was to determine the effect of exposure time on toxicity and the second type was to determine the effect of Pb concentration on the 24 h toxicity.

For the first type of toxicity tests (effect of exposure time), two test solutions were used: both contained 2,500 μg/L of Pb and 50 mg/L of nano-TiO_2 while one was supplemented with 1.8×10^5 cell/mL of algae. For each test solution, the survivorships of *C. dubia* neonates for different exposure times, ranging from 0 to 24 h, were monitored each hour. In brief, a total of twenty healthy neonates with an age of less than 24 h and four 30 mL medicine cup reactors that contained 15 mL test solution each were used. Five neonates were washed three times with the clean culture medium buffer to remove residual food on their surface before transferring to each test reactor. A plastic dropper with a 3 mm diameter opening was used to transfer neonates to prevent any damage to them. The survivorship of the neonates was visually monitored each hour and dead neonates were removed from the test reactor until 24 h.

For the second type of tests (effect of Pb concentration), two test kinds of test solutions were used: both contained Pb in a series of different concentrations (1,000, 1,500,

1,750, 2,000, 2,500, and 5,000 μg/L) and 50 mg/L of nano-TiO_2 while and one was supplemented with 1.8×10^5 cell/mL of algae. Procedures were same as that described in the first type of toxicity test (effect of exposure time), except for using 4 replicate reactors for each Pb concentration and the survivorship was examined only after 24 h of exposure.

3.5. ANALYTICAL METHOD

To determine the soluble Pb concentration in test solutions, 10 mL of the test solution was first centrifuged at 2,000 G for 10 min to remove particles. The supernatant was then collected, acidified, and analyzed using a graphite furnace atomic absorbance spectrophotometer (GFAA) (Perking Elmer AAnalyst 600), which has a Pb detection limit of 0.5 μg/L.

4. RESULTS AND DISCUSSION

4.1. Pb ACCUMULATION IN THE PRESENCE OF NANO-TiO_2

C. dubia neonates were used to conduct Pb toxicity tests according to EPA procedures.[16] Under the ideal conditions the same *C. dubia* neonates should be used to conduct Pb accumulation and depuration tests. However, because the neonates were extremely fragile and could not survive the multiple manual steps of the accumulation and depuration tests, it was not practical to use them for both of these tests. Instead, we used *C. dubia* adults (2 weeks old) to conduct the Pb accumulation and depuration tests.

Figure 2 shows the Pb accumulation data (filled circles) in *C. dubia* during the 6 h testing period. Equations (1) - (3) were used to fit the experimental data. The modeling

result (solid line) was in agreement with the experimental data (R^2=0.98). The related model parameters were also determined, shown in Table 1. By using the model parameters, we were able to estimate the Pb accumulation in different compartments of *C. dubia*. The dashed line was the estimated gut accumulation of Pb. The estimation indicates that gut Pb accumulation was very fast and took less than 2 h to reach the maximum value. The fast gut accumulation was caused by the active uptake of mostly the particulate Pb through the mouth.

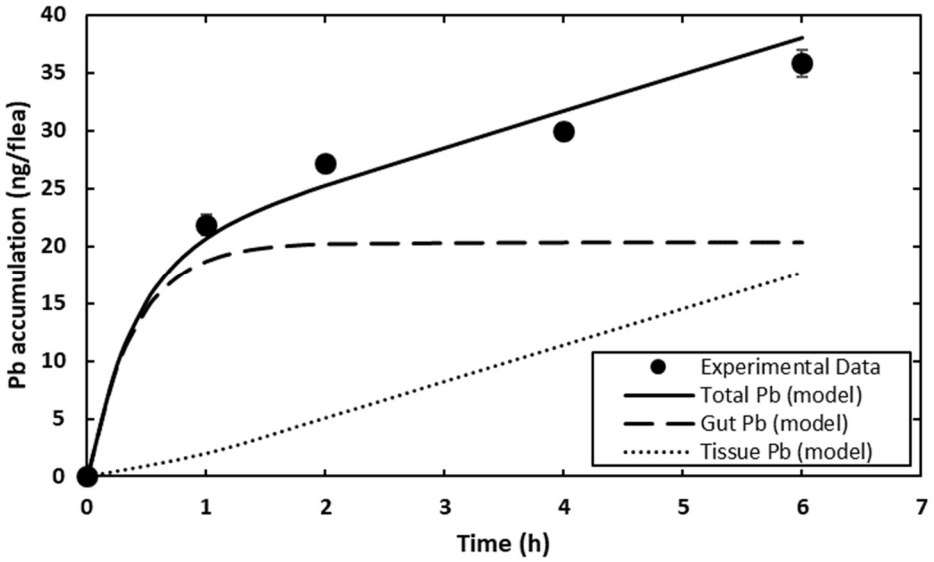

Figure 2. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2. Condition of the exposure medium: [Pb] = 2,500 μg/L; [nano-TiO_2] = 50 mg/L. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

In contrast, the tissue can accumulate heavy metals from both the surrounding test solution and the gut. The dotted line in Figure 2 shows the modeled Pb tissue accumulation, which gradually increased at a constant rate. Compared to gut accumulation, Pb tissue accumulation was much slower. Pb is a non-essential element, which does not have any

physiological function in cells and specific uptake pathways.[21] In addition, Pb uptake has been shown to be mainly through the calcium (Ca) ion channel[22] and our culture medium was a moderate hardness water, which had a much higher Ca concentration than Pb. Therefore, these resulted in low Pb accumulation rate in tissue.

Table 1. Two compartment accumulation model parameters.

Test Solution	k (mg/flea/h)	j (L/flea/h)	k_{12} (h^{-1})	k_{1e} (h^{-1})	k_{2e} (h^{-1})	AE* (%)	R^2
Before model simplification							
**Pb+nano-TiO_2	1.022×10^{-3}	0.001×10^{-3}	0.155	2.357	0.001	6.2	0.98
After model simplification							
**Pb+nano-TiO_2	1.022×10^{-3}	N.A.	0.155	2.357	N.A.	6.2	0.98
**Pb+nano-TiO_2+algae (1.8×10^5 cell/mL)	0.926×10^{-3}	N.A.	0.080	2.357	N.A.	3.2	0.93
**Pb+nano-TiO_2+algae (5.4×10^5 cell/mL)	0.693×10^{-3}	N.A.	0.080	2.357	N.A.	3.2	0.95

*AE = assimilation efficiency = $k_{12}/(k_{12}+k_{1e})$ [29]

**[Pb] = 2,500 μg/L; [Nano-TiO_2] = 50 mg/L

We also used Equations (1) – (3) to quantity the accumulation of iron (Fe), an essential element, using a test solution that contained 2,500 μg/L of Fe and 50 mg/L of nano-TiO_2 (Figure.S1). The purpose of this test was to determine the difference between non-essential elements and essential elements in terms of tissue accumulation. Experimental data showed that Fe had a similar accumulation pattern as Pb. However, Fe exhibited a significant higher tissue accumulation rate than Pb. Fe is an important component in the electron transport chain during the metabolic process.[23] Therefore, it had a higher tissue accumulation rate in *C. dubia* than Pb.

4.2. MODEL SIMPLIFICATION

Table 1 shows that the tissue accumulation constant j that denotes Pb diffusion from the surrounding test solution to the tissue was very small. In addition, the body depuration rate constant k_{2e} that indicates Pb diffusion from the tissue to the surrounding solution was also very small, much smaller than the gut depuration rate constant k_{1e}. Therefore, the tissue has very low ability to directly accumulate Pb from and directly depurate Pb to the surrounding solution. Consequently, we can simplify the tissue accumulation model by neglecting these two pathways:

$$\frac{dC_{tissue}(t)}{dt} = k_{12}C_{gut}(t) \tag{9}$$

the corresponding analytical solutions for the simplified version of the two compartment accumulation model (Equations 1, 2, and 9) are:

$$C_{gut}(t) = \frac{\frac{C_T}{C_{NP}} \times k}{k_{12}+k_{1e}} \times (1 - e^{-(k_{12}+k_{1e})t}) \tag{10}$$

$$C_{body}(t) = \frac{\frac{C_T}{C_{NP}} \times k \times k_{12} e^{-(k_{12}+k_{1e})t} (e^{(k_{12}+k_{1e})t} \times (k_{12}t + k_{1e}t - 1) + 1)}{(k_{12}+k_{1e})^2} \tag{11}$$

The curve fitting results and related constants using simplified Equations (10) and (11) are also presented in Figure 2 and Table 1. Note that the new curve from the simplified model completely overlaps with the curve based on the original model. Importantly, the new constants are also consistent with the ones obtained before simplification. Modeling results revealed that active ingestion through the mouth was the major Pb accumulation pathway not only for the gut but also for the tissue of *C. dubia*. In addition, nearly all Pb depuration occurred from the gut.

The direct tissue accumulation of Pb from solution was through a slow diffusion process.[17] In addition, in the presence of nano-TiO_2, the soluble Pb concentration was reduced to a very low level (< 10 μg/L) due to adsorption of Pb by nano-TiO_2, further reducing the driving force for diffusion. Therefore, the direct accumulation of Pb from solution was negligible compared that from the active mouth uptake and gut transfer. The direct tissue depuration of heavy metals to the surrounding solution is an ATP-based biological process, which involves specific transporters.[24] This resulted in a very low depuration rate, negligible compare to that from the gut depuration. This is supported by Gills et al.[12] and Wang et al.[17] who also reported very low heavy metal depuration rates from the tissue of *D. magna* and algae cells, respectively. In contrary, the heavy metal depuration from the gut was influenced by gut pH, which is 6.0 – 6.8 for *C. dubia*.[25] Pb could be desorbed from nano-TiO_2 within the gut and quickly flushed out of *C. dubia* through the anus. Furthermore, the newly ingested nano-TiO_2 could physically squeeze the previously accumulated nano-TiO_2 along with the adsorbed heavy metals out of the gut through anus.[12] Both processes led to a much greater Pb depuration rate from the gut than that from the tissue.

4.3. DEPURATION MODEL

We conducted a depuration experiment in a test solution that contained 1.8×10^5 cell/mL of algae. The algae was used to speed up the depuration process and support the life of *C. dubia*.[12] The depuration data were modeled using Equation (5) to determine the depuration kinetic constants. These constants were then used to calculate the Pb distribution in the *C. dubia* compartments at different depuration times including at the

beginning of the depuration. For this experiment, the *C. dubia* were initially exposed to a test solution that contained 2,500 μg/L of Pb and 50 mg/L of nano-TiO_2 for 2 h to accumulate Pb before placing them into the depuration solution. Therefore, Pb accumulation in both compartments after 2 h of exposure in this test solution can also be determined using the depuration model. Figure 3 shows the experimental Pb depuration results (filled circles), model calculations (smooth curves), and model parameters. It suggests that the depuration was mostly from the gut (dashed line), and there was no depuration from the tissue (dotted line). The high depuration from the gut was likely caused by the algae, which can quickly remove nano-TiO_2 along with the adsorbed Pb from the gut.[12] After 8 h of depuration, all Pb in the gut was eliminated. In contrast, tissue depuration of Pb is an ATP and specific transporter limited biological process,[24] which was extremely slow. Therefore, the reduced Pb content during the depuration process was mostly from gut elimination, and the remaining Pb after depuration was mostly in the tissue. The depuration modeling results indicated that at the beginning of the depuration process, e.g., after 2 h of exposure in the test solution containing 2,500 μg/L of Pb and 50 mg/L of nano-TiO_2, Pb accumulation in the gut and in the body were 22 ng/flea and 5.2 ng/flea, respectively. These results are in good agreement with that from the accumulation modeling results using Equations (10) and (11), which were 20.2 ng/flea in the gut and 5.2 ng/flea in the tissue, after the same 2 h exposure time. This confirms the two compartment accumulation model is appropriate to quantity Pb accumulation in each compartment of the *C. dubia*.

As indicated in Figure 3, the Pb in the gut was completely depurated in 8 h. However, the Pb in the tissue had no change in the 8 h depuration period. Therefore, the

Pb accumulation in the entire body after 8 h of depuration can be considered as the Pb tissue in the tissue throughout the entire depuration period, including at the beginning of the depuration process. The Pb accumulation in the gut at the beginning of the depuration can be estimated by subtracting the Pb accumulation in the tissue (e.g. the Pb in the entire body after 8 h depuration) from the total Pb accumulation in the entire body at the beginning of depuration. Therefore, by measuring Pb in the entire body of *C. dubia* at the beginning of the depuration and after 8 h of the depuration, respectively, we can estimate the Pb accumulation in the gut and that in the tissue at the beginning of depuration.

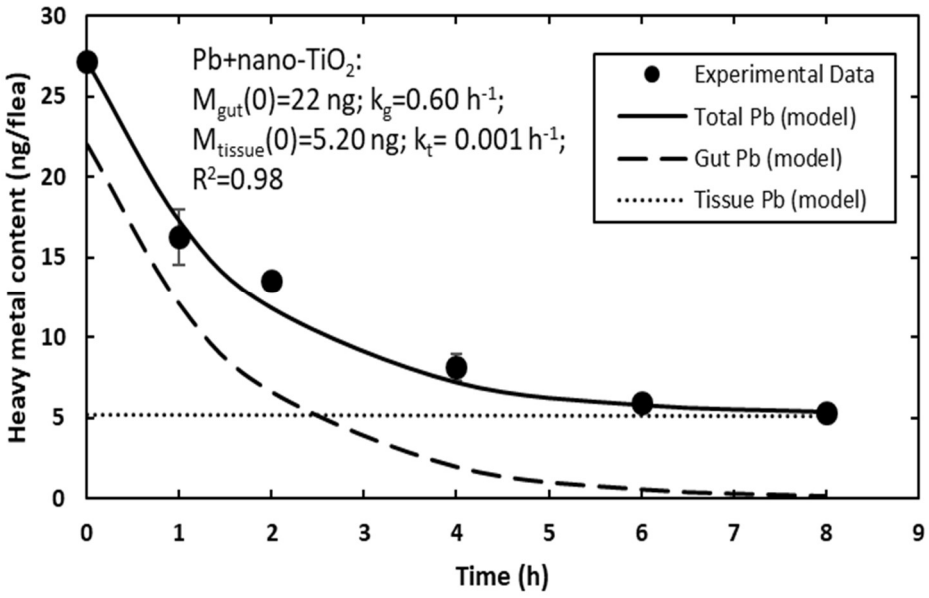

Figure 3. The Pb depuration from *C. dubia* in a culture medium that contained algae (1.8×10^5 cell/mL) after exposure in an exposure medium that contained Pb+NPs. Conditions of the exposure medium: [Pb] = 2,500 μg/L; [nano-TiO_2] = 50 mg/L; pre-exposure time before depuration: 2 h. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

4.4. ALGAE IMPACT ON Pb ACCUMULATION

Algae are a natural food source for aquatic organisms. Therefore, when investigating the toxicity of contaminants in a realistic environment, the effect of algae should be considered. Many studies have shown that algae can enhance toxic metal and/or NPs accumulation in organisms, therefore affect their toxicity.[8,26-28] Figure 4 shows the Pb accumulation data (filled circles) in the presence of 2,500 μg/L of Pb and 50 mg/L of nano-TiO_2, under two algae concentration, 1.8×10^5 cell/mL and 5.4×10^5 cell/mL. Through fitting

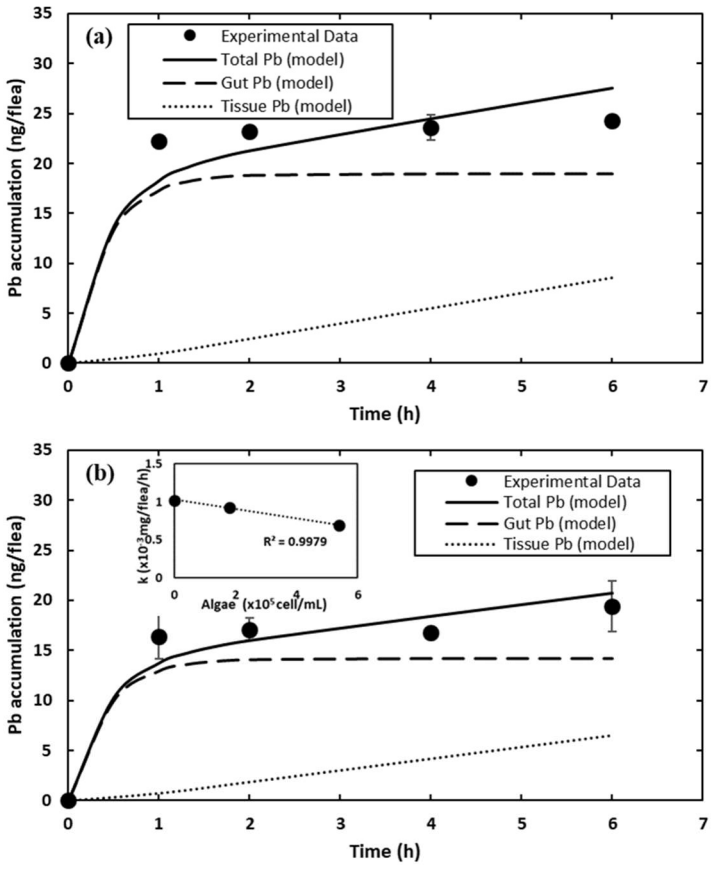

Figure 4. The Pb accumulation in *C. dubia* under different algae concentration conditions. (a) 1.8×10^5 cell/mL of algae; (b) 5.4×10^5 cell/mL of algae. Other condition of the exposure medium: [Pb] = 2,500 μg/L; [nano-TiO_2] = 50 mg/L. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

the experimental data using the simplified two compartment accumulation model, Equations (10) and (11), we can determine the accumulation kinetic constants, shown in Table 1. These constants were used to calculate Pb accumulations in the whole body, the gut, and the tissue, shown as solid, dashed, and dotted curves, respectively.

The Pb accumulation pattern was similar to that without algae. However, algae reduced Pb accumulation in both the gut and the tissue. We also validated the two compartment accumulation model that reflects the effect of algae by using the depuration experimental results. Before the depuration test, *C. dubia* were exposed in test solutions identical to those used for accumulation tests for 2 h to accumulate Pb. They were then placed in a depuration solution for elimination. The total Pb content at the beginning of the depuration test and that after 8 h of depuration time were determined to estimate the Pb distribution in the gut and the tissue at the beginning of depuration with different algae concentrations. For the *C. dubia* that were initially placed in the test solution containing 2,500 μg/L of Pb, 50 mg/L of nano-TiO_2, and 1.8×10^5 cell/mL of algae, the estimated beginning Pb accumulation in the gut was 19.24 ng/flea, and that in the tissue was 3.27 ng/flea. The Pb accumulation values calculated from the accumulation model for the same 2 h accumulation time for the gut and the tissue were 18.85 ng/flea and 2.36 ng/flea, respectively. For the *C. dubia* that were initially placed in the test solution containing 2,500 μg/L of Pb, 50 mg/L of nano-TiO_2, and 5.4×10^5 cell/mL of algae, the estimated beginning Pb accumulation in the gut was 14.31 ng/flea, and that in the tissue was 2.60 ng/flea, based on the depuration data. The Pb accumulation values calculated from the accumulation model for the same 2 h accumulation time for the gut and the tissue were

14.11 ng/flea and 1.85 ng/flea, respectively. The consistency of the data obtained from approaches suggests that the accumulation model was appropriate.

The model parameters in Table 1 suggest that the presence of algae significantly reduced the gut uptake constant k of Pb. Because algae has a much lower Pb adsorption capacity than nano-TiO_2[8] and they occupy some gut space after ingestion, the nano-TiO_2 and associated Pb accumulation in *C. dubia* was reduced. The small insert in Figure 4b shows that the Pb gut uptake constant k was linearly reduced with increase in algae concentration. The model parameters also indicate that, for both algae concentrations, algae reduced Pb transfer rate from the gut to the tissue by approximately 50%, and also reduced the assimilation efficiency (AE) of Pb by approximately 50%. Because the ingestion of algae can reduce the gut passage time of nano-TiO_2[30] and the retention time of the released Pb, the Pb transfer from the gut to the tissue and the Pb assimilation efficiency were reduced.

It is interesting to note that, for both algae concentrations, and even for the condition without algae, the gut depuration rate constant k_{1e} was the same. The gut depuration of heavy metals is a physical-chemical process. The adsorbed Pb on nano-TiO_2 was first released as soluble Pb ions within the gut and some of these ions were depurated from *C. dubia* through the anus. The desorption of Pb from nano-TiO_2 is controlled by the gut pH, which was relatively constant (pH = 6.0-6.8) under different conditions.[25] Apparently, the algae did not change the gut pH and thus the gut depuration rate constant k_{1e} was the same.

It is also important to note that, for both algae concentrations, the gut to tissue transfer rate constant k_{12} is the same. Algae reduced the Pb transfer rate from the gut to the

tissue due to dilution of the gut Pb by the algae. However, the Pb transfer from the gut to the tissue is a biological process, which is depended on ion channels and protein transporters.[18] It is possible that different concentrations of algae did not change the biological activity that is related to the rate of Pb transfer from the gut to the tissue.

4.5. Pb TOXICITY IN THE PRESENCE OF NANO-TiO_2

In a previous study we found that 2,500 μg/L of Pb alone had a very low toxicity, with a 24 h mortality of less than 10%, but nano-TiO_2 significantly increased Pb toxicity.[8] Gillis et al.[12] reported that heavy metals accumulated in the tissue are responsible for toxicity. Together, these findings suggested that nano-TiO_2 enhanced Pb accumulation in the tissue. After exposure to the test solution, *C. dubia* gradually accumulated Pb in the tissue until exceeding a threshold concentration and developing toxicity. Figure 5a shows that the observed survivorship of *C. dubia* decreases with the increase of exposure time. This is consistent with the Pb accumulation data shown in Figure 2, which indicate that Pb tissue accumulation in *C. dubia* adults increased over time.

Our previous research indicated that accumulations of heavy metals and NPs in adult *C. dubia* were positively correlated to toxicity in *C. dubia* neonates.[5,8,31] Therefore, we can correlate the projected Pb tissue accumulation in *C. dubia* adults during the 24 h exposure period ($C_{tissue}(t)$) with the experimental survivorship data from *C. dubia* neonates (S(t)) using the toxicodynamic model, Equation (8), to determine the relevant constants in the model. The calculated $C_{tissue}(t)$ during a 24 h accumulation period using Equation (11) based on parameters in Table 1 are shown as the dotted curve in Figure 5(a). The experimental S(t) for the same duration of exposure are shown as the filled circles in Figure

5(a). The solid curve in Figure 5(a) is the curve fitting results, which reflects the experimental survivorship data. Based on the model, the Pb threshold concentration was determined to be C_{TH} = 25.87 ng/flea, and the killing rate was determined to be $k_k = 5\times10^{-4}$ flea/ng/h. These constants are also listed in Figure 5(a).

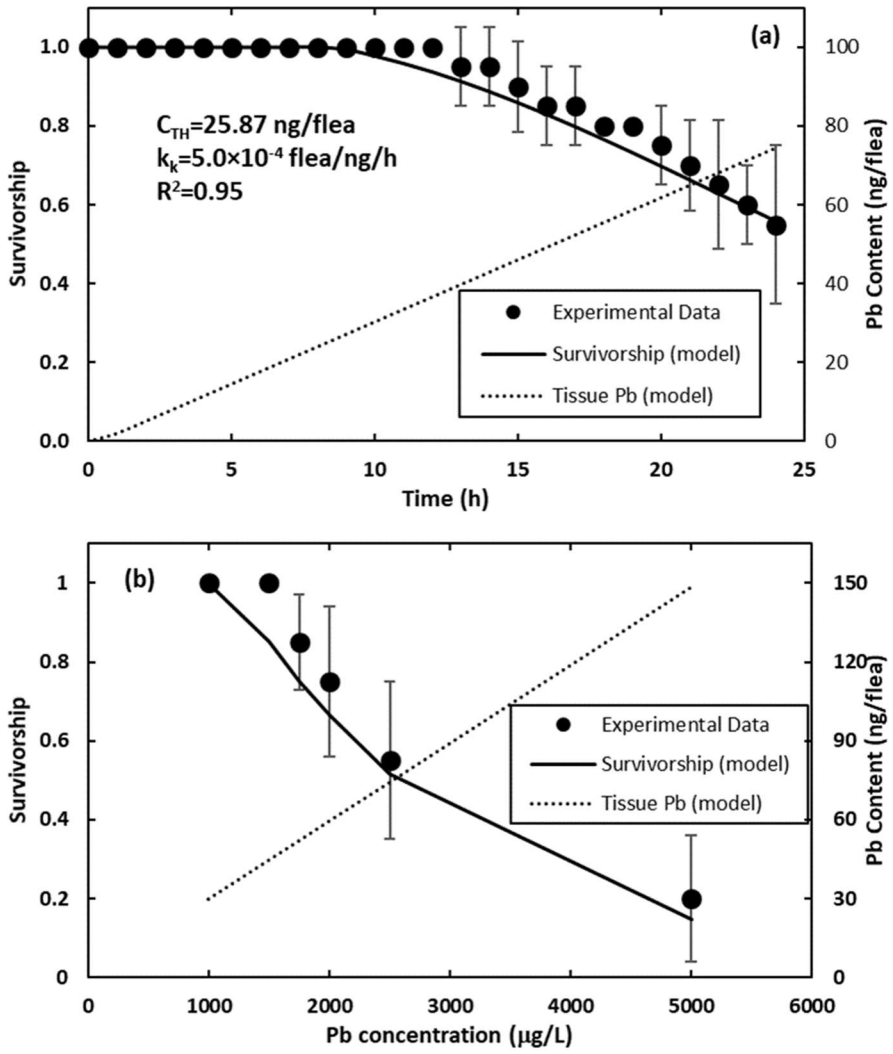

Figure 5. The survivorship of *C. dubia* in the presence of Pb and nano-TiO_2. (a) The survivorship at different exposure times using a test solution that contained 2,500 µg/L of Pb and 50 mg/L of nano-TiO_2. (b) The 24 h survivorship in test solutions that contained 50 mg/L of nano-TiO_2 and different concentrations of Pb (µg/L).

To validate the applicability of the two compartment toxicodynamic model, Equations (8) and (11), we conducted independent 24 h toxicity experiments using test solutions that contained 50 mg/L of nano-TiO_2 and different concentrations of Pb. Figure 5(b) shows the experimental 24 h toxicity data as a function of Pb concentration in filled circles.

The change in Pb concentration should proportionally alter the adsorption density of Pb on nano-TiO_2, thus changing the Pb accumulation in the gut due to its limited space to hold nano-TiO_2. This implies a change in Pb tissue accumulation and a consequential reduction of survivorship with increasing Pb concentration. To predict the 24 h survivorship under different Pb concentrations, we first used parameters developed in the Pb accumulation experiments in Table 1 to calculate Pb tissue accumulation using Equation (11), shown as the dotted line in Figure 5(b). We then used the same Pb threshold concentration C_{TH} and the killing rate k_k determined through modeling the time-dependent survivorship data (Figure 5(a)), and the toxicodynamic model Equation (8) to predict the 24 h survivorship of *C. dubia* under different Pb concentrations, shown as the solid curve in Figure 5(b). The prediction results described the experimental data in the entire Pb concentration range very well. Consequently, the two compartment toxicodynamic model, Equations (8) and (11), is appropriate to not only simulate Pb toxicity, but also predict Pb toxicity under different Pb concentrations, in the presence of 50 mg/L of nano-TiO_2. Note that Equation (11) can be combined with Equation (8) to directly calculate the survivorship of Pb as a function of NP concentration, Pb concentration, and accumulation time, without actually calculating $C_{tissue}(t)$ as an intermittent step.

4.6. ALGAE IMPACT ON THE COMBINED TOXICITY OF Pb AND NANO-TiO_2

Algae significantly reduced the combined toxicity of Pb and NPs. In this work, we quantified the algae effect on the combined toxicity of Pb and nano-TiO_2, using the two compartment modeling approach, Equations (8) and (11). The time-dependent experimental survivorship data over a 24 h period with 2,500 μg/L of Pb, 50 mg/L of nano-TiO_2, and 1.8×10^5 cell/mL of algae are shown in Figure 6(a) (filled circles). These experimental data were also directly modeled using the toxicodynamic model, Equation (8), to determine the threshold concentration C_{TH} and the killing rate k_k for this particular test solution. The curve fitting results for Equation (8) (solid curve) and the related model parameters are shown in Figure 6(a). The $C_{tissue}(t)$ used in Equation (8) was separately calculated using Equation (11) and the related constants in Table 1, shown as the dotted line in Figure 6(a).

The model results well described the experimental survivorship data. Compared to Figure 5(a), the test without algae, the presence of 1.8×10^5 cell/mL of algae significantly increased the survivorship of *C. dubia*. The parameters suggested that the ingestion of algae did not change the toxic metal threshold concentration C_{TH}, but reduced the killing rate k_k by approximately 15%. The major toxicity mechanism of heavy metals is the production of reactive oxygen species (ROS).[32] Algae, as a food source, can provide energy to elevate antioxidant levels in organisms to neutralize ROS.[33] In addition, some algae may directly serve as antixodiants.[34,35] Both of these effects could possibly mitigate the toxic effect of Pb and thus reduce the killing rate.

By comparing the parameters in the Pb accumulation modeling and the Pb toxicodynamic modeling, it was apparent that algae mitigated the combined toxicity of Pb

and nano-TiO_2 through two aspects: physical and biological. In the physical aspect, algae spatially reduced Pb accumulation in the tissue of *C. dubia*. In the biological aspect, algae provided energy to organisms and reduced the killing rate.

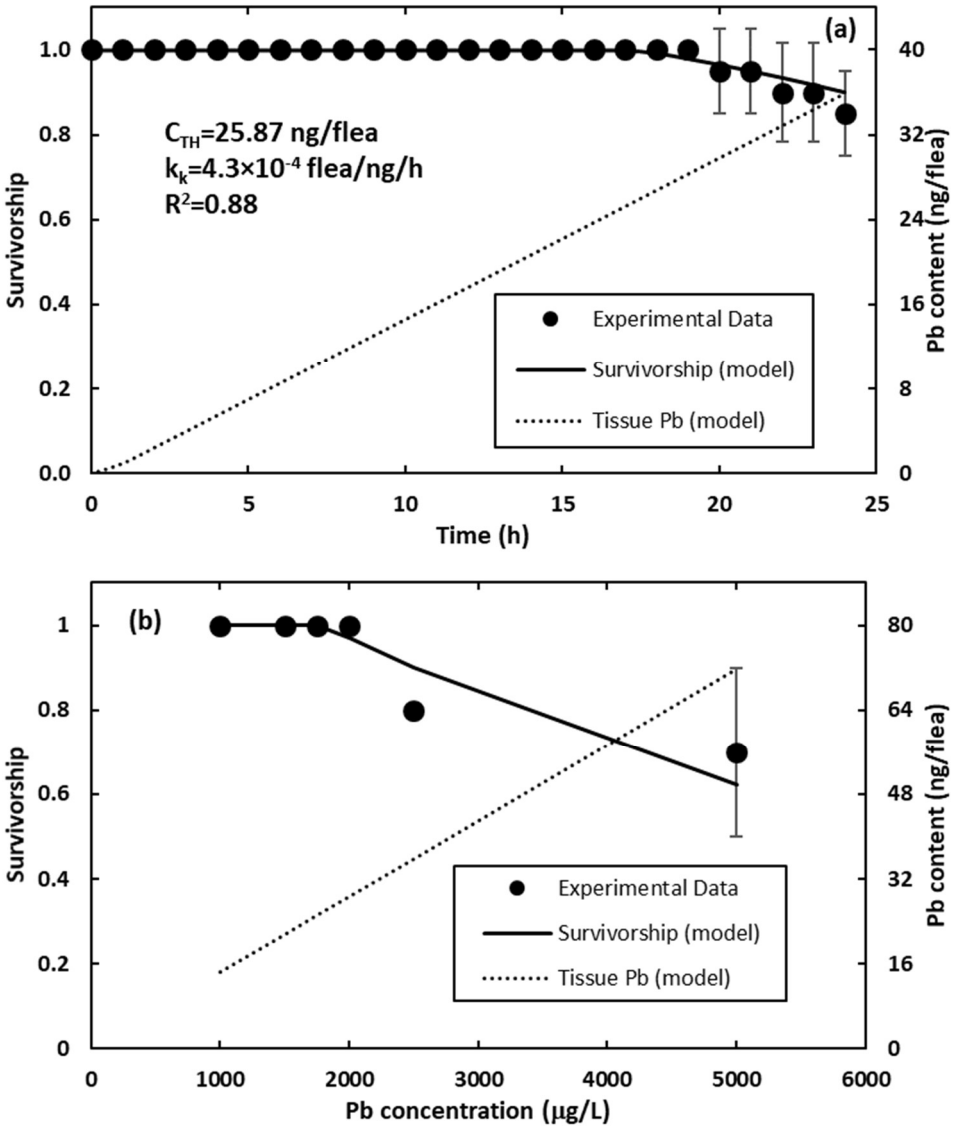

Figure 6. The survivorship of *C. dubia* in the presence of Pb, nano-TiO_2, and algae. (a) The survivorship at different exposure times using a test solution that contained 2,500 μg/L of Pb, 50 mg/L of nano-TiO_2, and 1.8×10^5 cell/mL of algae; (b) The 24 h survivorship in test solutions that contained 50 mg/L of nano-TiO_2, 1.8×10^5 cell/mL of algae, and different concentrations of Pb (μg/L).

In order to validate the two compartment toxicodynamic model, we analyzed the 24 h toxicity test using test solutions that contained 50 mg/L of nano-TiO_2, 1.8×10^5 cell/mL of algae, and various concentrations of Pb. Figure 6(b) shows the experimental data (filled circles) and the model prediction (solid curve). The model prediction was performed using Equations (8) and (11), based on parameters in Table 1 and Figure 6(a). Figure 6(b) shows that the model prediction was in agreement with the experimental data, indicating that the two compartment modeling approach can be used to predict the combined toxicity of Pb and nano-TiO_2 in the presence of algae. Collectively, this two compartment modeling approach provides a predictive tool for toxicity assessment under these complex conditions.

NPs could impact the toxicity of environmental toxins through multiple mechanisms, including adsorption/desorption, active uptake, diffusion, metabolic transfer, and many more. The presence of algae, a natural food source, makes this interaction more complex. Therefore, appropriate modeling is essential in integrating these impacts and providing a comprehensive understanding on toxicity mechanisms. This research links the two compartment accumulation model that predicts the metal distribution within the *C. dubia* with the toxicodynamic model that predicts the toxicity response of *C. dubia*. Consequently, advancement in interpreting the impacts of NPs in complex ecosystems can be achieved.

SUPPORTING INFORMATION

QUANTIFYING THE EFFECT OF NANO-TiO_2 ON THE TOXICITY OF LEAD ON *C. DUBIA* USING A TWO COMPARTMENT MODELING APPROACH

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1. METHOD

1.1. TEST SOLUTION PREPARATION

The stock solution of Fe(III) (1000 mg/L) was prepared by dissolving appropriate amounts of $Fe(NO_3)_3 \cdot 9H_2O$ into MQ water. The stock solutions were then acidified with trace metal grade nitric acid to make a pH of less than 4.

To extend the accumulation kinetic model application, test solutions containing 2,500 μg/L of Fe(III) and 50 mg/L of nano-TiO_2 were prepared. The accumulation test procedure was the same as section 3.2 in text, except Fe(III) was used instead of Pb (II).

The test solutions were mixed in a mechanical shaker for 24 h, and the final pHs of test solutions were in the range of 7.4-7.8.

2. SUPPLEMENTARY FIGURES

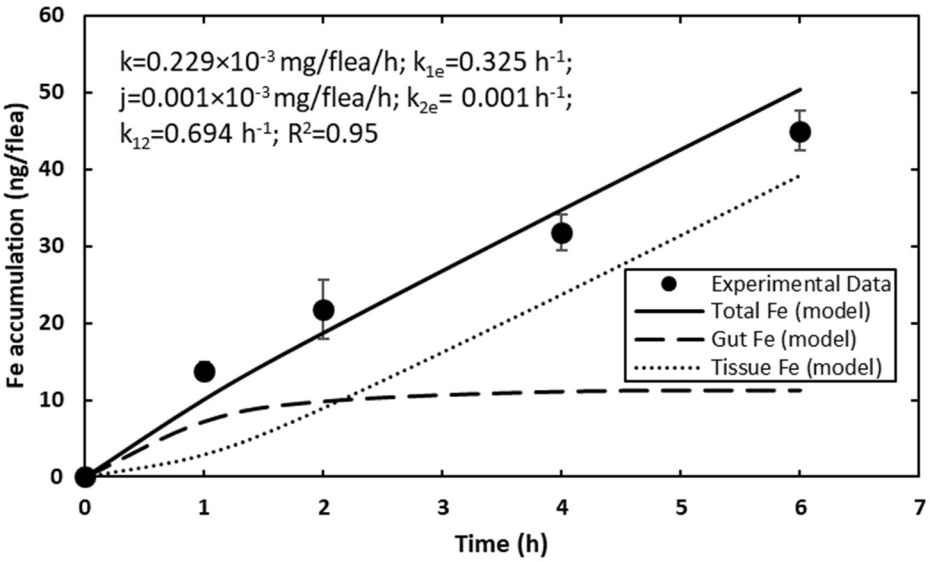

Figure S1. The Fe accumulation in *C. dubia* in the presence of nano-TiO_2. Condition of the exposure medium: [Fe] = 2,500 μg/L; [nano-TiO_2] = 50 mg/L.

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III. COMBINED TOXICITY OR SYNERGISTIC TOXICITY? UNDERSTANDING THE ROLE OF NANO-TiO_2 ON Pb TOXICITY THROUGH PREDICTIVE MODELING

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ABSTRACT

Nano-TiO_2 can significantly increase Pb toxicity on aquatic organisms. However, the type of the toxicity mechanism, combined or synergistic, has not been identified. In this research, we investigated the effect of nano-TiO_2 on the toxicity of Pb under different nano-TiO_2 concentrations using *Ceriodaphnia dubia* (*C. dubia*) as the model organism. The effect of algae, a natural food for aquatic organisms, on the toxicity of the binary mixture was also investigated. A two-compartment modeling approach was used to quantify the toxicity under these complex conditions. This two-compartment modeling approach can well describe the Pb accumulation in the gut and the body tissue of *C. dubia* under different nano-TiO_2 concentrations, with and without algae, and further, predict the toxicity on *C. dubia*. The parameters from a toxicodynamic model indicated that increasing nano-TiO_2

concentration reduced the tolerance level of Pb and also increased the killing rate of *C. dubia*. Therefore, nano-TiO_2 not only served as a carrier to enhance Pb accumulation, but also synergistically enhanced Pb toxicity. The modeling results also indicated that algae reduced both the Pb transfer from the gut to the body tissue and the killing rate, resulting in significant toxicity reduction. However, algae did not change the threshold level of Pb. The two-compartment modeling approach is essential in understanding the role of nanoparticles when coexisted with toxic elements in the environment.

Keywords: Two compartment toxicodynamic model, synergistic toxicity, lead, nano-TiO_2, algae

1. INTRODUCTION

The discharge of nanoparticle (NP) into the environment has received considerable attention due to their potential environmental and health risks.[1,2] Because nano-TiO_2 has a low toxicity,[3] it is widely used in commercial and industrial products.[4-6] Our previous study confirmed that nano-TiO_2 alone has very low toxicity on *C. dubia*.[7] However, many studies have also shown that nano-TiO_2 could interact with various background toxins, especially toxic metals, and alter their toxicity patterns, which is commonly described as the "Trojan Horse Effect".[7-10] The Trojan Horse Effect implies that the increase in toxicity is caused by the increased toxic metal accumulation, which increases the reactive oxidation species (ROS) production from the toxic metal.[11] This type of toxicity is characterized as combined, or additive toxicity. However, Li et al.[12] reported that nano-TiO_2 could also increase the ROS level in algae cells and inhibit the algae growth, adding another

dimension on the toxicity mechanism when both toxic metals and nano-TiO_2 co-exist. In addition, the adsorption of toxic metals on nano-TiO_2 could form a surface complex[13] and increase the toxic effect of the nano-TiO_2. Therefore, nano-TiO_2 could also contribute to the toxicity separately. These findings raised more questions on the effect of nano-TiO_2 on toxic metal toxicity: Does nano-TiO_2 simply serve as a carrier and enhance metal toxicity through more metal accumulation, or it provide an additional toxicity mechanism to synergistically enhance metal toxicity?

A process-based toxicokinetic (TK) - toxicodynamic (TD) model has been increasingly used to quantify the toxic effect of single or multiple toxins.[14-16] The assumption of this model is that the hazard starts to accumulate if the toxic element accumulation in body tissue exceeds a threshold value that organism could tolerate, and the probability of death increases.[14-16] The TK model quantifies the toxic element accumulation in organisms, and the TD model links the accumulation with the toxicity. In the TD model, two parameters, the threshold concentration and the killing rate are used to describe the lethality of the toxic element, and changing of these parameters reflects the toxicity change.[14] In our previous research we used a two-compartment modeling approach, including a two-compartment accumulation model and a body tissue accumulation based TD model, to quantify the Pb toxicity in the presence of 50 mg/L nano-TiO_2 on *C. dubia*.[17] Therefore, we expect that, by changing the concentrations of nano-TiO_2 in the toxicity test, we can develop experimental data that reflect the effect of nano-TiO_2 on the toxicity, and model these data to develop parameters that help us to better understand the role of nano-TiO_2 on the toxicity. The threshold concentration and the killing rate parameters in the TD model can provide mechanistic information regarding the effect of nano-TiO_2 on the

toxicity. In addition, algae could significantly reduce Pb toxicity in the presence of nano-TiO_2,[18] and the two-compartment modeling approach can quantify Pb accumulation and toxicity in the presence of both nano-TiO_2 and algae.[17] Consequently, the two-compartment TD model could also be used to reveal the role of algae on the toxicity. The objective of this study was to develop a fundamental understanding of the role of nano-TiO_2 on the toxicity of Pb through the two-compartment TD modeling of the experimental data obtained under different concentrations of nano-TiO_2 – is the toxicity combined or synergistic?

2. MATERIALS AND METHODS

2.1. CHEMICALS, NPS, AND ORGANISMS

The moderate hardness culture medium buffer (pH = 7.8 ± 0.2, hardness = 85± 5 mg/L as $CaCO_3$) was prepared by using $CaSO_4 \cdot 2H_2O$ (98%), Na_2SeO_4 (99%), $NaHCO_3$ (100.2%, Pb < 5 mg/kg), $MgSO_4$ (Pb < 0.001%), and KCl (99%) following the EPA standard method, EPA-821-R-02-012.[19] The 1,000 mg/L Pb stock solution was prepared by dissolving appropriate amount of $Pb(NO_3)_2$ into Milli-Q water (resistivity = 18.2 MΩ·cm), and then acidified to a pH of less than 4. Trace metal grade nitric acid was used for sample acidification and digestion. The certified Pb standard solution (1,000 mg/L) was diluted with Milli-Q water and used to develop standard calibration curves for the Pb analysis. All chemicals used above were acquired from Fisher Scientific (Fair Lawn, New Jersey, USA).

The nano-TiO_2 (5-10 nm, anatase, 99%) used in this research was purchased from Skyspring Nanomaterials Inc. (Houston, TX, USA). The nano-TiO_2 stock solution (200 mg/L) was prepared by adding an appropriate amount of dry nano-TiO_2 particles into a 100 mL culture medium in a 125 mL HDPE bottle, and then sealed and mixed in a mechanical shaker for 10 min. The nano-TiO_2 stock solution was freshly prepared in each test.

C. dubia was selected as the model organism for this research. The starter *C. dubia* was acquired from MBL Aquaculture (Sarasota, FL, USA), and cultured in the lab. The daily food, algae (*Raphidocelis*) and the mixture of yeast, trout chow, and cereal leaves (YTC) (Pb < 1μg/L), for *C. dubia* were purchased from ABS Inc. (Fort Collins, CO, USA). The concentrations of algae (*Raphidocelis*) and YTC in the stock food solutions were 3×10^7 cell/mL and 1,700 mg/L as total solid, respectively. The algae and YTC concentrations in the culture medium were 1.8×10^5 cell/mL and 6.8 mg/L as total solid, respectively, achieved by spiking appropriate volumes of the above stock food solutions into the culture medium buffer. The same algae (*Raphidocelis*) were also used in the accumulation, depuration, and toxicity test. A laminar flow hood (SVC-6AX, Streamline® laboratory products, Fort Myers, FL, USA), which was installed in in a temperature-controlled chamber (25 ℃), was used to conduct mass culture and all other tests. The light: dark cycle of the temperature-controlled chamber was set at 16 h light: 8 h dark to mimic the natural day light and night light circles.

2.2. ACCUMULATION TEST

The accumulation tests were used to determine the total Pb accumulation in *C. dubia* under different conditions (accumulation time, nano-TiO_2 concentration, the

absence/presence of algae), using 100 mL test solutions contained in 125 mL HDPE bottles. A total of 8 test solutions were prepared for the accumulation test. For each test solution, a total of 9 reactors, representing 9 exposure times, 1, 2, 4, 6, 8, 12, 16, 20, and 24 h, respectively, were used. All test solutions contained 2,500 μg/L of Pb. The first 5 test solutions also contained nano-TiO_2 at concentrations of 10, 20, 50, 100, and 200 mg/L, respectively. The rest of test solutions contained 1.8×10^5 cell/mL of algae and 3 different concentrations of nano-TiO_2, 50, 100, and 200 mg/L, respectively. A control group without contacting any test solution was also included, to determine the background Pb concentration in *C. dubia*. The experimental data were used to determine the accumulation kinetic constants through modeling, and further, to predict Pb distribution in *C. dubia*.

For each reactor used in the accumulation test, approximately 30 adult *C. dubia* (1 week old) were used. They were washed with a clean culture medium buffer three times before being transferred to the reactors. After being exposed for a pre-selected period of time, *C. dubia* were picked and washed with a clean culture medium buffer three times to remove the particles from the surface of *C. dubia*. The *C. dubia* were then filtered through a 0.297 mm standard sieve, counted, and transferred to a digestion vessel that contained five milliliter of nitric acid for digestion at 95°C for 12 h, using a hotblock digester with a Watlow mini controller (Watlow mini-0R10-000G). After digestion, the sample was diluted with Milli-Q water and analyzed for the soluble Pb concentration, which was then used to calculate Pb content in *C. dubia*. The result from the control group, 0.10 ng/flea, was used as the Pb background. The accumulated Pb was calculated by subtracting the Pb background from the total Pb content. All the accumulation experiments were conducted in duplicate.

A two-compartment accumulation modeling approach was used to quantify the Pb accumulation in the presence of NPs in *C. dubia*.[17] In brief, the *C. dubia* was characterized as the gut and the rest of body tissue (referred as "tissue" hereafter). The Pb gut accumulation was caused by the active mouth uptake, gut depuration, and transfer to the body tissue. The Pb tissue accumulation was caused by the transfer from the gut. The Pb diffusion in and out of the tissue through the body surface was negligible. The equations below express the Pb gut accumulation and tissue accumulation in the presence of nano-TiO_2.[17]

$$C_T(t) = C_{gut}(t) + C_{tissue}(t) \quad (1)$$

$$C_{gut}(t) = \frac{\frac{C_T}{C_{NP}} \times k}{k_{12}+k_{1e}} \times (1 - e^{-(k_{12}+k_{1e})t}) \quad (2)$$

$$C_{body}(t) = \frac{\frac{C_T}{C_{NP}} \times k \times k_{12} e^{-(k_{12}+k_{1e})t} (e^{(k_{12}+k_{1e})t} \times (k_{12}t + k_{1e}t - 1) + 1)}{(k_{12}+k_{1e})^2} \quad (3)$$

where $C_T(t)$ = Pb in the whole body at time t (ng/flea); $C_{gut}(t)$ = Pb in gut at time t (ng/flea); $C_{tissue}(t)$ = Pb in the tissue at time t (ng/flea); C_T = the total Pb concentration (ng/L); C_{NP} = the NP concentration (mg/L); k = the gut uptake constant through active mouth uptake (mg/flea/h); k_{12} = Pb transfer constant from gut to body tissue (h^{-1}); k_{1e}= Pb depuration constant from gut (h^{-1}); t = exposure time (h).

2.3. TOXICITY TEST

Eight series of 100 mL test solution, with five Pb concentrations (500, 1,000, 1,500, 2,000, and 2,500 μg/L) in each series, were prepared by diluting Pb stock with a mixture of culture medium buffer, nano-TiO_2 stock solution, and algae stock solution at a certain ratio in 125 mL HDPE bottles. The test solutions that had a Pb concentration of 2,500 μg/L

were prepared in duplicate. The ratio of clean culture medium, nano-TiO_2 stock solution, and algae stock solution (if needed) was determined based on the concentrations of nano-TiO_2 and algae (if needed) selected for the toxicity test. In addition to Pb, the first to fifth series also contained 10, 20, 50, 100, and 200 mg/L of nano-TiO_2, respectively. The sixth to eighth series of bottles also contained different concentrations of nano-TiO_2, 50, 100, and 200 mg/L, respectively, and the same concentration of algae, 1.8×10^5 cell/mL. The algae at this concentration could support the normal living of *C. dubia*.[19] All the test solutions were mixed in a mechanical shaker for 24 h. The final pH of all test solutions after 24 h mixing was 7.8 ± 0.2.

The EPA method was followed to determine the toxicity.[19] Initially, a time-dependent toxicity test was conducted using 8 test solutions that had a Pb concentration of 2,500 μg/L and different concentrations of nano-TiO_2, with and without algae. The experimental data were used to determine kinetic constants in the TD model. For each test solution, four parallel 30 mL reactors, with each reactor containing 15 mL of test solution, were included. Twenty healthy *C. dubia* neonates (<24 h) were used for each test solution, 5 for each reactor. The neonates were first washed with a clean culture medium buffer three times and transferred to the reactor by using a plastic dropper with a 3 mm diameter opening tip. The duration of the toxicity test was 24 h, and the survivorship of the neonates was checked hourly. The dead neonates were removed from the reactor every hour. The time-dependent toxicity data were fitted with the TD model.

Independent 24 h toxicity tests were also conducted using all 8 series of test solutions prepared above, to develop data and validate the TD model. The test procedures were the same as above, except that the survivorship was only checked at the 24^{th} h.

A TD model was used to predict the heavy metal toxicity based on the Pb tissue accumulation.[17]

$$S(t) = \begin{cases} 1 & if\ C_{tissue}(t) \le C_{TH} \\ e^{-k_k \times (C_{body}(t) - C_{TH})t} & if\ C_{tissue}(t) > C_{TH} \end{cases} \quad (4)$$

where S(t) = survival probability of the test organism at time t; k_k = killing rate (flea/ng/h); C_{TH} = toxic metal threshold concentration (ng/flea).

2.4. ANALYTICAL METHOD

The soluble Pb concentrations in the digested samples from accumulation and depuration tests were determined using a graphite furnace atomic absorbance spectrometer (GFAA) (Perking Elmer AAnalyst 600), which has a Pb detection limit of 0.5 μg/L.

3. RESULTS AND DISCUSSION

3.1. Pb ACCUMULATION IN THE PRESENCE OF NANO-TiO_2

Based on the EPA standard procedure, *C. dubia* neonates were used for the toxicity test. Ideally, the same neonates should be used to conduct the accumulation test. However, because the *C. dubia* neonates were extremely fragile, they could not sustain the multiple manual steps of the procedure used for the accumulation test. Therefore, we used adult *C. dubia* to conduct the accumulation test.[17,18,21] In this work, we used the time-dependent accumulation data during the 24 h accumulation period to fit the two-compartment accumulation model, Equations (1), (2), and (3). Figure 1 shows the Pb accumulation data (filled circles) in the presence of 100 mg/L of nano-TiO_2, with and without algae. Accumulation data under other nano-TiO_2 concentrations (10, 20, 50, and 200 mg/L) were

shown in Figures S1-S4 (supplementary information). The curve fitting results for the total Pb accumulation in the whole body were shown as the solid curve in Figure 1. The modeling results were in good agreement with experimental data. Table 1 lists the accumulation kinetic parameters under all experimental conditions. Based on these parameters, Pb accumulations in the gut and the tissue were calculated and shown as the dashed curve and dotted curve, respectively. The modeling results suggested that the Pb gut accumulation reached the maximum value in approximately 2 h. However, the Pb tissue accumulation increased gradually at a constant rate. The gut could regulate the nutrient transport to body tissue through active and passive mechansims.[22] Although Pb did not serve as a nutrient for *C. dubia*, it may use nutrient channels, e.g., the calcium (Ca) ion channel, to transfer to the body tissue continuously.[23] As a consequence, the tissue's Pb content gradually increased with time. Results showed that Pb was accumulated in the gut at a much faster rate compared to that in the tissue. This was because that the Pb gut accumulation was from active uptake which is a quick process. However, Pb lacks a specific uptake pathway from the gut to the tissue due to the non-physiological function of Pb in cells.[29] Although the Ca ion channel is one of the pathways for Pb transfer,[23] our test medium, moderate hardness water, had a high concentration of Ca, which outcompeted Pb and minimized Pb transfer from the gut to tissue.

As indicated in Table 1, the gut uptake constant was increased with nano-TiO_2 concentrations (10-200 mg/L). The same phenomenon was also reported by Tan and Wang[24] that the influx rate is concentration dependent. In addition to the gut uptake constant, we also found the rate of Pb transfer from gut to tissue and Pb depuration from gut were the same under different nano-TiO_2 concentrations. The Pb transfer from the gut

to the tissue is limited by ion channel and transporter.[22] Obviously, changing of nano-TiO_2 concentration would not change these biological activities to alter the Pb transfer rate. For the gut depuration of Pb, the adsorbed Pb was first released as Pb ion from nano-TiO_2 surface, and some of the released ions was depurated from the gut of *C. dubia*. The release of Pb from nano- TiO_2 is pH depended, and the gut pH of *C. dubia* is relative constant (pH = 6.0-6.8).[25] Therefore, the gut depuration of Pb under different nano-TiO_2 concentrations would be the same.

Table 1. Toxicokinetic model parameters.

NPs (mg/L)	Pb (mg/L)	Algae (cell/mL)	k ($\times 10^{-3}$ mg/flea/h)	k_{12} (h^{-1})	k_{1e} (h^{-1})	R^2
10	2.5	0	0.097	0.026	1.588	0.95
20			0.395			0.88
50			0.694			0.96
100			1.091			0.91
200			1.418			0.93
50		1.8×10^5	0.610	0.022	1.588	0.89
100			0.998			0.95
200			1.342			0.93

Nano-TiO_2 serves as a carrier for Pb, therefore, nano-TiO_2 concentration significantly affects the Pb accumulation. The small insert in Figure 1(a) shows the 24 h total Pb accumulation in the entire body under different concentrations of nano-TiO_2 without algae. It shows that the 24 h accumulation of Pb first increased with the increase in nano-TiO_2. However, when the nano-TiO_2 concentration was greater than 20 mg/L, the Pb accumulation decreased with the increase in nano-TiO_2. This was because that, at low nano-TiO_2 concentrations, increase in the nano-TiO_2 did not significantly change the

soluble Pb concentration therefore the adsorption density of Pb on nano-TiO_2. Thus, increasing nano-TiO_2 concentration increased the active uptake of nano-TiO_2 along with the adsorbed Pb, resulting in the increase in total Pb accumulation. However, when nano-TiO_2 concentration was further increased to above 20 mg/L, most of the Pb was adsorbed by nano-TiO_2.[17,18] Therefore, increasing nano-TiO_2 concentration linearly reduced Pb adsorption density, resulting in a linear decrease in total Pb accumulation in *C. dubia*. This can be referred as a dilution effect from nano-TiO_2.

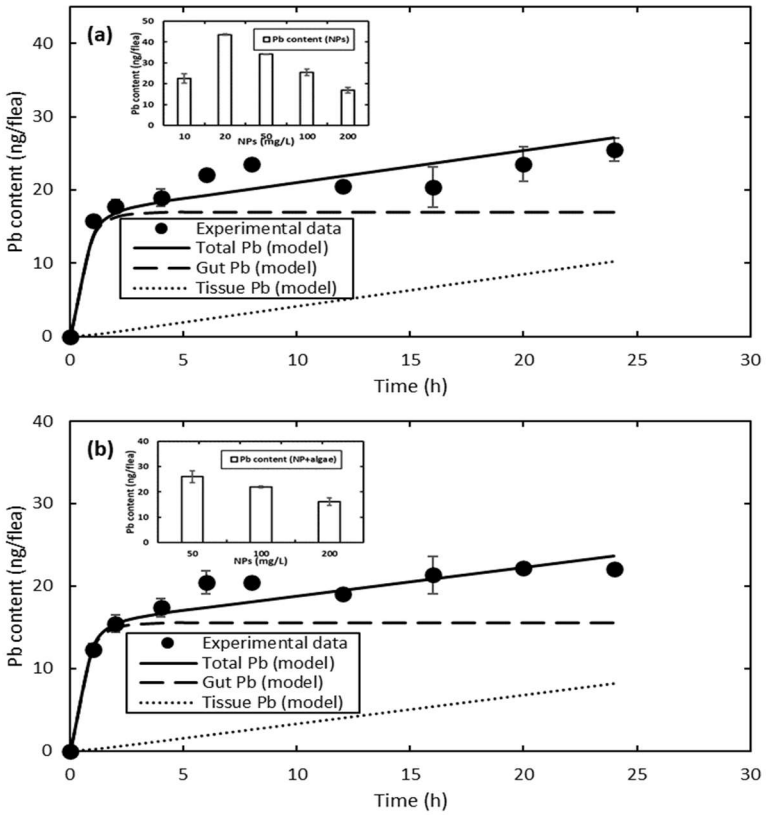

Figure 1. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2 with and without algae: (a) nano-TiO_2 + Pb; (b) nano-TiO_2 + Pb + algae. Condition of the exposure medium: [Pb] = 2,500 μg/L; NPs = 100 mg/L; algae = 1.8×10^5cell/mL. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

3.2. ALGAE IMPACT ON Pb ACCUMULATION

By comparing Figures 1(a) and 1(b), it is clear that the presence of algae reduced Pb accumulation in both the gut and the tissue. However, the accumulation patterns of Pb under both conditions were same. Algae could serve as a carrier of Pb,[26] but its adsorption capacity was much lower than nano-TiO_2.[18] Therefore, most of the Pb accumulation in *C. dubia* was carried in by nano-TiO_2. Algae could occupy some gut space and therefore reduce the nano-TiO_2 accumulation, resulting in less Pb accumulation. The model results suggest that the maximum Pb content in the gut was 16.9 ng without algae, it reduced to 15.5 ng in the presence of algae (1.8×10^5 cell/mL).

Figures S5 and S6 (supplementary information) show the accumulation data and the modeling results in the presence of algae at nano-TiO_2 concentrations of 50 and 200 mg/L, respectively. The kinetic constants for accumulation in the presence of algae are shown in Table 1. As demonstrated in Table 1, algae reduced the Pb uptake constant k for all nano-TiO_2 concentrations, which agree with the above assessment. Table 1 also shows that algae reduced Pb transfer rate k_{12} from the gut to the body tissue. This was because algae could reduce the gut passage time of nano-TiO_2, thereby reducing the Pb retention time,[27] resulting less Pb assimilation in the tissue. Interestingly, Table 1 shows that algae did not change the gut depuration constant. As discussed earlier, the Pb depuration from the gut is dominated by the gut pH, which is a constant (6.0-6.8) under different conditions.[25] The presence of algae might not change the gut pH of *C. dubia*. Therefore, the same gut depuration rate was observed. The small insert in Figure 1(b) also shows that Pb accumulation linearly reduced with the increase in nano-TiO_2 concentration under these nano-TiO_2 concentrations.

3.3. EFFECT OF NANO-TiO_2 ON Pb TOXICITY – EXPERIMENT DATA AND MODEL PREDICTION

Figure 2a shows the time-dependent survivorship of *C. dubia* exposed to 2,500 μg/L of Pb with different concentrations of nano-TiO_2 during a 24 h testing period. It was found that the survivorship was reduced with an increasing nano-TiO_2 concentration. From the insert in Figure 1a, it was noted that the 24 h total Pb accumulation in *C. dubia* first increased with increasing nano-TiO_2 concentration. However, if the nano-TiO_2 concentration exceeded 20 mg/L, the Pb accumulation was reduced due to a dilution effect – a higher nano-TiO_2 concentration reduced adsorption density of Pb on nano-TiO_2, resulting in less Pb accumulation in *C. dubia* (*C. dubia* gut has a fixed capacity to maintain nano-TiO_2). A similar trend was found for the predicted Pb tissue accumulation (dotted lines in Figures 1a, S1 – S4) as effects of nano-TiO_2 concentration. Apparently the trend of total or tissue accumulation of Pb was not consistent with the trend of the survivorship. Nano-TiO_2 must have provided additional toxic effect. Based on the experimental observation, for *C. dubia* exposed to 2,500 μg/L of Pb with low nano-TiO_2 concentrations (10-20 mg/L), the decreased survivorship was mainly caused by the increasing Pb tissue accumulation. However, when a higher nano-TiO_2 concentration (50-200 mg/L) was used, although the Pb content was decreased, the survivorship continued to decrease due to the high concentrations of the nano-TiO_2.

The toxicodynamic model, Equation (4), was used to quantify the toxicity process, based on the Pb tissue accumulation. The Pb tissue accumulation under different nano-TiO_2 concentrations was determined using Equation (3) based on the parameters in Table 1, shown as the dotted lines in Figures 1a, S1 – S4. The solid curves in Figure 2a were the toxicodynamic model results, and they were in the good agreement with the experimental

data. For 10 mg/L of nano-TiO_2 with 2,500 μg/L of Pb, the change in survivorship was negligible compared with the negative control group and was not fitted by the model. Table 2 shows the model parameters. The C_{TH} and kk were also plotted as a function of nano-TiO_2 concentration in the small insert in Figure 2a. It was found that C_{TH} was reduced and k_k was increased with an increasing nano-TiO_2 concentration. For example, when nano-TiO_2 concentration increased from 20 to 200 mg/L, the C_{TH} reduced approximated 75% and k_k increased 20 times. The C_{TH} reflected the tolerance of toxic elements, and k_k indicated that the rate of toxicity development if the toxin exceed C_{TH}.[14] Therefore, with a higher nano-TiO_2 concentration, the tolerance of Pb in *C. dubia* was reduced and, simultaneously, the Pb became more toxic. Our previous study shows that nano-TiO_2 alone would not cause the death of *C. dubia* in the test range.[7] However, it can enhance Pb toxicity by forming a surface complex, Ti-O-Pb^+ with the hydroxyl group in nano-TiO_2.[13] On one hand, the positive charged complex may have better interaction with the negatively charged biological tissue or membrane due to the charge effect.[30] On the other hand, the metal complex played an important role in toxicity,[31] and it could also change the toxicity compared with metal ions or NPs alone.[32] A higher nano-TiO_2 concentration could also increase the physical body burden of *C. dubia*,[33] resulting in less tolerance to other toxic elements. All of these effects could reduce the C_{TH} and/or increase the k_k of test organisms. As a result, nano-TiO_2 could serve as a carrier to enhance Pb accumulation, especially at low nano-TiO_2 concentration, and participate in the toxic process and synergistically enhanced the Pb toxicity, which was more evident at high nano-TiO_2 concentration.

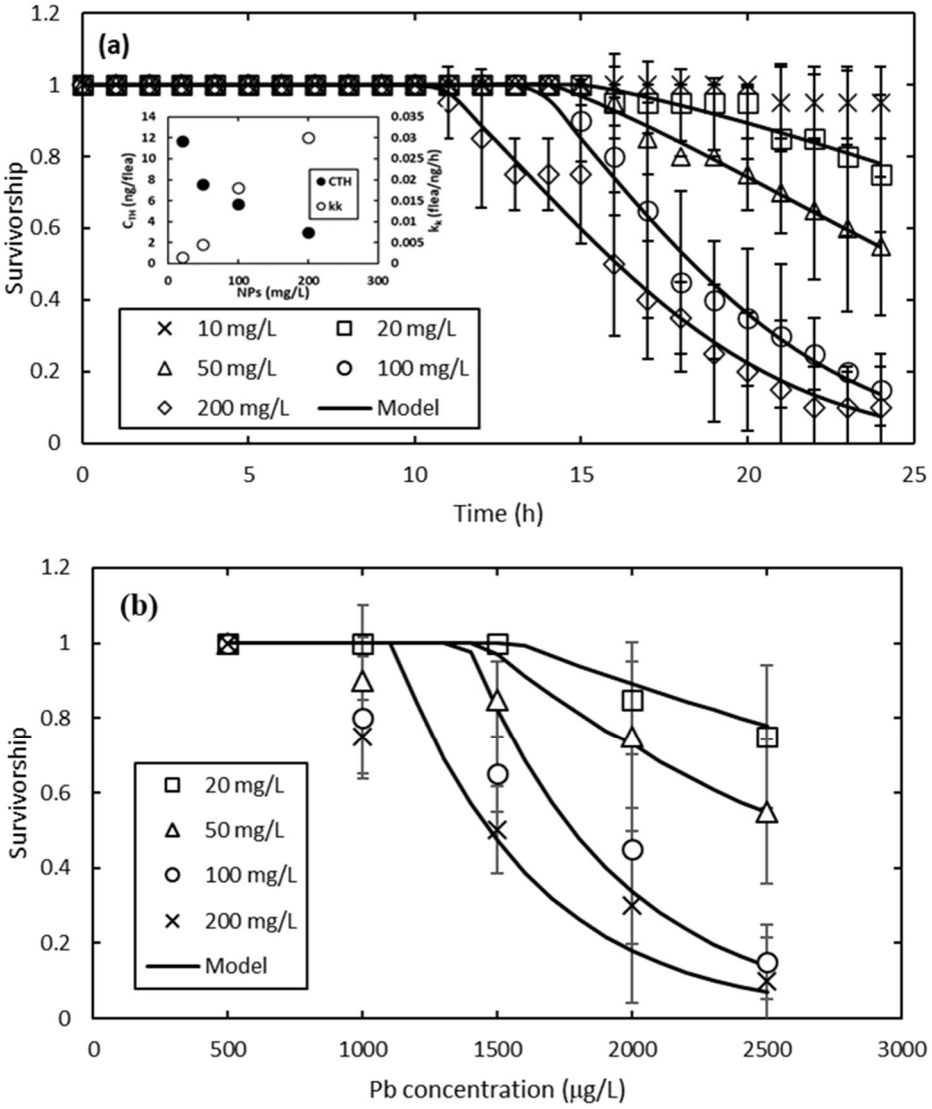

Figure 2. Survivorship of *C. dubia* in the presence of Pb and nano-TiO_2. (a) The survivorship during the 24 h exposure period in test solutions that contained 2500 µg/L of Pb and various concentrations of nano-TiO_2; (b) Comparison of observed and predicted 24 h survivorship for different concentrations of nano-TiO_2 and Pb.

Based on the C_{TH} and k_k values in Table 2, we can predict the survivorship of *C. dubia* under other Pb concentration conditions, for each nano-TiO_2 concentration. We can also conduct independent experiment to validate the model prediction. Figure 2b shows the experimental 24 h survivorship data (symbols) and predicted results (curves) under

different Pb concentrations, for each nano-TiO_2 concentration (20, 50, 100, and 200 mg/L, respectively). In this prediction we first used Equation (3) and related constants in Table 1 to calculate the 24 h Pb tissue accumulation. We then used Equation (4) and constants in Table 2 to calculate the survivorship. The predicted survivorship had a good agreement with the measured results, which suggested that the TD model parameters could accurately describe the toxicity process. The agreement between the experimental data and model production suggested that nano-TiO_2 not only increase toxic element accumulation, but also participate the toxic process to enhance the toxicity. Therefore, nana-TiO_2 synergistically enhanced Pb toxicity.

3.4. ALGAE IMPACT ON Pb-NANO-TiO_2 TOXICITY

Figure 3a shows the impact of algae on the toxicity of Pb and nano-TiO_2. The trend of survivorship was the same as in the absence of algae: the survivorship of *C. dubia* was reduced with increasing nano-TiO_2 concentration. Compared with Figure 2a, the presence of 1.8×10^5 cell/mL algae increased the 24 h survivorship by 0.35, 0.35, and 0.30 for 50, 100, and 200 mg/L of nano-TiO_2, respectively. To quantify the effect of algae on the toxicity of Pb and nano-TiO_2, we first used Equation (3) and related constants in Table 1 to calculate Pb tissue accumulation, and then used Equation (4) to model the time-dependent survivorship data to determine the threshold concentration C_{TH} and the killing rate k_k under each nano-TiO_2 concentration. The solid line in Figure 3a was the curve fitting results, and the parameters were listed in Table 2. Compared with the exposure condition without algae, the presence of algae did not change the threshold value of Pb, but it reduced the killing rate, k_k, approximately 43%, 61%, and 70%, respectively, for 50, 100, and 200

mg/L of nano-TiO_2, respectively. In heavy metals related toxicity process, the production of ROS is the main mechanism,[11] and the production of antioxidants, e.g., glutathione (GSH) and superoxide dismutase (SOD), which could neutralize ROS is energy related.[35]

Table 2. Toxicodynamic model parameters.

Test Solution *	Predicted C_{body}(24 h) (ng/flea)	C_{TH} (ng/flea)	k_k (flea/ng/h)	R^2
10 mg/L TiO_2**	9.13	N.A.	N.A.	N.A.
20 mg/L TiO_2	16.71	11.69	1.5×10^{-3}	0.94
50 mg/L TiO_2	13.07	7.53	4.6×10^{-3}	0.99
100 mg/L TiO_2	10.27	5.70	0.018	0.99
200 mg/L TiO_2	5.14	2.96	0.030	0.99
50 mg/L TiO_2 + algae***	9.73	7.53	0.002	0.95
100 mg/L TiO_2 + algae***	7.97	5.70	0.011	0.97
200 mg/L TiO_2 + algae***	4.17	2.96	0.021	0.97

* All test solutions contain 2,500 μg/L of Pb.

** For 10 mg/L TiO_2, the changing in survivorship is negligible compared with negative control which is not fitted by the toxicodynamic model.

***algae concentration = 1.8×10^5 cell/mL.

As a food source, algae could provide energy to support the living of *C. dubia* and also boost the production of antioxidants. In addition, some algae could directly serve as antioxidants when ingested by *C. dubia*.[36] Therefore, some of the Pb produced ROS was neutralized by algae, and the killing rate of Pb was consequently reduced. Although algae slightly reduced the accumulation of Pb (Figure 1), it did not change the C_{TH}, indicating that algae did not change the Pb tolerance for *C. dubia*. Considering the parameters for the accumulation model and the TD model, it can be conclude that algae can occupy some of the gut space and reduce Pb uptake. They can also reduce Pb transfer from gut to tissue where the toxicity effect occurred. Moreover, algae can mitigate the toxic strength of Pb,

probably through the elevation of the antioxidant level in *C. dubia* that neutralizes the ROS produced by Pb.

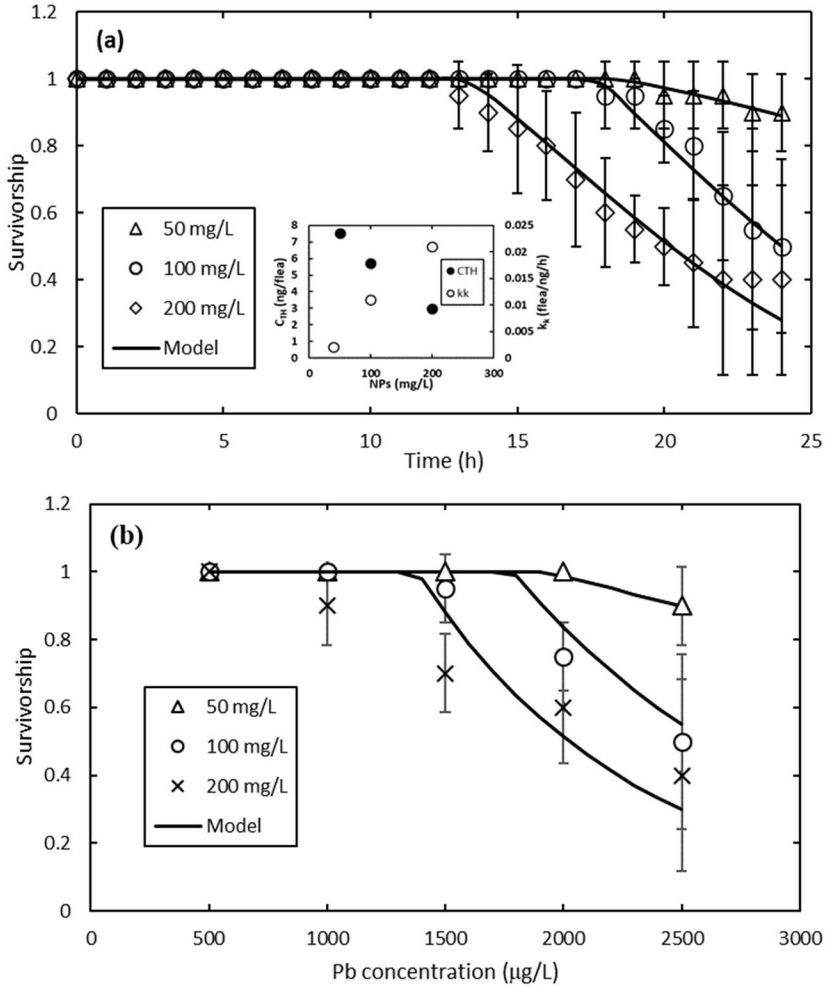

Figure 3. Survivorship of *C. dubia* in the presence of Pb, nano-TiO_2, and algae. (a) The survivorship during the 24 h exposure period in test solutions that contained 2500 µg/L of Pb, various concentrations of nano-TiO_2, and 1.8×10^5 cell/mL of algae; (b) Comparison of observed and predicted 24 h survivorship for different concentrations of nano-TiO_2 and Pb, and 1.8×10^5 cell/mL of algae.

To validate these TD parameters, we also conducted independent 24 h toxicity test using test solutions that contained 1.8×10^5 cell/mL of algae and different concentrations of

Pb and nano-TiO_2. Figure 5b shows the predicted and observed 24 h survivorship results, which agrees with each other. This agreement suggests that the two-compartment modeling approach is appropriate to predict the effect of nano-TiO_2 on the toxicity of toxic metals, with or without the presence of algae.

4. CONCLUSION

In this research, we found that increasing nano-TiO_2 concentration reduced Pb accumulation but increased Pb toxicity on *C. dubia*. We used a two-compartment modeling approach to quantify the toxicity of Pb under different concentrations of nano-TiO_2, with or without algae presence. Modeling results suggested that nano-TiO_2 participated the Pb toxicity process, and the increasing nano-TiO_2 concentration reduced the Pb tolerance level and increased the killing rate of *C. dubia*. Therefore, nano-TiO_2 not only served as carrier that change Pb accumulation, but also synergistically enhanced Pb toxicity. Algae could reduce the killing rate to reduce the toxicity, but it did not change the Pb tolerance level of *C. dubia*. Importantly, this two-compartment modeling approach provided a useful tool to understand the role of NPs on heavy metal related toxicity process.

SUPPORTING INFORMATION

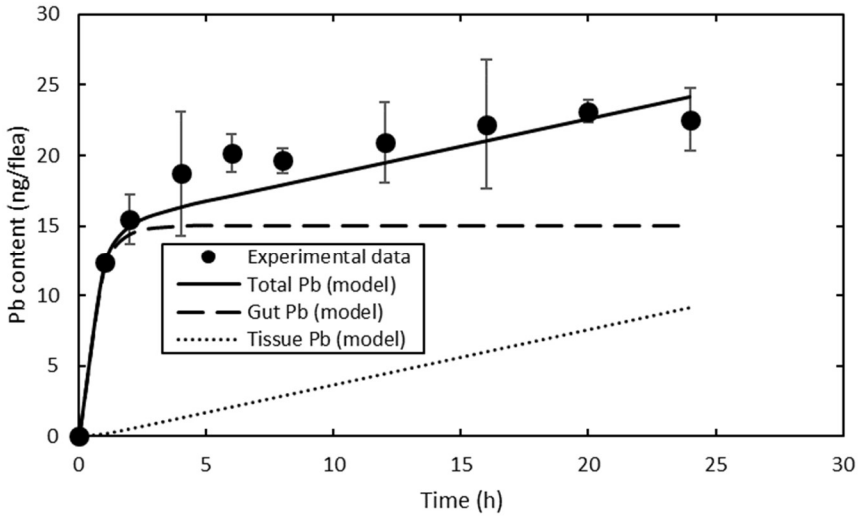

Figure S1. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2. Exposure conditions: [Pb] = 2500 µg/L; [NPs] = 10 mg/L; exposure time = 24 h. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

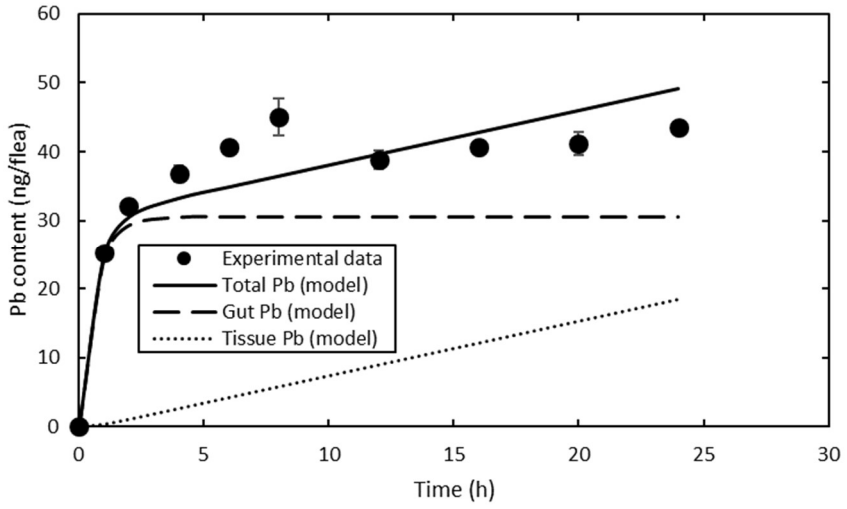

Figure S2. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2. Exposure conditions: [Pb] = 2500 µg/L; [NPs] = 20 mg/L; exposure time = 24 h. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

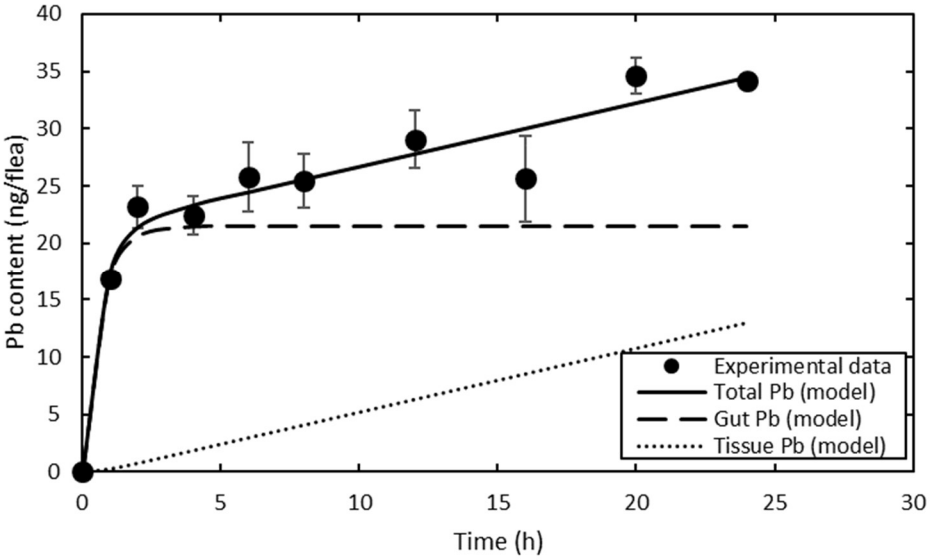

Figure S3. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2. Exposure conditions: [Pb] = 2500 μg/L; [NPs] = 50 mg/L; exposure time = 24 h. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

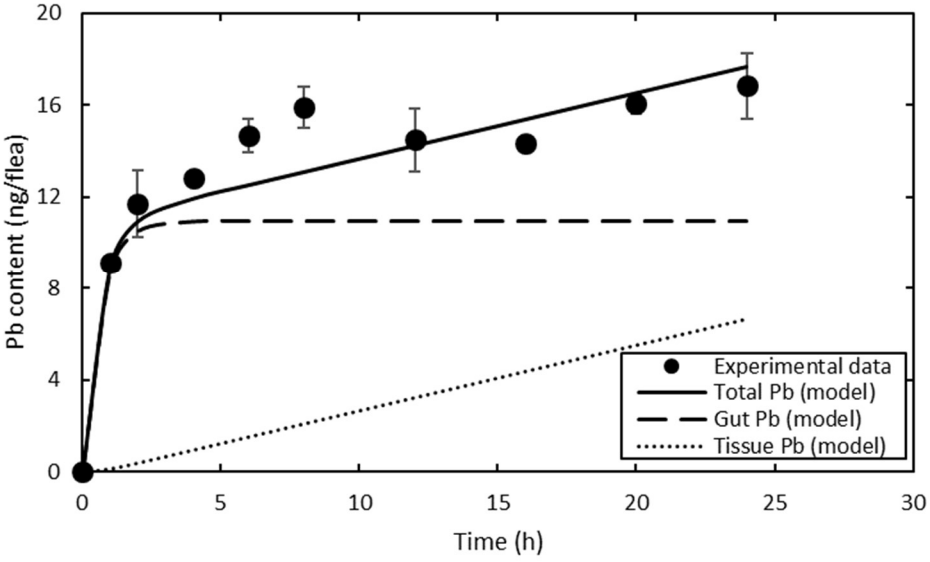

Figure S4. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2. Exposure conditions: [Pb] = 2500 μg/L; [NPs] = 200 mg/L; exposure time = 24 h. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

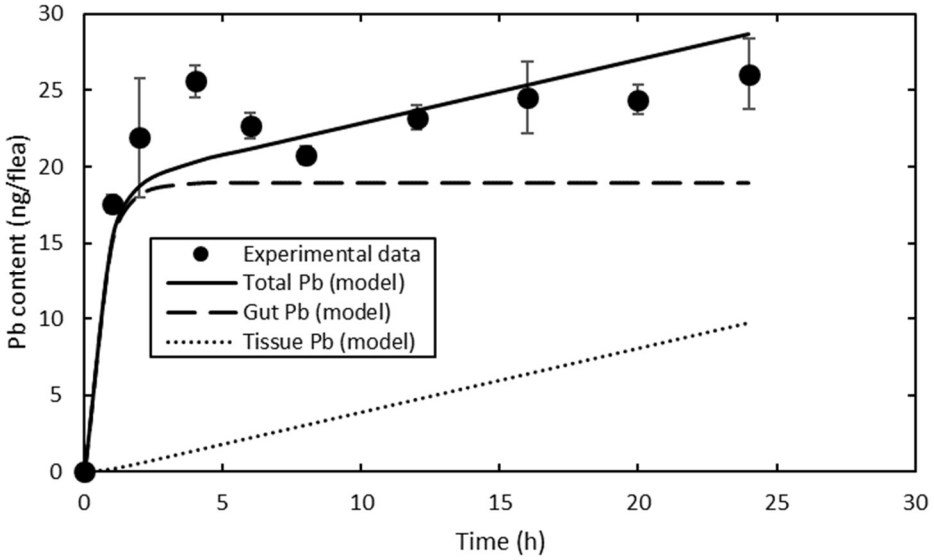

Figure S5. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2 and algae. Exposure conditions: [Pb] = 2500 μg/L; [NPs] = 50 mg/L; algae = 1.8×10^5 cell/mL; exposure time = 24 h. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

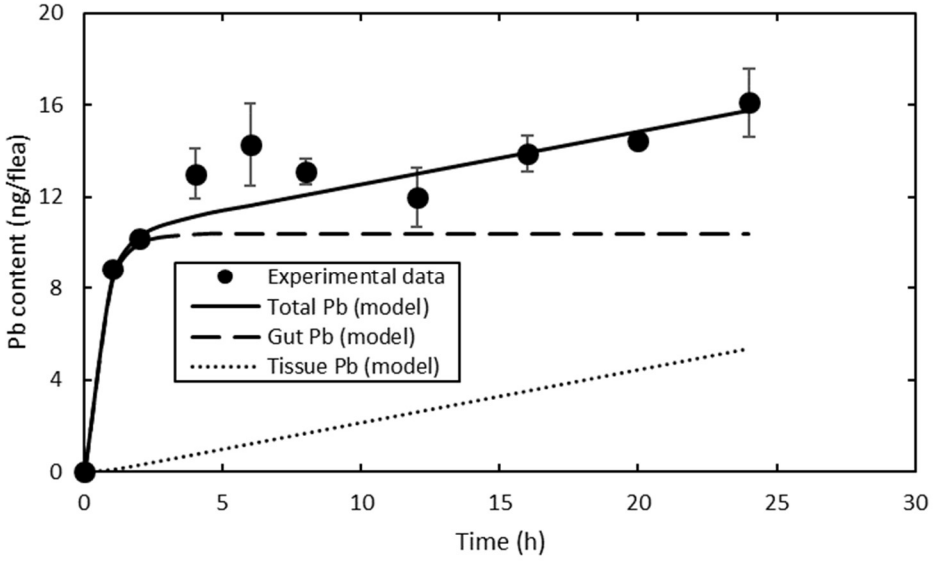

Figure S6. The Pb accumulation in *C. dubia* in the presence of nano-TiO_2 and algae. Exposure conditions: [Pb] = 2500 μg/L; [NPs] = 200 mg/L; algae = 1.8×10^5 cell/mL; exposure time = 24 h. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

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IV. ALGAE (*RAPHIDOCELIS*) MITIGATE COMBINED TOXICITY OF MICRO-PLASTIC AND LEAD ON *C. DUBIA*

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ABSTRACT

Microplastics (MPs) have been recognized as a new class of emerging contaminants in recent years. They not only directly impact aquatic organisms, but also indirectly impact these organisms by interacting with background toxins in the environment. However, under realistic environmental conditions, algae, a natural food for aquatic organisms, may alter the toxicity pattern related to MPs. In this research, we first examined the toxicity of MPs alone, and their effect on the toxicity of lead (Pb), using *C. dubia* as a model aquatic organism. Then, we investigated the effect of algae on the combined toxicity of MPs and Pb. We observed that, MPs significantly increased Pb toxicity in the absence of algae, and the increase in the toxicity is related to the increased soluble Pb concentration and the intake of Pb-loaded MPs, both of which increased the accumulation of Pb in *C. dubia*. The presence of algae mitigated the combined toxicity of MPs and Pb, although algae increased Pb accumulation. Therefore, the toxicity mitigation through algae uptake might come from

other mechanisms rather than reducing Pb accumulation, which will need further investigation.

Keywords: Micro-plastic, Lead, Toxicity, Algae, *C. dubia*

1. INTRODUCTION

Plastics have been used in numerous consumer products. In 2017, the global plastic production exceeded 348 million tons (Plastic Europe, 2018). This mass production inevitably increased the release of plastics into the environment, which can break into smaller sizes under various environmental conditions (Browne et al., 2007; Lambert et al., 2013; Brandon et al., 2016). Microplastics (MPs) are ordinary plastic debris with diameters from 100 nm to 5 mm (Alimi et al., 2018). Kazmiruk et al. (2018) found that the major environmental MPs are in fine sand levels (0.63-250 μm). In recent years, MPs have been recognized as a new class of emerging contaminants. Many studies have revealed that exposure to MPs can lead from sub-lethal to lethal effects on organisms in the aquatic food web, such as algae (Zhang et al., 2017), crustacean (Kim et al., 2018), mollusks (Van et al., 2015), and fish (Lei et al., 2018). Other than lone MP toxicity, MPs have the potential to interact with large amounts of environmental pollutants, and pose additional toxic effect on aquatic organisms. Batel et al. (2018) reported that MPs functioned as a transport media for polycyclic aromatic hydrocarbons (PAHs) and increase PAH uptake; Chen et al. (2017) demonstrated high combined toxicity of MPs and endocrine disrupting compounds (EDCs), also due to enhanced EDC accumulation through MPs; Town et al. (2018) revealed

that MPs increased the heavy metal toxicity. MPs could also carry heavy metals to higher trophic levels, and pose health threats to humans.

Different levels and types of toxic metals exist in the aquatic environment, which can adversely impact aquatic organisms (Gad, 1989; Ab Latif Wani and Usmani, 2015; Fan et al., 2019). Recent studies have shown that the toxicity of toxic metals can be significantly enhanced by metal oxide-based nanoparticles, and even these nanoparticles alone are considered non-toxic (Hu et al., 2011; Wang et al., 2011; Hu et al., 2012). This effect is characterized as the "Trojan-horse effect" (Hu et al., 2012). Collectively, nanoparticles serve as a carrier of toxic metals to facilitate their accumulation and toxicity (Hu et al., 2012; Liu et al., 2019). MPs may act in the same way as conventional nanoparticles and increase the toxicity of background toxic metals. However, because the surface property of MPs is significantly different from that of the conventional metal oxide-based nanoparticles, the toxicity mechanism of MPs will be different from that of conventional nanoparticles.

Algae is an essential food for most aquatic organisms in the natural environment. Previous research has indicated that algae exhibit different results in toxicity studies (Svensson, 2003; Petersen et al., 2009; Su et al., 2018). The interaction between algae and other toxins may affect the toxicity of these toxins through different pathways, such as accumulation, distribution, and bio-function (Luo et al., 2018; Fan et al., 2019). We previously discovered that algae could mitigate the combined toxicity of TiO_2 particles and lead (Pb) on *C. dubia* (Liu et al., 2019). A similar effect could also occur for the combined toxicity of MPs and toxic metals. The objectives of this research were to investigate the combined toxicity of MPs and Pb. In addition, in order to assess the toxicity of MPs and

Pb in real environment, the effect of algae, a realistic environmental condition, on this combined toxicity was investigated. The experiments were conducted to disclose the combined toxicity of MP and Pb and corresponding algae impact through three aspects (Figure.1a): 1) MP-Pb interaction, 2) MP accumulation, and 3) Pb accumulation

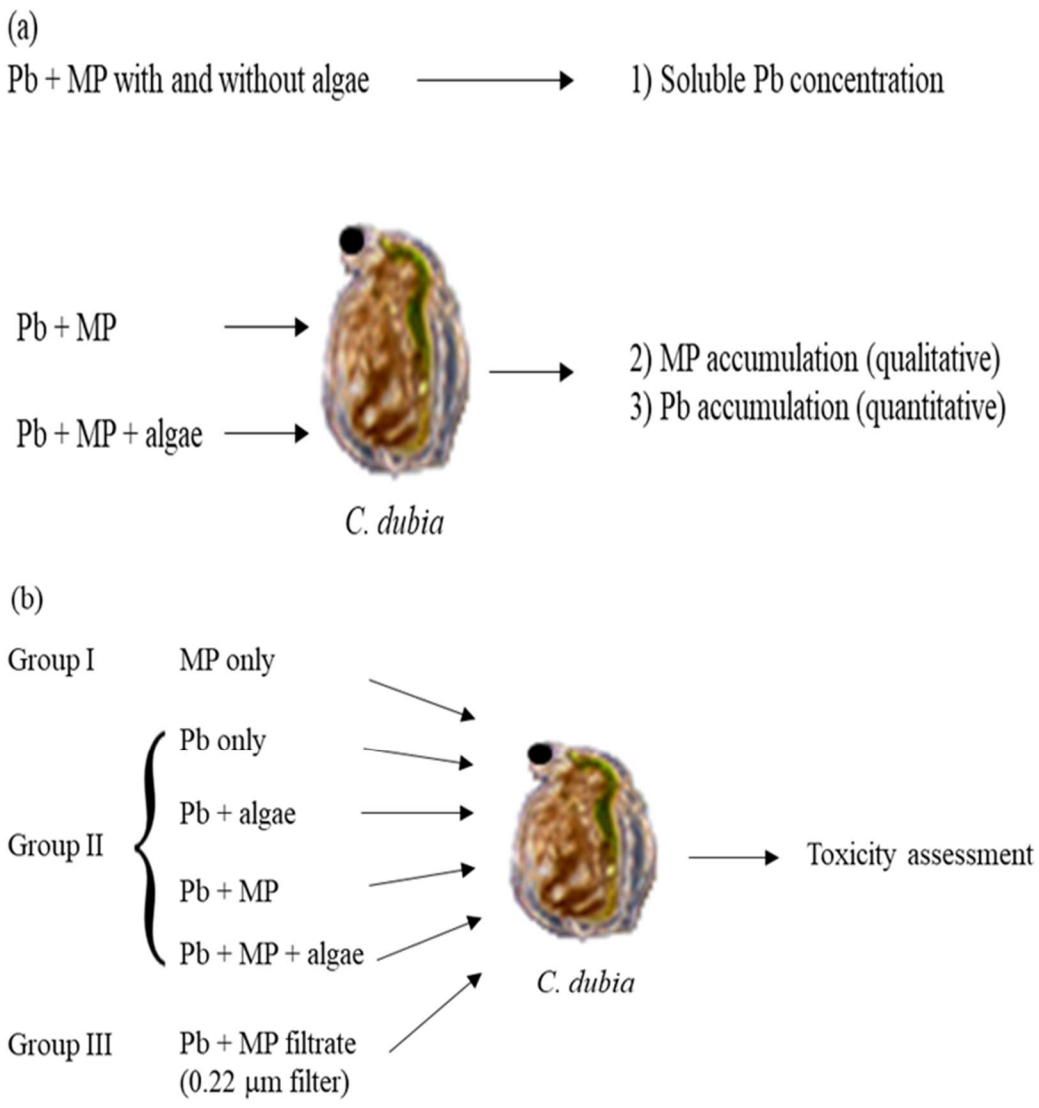

Figure 1. Experimental design for (a) Mechanism studies; (b) Actue toxicity studies.

2. MATERIALS AND METHODS

2.1. MPS, CHEMICALS, AND ORGANISMS

We selected MPs of polystyrene, a representative plastic in the environment (de Sá et al., 2018), and *Raphidocelis*, a representative algae species in freshwater, to evaluate the effect of algae on the combined toxicity of MPs and Pb on *C. dubia* based on the EPA standard test method (EPA, 2002). The carboxylate polystyrene microplastic (PS-COOH, d = 1.0 μm, 2.6% solid) was purchased from Polyscience Inc. (Warrington, PA, USA). The algae (*Raphidocelis*) stock solution were purchased from ABS Inc. (Fort Collins, CO, USA). The chemicals used in this research, including $CaSO_4 \cdot 2H_2O$ (98%), Na_2SeO_4 (99%), $NaHCO_3$ (100.2%, Pb < 5 mg/kg), $MgSO_4$ (Pb < 0.001%), KCl (99%), $Pb(NO_3)_2$, and trace metal grade nitric acid, were acquired from Fisher Scientific (Fair Lawn, New Jersey, USA). The Milli-Q (MQ) water (resistivity = 18.2 MΩ·cm) was used to prepare all the solutions.

The starter *C. dubia* was purchased from MBL Aquaculture (Sarasota, FL, USA) and continuously cultured in a laminar flow hood (SVC-6AX, Streamline® laboratory products, Fort Myers, FL, USA) in a temperature controlled chamber at 25°C and a light to dark cycle of 16 h : 8 h. The laminar flow hood was used to eliminate any airborne particles that could negatively impact the *C. dubia* during culturing, and an illuminating light was used to simulate the natural light cycles. The *C. dubia* was cultured in a culture medium of a synthetic water of moderate hardness (pH = 7.8 ± 0.2, hardness = 85 ± 5 mg/L as $CaCO_3$), following the EPA standard method, EPA-821-R-02-012 (EPA, 2002).

2.2. TEST SOLUTION PREPARATION AND Pb ADSORPTION TEST

The Pb stock solution (1,000 mg/L) was prepared by adding an appropriate amount of $Pb(NO_3)_2$ into MQ water, which was then acidified with a trace metal grade nitric acid to reduce the pH to less than 4. Eight series of 125 mL glass bottles, with different Pb concentrations from 0 to 2500 μg/L in each series, were prepared by diluting different volumes of the Pb stock solution with the culture medium to achieve a final volume of 70 mL. Glass bottles were used in this research to avoid any potential interference between MPs and plastic containers during the test (Van et al., 2015; Zhang et al., 2017; Kim et al., 2018; Lei et al., 2018). Different amounts of algae and/or MP particles were added to these glass bottles to obtain certain concentrations, respectively. In addition to Pb, the first, Pb-only series of bottles contained only the culture medium, without any algae or MP particles. The second, Pb+algae series of bottles contained 1.8×10^5 cell/mL of algae in each bottle that could support the living *C. dubia* (EPA, 2002). The third through fifth, Pb+MP series of bottles contained 5, 10, and 20 mg/L of MPs, respectively. The sixth through eighth, Pb+algae+MP series of bottles contained 1.8×10^5 cell/mL of algae in each bottle, and 5, 10, and 20 mg/L of MPs in series 6^{th}, 7^{th}, and 8^{th} bottles, respectively. All bottles were mixed at 250 rpm and 25°C for 24 h in an incubator shaker. After mixing, 60 mL of each test solution was used for a toxicity test, and the residual test solution was collected and filtered through a 0.22 μm nylon membrane filter. The filtrate was collected and acidified for soluble Pb analysis. For all tests, the final pH (after mixing for 24 h) was 7.8 ± 0.2.

2.3. ACUTE TOXICITY TEST

The acute toxicity was determined based on the EPA standard method, EPA-821-

R-02-012 (EPA, 2002). In brief, four replicate tests were conducted for each test solution. For each replicate test, five healthy neonates (aged 24 h or less) were transferred to a 25 mL glass reactor that contained 15 mL of test solution, and the 24 h mortality of these neonates was monitored. Before transferring to the test reactor, neonates were washed three times, with a fresh culture medium, to remove any food residual on the neonate surfaces. A plastic Pasteur pipette with a 3 mm-diameter opening was used to transfer neonates to prevent any damage to them. During the transfer, the fresh culture medium carried to each test reactor was approximately 0.2 mL, which was negligible as compared to the 15 mL of the test solution. No additional foods or particles were added to the reactor during the test. After a 24 h testing period, neonates were visually inspected for mortality. Control tests were also conducted using the culture medium, and the survival rate for all controls exceeded 90%.

In order to examine the toxic effect of Pb, MP, and their combinations, three groups of toxicity test were conducted (Figure. 1b). The preparation of all the test solutions were followed the procedure in Section 2.2. For group I, to test the toxicity of MPs alone, test solutions containing MP concentrations from 0 to 200 mg/L were used. The test solutions were prepared by adding different amounts of MPs into 70 mL of a culture medium. Based on this test, MP alone at concentrations of 50 mg/L and less did not result in significant toxicity on *C. dubia* (Figure. 2).

For group II, to test the combined toxicity of Pb and different particles (MP and/or algae), the test solutions included Pb only, Pb+algae, Pb+MP, and Pb+MP+algae. The Pb concentration varied from 0 to 2500 μg/L. The algae concentration was fixed at 1.8×10^5 cell/mL for corresponding tests. Three MP concentrations, 5, 10, and 20 mg/L, were used

in corresponding tests when testing the MP effect. The algae concentration used in the toxicity test was consistent with that in the individual culture reactor, which could support the living *C. dubia* (EPA, 2002).

During the research we have found that MPs significantly increased the soluble Pb concentration. In order to test the matrix effect of the solution on the toxicity after contacting with MPs, in group III, a series of test solutions containing 20 mg/L of MPs and various concentrations of Pb (ranging from 1500-2500 μg/L) were also prepared and filtered through 0.22 μm of nylon membrane filters. The filtrate was used to conduct the toxicity test. This test was used to isolate the effect of soluble Pb during the test involving MPs. The preparation procedure of the test solution was the same as that described in Section 2.2, except that all solutions were filtered after mixing for 24 h. The filtrates that contained only soluble Pb were used to conduct a toxicity test.

2.4. ACCUMULATION TEST

Accumulation of Pb in *C. dubia* is a key factor on Pb toxicity. Ideally, the same *C. dubia* neonates used for a toxicity text should be used to determine Pb accumulation. However, because the neonates can be easily damaged during the multiple manual steps of the test, *C. dubia* adults (7 day) were used for Pb accumulation test, to develop a trend of Pb accumulation by *C. dubia*. This method was used in previous research (Wang et al., 2011; Liu et al., 2019). In brief, approximately 50 adult *C. dubia* were washed three times with the clean culture medium, before placing in test solutions that contained Pb+MP and Pb+MP+algae. The concentrations of Pb, MP, and algae, in corresponding test solutions, were 2,500 μg/L, 20 mg/L, and 1.8×10^5 cell/mL, respectively. After designated exposure

time, *C. dubia* were picked and washed three times with the clean culture medium, and filtered through a 0.297 mm standard sieve, counted, and transferred to a digestion vessel that contained 5 mL of nitric acid. The solution was digested at 95°C for 12 h in a hot-block digester, with a Watlow mini controller (Watlow mini-0R10-000G). After digestion, MQ water was added to the digestion vessel to make up the volume lost due to evaporation. The final volume was made to 5 mL and then used for soluble Pb analysis, after appropriate dilution. The Pb accumulation in *C. dubia* was converted by using the Pb concentration of the digestion solution. A control group of *C. dubia* cultured in a clean culture medium was also included and used as background Pb content. The net accumulation was calculated by subtracting the Pb background from the total Pb accumulation. All Pb accumulation experiments were conducted in duplicate.

2.5. ANALYTICAL METHOD

A graphite furnace atomic adsorption spectrometer (GFAA) (Perking Elmer AAnalyst 600) was used to determine the soluble Pb concentration from different tests. The detection limit of the GFAA was 0.5 μg/L. The Pb adsorption on MPs was verified by X-ray photoelectron spectroscopy (XPS) (Kratos Axis 165). Dried particles of MPs, after 24 h of mixing with Pb, were analyzed through a survey scan with hybrid lens mode. To maintain an appropriate Pb peak strength, 5,000 μg/L of Pb were used in the XPS sample preparation. The images of *C. dubia* in different conditions were taken using phase contrast microscopy (Olympus CKX41SF). For each condition, at least 10 *C. dubia* were visually checked and the image of the most representative one was captured.

2.6. STATISTICAL ANALYSIS

Data from toxicity tests were presented as mean ± standard deviations. To determine whether the difference between various treatment groups was significant, or not, a two-way analysis of variance (ANOVA) was conducted. The "initial Pb concentration" and "type of particle" were used as factors to analyze mortality outcomes between different treatment groups. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. MICRO-PLASTIC TOXICITY

C. dubia can ingest particles that range in size from 100 nm to 5,000 nm (Geller and Müller, 1981). Figure. 2 shows that the test MPs (d = 1.0 μm) were ingested to *C. dubia* and caused 25% of the mortality when MP concentration was greater than 100 mg/L. However, at low MP concentrations (50 mg/L and less), no significant mortality was observed. Other than the concentration, the size of the plastic particles could also impact the toxicity, and this kind of impact was related to the distribution of particles within the organisms (Lee et al., 2019). In this research, we used plastic particles with a uniform size of 1.0 μm (Figure. S1a and Figure. S2). Although MPs of this size have low toxicity, they are the most commonly seen in the environment (Kazmiruk et al., 2018). Therefore, they were selected for this research. In addition to the concentration and size distribution, the surface property of plastic particles could also affect the toxicity pattern (Haegerbaeumer et al., 2019). Figure. S1b shows the surface morphology of the MPs we used in this research. They have a smooth surface and non-porous structure. Consequently, these MPs

have a small specific surface area to cause the toxicity (Braakhuis et al., 2016). Furthermore, functional groups on the MPs affect the toxicity pattern. MPs could cause histological changes (e.g., abscission, disintegration, and thickening) that damage the tissue through physical contact with cell membrane (Wang et al., 2019). Kim et al. (2017) reported that plastic particles with carboxylate groups exhibited a higher toxicity than plain plastic, presumably due to the presence of functional groups that affect the binding capacity of MP in organisms. Therefore, the carboxylate groups on the surface of the MPs that we used for this research could contribute to the death of *C. dubia*. Collectively, the concentration, size distribution, and surface properties of plastic particles should be considered when evaluating their adverse effect on aquatic organisms.

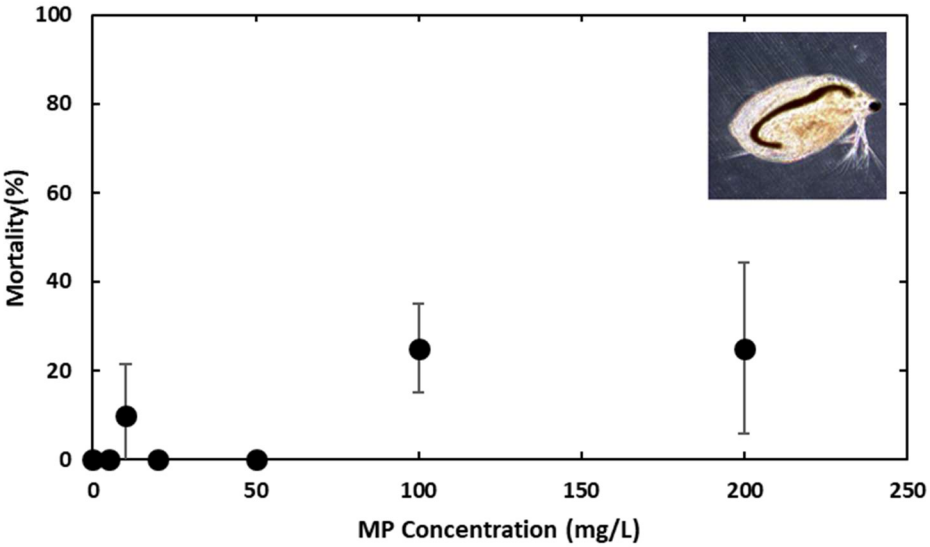

Figure 2. The 24 h mortality of *C. dubia* in the presence of MP. MP concentrations = 0-200 mg/L. Photo was *C.dubia* from 24 h uptake with MP (20 mg/L). Standard deviation is represented by an error bar attached to each point (N=4).

3.2. EFFECT OF MPS ON Pb TOXICITY

Figure. 3 shows the effect of MPs on the toxicity of Pb. It was found that Pb alone (up to 2,500 μg/L) did not show significant toxicity on *C. dubia*, which is consistent with our previous research (Liu et al., 2019). This is because the Pb toxicity is related to its solubility in water (Erten-Unal et al., 1998). In the culture medium (moderate hardness water) or the natural water, Pb forms precipitation with different anions, such as hydroxide, carbonate, and sulfate ions (Escudero-García et al., 2013), resulting in a very low solubility, and the Pb precipitates have much less toxicity than soluble Pb ions.

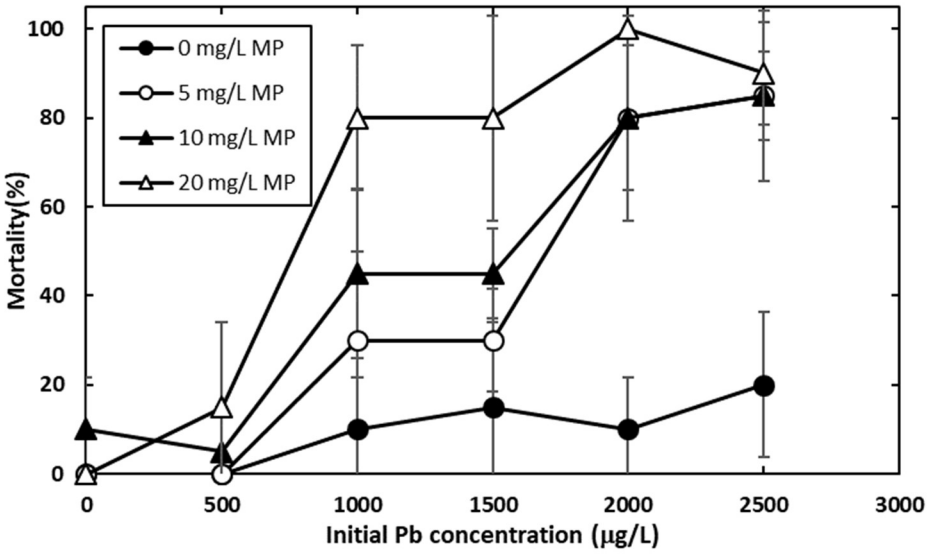

Figure 3. The 24 h mortality of *C. dubia* in the presence of Pb, with and without MP. MP concentration = 0-20 mg/L. Standard deviation is represented by an error bar attached to each point (N=4).

Remarkably, Figure. 3 shows that MP significantly increased Pb toxicity for all three MP concentrations used in this study (5, 10, and 20 mg/L) ($p < 0.05$), causing more than 85% mortality at the initial Pb concentration of 2,500 μg/L. In addition, a higher MP

concentration resulted in a greater Pb toxicity (between any two MP concentrations, $p < 0.05$). As indicated in Figure. 2, MPs alone had negligible toxicity at these three concentrations, but they enhanced Pb toxicity by several times. The same phenomenon was also observed in our previous research when other nanoparticles were mixed with toxic elements (Wang et al., 2011; Hu et al., 2012; Liu et al., 2019). Previously, we found that particles served as adsorbents to carry excessive amounts of toxic elements to organisms, resulting in an increase in toxicity (Liu et al., 2019). By the same token, MPs may also carry excessive amounts of Pb to *C. dubia* in this research. Other than excessive Pb accumulation, the deposition or adsorption of Pb on the MP surface could alter the surface property (Zhang et al., 2006; Zhou et al., 2009; Kong et al., 2019), thereby elevating MP toxicity (Haegerbaeumer et al., 2019). As a result, the excessive Pb uptake and the change in MP surface property could lead to an increase in the toxicity.

3.3. MICRO-PLASTIC AND Pb INTERACTION: SOLUBLE Pb CONCENTRATION

Previous research has indicated that the soluble heavy metal concentration played a key role in metal toxicity (Gad, 1989; Erten-Unal et al., 1998; Hu et al., 2012). Figure. 4a shows the effects of MPs on a soluble Pb concentration in the culture medium. It shows that Pb alone exhibited a very low soluble Pb concentration. Pb toxicity was also discussed in our previous research: the formation of Pb precipitation reduced Pb toxicity (Liu et al., 2019). The corresponding toxicity pattern can also be confirmed in this study (Figure. 3).

Figure. 4a also indicates that MP significantly increased the soluble Pb concentration in the culture medium, contradicting to our initial assumption that MPs could

serve as a Pb adsorbent to reduce the soluble Pb concentration. For example, when the initial Pb concentration is 2,500 μg/L, the presence of 20 mg/L MPs increased the soluble

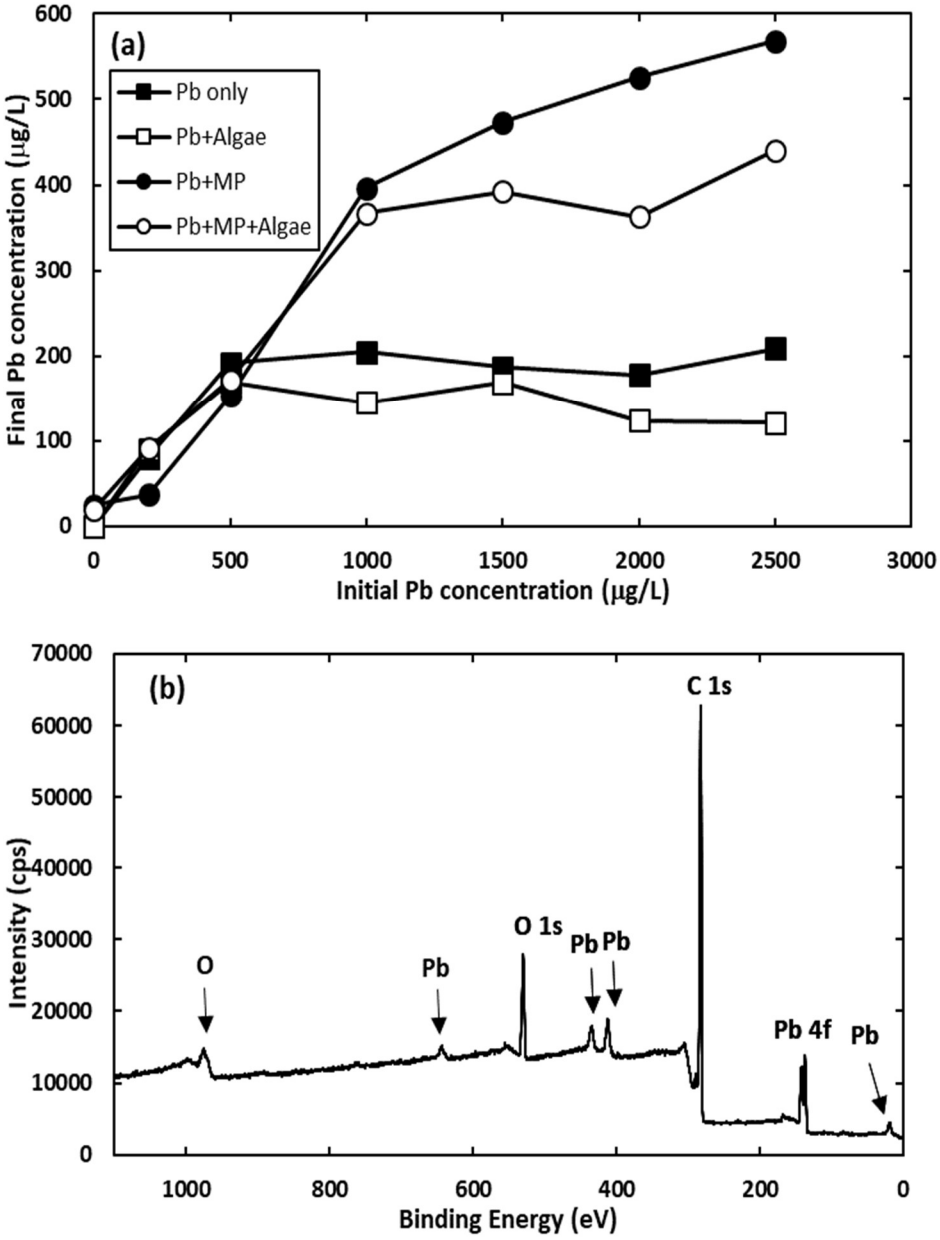

Figure 4. MP and Pb interaction in the culture medium. (a) Soluble Pb concentration with and without different particles (MP = 20 mg/L; Algae = 1.8×10^5 cell/mL); (b) XPS survey spectra of MP surface after Pb adsorption (MP =20 mg/L, Pb = 5000 μg/L).

Pb concentration from 208 μg/L to 568 μg/L. Jordan et al. (1997) and Korshin et al. (2005) found that the natural organic matter in the solution could form a complex with Pb to increase the soluble Pb concentration. In this study, MPs significantly increased the total organic carbon (TOC) in the culture medium (Table S1). The TOC could form complexes with Pb and increase the soluble Pb concentration, similar to that reported by Turner and Holmes (2015). Unlike the inorganic Pb ion, organic Pb can easily dissolve in the lipid bilayer, and then pass through cell membrane of *C. dubia*, resulting in a greater toxicity (Ab Latif Wani and Usmani, 2015). Therefore, both the increased soluble Pb concentration and the formation of organic Pb complex could contributed to the increased toxicity.

3.4. MICRO-PLASTIC AND Pb INTERACTION: Pb ADSORPTION

Small particles can serve as transport vector for toxic metals (Liu et al., 2019). As a consequence, the adsorption capacity of toxic metals on particles is always the focus in a nanoparticle toxicity study. Because Pb could form precipitates and MPs increased soluble Pb concentration in this study, we did not use the conventional subtraction method (the concentration difference between total metal and soluble metal) to determine the adsorbed Pb on MPs. Instead, we used XPS to directly examine if the Pb was adsorbed on MPs. Figure. 4b shows the XPS spectra of the MP surface after contacting with the Pb solution. The spectrum confirmed that Pb was present on the surface of the MP. The binding energy positions of ~18, ~137, ~142, ~412, and ~434 eV were assigned to a major Pb compound, according to the NIST X-ray Photoelectron Spectroscopy Database (NIST, 2012). Therefore, through MP and Pb interaction, MPs adsorbed some Pb on the surface. Apparently, the adsorbed Pb could be delivered to *C. dubia* through MP ingestion, resulting

in a greater Pb accumulation. Because the gut pH of *C. dubia* is typically in the range of 6.0-6.8, which is lower than culture medium (pH=7.8) (Ebert, 2005), the adsorbed Pb could desorb from the MP surface within the gut, making more Pb available for tissue uptake. Pb could increase reactive oxidative species (ROS) production and/or replace essential elements in enzyme to cause the death of organisms (Bray and Bettger, 1990; Ercal et al., 2001). In addition, as indicated earlier, toxic metal adsorption could change the surface properties of MPs and, therefore, induce toxicity. As a result, through the view of Pb adsorption, the excessive Pb uptake and the change in MP surface property led to the increase in the toxicity on *C. dubia*.

3.5. SOLUBLE Pb TOXICITY

As indicated earlier, through the interaction between MP and Pb, Pb could be divided into MP adsorbed Pb and soluble Pb (Figure. 4a and 4b). The presence of MPs increased soluble Pb concentration. Obviously, this increased soluble Pb concentration could elevate the toxicity. In order to examine the toxicity contribution from this soluble Pb after contacting with MPs, we filtered the MP+Pb test solution through a 0.22 μm filter and used the filtrate to conduct the toxicity test. Figure. 5 shows a comparison of the 24 h mortality of the original MP + Pb test solution and the filtrate that contains only soluble Pb. It appears that the soluble Pb contributed to less than 40% of the total mortality. For example, in the presence of 20 mg/L MP and 2,500 μg/L Pb, the overall toxicity resulted in 90% of the mortality. However, the filtrate of the test solution only resulted in 35% of the mortality. Apparently, MPs with the adsorbed Pb contributed 60% of the toxicity to the

C. dubia. In addition, the synergistic effect of soluble Pb and MP might have also contributed to the overall toxicity.

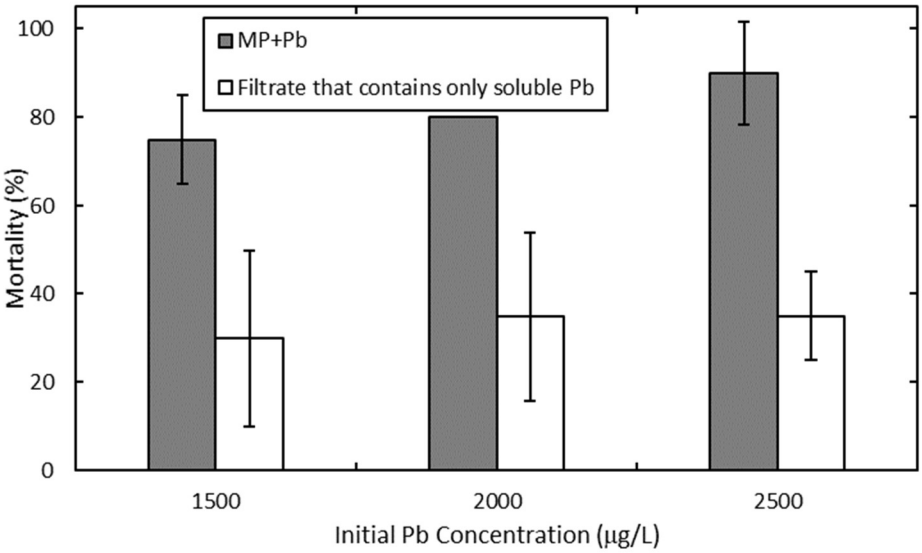

Figure 5. The toxicity of original MP + Pb test solution and the filtrate from the original test solution. The MP = 20 mg/L in the original test solution. Filtrate was collected by passing through 0.22 µm filter. Standard deviation is represented by an error bar attached to each point (N=4).

3.6. EFFECT OF ALGAE ON THE COMBINED TOXICITY OF MICRO-PLASTIC AND Pb

Algae are a natural food for most organisms. Many studies have shown that algae could impact the toxicity of other contaminants through different mechanisms (Svensson, 2003; Petersen et al., 2009; Luo et al., 2018; Su et al., 2019; Liu et al., 2019). Figure. 6 indicates that algae significantly reduced the combined toxicity of MPs and Pb (for all three MP concentrations, $p < 0.05$). For instance, at an initial Pb concentration of 2,500 µg/L with 20 mg/L of MP, the mortality of *C. dubia* was reduced from 90% to 45% as a result

of 1.8×10^5 cells/mL of algae. Algae also reduced the mortality of *C. dubia* to less than 20% for the other two lower MP concentrations.

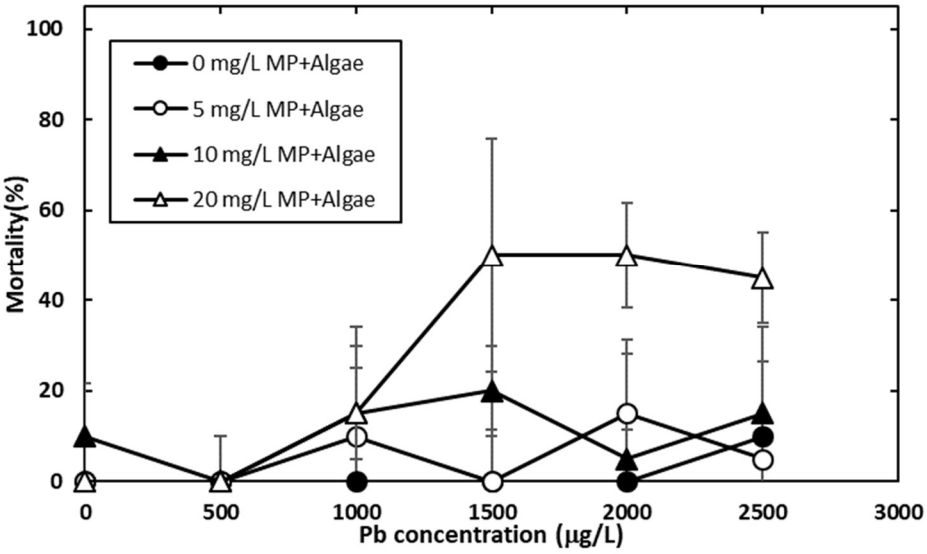

Figure 6. The 24 h mortality of *C. dubia* in the presence of Pb and algae, with and without MP. MP concentration = 0-20 mg/L; Algae = 1.8×10^5 cells/mL. Standard deviation is represented by an error bar attached to each point (N=4).

As indicated in Figure. 4a, algae could adsorb Pb and further reduce the soluble Pb concentration, in the absence and presence of MPs. Our previous research found, if only algae present with Pb, the ingestion of algae could increase metal accumulation in *C. dubia*, but the energy produced from the consumption of algae could mitigate this toxicity (Liu et al., 2019). Our previous research also revealed that algae actually reduced the combined toxicity of nano-TiO_2 and Pb on *C. dubia* (Liu et al., 2019). This is because that, as a food source, algae could provide energy for organisms to boost antioxidant synthesis or directly serve as an antioxidant to mitigate the toxicity (Romay et al., 1998; Poljsak et al., 2013). On the other hand, because algae exhibited lower metal adsorption capacity than

nanoparticles, algae could occupy some gut space and reduce the uptake of nanoparticles that are loaded with toxic metals in *C. dubia* (Wang et al., 2011; Liu et al., 2019). Both factors could contribute to the reduction in the combined toxicity of MPs and Pb in this research.

3.7. MP ACCUMULATION IN *C. DUBIA*

The accumulation of particles in organisms is one focus when investigating particle related toxicity. Prior to MP accumulation test, the *C. dubia* were fed with algae. They were then placed into the test solution (MP+Pb) that did not contain algae. Figure. 7 shows photos taken at different time periods showing MP accumulation results. It shows that MPs filled up the entire gut of *C. dubia* in 0.25 – 0.5 h. Within this time period, *C. dubia* could actively uptake particles through the mouthparts, and accumulate them in the gut. The typical passage time of particles in the gut ranges from 2 to 55 min (Cauchie et al., 2000). However, it is interesting to note that MP accumulation was significantly reduced after 0.5 h (Figure. 7), while most algae accumulated at the beginning of this test still stayed in the gut. This test suggests that, after 0.5 h of culturing, the *C. dubi*a reduces the uptake of MPs from the test solution.

The MP accumulation pattern in this study may be caused by a change in the feeding behavior of *C. dubia*. As a filter feeder organism, *C. dubia* use an appendage to filter water and particles (Geller and Müller, 1981). The appendage beat frequency could be significantly affected by toxic element exposure (Porter, 1977; Lovern et al., 2007). Therefore, *C. dubia* could selectively ingest some particles, on the basis of size, shape, and texture (Porter, 1977), and also reject particles, even food, from their mouthparts based on

the taste and/or toxicity (Porter, 1977). Figure. S3 shows that *C. dubia* exhibited a solid accumulation of MPs in the gut through the entire exposure period, if there is no Pb present in the test solution. As a result, the presence of Pb, in particular, the adsorbed Pb on the MP surface, reduced MP ingestion by *C. dubia*.

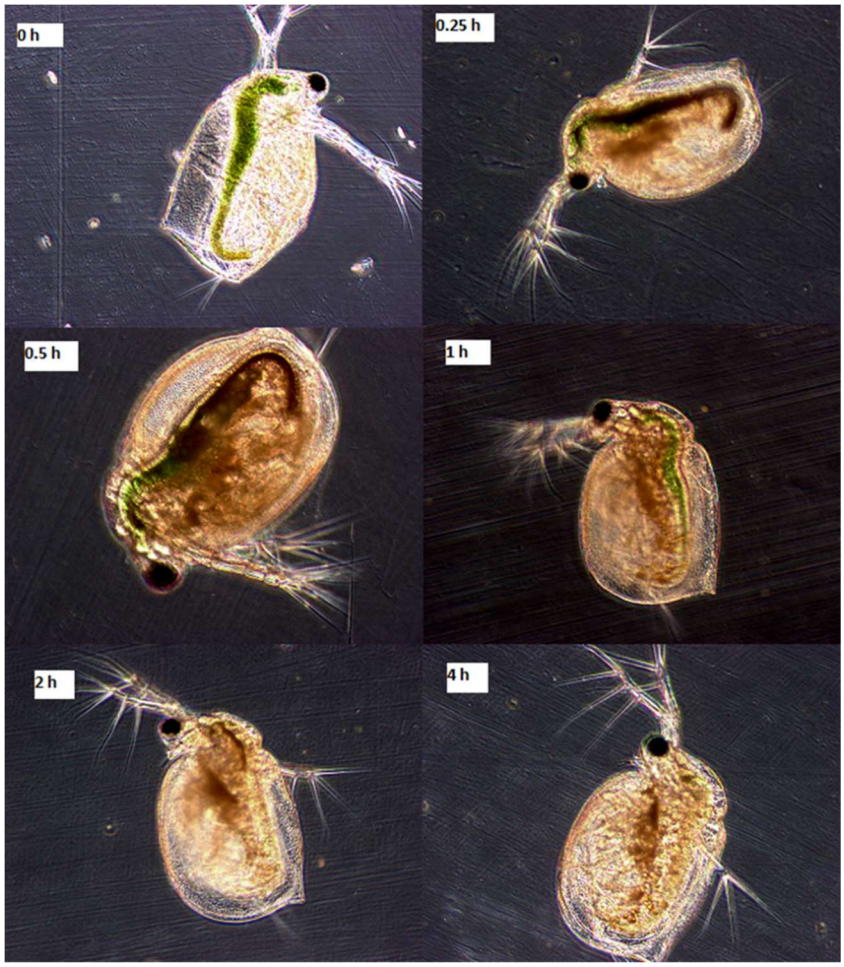

Figure 7. MP accumulation in *C. dubia* in the presence of Pb. Conditions of the exposure medium: [Pb] = 2,500 µg/L; MP = 20 mg/L. Photos were *C. dubia* from 0, 0.25, 0.5, 1, 2, 4 h of exposure.

Figure S4 shows the MP accumulation results in the medium that contained MPs, Pb, and algae. The MP accumulation pattern was similar to *C. dubia* exposed to MP+Pb.

Therefore, the presence of algae did not reduce the combined toxicity of MP and Pb through altering the MP accumulation. However, we also observed that, in the presence of algae, *C .dubia* was continuously accumulate algae in the gut, although MPs ingestion was reduced.

3.8. Pb ACCUMULATION IN *C. DUBIA*

The accumulation of Pb in *C. dubia* directly impacts its toxicity. In the presence of MPs, Pb has been divided into two portions: the adsorbed Pb and the soluble Pb (Figure. 4a and 4b). Therefore, the accumulation of MPs would increase the accumulation of the adsorbed Pb in *C. dubia*. As shown in Figure. 7, *C. dubia* could uptake MPs at the beginning of exposure, while the accumulation of MP was significantly reduced after 0.5 h of exposure. Therefore, the corresponding accumulation of MP-adsorbed Pb would initially increase and then decrease after 0.5 h. The soluble Pb accumulation would consistently increase, due to the relatively stable soluble Pb concentration. Collectively, the overall Pb accumulation in *C. dubia* would increase at the beginning of the exposure to MP+Pb. After this initial increase, the Pb accumulation in *C. dubia* might decrease, due to the depuration of MPs, and then gradually increase, caused by a consistent soluble Pb concentration.

Figure. 8 shows the Pb accumulation in the presence of MPs, with and without algae. Our previous research indicated that the net accumulation of Pb in *C. dubia* was approximate 1 ng/flea, if 2,500 μg/L of Pb was present in the culture medium, without any other particles (Liu et al., 2019). This value was significantly lower than that in the presence of MPs. Evidently, MPs significantly increased Pb accumulation in *C. dubia*. Results also indicated that, in the first 2 h, *C. dubia* rapidly accumulated Pb. Then, the Pb content in the

treatment group of Pb+MP decreased significantly (for Pb content at 2 h and 4 h, $p < 0.05$). The Pb content gradually increased again after 4 h. The Pb content curve lagged the MP accumulation information (Figure. 7), and this lag could be caused by the retardation effect. MPs carried adsorbed Pb into the gut of *C. dubia*, and released some of it due to the reduced gut pH (Ebert, 2005). Therefore, more soluble Pb was available for tissue uptake. Gillis et al. (2005) found that the removal of toxic metals from the gut of a water flea would take a longer time than the removal of particles. Therefore, we observed that the Pb content decreased after 2 h of exposure, rather than 0.5 h after the maximum amount of MPs was accumulated. At the end of the accumulation process, the Pb content decreased again. At this point we also observed a dead *C. dubia* with a broken body structure. It is possible that the *C. dubia* have lost cell membrane integrity at the end of the test, resulting in the release of cytoplasmic content, include accumulated Pb (Elmore, 2007).

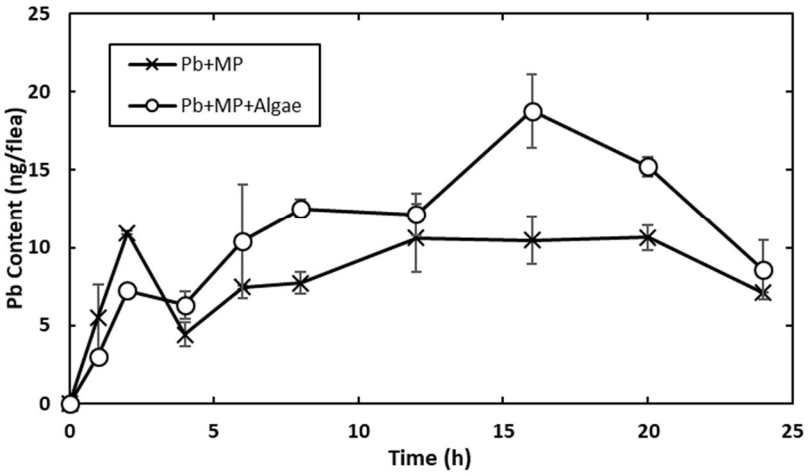

Figure 8. Pb content in *C. dubia* body in the presence of MP, with and without algae. Conditions of the exposure medium: [Pb] = 2500 μg/L; MP = 20 mg/L; Algae = 1.8×10^5 cell/mL. * $p<0.05$ compared to Pb content at 2 h and 4 h for both Pb+MP and Pb+MP+Algae groups. Each point represents the average value of data (N=2), error bar attached to each point represents the range of data.

Figure. 8 also shows the algae impact on Pb accumulation in the presence of MPs. The Pb accumulation pattern was similar to that of Pb+MP. However, in the first 2 h, algae reduced Pb accumulation in *C. dubia*. The SEM image shows that algae (*Raphidocelis*) and MPs have a comparable size range (1-10 μm) (Figure. S1c). Therefore, the ingested algae occupied some gut space to reduce MP uptake and Pb accumulation. However, after 2 h, the Pb content in the algae group exceeded that without algae. This Pb accumulation may be caused by selective uptake algae, which carried adsorbed Pb to the gut of *C. dubia*. Consequently, algae could induce more Pb into *C. dubia* to increase Pb accumulation (Liu et al., 2019). As indicated earlier, *C. dubia* could selectively uptake some particles (Porter, 1977). As indicated in Figure. 7, for the treatment group of Pb+MP, no algae were observed after 2 h of exposure. In contrast, for the treatment group of Pb+MP+algae, *C. dubia* could continuously accumulate algae after 2 h of exposure (Figure. S4). Therefore, in the presence of algae, *C. dubia* rejected some MP after the initial accumulation, but continuously ingested algae alone with the adsorbed Pb during the entire accumulation period, thereby accumulated more Pb than those without algae. Therefore, the reduction of toxicity in the presence of algae was not from the accumulated mass of Pb.

The 24 h mortality of Pb+MP and Pb+MP+algae were 90% and 45%, respectively (Figure. 3 and Figure. 6). However, the presence of algae increased Pb content in *C. dubia*. A similar finding was reported in the absence of MPs (Liu et al., 2019). This could be caused by the biological function of algae. The major toxicity mechanism of toxic metals is ROS production (Ercal et al., 2001). Organisms could produce antioxidants to neutralize ROS, or increase metal binding protein to immobilize metal ions. The production of antioxidants, such as glutathione and superoxide dismutase, and metal binding protein,

such as metallothionein, were energy related (Moltedo et al., 2000; Poljsak et al., 2013). Algae could produce needed energy through the metabolic process and, therefore, reduce toxicity. In addition, some algae may also contain a natural antioxidant (Romay et al., 1998). Thus, algae could also directly serve as an antioxidant to reduce toxicity. Collectively, the toxicity mitigation caused by algae was likely from the energy related pathways, rather than the mass of Pb accumulation

4. CONCLUSIONS

In this research we observed that MPs alone had low toxicity on *C. dubia*. However, MPs could interact with Pb and significantly enhance the Pb toxicity through increasing soluble Pb concentration and the uptake of Pb-loaded MPs. Importantly, we found that algae, a natural food for aquatic organisms, could significantly reduce the combined toxicity of MPs and Pb. In the presence of 20 mg/L of MPs and 2,500 μg/L Pb, 1.8×10^5 cells/mL of algae reduced the mortality of *C. dubia* from 90% to 45%, although algae increased Pb accumulation in *C. dubia*. The reduction in the toxicity might be related to the energy and antioxidant related pathways which need further investigation. Importantly, this research indicated that when evaluating the toxic effect of particles, environmental factors must be included during the investigation.

SUPPORTING INFORMATION

ALGAE (*RAPHIDOCELIS*) MITIGATE COMBINED TOXICITY OF MICRO-PLASTIC AND LEAD ON *C. DUBIA*

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1. ANALYTICAL METHOD

1.1. SCANNING ELECTRON MICROSCOPY (SEM) IMAGING

The surface morphology of micro-plastic (MP) was observed by using a Hitachi S-4700 field emission microscope. Samples that contained MP or MP+Algae were collected, and dried for SEM imaging. Samples were sputter-coated with Au before loading in SEM.

1.2. HYDRODYNAMIC SIZE

The hydrodynamic size of MP under different conditions was measured by a Nano-ZS90 Zetasizer (Malvern Instruments Ltd, UK). These particle suspensions were prepared, as described in section 2.2 in the text. For each condition, 1 mL of test solution was sampled at 2 cm below the suspension surface (total depth was about 5 cm). To investigate the size

change of MP during the experiment, three sampling times, which could cover the entire process, were selected. The first two samples were collected after 1 min and 24 h mixing, respectively. After the 24 h mixing, the suspensions were allowed to settle for 24 h, and, then, the last sample was collected. All samples were analyzed immediately after collection, and each sample was measured in triplicate.

1.3. TOTAL ORGANIC CARBON (TOC) ANALYSIS

The TOC was measured by a Shimadzu TOC-L with an ASI-L liquid auto-sampler. Samples that contained Pb, MP, and Pb+MP were prepared in the culture medium, respectively. The concentrations of Pb and MP, in a corresponding solution were 2,500 μg/L and 20 mg/L, respectively. A control sample, that only contained a culture medium, was also included. These samples were prepared, as described in section 2.2 in the text. After mixing, the test solution was filtered through a 0.22 μm nylon membrane filter. All of the TOC tests were conducted in duplicate.

2. SUPPLEMENTARY FIGURES

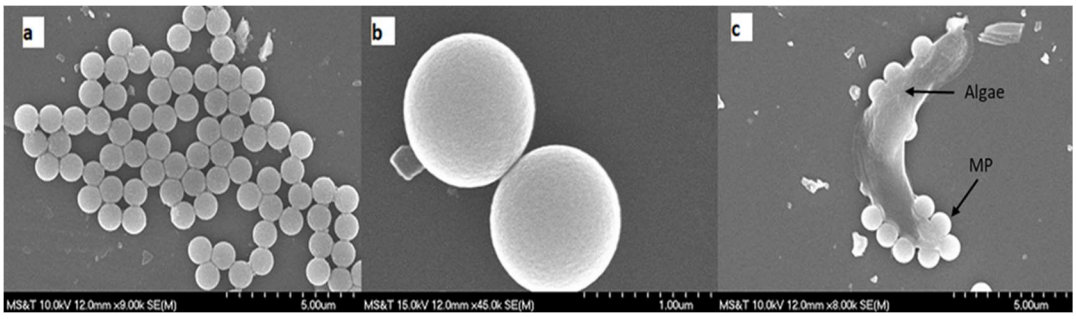

Figure S1. SEM image of (a) MP; (b) MP surface morphology; (c) MP and algae.

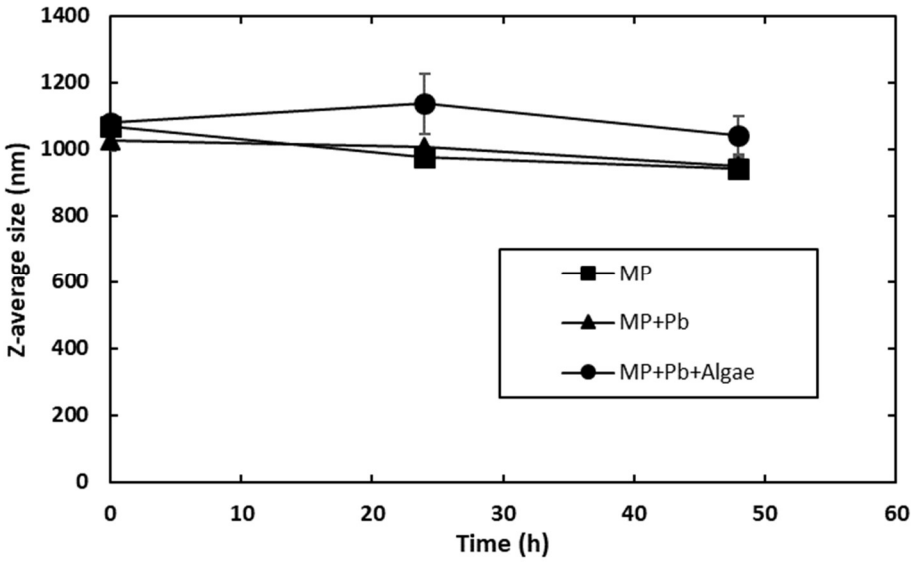

Figure S2. Hydrodynamic size of MP in a culture medium during the experiment. Conditions of exposure medium: MP = 20 mg/L; [Pb] = 2,500 μg/L; Algae = 1.8×10^5 cell/mL. Standard deviation is represented by an error bar attached to each point (N=3).

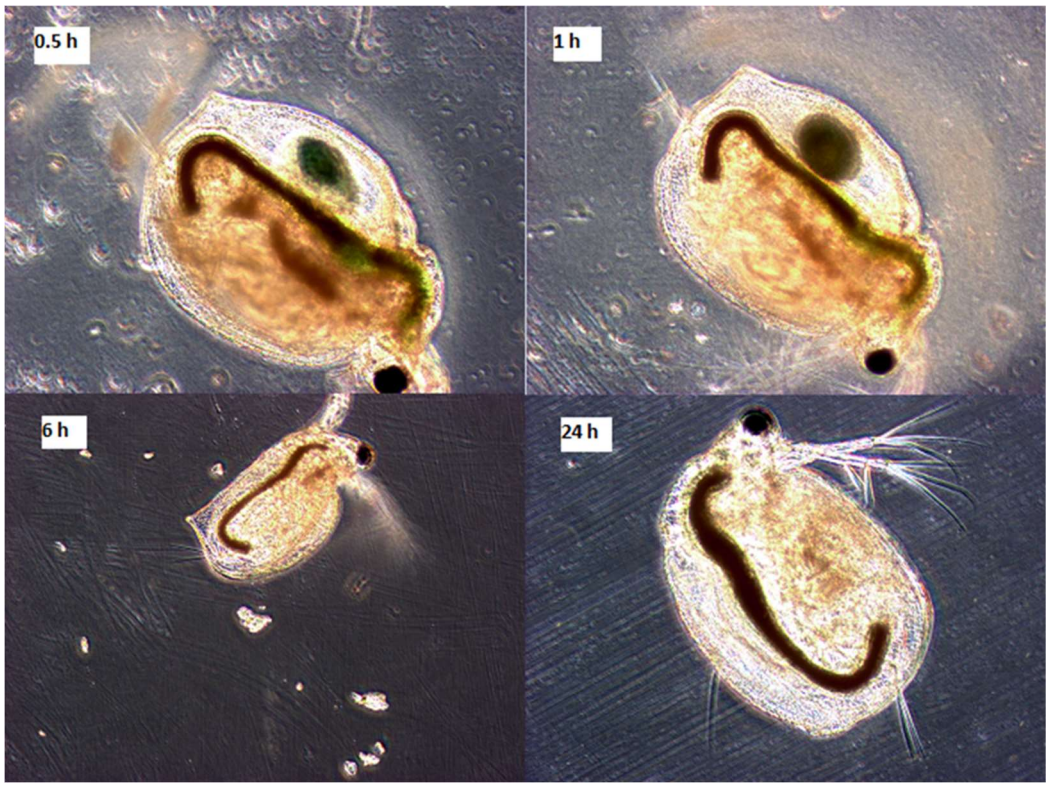

Figure S3. MP accumulation in *C. dubia*. Conditions of the exposure medium: MP = 20 mg/L. Photos were *C. dubia* from 0.5, 1, 6, 24 h of exposure.

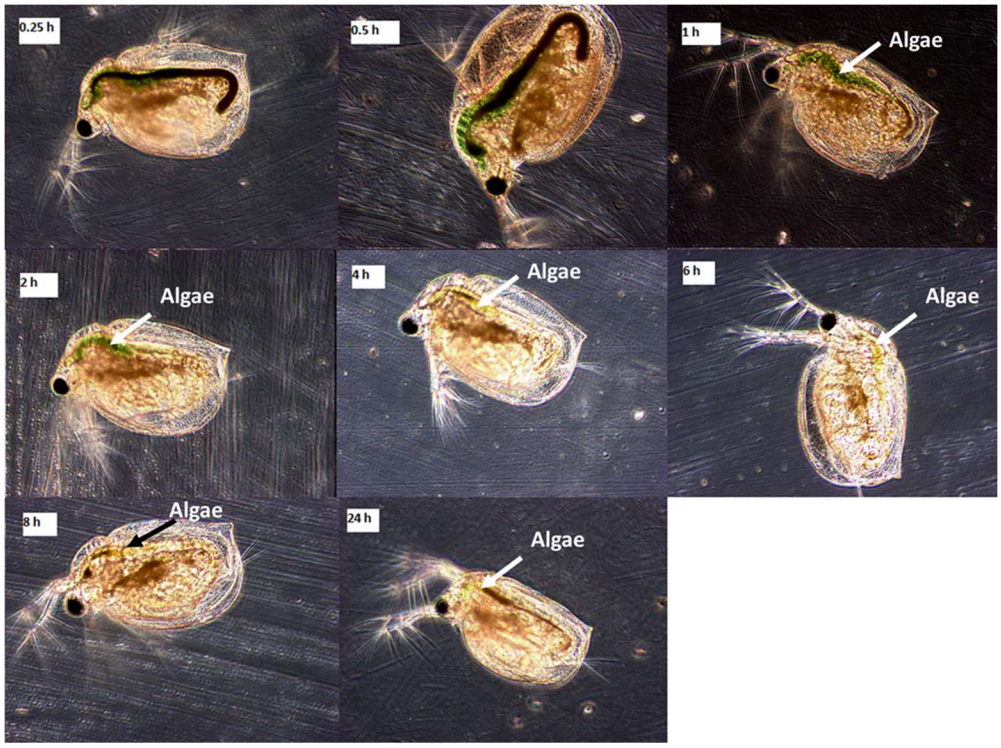

Figure S4. MP accumulation in *C. dubia* in the presence of Pb + algae. Conditions of the exposure medium: [Pb] = 2,500 µg/L; MP = 20 mg/L; Algae = 1.8×10^5 cell/mL. Photos were *C. dubia* from 0.25, 0.5, 1, 2, 4, 6, 8, 24 h of exposure.

3. SUPPLEMENTARY TABLES

Table S1 TIC and TOC of different solutions

Sample ID	TIC (mg/L)	TOC (mg/L)
Culture medium	12.68±0.05	2.27±0.60
Pb[a]	11.73±0.13	2.73±0.12
MP[b]	12.74±0.01	3.39±0.22
MP+Pb[c]	11.55±0.16	3.55±0.89

a. [Pb] = 2500 µg/L;
b. MP = 20 mg/L;
c. MP = 20 mg/L and [Pb] = 2500 µg/L.

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SECTION

2. CONCLUSIONS

The combined toxicity of heavy metals and NPs and the effect of algae (*Raphidocelis*) on this combined toxicity were studied in this work by using *C. dubia* as a model organism. The heavy metal accumulation and toxicity mechanisms with and without algae were also quantified by the model approach. This study shows that environmental conditions, specifically algae, significantly influence the combined toxicity of heavy metals and NPs. In addition, this work also provides a path to evaluate the environmental impact on the combined toxicity.

In paper I, the effect of algae on the combined toxicity of Pb and nano-TiO_2 is introduced. YTC, a type of food mixture that exhibits similar functions as algae are also tested. In the presence of algae or YTC, the combined toxicity of Pb and nano-TiO_2 can be significantly reduced. From the view of Pb accumulation, algae slightly reduce Pb uptake by *C. dubia*, but do not change the Pb depuration rate and distribution. Therefore, the significant toxicity reduction in the presence of algae is not from the change of Pb mass and speciation. The possible toxicity reduction might be from the biological function of algae, which needs further investigation.

In Paper II, a two-compartment kinetic model is developed to predict Fe and Pb accumulation in *C. dubia* in the presence of nano-TiO_2. The model differentiates heavy metal accumulation to toxicity-relevant (body tissue) and irrelevant (gut) parts of organisms. Algae, as an environmental condition, are also incorporated in this kinetic

model. The model reveals that major heavy metal uptake and depuration occur in the gut of *C. dubia*. Further, heavy metals accumulated in the body tissue of *C. dubia* are mainly transferred from the gut, and an essential element (Fe) has higher transfer rate than a non-essential element (Pb). The model results also suggest algae can reduce heavy metal uptake rate in the gut and the transfer rate to body tissue, but algae do not change the heavy metal depuration rate from the gut. We also connected the two-compartment kinetic model with toxicity results from nano-TiO_2 and Pb. It has shown this model can be used to quantify the combined toxicity of nano-TiO_2 and Pb.

In Paper III, a TK-TD model is used to reveal the role of nano-TiO_2 and the effect of algae in the combined toxicity of Pb and nano-TiO_2. The Pb accumulation in *C. dubia* depends upon nano-TiO_2 concentrations. The Pb accumulation increase with increasing nano-TiO_2 concentration if the saturated nano-TiO_2 uptake rate is not reached. Once saturated nano-TiO_2 uptake rate is reached, further increased nano-TiO_2 concentration can reduce Pb accumulation in *C. dubia*. However, the mortality keep increasing with increasing nano-TiO_2 concentration. The model results suggest nano-TiO_2 not only serves as a carrier of Pb, but also decreases the Pb tolerance and increases the killing rate in *C. dubia*. Further, the model results indicates algae can decrease the killing rate to reduce the toxicity when mixed with Pb and nano-TiO_2.

In Paper IV, the combined toxicity mechanisms of Pb and MP and corresponding toxicity mitigation by algae are studied. In the presence of Pb with MP, the Pb toxicity is significantly increased. Further study indicates the combined toxicity of Pb and MP comes from increasing soluble Pb concentration, excessive Pb accumulation, and the change in MP surface property. In the presence of algae, the combined toxicity of Pb and MP is

significantly reduced, although algae increases Pb accumulation in *C .dubia*. The possible combined toxicity reduction by algae might come from an energy-related pathway, which needs further investigation.

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