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The Chronic Toxicity of Titanium Dioxide Nanoparticles to the Freshwater Amphipod *Hyalella azteca*

By

Gurkirpal S. Malhi

(BSc., Wilfrid Laurier University, 2009)

THESIS

Submitted to the Department of Biology

Faculty of Science

in partial fulfillment of the requirements for the

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2011

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Abstract

There has been an increased use of metal-oxide nanoparticles in both commercial and consumer products. The use of these products and waste generated during manufacture may ultimately be released into the aquatic environment and the potential for these contaminants to cause impacts must be assessed. This study examines the effect of titanium dioxide (TiO_2) nanoparticles (NPs) on *Hyalella azteca*. Chronic toxicity exposures were conducted in 400mL of spiked test solution and contained 20 neonates. Samples collected for characterization of Ti in TiO_2 NP exposure solutions were digested using ammonium persulfate as an oxidizing agent and dissolved in 2% HNO_3, organisms were digested using 70% HNO_3 and 30% H_20_2. Dissolved Ti LC_{50} was 1404 µg Ti/L, all NP exposure's had LC_{50} values above 100 mg TiO_2/L. The IC_{50} value of dissolved Ti on growth was 914 µg Ti /L, while uncoated TiO_2 NPs P25, PC105, NM101, and NM105 yield IC_{50} values of 23.4, 31.2, 16 and 14.5 mg TiO_2/L respectively. NM103 (hydrophobic) and NM104 (hydrophilic) yielded IC_{50} values of 36.4 and 6.5 mg TiO_2/L respectively. Testing was done to assess the impact dispersion methods of NPs would have on the toxicity to *H.azteca*. Organisms were exposed to NP solutions that had been dispersed by a 24h spin or were dispersed by a 24h spin and 5 minute sonication step. Organisms exposed to sonicated solutions showed lower dry weight than those exposed to stirred solutions. *H.azteca* were exposed to a 'low' and 'high' cadmium concentration in the presence and absence of P25 TiO_2 NPs to determine the potential for NPs to acts a ligand to Cd. There was significantly lower bioaccumulation of Cd in organisms exposed in the presence of P25 TiO_2 NPs in both concentrations. These results show dissolved Ti has greater impact on *H.azteca* than TiO_2 NPs and it is difficult to relate physical particle

characteristics to biological effect. TiO_2 NPs with hydrophilic surface modification are more toxic than those with hydrophobic surface modifications. NP solutions dispersed by sonication are more toxic than those that are stirred. TiO_2 NPs acted as a negative vector for cadmium, limiting Cd bioavailability.

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Authors Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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Chapter 1

Introduction

1.1 Nanoparticles

In recent years nanotechnology has received a great deal of attention from the media and scientific communities for its amazing potential ranging from unique optical properties, magnetism, increased strength, flexibility, reactivity, electrical conductivity and more. However there are growing concerns about the safety of nanoparticles (NPs). Nanotechnology deals with the generation and manipulation of NPs, defined as having all three dimensions less than 100nm (Handy *et al.,* 2008a, b; Hoet *et al.,* 2004). Not only does this mean that manufactured NPs have unique physical properties but they may also exhibit unique biological interactions (Wigginton *et al.,* 2007; Farre *et al.,* 2008; Ling *et al.,* 2009; Sharma 2009).

NPs lie in a transition zone between their bulk counterparts and their atomic structures, yielding characteristics and behavior that may not be predicted based on conventional models (Wigginton *et al.,* 2007; Sharma 2009). Some NPs exhibit fluorescence due to quantum confinement causing excitation of electrons that is not observed in bulk forms of the same substances (Hardman 2006; Gagne *et al.,* 2007; Gagne *et al.,* 2008b). The very small size of NPs can also produce unique bioavailability properties as they may cross membranes and travel to regions of the body unreachable by larger molecules, for example some may be able to cross the blood brain barrier (Lockman *et al.,* 2003; Huang *et al.,* 2009; Prencipe *et al.,* 2009, Ramsden *et al.,* 2009). These bioavailability properties may provide for new medical applications of NPs (Gupta and Gupta 2004; Ling *et al.,* 2009). Other potential uses are varied and include use as food and drink additives, and as reactive substance for environmental remediation (Fujishima *et al.,* 2000; Esterkin *et al.,* 2005; Zhang and Elliot 2006; Perez 2007; Yu *et al.,* 2008; Kadar *et al.,* 2010).

Our current understanding of NPs to cause environment impacts is still considered to be in its infancy, despite the fact that there are already over 800 commercially available products that contain NPs in the market. The number of products is expected to rise quickly and it is estimated that the nanotechnology industry will grow to $1 trillion dollars before 2015 (Rocco 2003; Nel *et al.,* 2006; Gagne *et al.,* 2007). The study of the effects of NPs in water and sediments is of particular interest, as lakes and rivers are the receiving environment for domestic and industrial wastewaters and there is a growing potential for NP contamination in these discharges (Farre *et al.,* 2008; Handy *et al.,* 2008a; Klaine *et al.,* 2008).

1.2 Environmental Nanoparticles (ENPs)

Although research in the field of nanotoxicology is in its early years, NPs are naturally occurring in the environment for billions of years (Wigginton *et al.,* 2007; Handy *et al.,* 2008a, b; Simonet and Valcarcel 2009). ENPs are formed as a result of weathering, neoformation in saturated fluids, geothermal and/or hydrothermal activity and biogenic production from the activity of microorganisms (Wigginton *et al.,* 2007). Many organic entities such as proteins, DNA, ATP, and viruses are considered ENPs based on their size in the nanometer range (Handy *et al.,* 2008a). Organisms have evolved and lived in the presence of NPs, however, it is unknown whether or not manufactured NPs affect an organism's ability to survive and reproduce.

ENPs occasionally act as carriers of elements and compounds over long distances and contribute to soil genesis, water quality, element cycling and account for a large and potentially reactive surface area in the environment. Evidence from Clark Fork River, Montana, USA showed transport of As, Pb, Zn and Cu up to 500km downstream from

old mining sites and smelter operations near the headwaters of the river (Wigginton *et al.,* 2007). It is suspected that these contaminants were sorbed onto particles to move such a distance, since aqueous phase transport was highly unlikely. Analysis of water samples taken up to 500km downstream in the river revealed TiO_2 NPs between 5 – 15nm that were believed to have aided in transport of contaminants, they were likely ENPs. In water, ENPs in are typically transient and over time will aggregate to larger less bioavialable forms or break down into dissolved ions (Acosta 2008; Domingos *et al.,* 2009). Manufactured NPs differ from ENPs in that they may not dissolve into the aqueous phase, and/or can be manufactured to exist as colloid dispersion of monodispered particles in the water column (Lead and Wilkinson 2006). If ENPs can potentially bind and carry other substances acting as a ligand, similar behavior may be seen with manufactured NPs. By introducing manufactured NPs in the environment that are made to remain suspended this may alter fate and behavior of other contaminants (Benn and Westerhoff 2008).

1.3 Bioavailability and Potential Risk of NPs

Conventionally when looking at metal toxicity, free metal ions are considered to be the most toxic form of a metal, often taken up directly by ion channels and transporters. The potential toxicity of NPs may be related to physical characteristic and different mechanisms of toxicity, Figure 1.1.

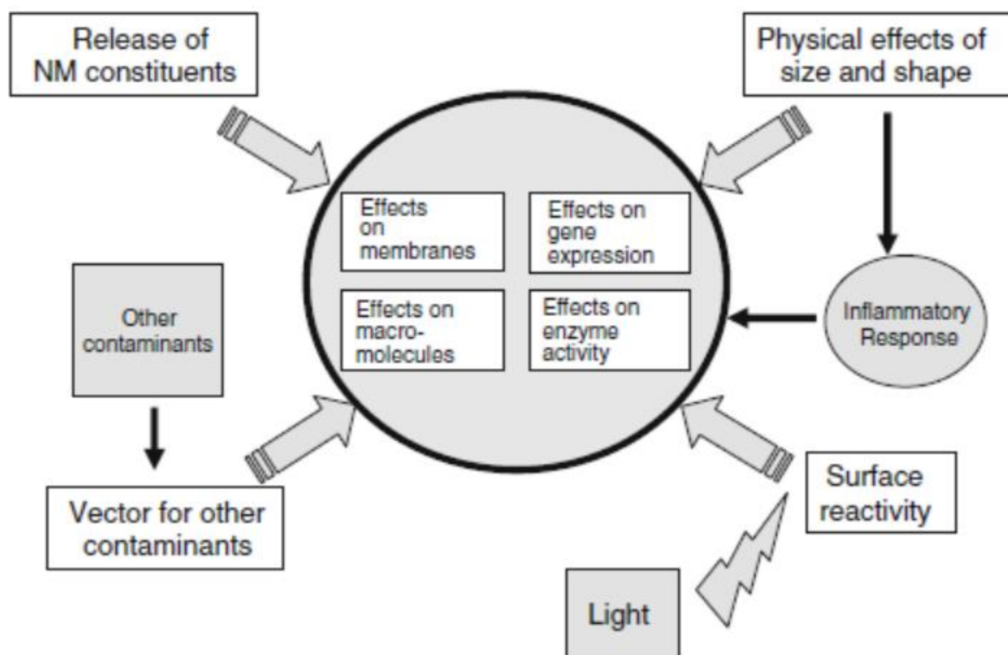


Figure 1.1: Once NPs enters an organism it may exert toxicity in one or a combination of up to four mechanisms. The first is release of NP constituents by particle dissolution and exerts toxicity as a result of dissolved ion. The second is the physical features of NPs which cause interference with biological processes. The third involves toxicity as a result of surface properties and reactivity. The fourth is NPs ability to act as vectors for the transport of toxic substances to sensitive tissues (Linkov *et al.,* 2009)

A high portion of the atoms of NPs are at the surface of the particle, giving rise to high surface reactivity relative to their bulk forms, and suggesting that specific surface area may be a more accurate measure to assess the reactivity of NPs (Nel *et al.,* 2006; Soto *et al.,* 2007; Farre *et al.,* 2009). NPs are capable of entering biological systems via different mechanisms ranging from, ingestion of particles into cells and adhesion onto biological surfaces (Asharani *et al.,* 2008; Yeo and Kang 2008; Laban *et al.,* 2010). Also it has been shown that internalization of NPs can lead to *in vivo* nanotoxicity by forming free radicals and inducing oxidative stress (Aillon *et al.,* 2009; Kadar *et al.,* 2010). This increased interaction of NPs with biological systems may lead to adverse effects

previously not seen with dissolved metals (Farre *et al.,* 2008). Examples of different internalization mechanisms of NPs are seen in studies with Zebrafish and Fathead minnow embryos. Ag-NPs were shown to be taken up by fathead minnow embryo (Laban *et al.,* 2010) as well as zebra fish embryo (Yeo and Kang 2008). In both studies there were developmental abnormalities associated with the internalization of Ag-NPs.

The size of the NPs may also influence their toxicity to aquatic organisms and the bioavailability of the NPs to an organism. It has been shown that smaller NPs are more bioavailable and as a direct result may exert a higher degree of toxicity (Hund-Rink and Simon 2006; Franklin *et al.,* 2007). NPs have been observed to cross the blood brain barrier of many higher level organisms causing oxidative stress in the brain tissue and other regions of the body (Lockman *et al.,* 2003; Huang 2009). Some NPs have the ability to sorb other substances to their surface which could potentially lead to them acting as ligands or competitors for other contaminants. As shown by Zhang *et al.,* (2007), internalization of Cd in the presence of TiO_2 NPs in comparison to sediment particles was increased by 146% and showed a positive correlation between Cd and TiO_2 concentrations. Considerable amount of Cd and TiO_2 accumulated in viscera and gills of carp.

1.4 Particle Characterization

In an attempt to understand the various mechanisms through which NPs may exert their toxicity, NPs physical characteristics must be well defined in order to associate potential effect to specific physical parameters. Without a set standard of characterization criteria NP studies are prone to anecdotal findings (Boverhof & David 2010). Minimum information of particle characteristics (MINChar) initiative set a

variety of parameters in order to raise the quality of research, summarized in Table 1.1. Many of these characterization details can be found on the MSDS provided by suppliers of NPs. These concerns are raised due to varying results founds in current NP research and discrepancies or differences arising as a results of NPs physical characteristics, which is why dose of exposure may not accurately correlate to toxic effect observed. In some instances surface area may be a more accurate predictor of potential toxicity as it is the greatly increased surface area's that account for potential increased biological interaction (Handy *et al.,* 2008a). Testing strategies for chemicals do not always apply to NPs as they are composites of multiple molecules and stability of nanoparticles is another factor we must consider (Xia *et al.,* 2008). NPs with lower stability may dissolve into aquatic medium and exert toxicity as a result of the dissolved ion. NPs with high stability may be more persistent (an inability to eliminate NPs from biological system) and thus impact biological response. Generally particles that are more stable are less likely to generate toxic response because individual atoms are released slowly (Boverhof & David, 2010).

Table 1.1: Recommended minimum physical and chemical parameters for characterizing nanoparticles in toxicology studies. Developed at a workshop on ensuring material characterization in nanotoxiclogy studies in Washington, DC, USA in October 2008. http://www.characterizationmatters.org (Boverhof & David 2009).

Interaction of nanoparticles with biological medium
What does the material look like? • Particle size / size distribution • Agglomeration state / aggregation • Shape
What is the material made of? • Overall composition (including chemical composition and crystal structure) • Surface composition • Purity (including levels of impurities)
What factors affect how material interacts with its surroundings • Surface area • Surface chemistry, including reactivity, hydrophobicity • Surface Charge
Overarching considerations to take into account when characterizing engineered nanoparticles in toxicity studies: • Stability – how do material properties change with time (dynamic stability), storage, handling, preparation, delivery, etc.? Include solubility, and the rate of material release through dissolution • Context/media – how do material properties change in different media; i.e., from bulk material to dispersion to material in various biological matrices? ("as administered" characterization is considered to be particularly important) • Where possible, materials should be characterized sufficiently to interpret the response to the amount of material against a range of potentially relevant dose metrics, including mass, surface area and number concentration

A common concern when conducting toxicity tests with NPs is their tendency to aggregate in solution. The aggregation of NPs is caused by 3 fundamental processes. The first is simple Brownian motion of particles, which will lead to perikinetic aggregation. Second is particles travelling at different velocities leading to orthokinetic aggregation. Lastly particles of different size or density will undergo settling with time

(Handy *et al.,* 2008a). Once the aggregate exceeds three dimensions over 100nm they are no longer classified as nanoparticles. However toxicity testing of NPs this early in research should consider aggregates in testing as they are still composed of individual NPs with their individual dimension still less than 100nm (Karla *et al.,* 2007; Soto *et al.,* 2007). In addition to behavior of the NPs, we must consider physical and chemical characteristics of the aquatic medium the NPs are in to understand fate and behavior (Guzman *et al.,* 2006). As the pH of the solution reaches the point of zero charge (pH_{ZPC}) aggregation of NPs will increase as repulsion between the surfaces of NPs will decrease since the surface charge on the particles is near zero (Adams *et al.,* 2006; Domingos *et al.,* 2009). The aggregation behavior during toxicity testing of NPs may reduce the specific surface area of the NPs. NP aggregates will likely deposit in sediments, hence the use of benthic organisms should be of particular interest for toxicity testing (Handy *et al.,* 2008a, b).

In order to assess the worst case scenario for NPs we must observe situations where they exist in a monodispersed solution with limited aggregates. To limit aggregation the most common methods employed are the use of solvents or surfactants, sonication, or prolonged stirring of samples. The most effective methods to maintain a monodispered solution is to introduce solvents or surfactants; however, this raises issues during toxicology testing of the impact the suspending agent has on the test organism. As shown by Zhu *et al.,* (2006), *Daphnia magna* 48 h LC_{50} values for C_{60} fullerenes decreased from > 35mg-L^{-1} to 0.8 mg-L^{-1} when in the presence of tetrahydrofuran (THF) as a dispersing agent, however differences in toxicity were likely associated with residual amount of THF trapped in the centre of the particles. Aggregation of NPs can be hindered in the

presence of humic substances and Suwannee River Fulvic Acid (SRFA) and may lead to seeing NPs with smaller hydrodynamic diameters in certain environments than can be predicted by laboratory measurements. In the presence of SRFA at concentration of 1mg-L^{-1} and a TiO_2 stock of 1mg-L^{-1} showed a hydrodynamic diameter of ≈ 3nm for particles of 5nm nominal measurements, likely a result of steric stabilization (Domingos *et al.,* 2009). In the absence of SRFA systems become more aggregated and this may affect toxicity of NPs.

1.5 Aquatic Invertebrates

Hyalella azteca is a freshwater epibenthic amphipod that is ubiquitous to streams, lakes and other freshwaters bodies that attain a summer surface temperature of at least 10°C (EC 1997). *H. azteca* is a member of the talitroidean amphipod family, Hyalellidae (kelp grazers) and are often used to assess environmental health as they are very sensitive to contaminants. An adult male can range up to 8mm, and females can be up to 6mm in length. *H. azteca* have been recorded from central Mexico to roughly the tree line in Canada and Alaska. They prefer lentic waters where vegetation provides food and cover (EC 1997). The life cycles of *H. azteca* are annual, they reproduce sexually and females can produce up to 30 eggs from the brood pouch under ideal conditions. Males search out and mate with females, males will use the first gnathopod and lock into the copulatory notch on the female leaving the second gnathopod free to fend of other males; males may remain attached for hours or days (Othman and Pascoe 2001).

H.azteca are been used extensively for toxicity testing in North America and can be used for water-only or sediments tests (Borgmann *et al.,* 2005). They can be held in

natural waters or artificial media, however in the latter case bromide must be added (Borgmann 2002). Tests should be conducted on organisms that are between 2 – 9 days of age (EC1997). If NPs are not suspended in solution they will eventually settle and collect along the bottom of their holding containers where *H. azteca* spend most of their time. Chronic exposure to the NPs may cause adverse health effects ranging from mortality to sub-lethal effects such as growth inhibition.

1.6 Research Goals & Objectives

The goal of this research is to contribute towards the understanding of the potential for NPs to cause environmental impacts. Hypotheses that will be tested in the project are:

1) There is correlation between NPs physical characteristics and biological effect.

2) TiO_2 NPs are more toxic than dissolved Ti in solution.

3) Particle dispersion methods will influence toxicity.

4) P25 TiO_2 NPs will act as a ligand to Cd and enhance bioaccumulation of Cd.

In order to test listed hypotheses the following objectives must be met:

1) Determine chronic toxicity of TiO_2 NPs on *H. azteca*

2) Determine chronic toxicity of dissolved Ti on *H. azteca*

3) Expose *H. azteca* to stirred and sonicated mixtures of TiO_2 NPs at determined IC_{50}'s.

4) Determine bioaccumulation of Cd after chronic exposure to Cd in the presence and absence of P25 TiO_2 NPs.

Chapter 2

Chronic Toxicity of Ti & TiO_2 NPs

2.1 Introduction

Titanium is a very useful metal as it is resistant to corrosion, and has an extremely high strength to weight ratio compared to other metals (Diebold 2003). TiO_2 coatings have been applied as self cleaning surfaces, antifogging agents on glasses, and on medical instruments (Fujishima *et al.,* 2000). Research into TiO_2 began in the late 1960's with the photoelectrochemical solar energy conversion and eventually to environmental photocatalysis (Diebold 2003). This introduced unique properties of self cleaning surfaces and more recently photoinduced hydrophilicity (Fujishima *et al.,* 2000). Surfaces coated with TiO_2 upon receiving light intensities as low at 10μW /cm^2 can remove a hydrocarbon layer up to 1μm thick per hour. This has lead to development of TiO_2 coated films and glass that does not need to be cleaned as regularly. TiO_2 that has been activated by illumination and has even been shown to kill tumor cells up to a certain size (Fujishima *et al.,* 2000).

These properties along with a strong oxidizing power have led to TiO_2 use in air and water purification applications (Matthews 1986, 1990; Ireland *et al.,* 1993; Fujishima *et al.,* 2000; Hong *et al.,* 2005; Esterkin *et al.,* 2006; Hund-Rink and Simon 2006). With the variety of applications of micron sized TiO_2 many applications are being found and used for nanoscale TiO_2. TiO_2 NPs have found a wide range of applications in consumer products such as use in photovoltaic cells, self cleaning surfaces, cosmetics and pigments. They have been found to limit organic build up when coated upon a material and can filter ultraviolet radiation with high efficiency. (Nohynek *et al.,* 2007; Margez *et al.,* 2009). Studies with illuminated TiO_2 NPs in water were able to remove up to 70% of the total organic carbon and are being suggested for use in waste water treatment (Le-Clech

et al., 2006).

Preliminary findings in literature have shown differences in toxicity of TiO_2 NPs can be directly related to the dimensions of the NPs. Wang *et al.,* (2009) have shown the toxicity of TiO_2 NPs to *Caenorhbtidis elegans* during 24 hour exposures to NPs of 7.3nm diameter yielded LC_{50} Values of 80mg-L^{-1} as opposed to their bulk counterparts with 285nm diameter showing LC_{50} values of 136mg-L^{-1}. Similarly Lovern and Klapper (2006) have shown 48 hour exposures to *Daphnia magna* to TiO_2 NP filtrate (<220nm) yield LC_{50} values for daphnia magna of 5.5mg-L^{-1}, without filtration there was no toxicity determined. If the same filtered solutions are illuminated prior to exposure LC_{50} values drop between 1.5 – 3mg-L^{-1}, likely a result of additional reactive oxygen species formation. Given our current understanding of nanotoxicology it is difficult to predict the effects of TiO_2 NP exposures.

The objective of this chapter will be to determine the toxicity of dissolved Ti and TiO_2 NPs. Biological response to dissolved Ti will be used as a proxy to potential dissolution of NPs in solution. It is hypothesized that TiO_2 NPs will exert more toxic effect than dissolved Ti. Chronic toxicity from TiO_2 NPs will be used to determine if a relationship exists between physical particle characteristic and biological response. It is hypothesized that as the size of the NPs decreases there will be an increase in toxicity.

2.2 Materials & Methods

2.2.1 Invertebrate Husbandry

An initial *H. azteca* culture was obtained from Aquatic Research Organisms (ARO; Hampton, NH, U.S.A.) and cultured following protocols from Borgmann (2002). The culture arrived with mixed age organisms and was split into sets of 30 adults per 1L high density polyethylene beakers. An artificial culture medium was used and made with deionized water to obtain a hardness of 130 mg $CaCO_3$/L (1mM $CaCl_2$-$2H_20$, 1mM $NaHCO_3$, 0.01mM NaBr, 0.05mM KCl, and 0.25mM of $MgSO_4$-$7H_2O$ (Sigma-Aldrich Inc. St. Louis, MO)) (Borgmann 2002). A 24h presoaked sterile piece of cotton gauze (5 cm X 5 cm) was placed in each beaker as a substrate for the *H. azteca*. The cotton gauze is preferred as a substrate over other materials as it promotes growth, and reproduction (Borgmann *et al.,* 1989). Temperature was held at 22°C ± 1 °C with 16h light and 8h dark photo period, fluorescent lighting was held 30cm above cultures. Tetramin™ flakes (Tetra Werke**,** Blacksburg, VA, U.S.A.) were ground up and passed through 500µm sieve, organisms received 5mg of dry Tetramin™ flakes 3 times per week, which was sprayed down with MilliQ ultrapure water to ensure food is accessible to organisms. Water renewals were done weekly, during this time neonates were enumerated as needed from the beakers and transferred to a mixed age holding aquarium.

2.2.2 *H. azteca* Chronic Exposure System

H. azteca chronic toxicity tests (28d) were carried out according to EPS/11RM/33. Exposure conditions were maintained at 22°C ± 1°C with 16h light and 8h dark

photoperiod fluorescent lighting was held above 30cm above cultures. A 5 cm X 5 cm piece of cotton gauze was used as substrate and each beaker received 5mg of dry Tetramin™ flakes 3 times per week, which was sprayed down with MilliQ ultrapure water. Organisms used for testing were removed from cultures at 0 to 7 days of age. They were held for 2 days in unspiked test media prior to being placed in the exposure system. Unspiked test media was made by dissolving 0.31mM $CaCl_2$-$2H_20$, 0.31mM $NaHCO_3$, 0.003mM NaBr, 0.02mM KCl, and 0.08mM of $MgSO_4$-$7H_2O$ (Sigma-Aldrich Inc. St. Louis, MO) with a pH of 7.3 ± 0.1 and a final hardness of 40 mg $CaCO_3$/L. Exposures were done in duplicate and were static renewal tests with 100% of water volume being replaced weekly. Polypropylene beakers were used for exposures and held 400mL of spiked medium.

2.2.3 Exposure Details

2.2.3.1 Exposure to Dissolved Ti & TiO_2 NPs

Twenty *H. azteca* of 2 – 9d of age were exposed to Ti from AAS standards (Sigma-Aldrich Inc. St. Louis, MO), which is called 'dissolved Ti' at nominal concentrations of 0, 0.1, 0.3, 0.75, 1.5 and 3 mg/L in duplicate. Stock dissolved Ti solution had a concentration of 1g Ti /L. Water was spiked with dissolved Ti and pH was adjusted as needed with 1M KOH solution made by dissolving KOH pellets (Sigma-Aldrich Inc. St. Louis, MO) in MilliQ ultrapure water.

Twenty *H. azteca* 2 – 9d of age were exposed to TiO_2 NPs at nominal concentrations of 0, 1, 5, 10, 20, 50 and 100 mg/L in duplicate, with NP details on Table 2.1. Stock

TiO_2 NP solutions were made by adding 1g of TiO_2 NP powder to 1L of test medium to yield final concentrations of 1g TiO_2 /L. Exposures were all in static renewal with 100% water changes performed weekly.

Table 2.1: NPs source, size, surface area, surface modification and crystal structure.

Name	Source	Average Particle Diameter (nm)	Specific Surface Area (m^2/g)	Surface Modification	Crystal Structure
P25	Commercial	25	50	None	Rutile-Anatase
PC105	Commercial	20	85	None	Anatase
NM101	OECD	7	320	None	Anatase
NM103	OECD	20	60	Dimethicone (2%)	Rutile
NM104	OECD	20	60	Glycerine	Rutile
NM105	OECD	22	61	None	Rutile-Anatase

2.2.3.2 Nanoparticle Dispersion by Sonication

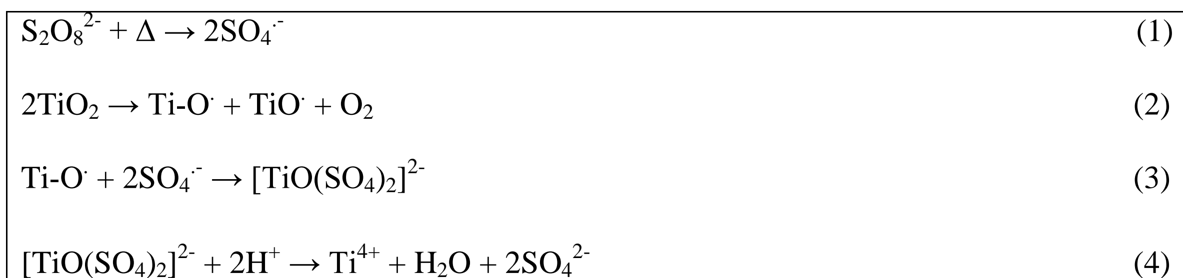
In order to achieve a monodispered solution, NPs were placed in test media and were dispersed in a two step method. The first step involved mixing of stock solutions using a stir bar for 24h (Wiench *et al.,* 2009). Secondly a sonication step was performed. 186 mL of stock solution were sonicated using a probe sonicator (QSonica, Sonicator 4000, Newton, CT) for 5 minutes at 20 kHz, 20mm, 0.5 inch Ti horn prior to addition into exposure system (Wiench *et al.,* 2009, Termnak 2007).

2.2.4 Sampling and Sample Digestion

2.2.4.1 Water Sampling and Digestion

Water samples were taken before organisms were added to exposure system and before weekly renewals. Each water sample was drawn up using a 20mL disposable syringe, 10mL water samples were filtered (0.45 µm syringe filter; Acrodisc HT tuffryn membranes, Pall Corporation, Ann Arbor, MI) an additional 10mL were unfiltered, samples were stored in 20mL scintillation vials. Water samples were acidified to 2% by adding 200µL of 70% HNO_3 (Trace Metal Grade, Fisher Scientific, Mississauga, ON) to final volume.

All TiO_2 NPs were digested with the use of ammonium persulfate as an oxidizing agent with the heat of an open flame and dissolved in 2% HNO_3 to yield Ti^{4+} ion, with reaction scheme in Figure 2 according to the methods of Khosravi *et al.,* 2011. Water samples containing TiO_2 NPs were diluted and transferred to porcelain annealing cups. Samples were evaporated at 80^oC for 1 hour until completely dry. Ammonium persulfate (1 gram) was placed in each dry annealing cup and spread to cover the bottom of the cup completely. Annealing cups were then suspended over a Bunsen burner (using a wire mesh) until fuming ceased (approx. 15 min), at which point $[TiO(SO_4)_2]^2$ has formed (Step 3 in Figure 2.1). Cups were cooled at room temperature, then 5 mL of 2% nitric acid (trace metals grade, Fisher Scientific, Mississauga ON) was added along with a micro stir bar and then they were placed on a hot plate and the mixture gently boiled for approximately 10 minutes. The resulting solution with TiO_2 NP converted to Ti^{4+} was then saved and subsequently analyzed for total Ti content by graphite furnace atomic absorption spectroscopy (GF-AAS).



$$S_2O_8^{2-} + \Delta \rightarrow 2SO_4^{\cdot -} \quad (1)$$

$$2TiO_2 \rightarrow Ti\text{-}O^{\cdot} + TiO^{\cdot} + O_2 \quad (2)$$

$$Ti\text{-}O^{\cdot} + 2SO_4^{\cdot -} \rightarrow [TiO(SO_4)_2]^{2-} \quad (3)$$

$$[TiO(SO_4)_2]^{2-} + 2H^+ \rightarrow Ti^{4+} + H_2O + 2SO_4^{2-} \quad (4)$$

Figure 2.1: Reaction scheme for TiO_2 NP digestion. (1) Ammonium persulfate is heated and decomposes to produces sulfate radicals. (2) Energy released during formation of sulfate radicals provides sufficient energy to break down titanium-oxygen bonds forming titanium oxide radicals. (3) Titanium oxide radicals then react with the excess sulfate radicals to form titanium oxosulfate anion. (4) Titanium oxosulfate anion is soluble and readily dissociates in 2% HNO_3 to generate Ti^{4+} and sulfate anions (Khosravi *et al.,* 2011).

2.2.4.2 Organism Sampling and Digestion

At test termination all living *H. azteca* were removed from exposure system using a disposable pipette and placed in clean culture water. Organisms were given 6 hours for gut clearance, transferred and blotted dry before being placed (with a fine tip paint brush) in a 0.6 mL ultracentrifuge tube to be dried for 48 hours at 80°C. After drying was complete individual organisms were weighed using a Sartorious SE2 Ultra Micro Balance, with averages being taken per concentrations (Sartorius Mechantronics Corp., Bohemia, NY, U.S.A)

After measuring dry weight, individual organisms were placed in 25 μL of 16N trace-metal grade HNO_3 for 6 days at room temperature and then 20 μL of 30% H_2O_2 were added for 24h and lastly diluted to a final volume of 250μL using MilliQ ultrapure water (Borgmann and Norwood, 1997)

2.2.5 Statistical Analysis

Data are all expressed as mean ± 1 standard error of the mean (SEM) and statistical analysis was performed using SigmaPlot 11.0 computer software (Systat Software, Inc., San Jose, CA). Dry weight of organism during standard toxicity tests was subjected to a one-way analysis of variance (ANOVA) using Dunnet's post hoc test to detect significant difference of growth relative to control (unexposed) groups. All effect concentration values were calculated using Spearman-Karber analysis using the Comprehensive Environmental Toxicity Information System software (CETIS V1.6.1 rev C) and statistical significance was taken as $P<0.05$.

2.3 Results

2.3.1 Mortality and Dry Weight After 28d Exposure to Dissolved Ti

During 28d chronic exposures to dissolved Ti of nominal concentration of 300, 750, 1500 and 3000 µg Ti /L correspond to measured concentration of 278 ± 27.5, 501 ± 77.6, 595 ± 109, and 2349 ± 527 µg Ti /L respectively (n = 8). Survival decreased with increasing dissolved Ti exposure concentrations (Figure 2.2). A LC_{50} value of 1404 ± 347 µg Ti /L was calculated.

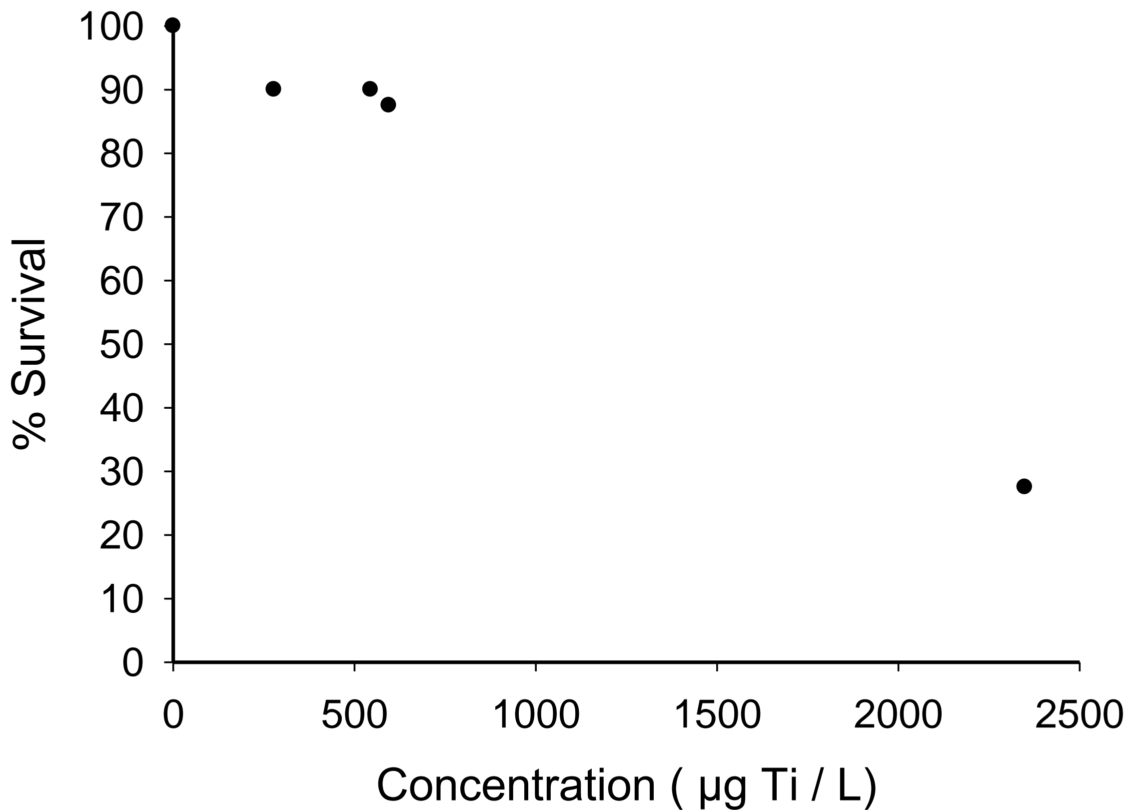


Figure 2.2: Average percent survival of *H. azteca* after 28d exposure to dissolved Ti from AAS standards.

There was significant impaired growth based on dry weight per organism at exposure concentrations above 501 µg Ti /L of dissolved Ti (Figure 2.3). An IC_{50} of 914 ± 369 µg Ti /L was calculated.

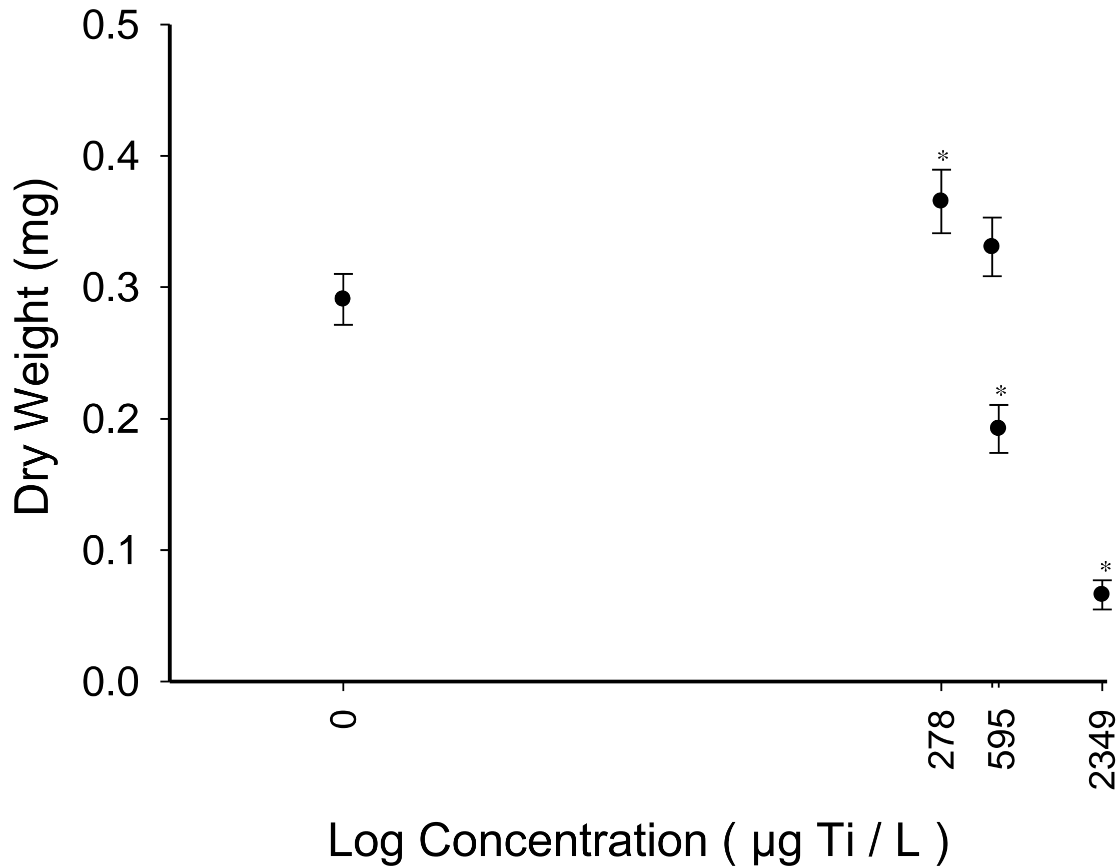


Figure 2.3: Mean dry weight of *Hyalella azteca* (± 95% CI, n= 11-40) after 28d of exposure to dissolved Ti from AAS standards. A group of control (unexposed) organisms are also included and a * indicates significant difference in mean dry weight relative to unexposed Hyalella, ANOVA; P < 0.05.

2.3.2 Exposure to Uncoated Sonicated TiO_2 NPs

2.3.2.1 Mortality and Dry Weight of *H. azteca* After 28d Exposure to P25 TiO_2 NPs

Hyalella azteca chronically (28d) exposed to sonicated solutions of P25 TiO_2 NPs of nominal concentration of 20, 50, and 100 mg TiO_2 /L which correspond to measured concentration of 8.4 ± 2.2, 23.9 ± 4.2, and 51.5 ± 18.4 mg TiO_2 / L respectively (n=8). Concentrations were measured as total Ti and converted to TiO_2 concentrations. An LC_{50} could not be calculated since exposure at tested concentration did not greatly impact survival. There was however significant reduction in dry weight with increasing TiO_2 additions (Figure 2.4). An IC_{50} value of 23.4 ± 9.4 mg TiO_2 /L and IC_{20} value of 6.3 ± 2.2 mg TiO_2 /L were calculated.

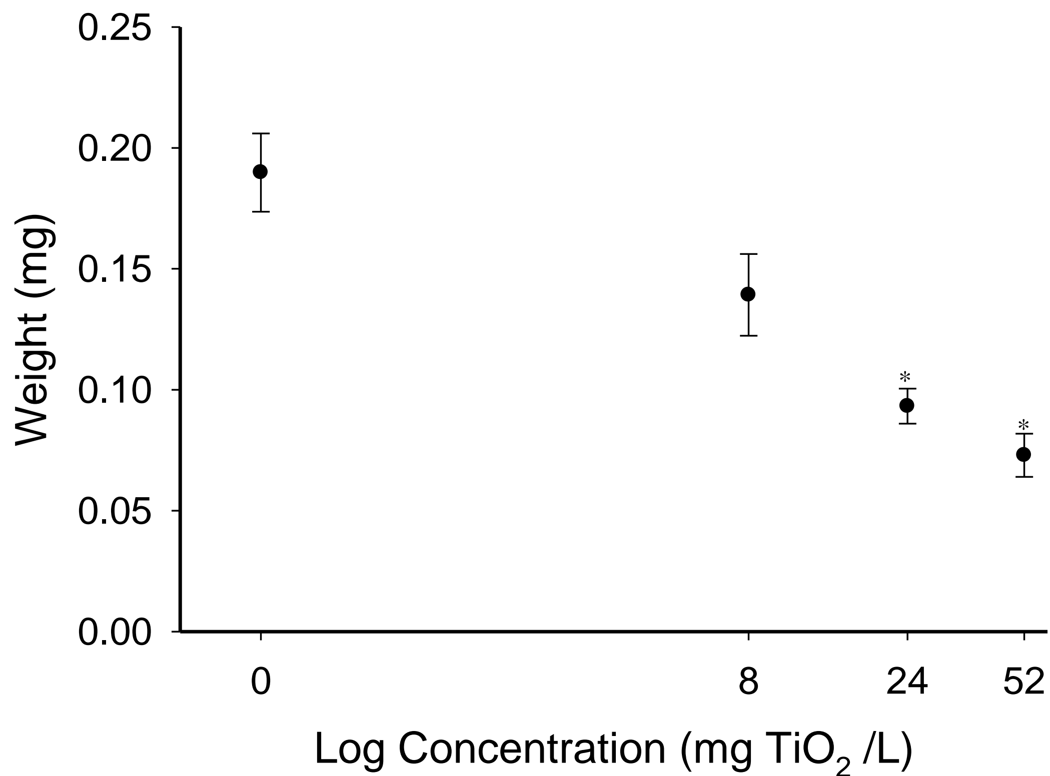


Figure 2.4: Mean dry weight of *Hyalella azteca* (± 95% CI, n = 19-35) after 28d of exposure to P25 TiO_2 NPs. A group of control (unexposed) organisms are also included and a * indicates significant difference in mean dry weight relative to unexposed Hyalella group, ANOVA; $P < 0.05$.

2.3.2.2 Mortality and Dry Weight of H. azteca After 28d Exposure to PC105 TiO_2 NPs.

Hyalella azteca chronically (28d) exposed to sonicated solutions of PC105 TiO_2 NPs of nominal concentrations of 20, 50 and 100 mg TiO_2 /L which correspond to measured concentration of 9.99 ± 3.2, 25.6 ± 3.6, and 42.1 ± 10.4 mg TiO_2 /L respectively (n=8). Concentrations were measured as total Ti and converted to TiO_2 concentrations. An LC_{50} could not be calculated since exposure at tested concentration did not greatly impact survival. There was however significant reduction in dry weight with increasing TiO_2 additions (Figure 2.5). An IC_{50} value of 31.2 ± 2.3 mg TiO_2 /L and IC_{20} value of 11.8 ± 4.4 mg TiO_2 /L were calculated.

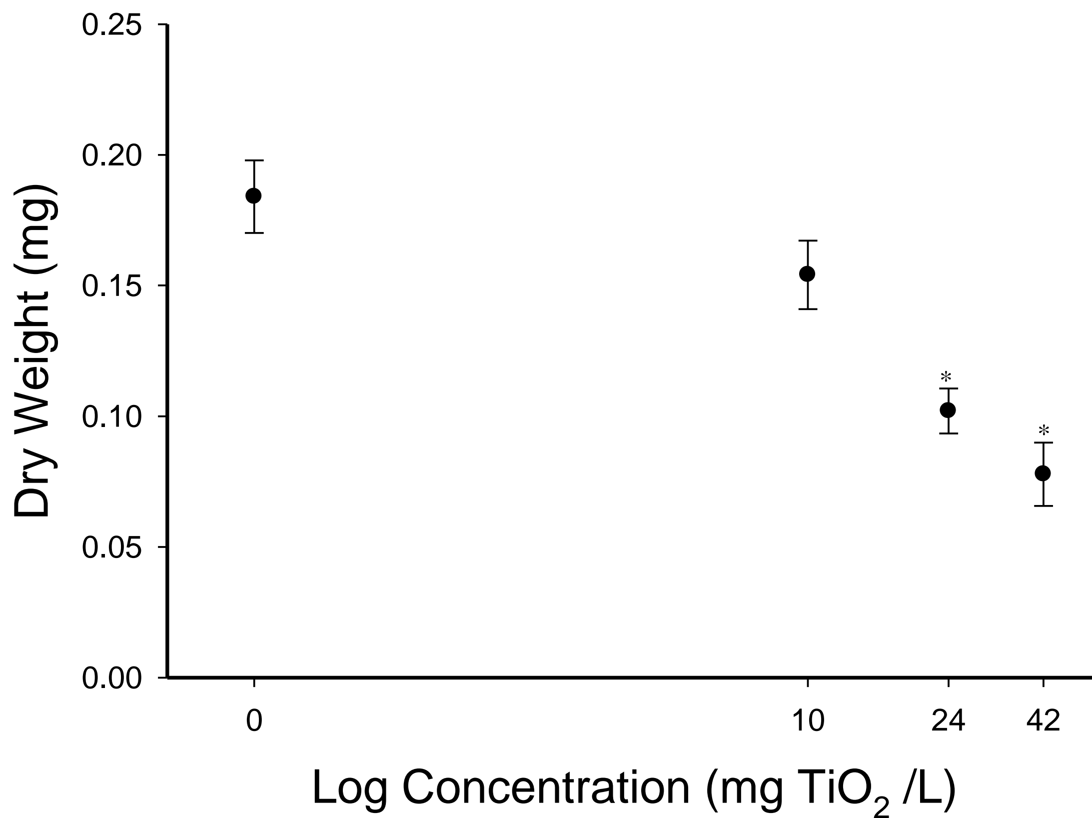


Figure 2.5: Mean dry weight of *Hyalella azteca* (± 95% CI, n = 19-33) after 28d exposure to PC105 TiO_2 NPs. A group of control (unexposed) organisms are also included and a * indicates significant difference in mean dry weight relative to unexposed Hyalella, ANOVA; $P < 0.05$.

2.3.2.3 Mortality and Dry Weight of *H. azteca* After 28d Exposure to NM101 TiO_2 NPs

Hyalella azteca chronically (28d) exposed to sonicated solutions of NM101 TiO_2 NPs of nominal concentration of 20, 50, and 100 mg TiO_2 /L which correspond to measured concentration of 7.8 ± 1.6, 20.4 ± 1.6, and 48.6 ± 2.97 mg TiO_2 /L respectively (n=8). Concentrations were measured as total Ti and converted to TiO_2 concentrations. An LC_{50} could not be calculated since exposure at tested concentration did not greatly impact survival. There was however significant reduction in dry weight with increasing TiO_2 additions (Figure 2.6). IC_{50} value of 15.98 ± 1.4 mg TiO_2 /L and an IC_{20} value of 8.8 ± 2.8 mg TiO_2 /L were calculated.

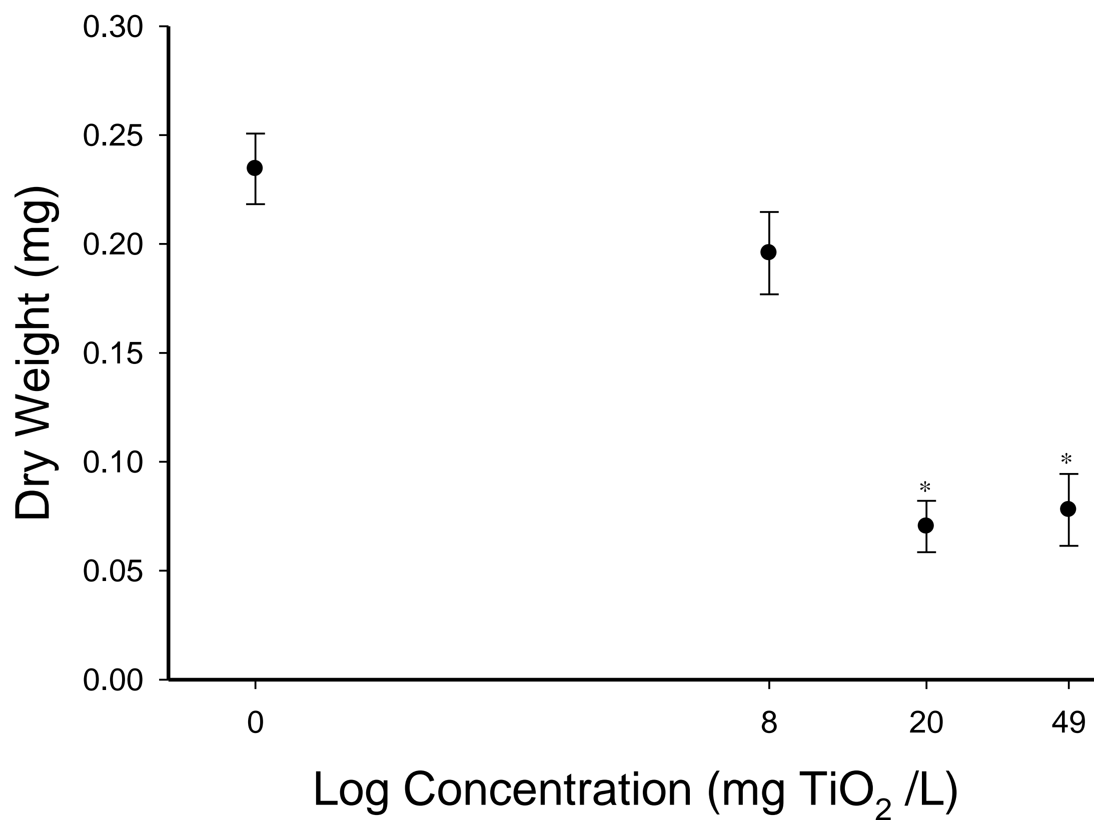


Figure 2.6: Mean dry weight of *Hyalella azteca* (± 95% CI, n = 23-35) after 28d exposure to NM101 TiO_2 NPs. A group of control (unexposed) organisms are also included and a * indicate significant difference in mean dry weight relative to unexposed Hyalella, ANOVA; $P < 0.05$.

2.3.2.4 Mortality and Dry Weight of *H. azteca* After 28d Exposure to NM105 TiO_2 NPs

Hyalella azteca chronically (28d) exposed to sonicated solutions of NM105 TiO_2 NPs of nominal concentration of 20, 50, and 100 mg TiO_2 /L which correspond to measured concentration of 9.3 ± 2.3, 26.1 ± 5.3, and 48.5 ± 6.4 mg TiO_2 /L respectively (n=8). Concentrations were measured as total Ti and converted to TiO_2 concentrations. An LC_{50} could not be calculated since exposure at tested concentration did not greatly impact survival. There was however significant reduction in dry weight with increasing TiO_2 additions (Figure 2.7). An IC_{50} value of 14.5 ± 4.99 mg TiO_2 /L and an IC_{20} value of 4.3 ± 0.6 mg TiO_2 /L were calculated.

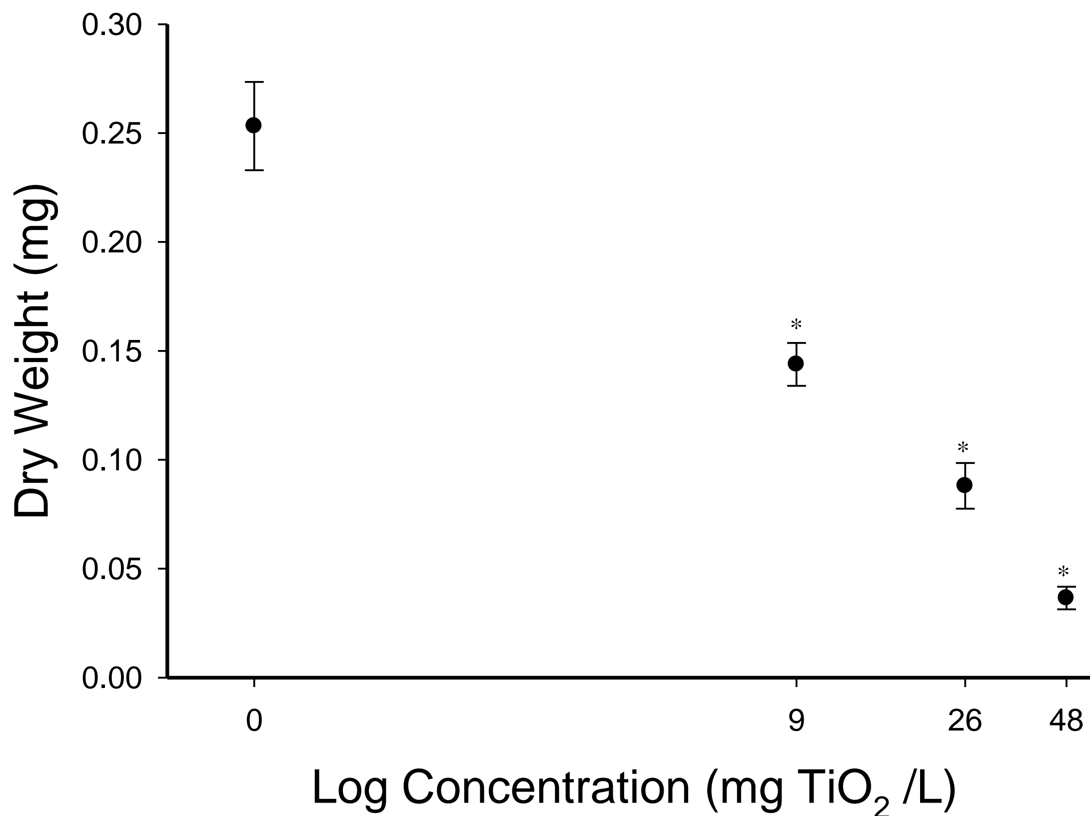


Figure 2.7: Mean dry weight of *Hyalella azteca* (± 95% CI, n = 21-36) after 28d exposure to NM105 TiO_2 NPs. A group of control (unexposed) organisms are also included and a * indicate significant difference in mean dry weight relative to unexposed Hyalella, ANOVA; $P < 0.05$.

2.3.2.5 Exposure Concentrations of Uncoated TiO_2 NPs

Organisms were all exposed to uncoated TiO_2 NPs that showed no significant difference in concentrations at nominal concentrations of 20, 50 and 100 mg TiO_2 /L, which correlate to measured concentrations on Table 2.1. Concentrations were measured as total Ti and converted to TiO_2 concentrations. NP solutions are therefore not different based on concentration and can be compared based on particle characteristics.

Table 2.1: Measured concentration of sonicated total Ti (unfiltered) water samples during 28d chronic exposure to sonicated solutions. Concentrations were measured as Total Ti and converted to TiO_2. Values are expressed as means ± 1 SEM (n = 8, for each concentration).

Nominal Exposure	P25	PC105	NM101	NM105
20 mg TiO_2 / L	8.41 ± 2.2	9.99 ± 3.15	7.81 ± 1.62	9.32 ± 2.3
50 mg TiO_2 / L	23.92 ± 4.17	23.58 ± 3.59	20.39 ± 1.55	26.12 ± 5.34
100 mg TiO_2 / L	51.29 ± 18.41	42.08 ± 10.34	48.26 ± 2.97	48.47 ± 6.36

2.3.3 Exposure to Surface Modified Sonicated TiO_2 NPs

2.3.3.1 Mortality and Dry Weight of *H. azteca* After 28d Exposure to NM103 TiO_2 NPs

Hyalella azteca were chronically (28d) exposed to sonicated mixtures of NM103 TiO_2 NPs which have a slightly hydrophobic surface by treatment with dimethicone (2%). Exposure to nominal concentration of 20, 50, and 100 mg TiO_2 /L which correspond to measured concentration of 6.6 ± 1.1, 19.6 ± 1.1, and 53.7 ± 6.2 mg TiO_2 /L respectively (n=8). Concentrations were measured as total Ti and converted to TiO_2 concentrations. An LC_{50} could not be calculated since exposure at tested concentration did not greatly impact survival. There was however significant reduction in dry weight with increasing TiO_2 additions (Figure 2.8). An IC_{50} value of 36.4 ± 2.8 mg TiO_2 /L and an IC_{20} value of 5.5 ± 0.8 mg TiO_2 /L were calculated.

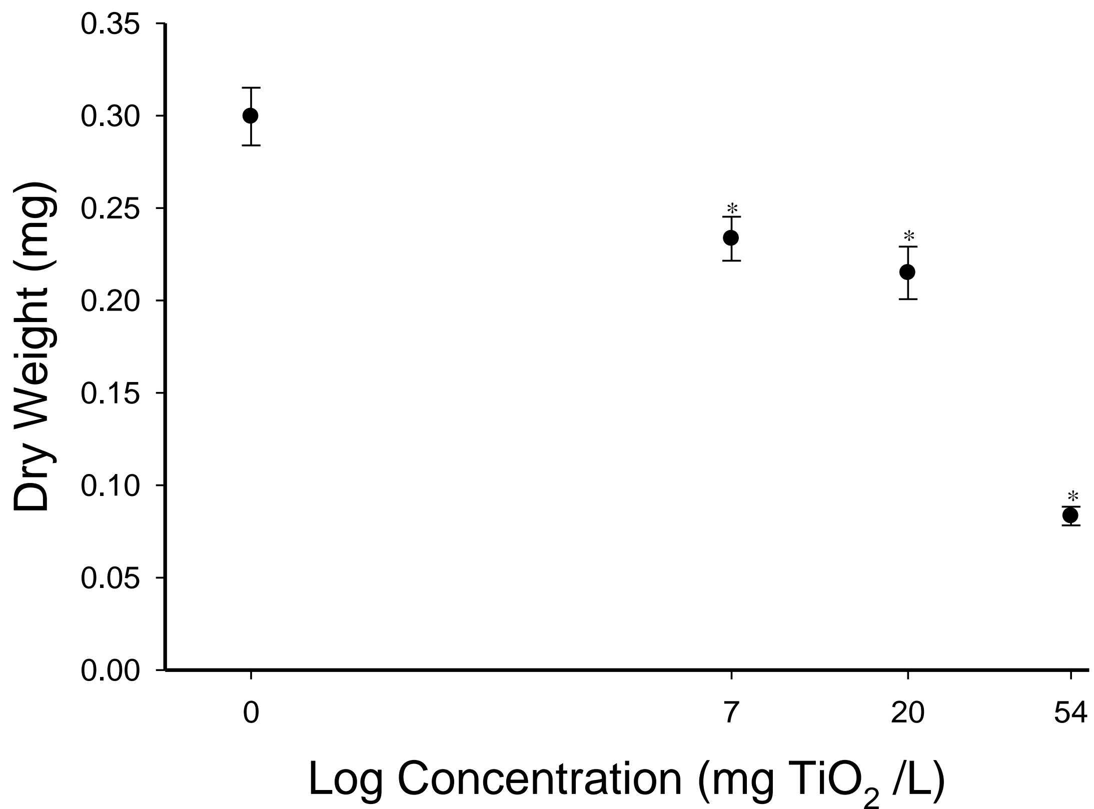


Figure 2.8: Mean dry weight of *Hyalella azteca* (± 95% CI, n = 27-39) after 28d exposure to NM103 TiO_2 NPs. A group of control (unexposed) organisms are also included and a * indicates significant difference in mean dry weight relative to unexposed Hyalella, ANOVA; $P < 0.05$.

2.3.3.2 Mortality and Dry Weight of *H.azteca* After 28d Exposure to NM104 TiO_2 NPs

Hyalella azteca were chronically (28d) exposed to sonicated mixtures of NM104 TiO_2 NPs which have a hydrophilic surface by treatment with glycerine. Exposure to nominal concentration of 20, 50, and 100 mg TiO_2 /L which correspond to measured concentration of 4.48 ± 0.4, 22.3 ± 1.97, and 49.3 ± 4.6 mg TiO_2 /L respectively (n=8). Concentrations were measured as total Ti and converted to TiO_2 concentrations. An LC_{50} could not be calculated since exposure at tested concentration did not greatly impact survival. There was however significant reduction in dry weight with increasing TiO_2 additions (Figure 2.9). An IC_{50} value of 6.5 ± 1 mg TiO_2 /L and an IC_{20} value of 1.9 ± 0.1 mg TiO_2 /L were calculated.

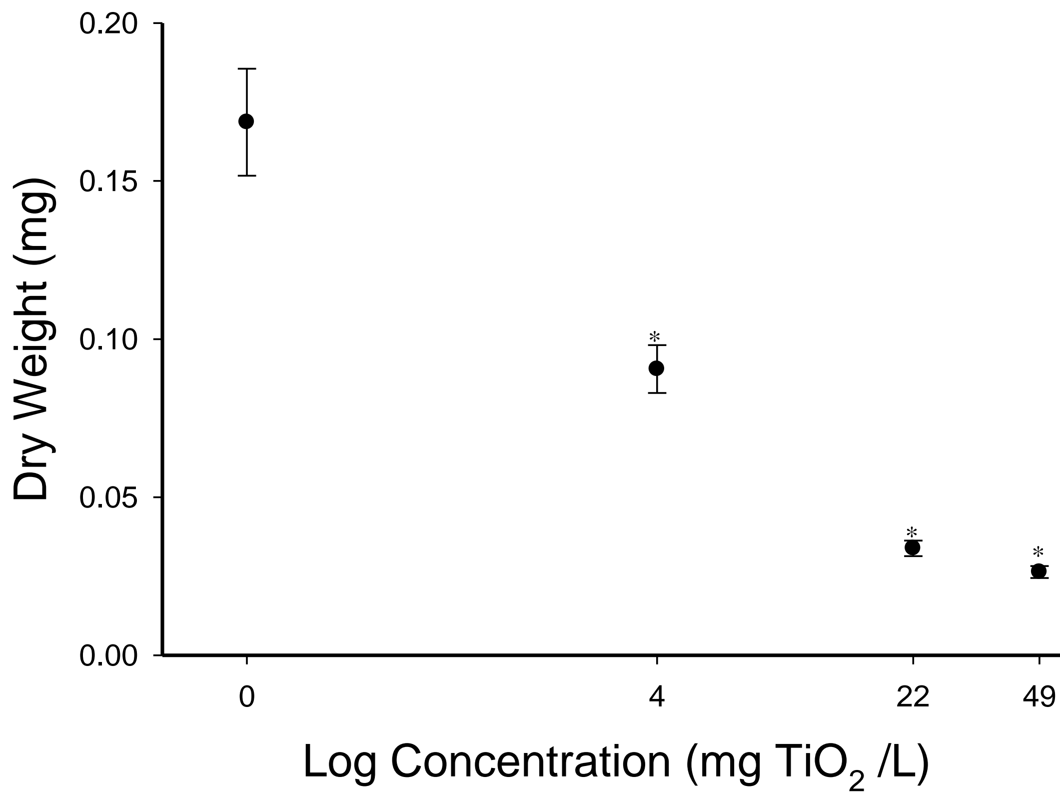


Figure 2.9: Mean dry weight of *Hyalella azteca* (± 95% CI, n = 24-35) after 28d of exposure to 4.48 ± 0.4, 22.3 ± 1.97, and 49.3 ± 4.6 mg TiO_2 /L from NM104 NPs. A group of control (unexposed) organisms are also included and a * indicate significant difference in mean dry weight relative to unexposed Hyalella, ANOVA; $P < 0.05$.

2.3.3.3 Exposure Concentration of Surface Modified TiO_2 NPs

Organisms were all exposed to coated TiO_2 NPs that showed no significant difference in concentrations at nominal concentrations of 50 and 100 mg TiO_2 /L, which correlate to measured concentrations on table 2.2. Concentrations were measured as total Ti and converted to TiO_2 concentrations. Measured concentrations showed significant difference at nominal concentration of 20mg TiO_2 /L, with NM103 significantly higher than NM104.

Table 2.2: Measured concentration of total Ti (unfiltered) during 28d chronic exposure to sonicated solutions. Concentrations were measured as total Ti and converted to TiO_2. Values are expressed as means ± 1 SEM (n = 8, for each concentration).

Nominal Exposure	NM103	NM104
20 mg TiO_2 / L	6.64 ± 1.07	4.48 ± 0.41
50 mg TiO_2 / L	19.63 ± 1.14	22.34 ± 1.97
100 mg TiO_2 / L	53.67 ± 6.17	49.32 ± 4.55

2.4 Discussion

LC_{50} values could not be calculated as the maximum exposure concentrations (100 mg TiO_2 /L) were insufficient to cause mortality great than 50%. Also with respect to growth inhibition all TiO_2 NPs were significantly less toxic than dissolved Ti. At lower exposure concentrations of dissolved Ti a hormetic effect was observed as average dry weight increased above control groups. IC_{50} values were between 8.6 and 18.5 mg Ti/L for uncoated TiO_2 NPs which shows *H. azteca* were between 9.4 to 20.2 times more sensitive to dissolved Ti than they are to uncoated TiO_2 NPs (Figure 2.10). For TiO_2 NPs with hydrophilic and hydrophobic surface modification IC_{50} values were 3.9 and 21.8 mg Ti /L, which are 4.3 and 23.9 times more sensitive to dissolved Ti than modified TiO_2 NPs respectively. Given the differences between IC_{50} values it is likely that NPs, coated and uncoated, were very stable and dissolution of ions into solution contributes very little or not at all to toxicity (Xia *et al.,* 2008). Chronic exposures to TiO_2 NPs hinder growth of organisms. Significant growth reductions in exposed groups may be due to abnormal food intake as NPs may line organism's digestive tract and lead to malnutrition (Zhu *et al.,* 2010).

There was a weak correction ($R^2 = 0.16$) between the IC_{50} values and particle size of uncoated TiO_2 NPs (Figure 4.2). Aggregation behavior of particles was not measured however it is likely that particles have formed larger, less bioavailable aggregates which may account for the lack of correlation (Domingos *et al.,* 2009). This result was not expected based on nominal sizes of particles, however interpretation may change if particle size distribution was measured in exposure system. If toxicity is compared based on surface area then NM101 (specific surface area of $320m^2/g$) should be the most toxic, however it is not significantly different from NM105 (specific surface area of $61m^2/g$). NM101 has the highest total surface area of $2m^2$ at its IC_{50} value of 16mg TiO_2 /L, and NM105 has the lowest total surface area of $0.4m^2$ at its IC_{50} value of 14.5 mg TiO_2 /L, again the correlation between particle characteristic and biological effect is weak. Based on visual observations, the majority of TiO_2 NPs fell out of solution within 24hours, which may limit mobility and bioavailability of NPs. Settling of particles may also be a cause of aggregation and observed low toxicity values. Given the Hyalella are benthic organisms and much of food particles settle to the bottom of exposure system this may be a more accurate exposure scenario. Chronic toxicity tests of NM103 with hydrophobic surface modification and NM104 with hydrophilic surface modification suggest that hydrophilic surface modifications are roughly 6 times more toxic than hydrophobic surface modifications. The toxicity of the modifying agents was not tested individually and may account for differences seen in toxicity. Although not measured this may be a result of better particle dispersion in water or possibly differences in adherence along the organism's digestive tract. It may also be a result of particles interacting with gill

surfaces and organisms expending energy on detoxification rather than growth, however visual observation of particle accumulation were not made.

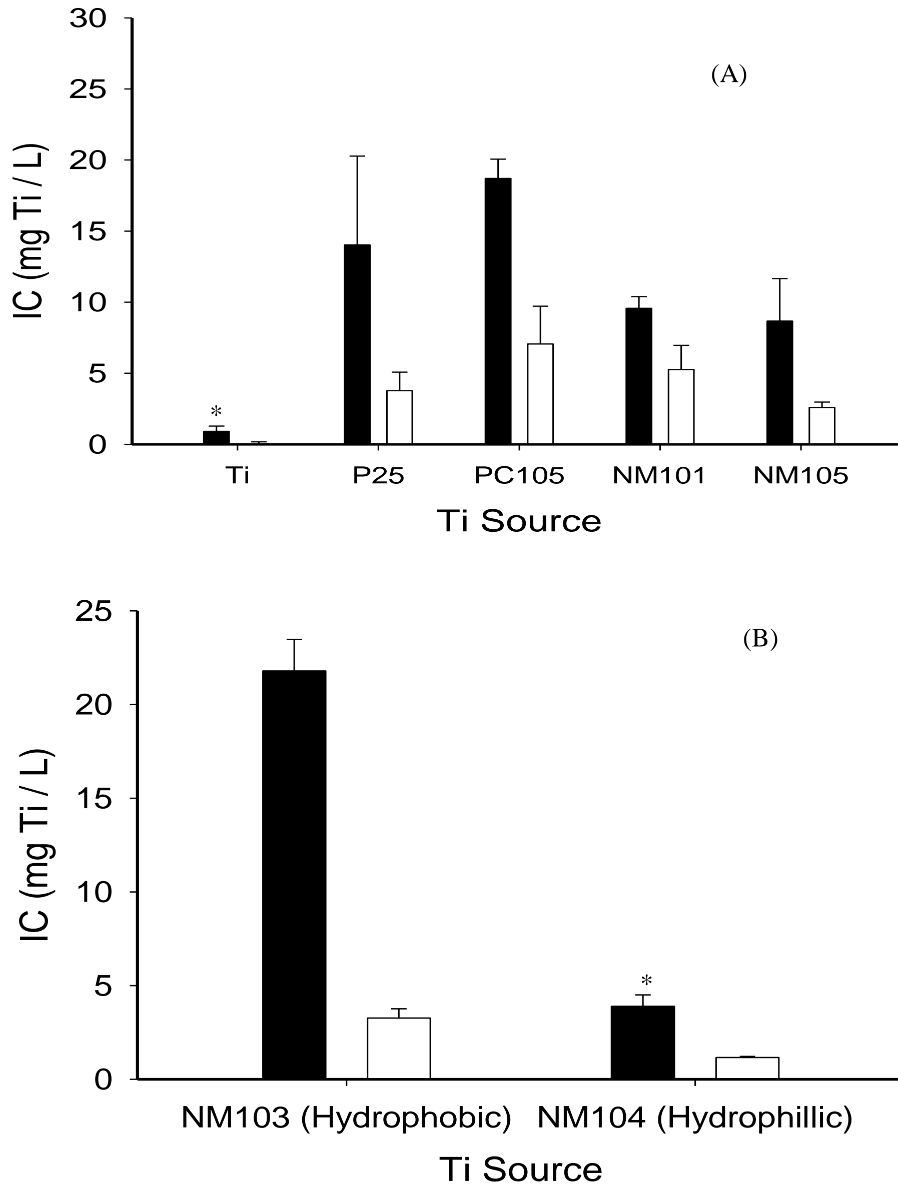


Figure 2.10: (A) Calculated IC_{50} (Black) and IC_{20} (White) values of uncoated TiO_2 NPs and dissolved Ti (± 95% CI). (B) IC_{50} and IC_{20} values of surface modified TiO_2 NPs (± 95% CI).

TiO_2 NPs were less toxic than dissolved Ti however the mechanisms of toxic action are unknown for Ti (dissolved or NP forms). It is likely that toxicity is a result of NPs physically interfering with biological processes. Similar trends are seen with other metal nanoparticles in that the dissolved form of the metal exerts more toxic response than NP counterparts (Navarro *et al.,* 2008; Xia *et al.,* 2008; Blinova *et al.,* 2010). Daphnia are used in many studies and appear to be more sensitive than *H. azteca* as LC_{50} were attained and IC_{50} values were in the low mg TiO_2 /L concentration range (Lovern & Klapper 2006; Zhu *et al.,* 2010). Differences in effect concentrations between published data are likely associated with aggregation and settling of particles, less frequent water changes, and differences in exposure systems.

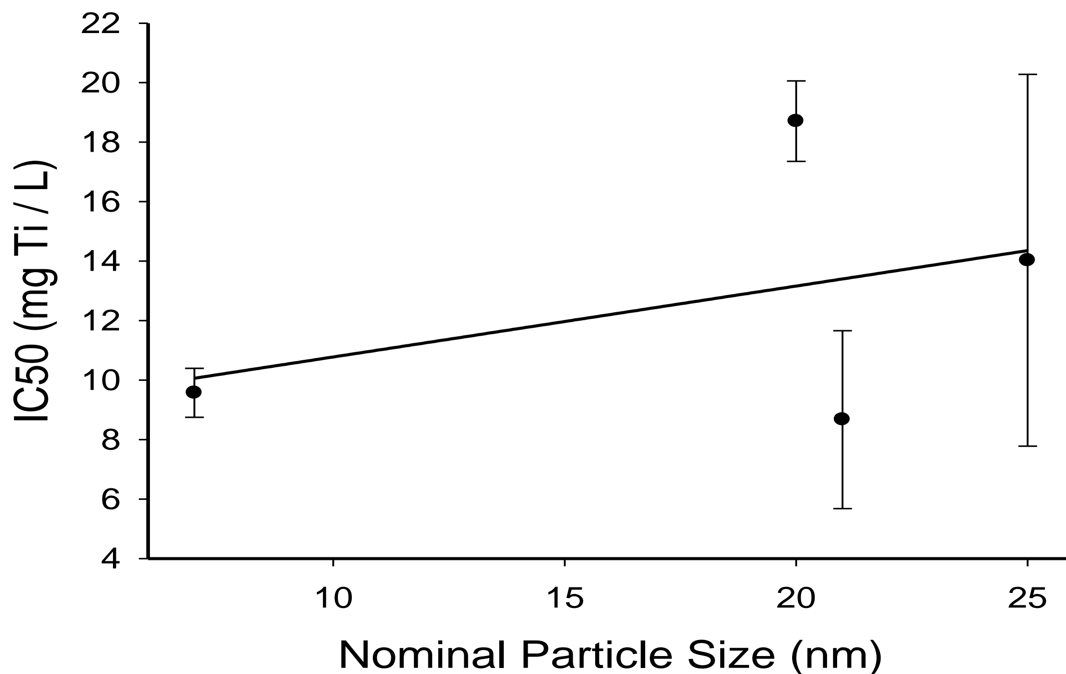


Figure 2.11: IC_{50} values of uncoated TiO_2 NPs in relation to nominal particle size. Error bars represent 95% confidence intervals

Chapter 3

Exposure to Stirred vs. Sonicated TiO_2 NPs

3.1 Introduction

Sonication refers to the use of ultrasound between 20 kHz to 10 MHz (Gedanken 2004). Upon introduction in a liquid medium the ultrasound is distributed through the medium by causing vibrational motion of the medium and all contents in it. It causes a compression and stretch of the molecular structure of the medium. As the intensity of the ultrasound being applied to the medium increases, there is a point where intermolecular forces that hold the molecular structure fail (Gedanken 2004). The process of breaking intermolecular forces by sonication is called acoustic cavitation, the rapid formation and collapse of bubbles formed in the liquid that cause the breakdown of larger particles. It has been estimated that temperature inside the bubble can range from 5000 to 25,000 Kelvin with pressures between 300 – 500atm. (Gadanken 2004; Kis-Csitari *et al.,* 2008). Sonication has also been used to break up micron sized TiO_2 aggregates, and reduced particle size reduction nearly 10 fold. The particles do no agglomerate back to clusters (Lorimer *et al.,* 1991).

Sonication is used in the field of nanotechnology for dispersion of NPs and the breakdown of aggregates that may have formed under experimental conditions (Lorimer *et al.,* 1991; Karthikeyan *et al.,* 2008). This application is used for toxicology studies to limit aggregation of NP in exposure system. However if sonication is carried out for an extended period of time this can lead to erosion of particles leading to the production of smaller particles or inversely the reformation of aggregates (Wang *et al.,* 2004). It has been shown that exposure to sonicated nanoparticle solutions can increase the toxicity compared to solutions that are not (Laban *et al.,* 2010). LC_{50} values for fathead minnows when exposed to Ag-NPs that were stirred was 9.5mg-L^{-1}, however if the same solution is

sonicated for 5 minutes the LC_{50} values drop to 1.25mg-L^{-1} (Laban *et al.,* 2010). Sonication is also useful in nanotoxicology as it does not introduce new substances such as dispersing or capping agents to an exposure system, which may complicate interpretation of results.

The objective of this chapter is to determine if particle dispersion methods influence toxicity of TiO_2 NPs. It is hypothesized that solutions dispersed by stirring and sonication will be more toxic than solutions dispersed only by stirring.

3.2 Materials & Methods

3.2.1 Exposure Details

Exposure systems will follow procedure's listed in chapter 2.2.3. Organisms were exposed to uncoated TiO_2 NPs P25, PC105, NM101 and NM105 at nominal concentrations of 41.5, 70.7, 38.3 and 27.7 mg TiO_2 /L respectively. Organisms were also exposed to surface modified TiO_2 NPs NM103 and NM104 at nominal concentrations of 74.7 and 21.5 mg TiO_2 /L respectively. Exposures were all done in duplicate for both sonicated and stirred solutions at listed concentrations.

3.2.2 Particle Dispersion

Exposures to stirred solutions were dispersed by mixing of stock solutions using a stir bar for 24h (Wiench *et al.,* 2009). Solutions that were sonicated were first mixed using a stir bar for 24h. Secondly a sonication step was performed. 186 mL of stock solution were sonicated using a probe sonicator (QSonica, Sonicator 4000, Newton, CT)

for 5 minutes at 20 kHz, 20mm, 0.5 inch Ti horn prior to addition into exposure system (Wiench *et al.,* 2009, Termnak 2007).

3.2.3 Statistical Analysis

Data are all expressed as mean ± 1 SEM and statistical analysis was performed using SigmaPlot 11.0 computer software (Systat Software, Inc., San Jose, CA). Comparison made between dry weights of organisms that were exposed to stirred or sonicated solutions were subjected to a t-test with statistical significance was taken as $P<0.05$.

3.3 Results

3.3.1 Growth Inhibition of *H. azteca* After 28d Exposure to uncoated TiO_2 NPs

Hyalella azteca chronically (28d) exposed to sonicated solutions of P25, PC105, NM101 and NM105 TiO_2 NPs at nominal IC_{50} concentration of 41.5, 70.7, 38.3 and 27.7 mg TiO_2 /L which correspond to measured concentrations of 29.9 ± 2.5, 55.8 ± 8.5, 12 ± 2.9, and 37.5 ± 2.9 mg TiO_2 /L. Exposure to these concentration yield 41.2, 50.3, 55.7 and 58.7% average growth inhibition relative to control groups (Figure 3.1).

Hyalella azteca chronically (28d) exposed to stirred solutions of P25, PC105, NM101 and NM105 TiO_2 NPs at nominal IC_{50} concentration of 41.5, 70.7, 38.3 and 27.7 mg TiO_2 / L which correspond to measured concentrations of 18.8 ± 4.4, 29.5 ± 8.1, 19.4 ± 3.5, and 29.4 ± 2.9 mg TiO_2 / L. Exposure to these concentration yield 28.9, 28.9, 40.4, and 11.6% average growth inhibition relative to control group (Figure 3.1).

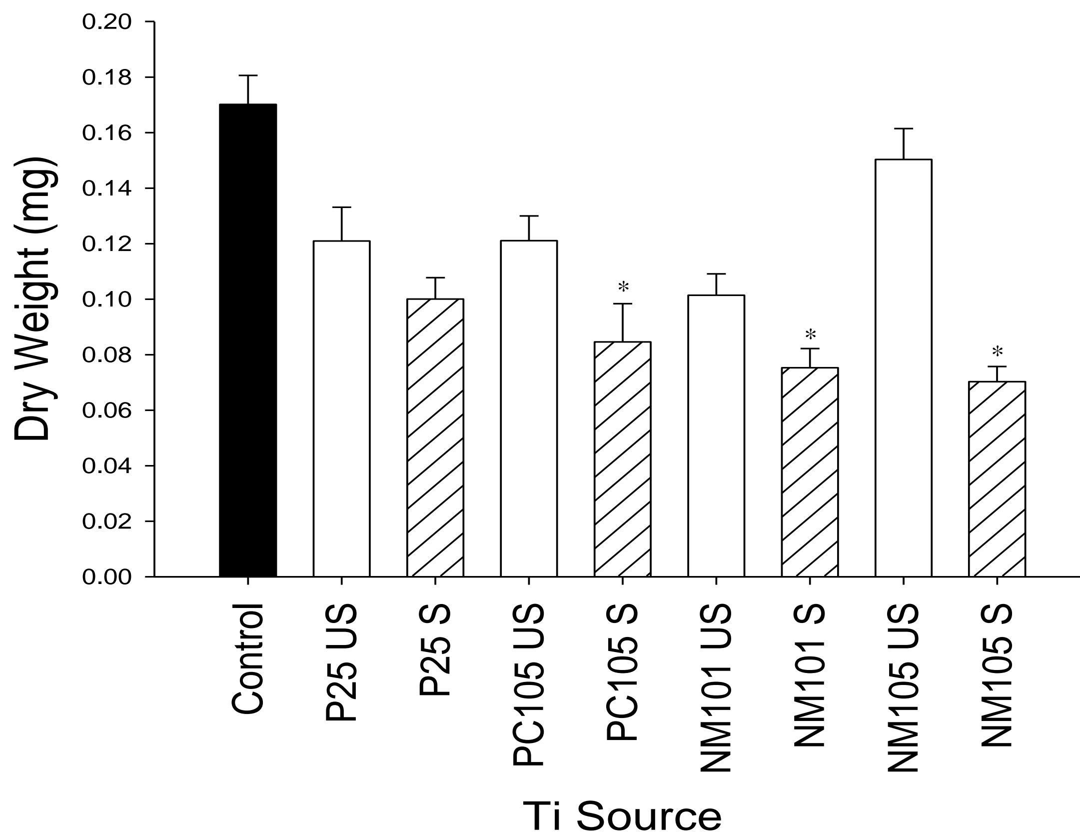


Figure 3.1: Mean dry weight of *Hyalella azteca* (± 95% CI) after 28d of exposure to stirred solutions of P25 US, PC105 US, NM101 US, and NM105 US at measured concentrations of 18.8 ± 4.4, 29.5 ± 8.1, 19.4 ± 3.5, and 29.4 ± 2.9 mg TiO_2 /L respectively (White). Organisms were also exposed to sonicated solution of P25 S, PC105 S, NM101 S and NM105 S at measured concentrations of 29.9 ± 2.5, 55.8 ± 8.5, 12 ± 2.9, and 37.5 ± 2.9 mg TiO_2 /L respectively (Striped) A group control (unexposed organisms) organisms are also included (Black). A * Indicates significant difference in mean dry weight of sonicated compared to stirred groups; ANOVA $P < 0.05$.

3.3.2 Growth Inhibition of *H.azteca* After 28d Exposure to surface modified TiO_2 NPs

Hyalella azteca chronically (28d) exposed to sonicated solutions of NM103 and NM104 TiO_2 NPS at nominal IC_{50} concentration of 74.7 and 21.5 mg TiO_2 /L which correspond to measured concentrations of 25.6 ± 2.7, and 2.1 ± 0.04 mg TiO_2 /L. Exposure to these concentration yield 77.2 and 26.4% average growth inhibition relative

to control group (Figure 3.2).

Hyalella azteca chronically (28d) exposed to stirred solutions of NM103 and NM104 TiO_2 NPs at nominal IC_{50} concentration of 74.7 and 21.5 mg TiO_2 /L which correspond to measured concentrations of 37.8 ± 6, and 2.95 ± 1.95 mg TiO_2 / L. Exposure to these concentration yield 69.2 and 13.2% average growth inhibition relative to control group (Figure 3.2).

3.3.3 Exposure Concentrations of Sonicated and Stirred TiO_2 NPs

Organism were exposed to sonicated solution of P25, PC105 and NM101 show significant difference in concentration compared to stirred solutions. Concentrations were measured as total Ti and converted to TiO_2 concentrations. Sonicated solutions of NM103, NM104 and NM105 do not show significant difference compared stirred solutions (Table 3.1).

Table 3.1: Measured concentration of total (unfiltered) TiO_2 NPs during 28d chronic exposure to stirred and sonicated solutions. Concentrations were measured as total Ti and converted to TiO_2. Values are expressed as means ± 1 SEM (n = 8, for each concentration).

	Exposure Concentration (mg TiO_2 /L)					
	P25	**PC105**	**NM101**	**NM105**	**NM103**	**NM104**
Nominal	41.5	70.7	38.3	27.7	74.7	21.5
Stirred	18.8± 4.4	29.5 ± 8.1	19.6 ± 3.5	29.4 ± 2.9	37.8 ± 6.0	3.0 ± 2.0
Sonicated	29.9 ±2.5	55.8 ± 8.5	12.0 ± 2.9	37.5 ± 2.9	25.6 ± 2.7	2.1 ± 0.0

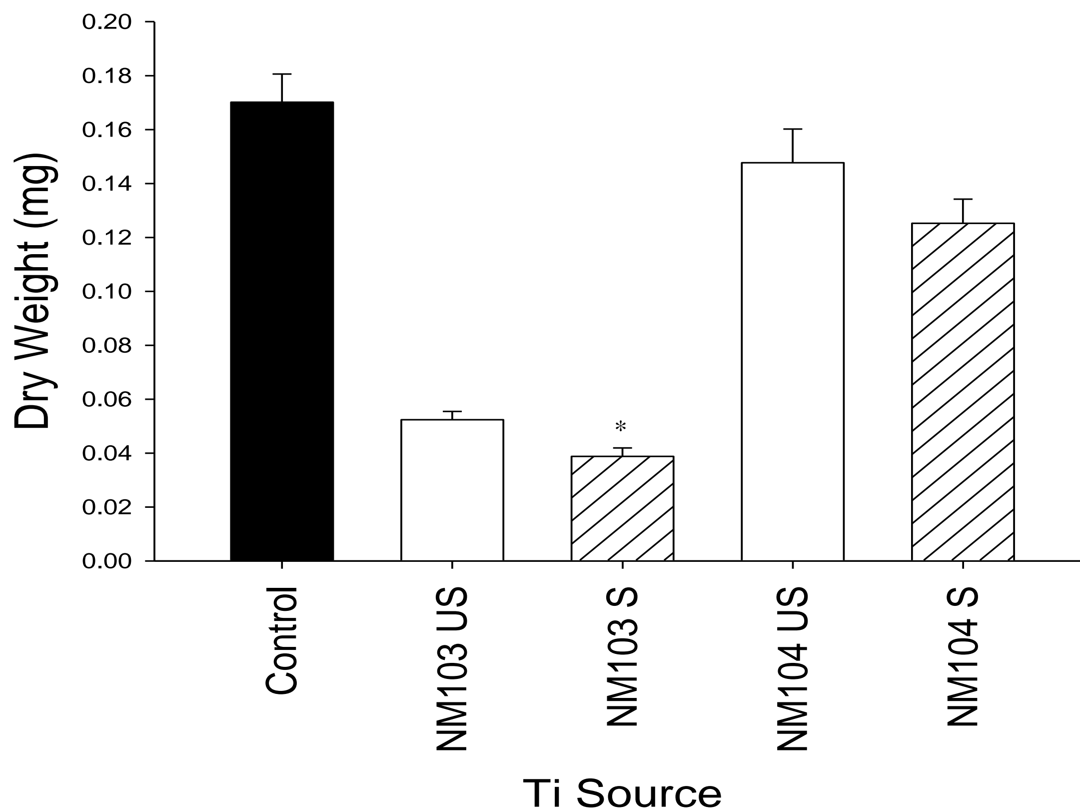


Figure 3.2: Mean dry weight of *Hyalella azteca* (± 95% CI) after 28d of exposure to stirred solutions of NM103 US, and NM104 US at measured concentrations of 37.8 ± 6, and 2.95 ± 1.95 mg TiO_2 /L respectively (White). Organisms were also exposed to sonicated solution of NM103 S and NM104 S at measured concentrations of 25.6 ± 2.7, and 2.1 ± 0.04 mg TiO_2 /L respectively (Striped) A group control (unexposed organisms) organisms are also included (Black). A * indicates significant difference in mean dry weight of sonicated compared to stirred groups; ANOVA $P < 0.05$.

3. 4 Discussion

During all standard toxicity tests NP solution were sonicated, which was done in an attempt to limit aggregates that may have formed in stock solutions. Particle dispersion methods are often a source of controversy when interpreting results from NP exposures as they may alter mechanism of action and NP behaviour (Handy *et al.,* 2008a). For NM103, NM104 and NM105 measured exposure concentrations were not significantly different for stirred solutions compared to sonicated solution. Since the organisms were exposed within a system that did not have significantly different exposure concentrations the differences in dry weight are likely associated with differences in dispersion method. For P25, PC105, and NM101 the measured sonicated concentrations were significantly different from the stirred solutions. It may be argued that the differences in dry weight were a result of exposure concentrations however the average dry weight of organisms at day 28 were lower in all sonicated groups than stirred groups. Exposure to PC105, NM101, NM103, and NM105 showed significantly lower dry weight in sonicated groups than stirred groups. Exposure to NM105 and P25 did not show significantly lower dry weights in sonicated groups and stirred groups, which may be linked to better particle stability or an inability to breakdown aggregates. It was expected that exposure to solutions that had been sonicated would exert higher toxicity than stirred solutions. Particle stability was not measured in stirred or sonicated groups however it is likely that sonication caused decreased aggregation or potentially the erosion of NPs in suspension, yielding TiO_2 NPs fragments that may exert more toxic effect (Wang *et al.,* 2004).

Chapter 4

P25 TiO_2 NPs as a Ligand to Cd

4.1 Introduction

NPs may exert toxicity through one or a combination of four mechanisms (Figure 1.1). One of the mechanisms of action is the ability for NPs to aid in the transport of contaminants in aquatic systems by adsorbing contaminants onto particle surface. Little is known about transfer and fate of NPs and their potential influence on the transfer and fate of other contaminants (Wigginton *et al.,* 2007). As shown by Zhang *et al.,* 2007 P25 TiO_2 NPs showed a strong adsorption for Cd and caused a significant increase of Cd accumulation in carp. TiO_2 NPs and Cd reach equilibrium within 30 minutes which may be explained by particle size and large surface area. TiO_2 NPs have the potential to act as ligands to Cd and potentially enhance bioaccumulation of Cd in aquatic organisms (Zhang *et al.,* 2007).

The objective of this chapter is to determine the bioaccumulation of Cd in the presence and absence of P25 TiO_2 NPs at two Cd concentrations. It is hypothesized that organisms exposed to Cd in the presence of P25 TiO_2 NPs will show enhanced bioaccumulation of Cd compared to those exposed to Cd in the absence of P25 TiO_2 NPs.

4.2 Materials & Methods

4.2.1 Exposure System

Exposure systems will follow procedure's listed in chapter 2.2.3.

4.2.2 Chronic Cd Exposures

Fifty *H. azteca* of 2 – 9d of age were exposed to Cd at 'low' and 'low +' concentrations which both correspond to a nominal concentration of 1 µg Cd /L in duplicate. The second concentration was labeled 'high' and 'high +' which both correspond to nominal concentrations of 3 µg Cd /L in duplicate. Water was spiked with Cd from a stock solution of 1g Cd /L made from $CdCl_2$ (Sigma-Aldrich Inc. St. Louis, MO). 'low +' and 'high +' contained P25 TiO_2 NPs at nominal concentrations of 16 mg TiO_2 /L, made from stock solutions of 1 mg TiO_2 /L that were sonicated for 5 minutes at 20 kHz, 20mm, 0.5 inch Ti horn prior to addition into exposure system. Organisms were sampled on day 0, 1, 7, 14 and 28 for dry weight and total Cd bioaccumulation.

4.2.3 Statistical Analysis

Data are all expressed as mean ± 1 SEM and statistical analysis was performed using SigmaPlot 11.0 computer software (Systat Software, Inc., San Jose, CA). Accumulation data during Cd exposures was subjected to one away ANOVA with a Tukeys post hoc test to determine significant difference in Cd body burden between groups exposed in the presence and absence of TiO_2 with statistical significance was taken as $P<0.05$.

4.3 Results

4.3.1 Growth Inhibition of *H. azteca* exposed to Cd with or without P25 TiO_2 NPs

Hyalella azteca chronically exposed to total Cd at 'low' concentrations of 1.1 ± 0.1 and 'high' 3.1 ± 0.2 µg Cd /L (n=8) yield average growth inhibitions of 18.1 and

68.5% respectively relative to control group not exposed to Cd or P25 TiO_2 NPs. Organisms exposed to Cd at 'low +' concentrations of 1.9 ± 0.3 and 'high +' 3.2 ± 0.4 µg Cd /L in the presence of 8.3 ± 1.7 mg TiO_2 /L (n = 8) showed 39.9 and 45.1% growth inhibition relative to control group that have been exposed only to P25 (Figure 4.1). Changes in total Cd concentrations during exposure are listed in Table 4.1.

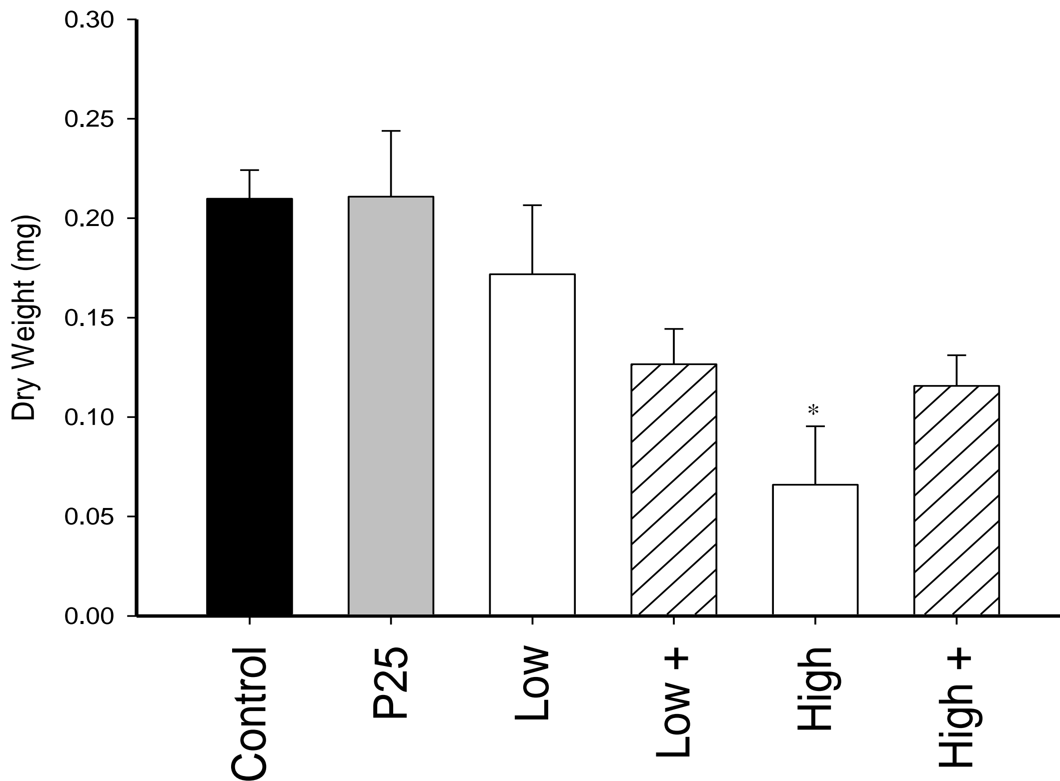


Figure 4.1: Mean dry weight of *Hyalella azteca* (± 95% CI) after 28d of exposure to P25 TiO_2 NPs (Grey), 'low' and 'high' Cd concentrations of 1.1 ± 0.1 and 3.1 ± 0.2 µg Cd /L respectively (White) and organisms that have been exposed to 'low+' and 'high+' Cd concentrations of 1.9 ± 0.3 and 3.2 ± 0.4 µg Cd /L respectively in the presence of 8.3 ± 1.7 mg TiO_2 /L (Striped). Values are means ± 1 SEM. A group of control (unexposed) organisms are also included (Black). A * indicates significant difference in mean dry weight compared to control group (Black), ANOVA; $P < 0.05$.

Table 4.1: Measured concentration of total (unfiltered) Cd in exposure solutions during 28d of chronic static renewal exposures to either Cd alone (low or high) with or without P25 TiO_2 NPs. Initial refers to the concentration of new test solutions and final refers to the concentration after a week and before renewal of solutions. The mean values (n=8) are shown with ±1 SEM.

	Low Cd		**Low Cd + NP**		**Hi Cd**		**Hi Cd + NP**	
	Initial	***Final***	***Initial***	***Final***	***Initial***	***Final***	***Initial***	***Final***
Week 1	1.61	0.9	2.55	1.51	3.69	2.13	3.91	3.01
Week 2	1.45	0.83	2.6	2.12	3.68	2.74	3.79	3.14
Week 3	1.42	1.28	2.56	0.53	3.7	2.44	3.84	1.06
Week 4	0.98	0.64	2.8	0.61	3.8	2.95	3.92	2.58
Mean	1. 14 ± 0.1		1.91 ± 0.3		3.14 ± 0.2		3.16 ± 0.4	

4.3.2 Cd Accumulation in *H.azteca* in the Presence and Absence of P25 TiO_2 NPs

Hyalella azteca control groups showed low presence of Cd accumulation. Organism's exposed to P25 TiO_2 NPs showed significantly different accumulation values to control organisms in clean test medium on day 1, and 21 (Figure 4.2). However control values are all significantly lower than exposed organisms. Organisms exposed to 'low' Cd concentration had a measured total water concentration of 1.13 ± 0.12 µg Cd / L and showed significantly higher Cd body burden than organisms exposed to 'low +' with measured water concentrations of 1.91 ± 0.3 µg Cd / L with 8.30 ± 1.69 mg P25 TiO_2 / L on day 1, 14, 21 and 28 of exposure (Figure 4.3). Likewise organisms exposed to 'high' Cd concentrations had a measured total water concentration of 3.14 ± 0.23 µg Cd / L and showed significantly higher Cd body burden than organisms exposed to 'high +' with measured water concentration of 3.16 ± 0.35 µg Cd / L with 8.30 ± 1.69 mg P25 TiO_2 /L on day 1, 7, 14, 21 and 28 of exposure (Figure 4.3). Cd body burden concentrations are listed in Table 4.2.

Table 4.2: Measured Cd body burden concentrations (µg Cd /g dry weight) on day 0, 1, 7, 14, 21 and 28 (± 95% CI, n = 6, for each measurement)

Day	Control	P25	Low	Low +	High	High +
0	0.31 ± 0.12	0.31 ± 0.12	0.31 ± 0.12	0.31 ± 0.12	0.31 ± 0.12	0.31 ± 0.12
1	1.1 ± 0.43	3.2 ± 0.75	18.7 ± 3.52	8.9 ± 1.3	20 ± 1.8	13.6 ± 1.2
7	1.9 ± 0.38	3.1 ± 0.9	63.2 ± 7.9	43.5 ± 6.2	137.7 ± 6.1	60.9 ± 3.5
14	1.6 ± 0.65	2.5 ± 0.37	122.1 ± 9.1	33 ± 4	222 ± 21.3	76.5 ± 7.9
21	0.75 ± 0.14	1.6 ± 0.1	163.5 ± 22.5	49 ± 1.4	278 ± 32.6	85.9 ± 2.8
28	0.55 ± 0.08	0.69 ± 0.18	165.4 ± 10.9	26.3 ± 1.9	327 ± 32.3	103.4 ± 8.96

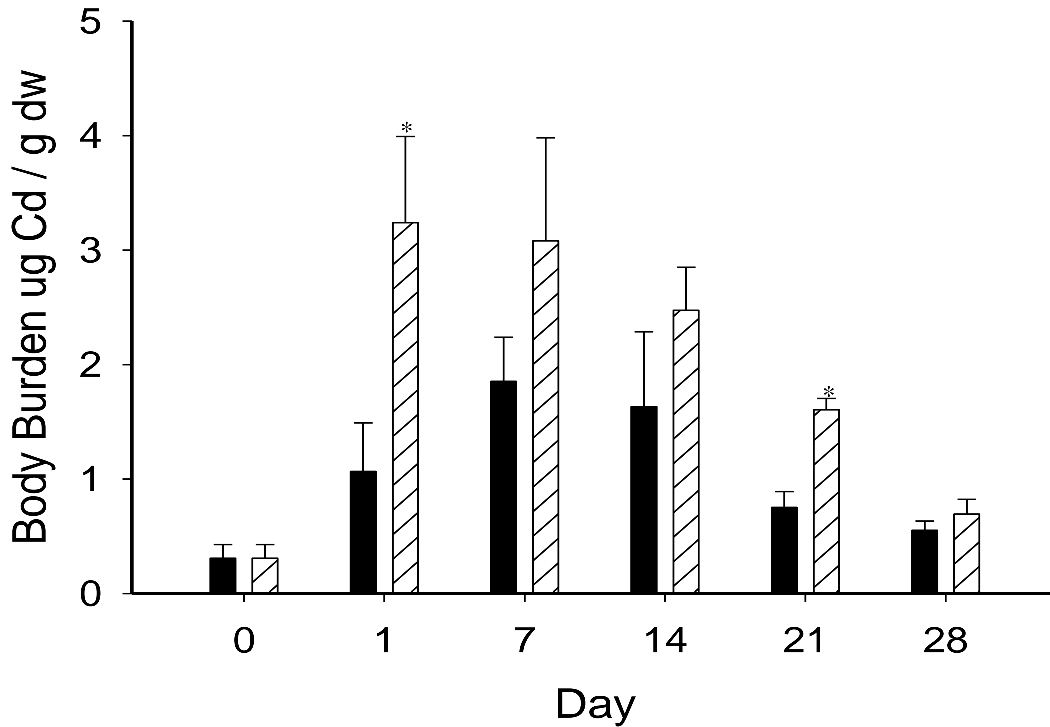


Figure 4.2: Cd accumulation in *H. azteca* during 28 d exposure to P25 TiO_2 NPs at measured concentration of 8.3 ± 1.7 mg TiO_2 /L (striped). Values are means ± 1 SEM (µg/g dry wt). A group of control (unexposed) organisms are also included (black). A * indicates significant difference in Cd accumulation of organisms exposed to P25 compared to control group, ANOVA; $P < 0.05$.

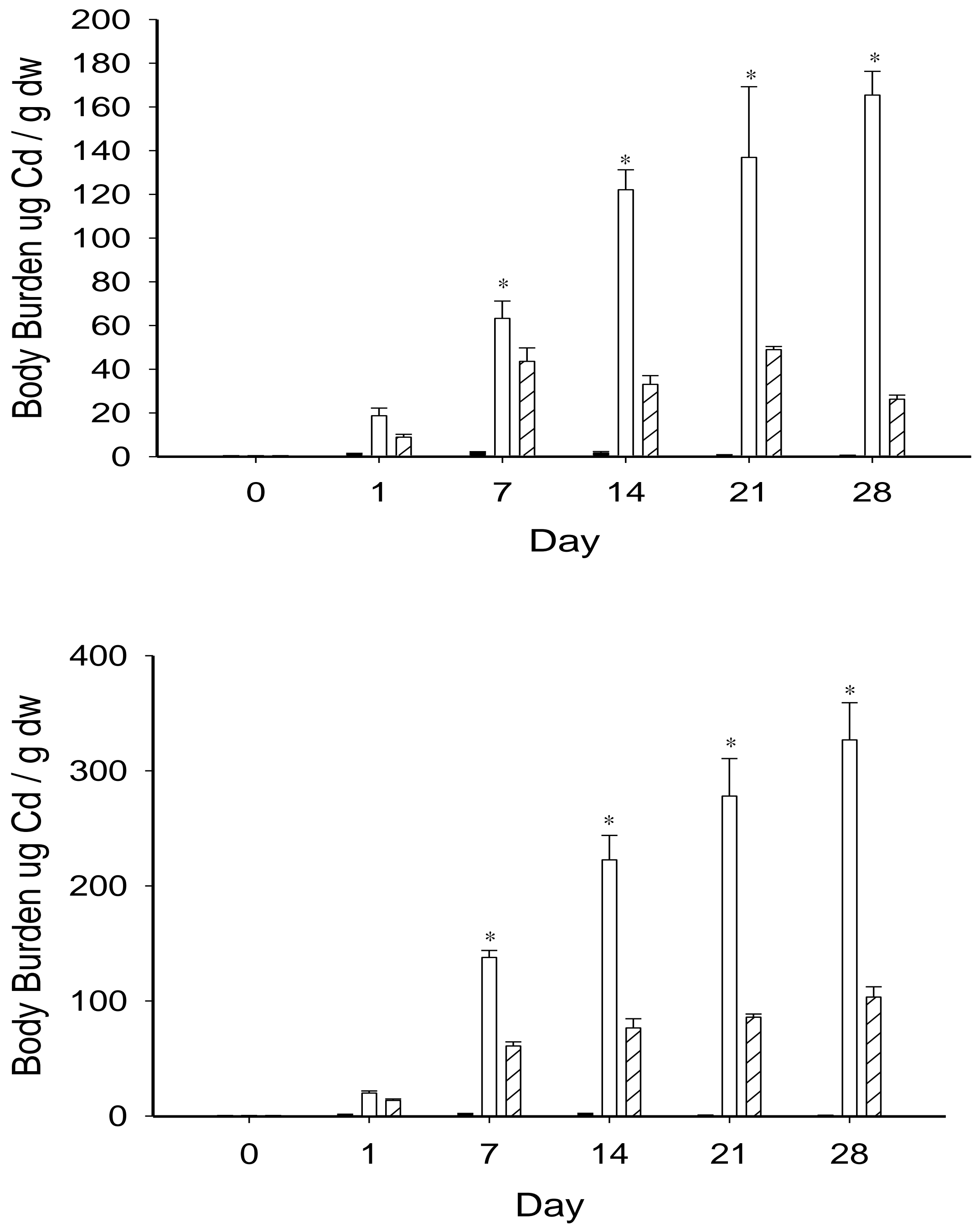


Figure 4.3: Cd accumulation in *H. azteca* during 28 d exposure to either low (upper panel) or high (lower panel) Cd concentrations (open bars) or Cd with P25 TiO_2 added (striped bars). Values are means ± 1 SEM (µg/g dry wt). A group of control (unexposed) organisms are also included (black). A * indicates significant difference in Cd accumulation of between group with and without added NPs $P < 0.05$.

4.4 Discussion

One of the other potential mechanism's of NP toxicity is the ability for NPs to act as vectors for other contaminants (Wigginton *et al.,* 2007, Linkov *et al.,* 2009). The group exposed to P25 TiO_2 NPs shows no significant difference in dry weight versus unexposed control group holding consistent with earlier exposures from chapter 2. Those exposed to high Cd concentrations showed significant differences in dry weight on day 28 of exposure which can be attributed to the toxic effect of Cd. Both low+ and high+ did not show significantly different dry weights from each other which is likely a result of Cd being bound to P25 NPs and hindering the toxicity of Cd.

Control groups showed very low levels of Cd bioaccumulation and all significantly lower than exposed groups. In the low exposure group bioaccumulation reaches a steady state and does not significantly change from day 14 onwards. In the low+ exposure group a steady state is reached by day 7 and does not significantly change onwards. Likewise in the high exposure group bioaccumulation reaches a steady state and does not significantly change from day 14 onwards. In the high + exposure group a steady state is reached by day 7 and does not significantly change onwards. In both concentrations those exposed in the presence of P25 showed significantly lower bioaccumulation of Cd. This may be an artifact if Cd is bound too strongly to P25 and is not giving an accurate representation of body burden in organisms. The protective effects of P25 can be seen at this point and will likely be seen with other contaminants that resemble Cd or that may interact similarly with particle surface. This protective effect is likely due to NPs ability to sorb Cd onto the surface of the NPs and making it less bioavailable (Zhang *et al.,* 2007).

Chapter 5

General Discussion & Integration

5.1 General Discussion

Standard toxicity testing of TiO_2 NPs shows there is a weak correction between particle toxicity and physical parameters of NPs. It was expected that as particle diameter decreased there would be an increase in toxicity as stated in hypothesis 1, however this was not observed. *H. azteca* were more sensitive to dissolved Ti than to metal-oxide NP forms with respect both lethality and chronic toxicity. It was expected from hypothesis 2 that NPs would be more toxic than dissolved Ti however dissolved Ti was more toxic than TiO_2 NPs. There were however significant differences in the toxicity of NP depending on surface modifications. NPs with hydrophilic surface modification (2% dimethicone) were more toxic NPs with hydrophobic surface modifications (glycerine). With respect to hypothesis 3, this study showed that particle dispersion methods influence toxicity and sonication of NP stock solutions increases toxicity compared to stirred solutions. This may occur due to either decreased aggregation or potentially formation of smaller NPs through surface erosion, however this was not studied. It was expected by hypothesis 4 that there will be an increase in Cd bioaccumulation in the presence of P25 TiO_2 NPs. The NPs do act as a ligand to Cd however limit Cd bioaccumulation in the presence of P25 TiO_2 NPs. Based on these studies, the TiO_2 NPs tested are not likely to cause adverse effects to sensitive aquatic invertebrates such as Hyalella. Future research on the toxicity of NPs should focus on the mechanisms of toxicity to further understand the potential risks from NPs.

5.2 Using an Integrative Approach

With the advances in biology there has become a tendancy to overspecialize in one subject and can lead to losing sight of the 'big picture'. An integrative approach produces students with a more holistic understanding of biology who are able to utilize a wide variety of biological tools to solve problems. As mentioned, the field of nanotechnology is rapidly expanding, as is the production and use of NPs. There were many uncertainties and complexities to this project, and an integrative approach improved our understanding of the subject. The goal of this research project was to contribute towards the understanding for NPs to cause environmental impact. The objectives of the project were to 1) observe if there is a relationship with particle characteristic and biological effect, 2) determine the chronic toxicity of dissolved Ti on *H. azteca,* 3) observe the effect that dispersion method of particles would have on *H. azteca* survival and growth, 4) determine bioaccumulation of Cd in the presence and absence of TiO_2 NPs.

The project was focused on NPs of varying size and surface modifications which is an active area of research. A broad range of skills and applications were utilized from various NP studies to conduct my experiments and gain a clearer understanding of the results. Approaches from this study can also be applied to other NP studies as well. It has been suspected that smaller particles are more bioavailable and could cause greater impact as a result in increased bioavailability, however my results showed no clear relationship for TiO_2 NPs. The effects of dissolved Ti showed more toxic response compared to TiO_2 NPs. These results suggest high particle stability and toxicity NP dissolution unlikely to contributing to toxic effects observed. Analyzing particle

dissolution and toxic effect from NP would be a useful future step in NP research. Sonication is used in many facets of nanotechnology, most frequently in order to disperse NPs. As shown in this project, sonication caused a more toxic response compared to stirring of NPs. TiO_2 are very stable and show a tendency to aggregate, sonication may have lead lowered particle aggregations, caused particle fragmentation, or increased particle dissolution, similar trends may be seen in other NPs. The exposure of Cd in the presence and absence of P25 TiO_2 NP integrated dissolved metal toxicology and NP toxicology into a common exposure. Seldom in a natural environment are organisms exposed to a single contaminant, and contaminants may show different effects when in combination would other environment factors. The exposure with TiO_2 NPs and Cd exposure provides an understanding how TiO_2 NPs could interact with other contaminants and how this can affect the biological response. As shown they hinder bioaccumulation of Cd and suggest a protective effect however this is not known for all NPs.

The goal of toxicology studies is to provide data that may be used to understand the biological response of sensitive and widely distributed organisms in an ecosystem. Amphipods are widely distributed and are sensitive to metal contaminants in their environment. The use of amphipods for preliminary toxicity testing of NPs can provide an accurate portrayal for sensitive organisms in many environments. Proficiency in invertebrate husbandry was a major focus on this project which lead to further my understanding of the physiology and life cycle of the organisms. My studies have shown TiO_2 NPs were capable of hindering organism growth however there are additional endpoints which may be more sensitive measures of impact. Supplementary sub-lethal

measures may include reproduction and the recovery of organisms after chronic exposures. If organism development is affected this may delay reproductive age and the number of offspring produced. By understanding the organism's life cycle, tests duration could be extended to observe if organisms can recover in clean culture medium after chronic exposures to TiO_2 NPs. Along with extended studies it would also be beneficial to track NPs in *H. azteca* with respect to accumulation / depuration kinetics and potential translocation of NPs in the organisms.

In addition to the integration of biological fields, integrating significant expertise of chemistry into this project was essential. The behavior of NPs in aquatic medium that could help better understand my results would be knowing more about particle stability and aggregation behavior. With the aid of analytical techniques and tracking how the NPs change in aquatic medium by using dynamic light scattering, settling patterns and aggregation measurements this may provide information to the mechanisms of action and differences in toxicity as a result of physical parameters. An understanding of these factors could provide further insight into particle behavior and interpreting toxicity results. This project has also lead to networking and learning a novel digestion technique with the help of Dr. Metcalfe and Dr. Kosravi from Trent University. Understanding invertebrate husbandry was facilitated with guidance from Dr. Norwood from the Canadian Centre of Inland Waters. With the collaboration of other scientists and integration of a broad array of approaches we were able to quantify the effects of TiO_2 NPs.

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Appendix A: IC_{50} Summary Table

Ti Source	IC_{50} (mg TiO_2 /L)	Lower Confidence Limit (mg TiO_2 /L)	Upper Confidence Limit (mg TiO_2 /L)
P25	23.4	21.3	32.8
PC105	31.2	28.9	34.5
NM101	16	13.2	17.35
NM105	14.5	10.3	19.5
NM103	36.4	32.8	39.2
NM104	6.5	5.5	7.5
Dissolved Ti	0.9	0.6	1.3

Table 1: IC_{50} concentrations for growth with upper and lower confidence limits.

Appendix B: Measured Ti Concentrations & Average Dry Weights of Chronic Toxicity Tests

Dissolved Ti	0.3 mg Ti /L		0.75 mg Ti /L		1.5 mg Ti /L		3.0 mg Ti /L	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Final*
Week 1	0.3	0.2	0.3	0.3	0.6	0.4	4.4	0.3
Week 2	0.3	0.2	0.7	0.4	0.6	0.3	3.2	2.1
Week 3	0.3	0.4	0.4	x	0.8	0.3	3.3	1.2
Week 4	0.3	0.2	0.9	0.5	1.2	0.4	3.6	0.7

Table 1: Measured Ti concentrations of dissolved Ti. X represents an outlier.

P25	20 mg TiO_2 /L		50 mg TiO_2 /L		100 mg TiO_2 /L	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*
Week 1	7.2	6.3	25.1	30.1	68.3	12.3
Week 2	8.1	6.1	34.8	16.4	22.6	12.7
Week 3	15.5	6.3	25.3	15.3	55.4	83.7
Week 4	12.7	5.1	26.9	17.6	81.9	73.3

Table 2: Measured Ti concentrations of P25 NPs converted to TiO_2 concentrations.

PC105	20 mg TiO_2/L		50 mg TiO_2/L		100 mg TiO_2/L	
	Initial	***Final***	***Initial***	***Final***	***Initial***	***Final***
Week 1	5.8	4.8	16.6	15.4	37.2	9.5
Week 2	15.0	6.0	22.2	24.7	69.8	52.8
Week 3	13.6	5.6	33.0	21.0	43.0	38.3
Week 4	18.8	10.2	28.0	27.8	35.7	50.3

Table 3: Measured Ti concentrations of PC105 NPs converted to TiO_2 concentrations.

NM101	20 mg TiO_2/L		50 mg TiO_2/L		100 mg TiO_2/L	
	Initial	***Final***	***Initial***	***Final***	***Initial***	***Final***
Week 1	9.9	4.2	26.0	17.3	46.7	43.6
Week 2	11.4	4.5	18.7	20.1	51.3	51.4
Week 3	9.0	10.0	20.6	19.7	49.7	42.0
Week 4	5.9	7.6	19.3	21.5	56.9	44.6

Table 4: Measured Ti concentrations of NM101 NPs converted to TiO_2 concentrations.

NM105	20 mg TiO_2/L		50 mg TiO_2/L		100 mg TiO_2/L	
	Initial	***Final***	***Initial***	***Final***	***Initial***	***Final***
Week 1	14.5	5.5	25.4	12.5	54.0	54.1
Week 2	11.8	5.6	24.0	19.1	41.7	42.3
Week 3	7.7	5.3	24.2	28.5	35.3	37.4
Week 4	13.6	10.7	33.0	42.2	59.3	63.6

Table 5: Measured Ti concentrations of NM105 NPs converted to TiO_2 concentrations.

NM103	20 mg TiO_2/L		50 mg TiO_2/L		100 mg TiO_2/L	
	Initial	***Final***	***Initial***	***Final***	***Initial***	***Final***
Week 1	5.6	6.3	19.8	18.1	55.7	52.3
Week 2	6.9	4.3	17.8	18.3	49.8	45.1
Week 3	8.5	4.9	22.2	17.9	60.9	73.8
Week 4	7.1	9.6	22.4	20.5	51.6	40.0

Table 6: Measured Ti concentrations of NM103 NPs converted to TiO_2 concentrations.

NM104	20 mg TiO_2/L		50 mg TiO_2/L		100 mg TiO_2/L	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*
Week 1	4.6	3.9	20.4	18.8	48.4	43.6
Week 2	4.4	3.4	21.4	23.3	66.3	51.4
Week 3	5.1	4.2	27.3	19.9	48.4	42.0
Week 4	5.7	4.5	27.2	20.3	49.9	44.6

Table 7: Measured Ti concentrations of NM104 NPs converted to TiO_2 concentrations.

P25 Exposure Concentration (mg TiO_2 /L)	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	90	0.19	0.02
1	85	0.23	0.01
5	83	0.21	0.02
10	83	0.20	0.02
20	80	0.14	0.02
50	78	0.09	0.01
100	65	0.07	0.01

Table 8: Average survival and dry weights of organisms exposed to P25 TiO_2 NPs.

PC105 Exposure Concentration (mg TiO_2 /L)	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	83	0.18	0.01
1	90	0.17	0.02
5	85	0.16	0.01
10	73	0.15	0.01
20	85	0.15	0.01
50	80	0.10	0.01
100	43	0.08	0.01

Table 9: Average survival and dry weights of organisms exposed to PC105 TiO_2 NPs.

NM101 Exposure Concentration (mg TiO_2 /L)	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	87.5	0.23	0.02
1	90	0.19	0.02
5	95	0.28	0.02
10	100	0.25	0.02
20	83	0.20	0.02
50	65	0.06	0.01
100	58	0.08	0.02

Table 10: Average survival and dry weights of organisms exposed to NM101 TiO_2 NPs.

NM105 Nominal Exposure Concentration (mg TiO_2 /L)	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	90	0.25	0.02
1	100	0.26	0.02
5	93	0.20	0.01
10	88	0.18	0.01
20	88	0.14	0.01
50	73	0.10	0.01
100	53	0.04	0.01

Table 11: Average survival and dry weights of organisms exposed to NM105 TiO_2 NPs.

NM103 Nominal Exposure Concentration (mg TiO_2 /L)	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	98	0.30	0.02
1	98	0.26	0.01
5	98	0.30	0.01
10	90	0.29	0.02
20	98	0.23	0.01
50	90	0.21	0.01
100	68	0.08	0.01

Table 12: Average survival and dry weights of organisms exposed to NM103 TiO_2 NPs.

NM104 Nominal Exposure Concentration (mg TiO_2 /L)	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	88	0.17	0.02
1	100	0.18	0.01
5	93	0.17	0.01
10	85	0.14	0.01
20	98	0.09	0.01
50	73	0.03	0.00
100	60	0.03	0.00

Table 13: Average survival and dry weights of organisms exposed to NM104 TiO_2 NPs.

Dissolved Ti Nominal Exposure Concentration (mg TiO_2 /L)	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	90	0.29	0.02
0.1	85	0.22	0.02
0.3	90	0.36	0.02
0.75	83	0.33	0.02
1.5	90	0.19	0.02
3.0	25	0.07	0.01

Table 14: Average survival and dry weights of organisms exposed to dissolved Ti.

Appendix C: Measured Ti Concentration of Sonicated v.s. Stirred TiO_2 Exposures and Average Dry Weights

Sonicated	P25 mg TiO_2 /L		PC105 mg TiO_2 /L		NM101 mg TiO_2 /L		NM105 mg TiO_2 /L	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Final*
Week 1	27.4	18.5	97.3	x	30.2	16.0	43.9	46.1
Week 2	34.9	22.2	42.2	76.7	9.4	12.0	37.2	22.1
Week 3	35.5	38.3	44.1	46.1	5.1	8.8	43.3	36.5
Week 4	33.5	28.6	37.3	46.7	6.2	8.5	41.2	29.9

Table 1: Measured Ti concentrations of sonicated uncoated NPs converted to TiO_2 concentrations

Stirred	P25 mg TiO_2 /L		PC105 mg TiO_2 /L		NM101 mg TiO_2 /L		NM105 mg TiO_2 /L	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Final*
Week 1	16.7	8.0	17.1	35.8	23.9	13.8	29.7	18.7
Week 2	17.7	8.1	13.0	22.8	29.1	38.7	23.1	45.6
Week 3	16.2	18.6	17.1	83.0	11.7	13.7	25.8	29.2
Week 4	47.7	17.7	30.1	17.0	14.1	12.2	28.4	34.8

Table 2: Measured Ti concentrations of stirred uncoated NPs converted to TiO_2 concentrations

Sonicated	NM103 mg TiO_2/L		NM104 mg TiO_2/L	
	Initial	***Final***	***Initial***	***Final***
Week 1	22.2	13.9	2.0	2.2
Week 2	27.1	20.2	2.0	2.0
Week 3	24.9	39.4	2.0	2.3
Week 4	30.8	26.1	2.0	2.0

Table 3: Measured Ti concentrations of sonicated surface modified NPs converted to TiO_2 concentrations

Stirred	NM103 mg TiO_2/L		NM104 mg TiO_2/L	
	Initial	***Final***	***Initial***	***Final***
Week 1	24.8	48.2	9.3	0.0
Week 2	24.3	37.2	0.0	0.0
Week 3	40.7	27.6	0.9	14.0
Week 4	25.7	74.1	0.2	0.0

Table 4: Measured Ti concentrations of stirred surface modified NPs converted to TiO_2 concentrations

Stirred Particles	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	85	0.17	0.01
P25	78	0.12	0.01
PC105	60	0.12	0.01
NM101	78	0.5	0.00
NM105	88	0.15	0.01
NM103	75	0.10	0.01
NM104	93	0.15	0.01

Table 5: Average dry weights of organisms exposed to stirred TiO_2 NPs.

Sonicated Particle	Average % Survival (/40)	Average Dry Weight (mg)	Standard Error (mg)
Control	85	0.17	0.01
P25	70	0.10	0.01
PC105	33	0.08	0.01
NM101	63	0.04	0.00
NM105	80	0.07	0.01
NM103	70	0.07	0.01
NM104	95	0.13	0.01

Table 6: Average dry weights of organisms exposed to sonicated TiO_2 NPs.

Appendix D: Average Dry weights of organisms exposed to Cd in the presence and absence of P25

Day	Control(mg)	Standard Error	P25 (mg)	Standard Error
0	0.01	0.001	0.01	0.001
1	0.01	0.001	0.02	0.001
7	0.02	0.002	0.02	0.004
14	0.04	0.003	0.04	0.005
21	0.05	0.007	0.10	0.015
28	0.20	0.014	0.21	0.033

Table 1: Average dry weight and standard error of control organisms

Day	Low (mg)	Standard Error	Low + (mg)	Standard Error
0	0.01	0.001	0.01	0.001
1	0.02	0.002	0.01	0.003
7	0.02	0.001	0.01	0.000
14	0.03	0.004	0.02	0.002
21	0.04	0.013	0.07	0.011
28	0.17	0.035	0.13	0.018

Table 2: Average dry weight and standard error of organisms exposed to low and low+ Cd

Day	High (mg)	Standard Error	High + (mg)	Standard Error
0	0.01	0.001	0.01	0.001
1	0.01	0.001	0.01	0.002
7	0.02	0.003	0.02	0.002
14	0.02	0.004	0.02	0.002
21	0.03	0.011	0.03	0.003
28	0.07	0.03	0.12	0.015

Table 3: Average dry weight and standard error of organisms exposed to high and high+ Cd