PHOTOTOXIC EFFECTS OF TITANIUM DIOXIDE
NANOPARTICLES ON *Daphnia magna*

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Titanium dioxide nanoparticles (TiO$_2$-NP) are one of the most abundantly utilized nanomaterials in the world. Studies have demonstrated the mechanism of acute toxicity in TiO$_2$-NP to be the production of reactive oxygen species (ROS) leading to oxidative stress and mortality in exposed organisms. It has also been demonstrated that the anatase crystalline conformation is capable of catalyzing the cleavage of water molecules to further increase the concentration of ROS in the presence of ultraviolet radiation. This photoenhanced toxicity significantly lowers the toxicity threshold of TiO$_2$-NP to environmentally relevant concentrations (ppb). The goal of this study was to determine whether dietary uptake and accumulation of TiO$_2$-NP in the aquatic filter feeder *Daphnia magna* resulted in photoenhanced toxicity. *D. magna* and *S. capricornatum* were exposed to aqueous solutions of 20ppm and 200ppm TiO$_2$-NP for 24hrs and then transferred to clean moderately hard water. Samples were taken at various time points, dried, and TiO$_2$ quantified using ICP-MS. Toxicity assays were run on *D. magna* using three TiO$_2$-NP (20ppm, 200ppm) exposure protocols and two ultraviolet radiation treatments. The first exposure group was exposed to aqueous solutions of TiO$_2$-NP for the duration of the test. The second exposure group was exposed to TiO$_2$-NP for an hour and then transferred to clean water. The third exposure group was fed *S. capricornatum* that had been allowed to adsorb TiO$_2$-NP. All samples were then placed in an outdoor UV exposure system and exposed to either full spectrum sunlight (with UV) or filtered sunlight (no UV). Here we show that TiO$_2$ uptake
peaked at one hour of exposure likely due to sedimentation of the particles out of suspension, thus decreasing bioavailability for the duration of the test. Interestingly, when *D. magna* were moved to clean water, aqueous concentrations of TiO$_2$ increase as a result of depuration from the gut tract. Data also suggests these excreted particles were bioavailable and re-consumed by *D. magna*. These data will contribute to the understanding of TiO$_2$-NP environmental fate and toxicity.
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CHAPTER I

INTRODUCTION

Nanoparticles are defined as materials with at least one dimension between 1nm and 100nm (Klaine et al., 2008; Moore et al., 2006). Nanoparticles demonstrate unique physiochemical properties when compared to their corresponding bulk material counterparts (EU-OSHA, 2005). As the particle size becomes smaller, the surface area to mass ratio increases (Figure 1). The result is a large surface area of reactive particles that are capable of increased rates of bio reactivity.

![Figure 1. Relationship of particle diameter and surface area. As particle diameter decreases, this implies an exponential increase in percent surface area. (Adapted from Oberdorster et al., 2005, Nel)](image)

Nanotechnology is hailed as the next technical revolution. As of 2012, the world’s governments invested $10 billion per year in nanotechnology development (Harper, 2011). Nanotech is already being used in industries such as; sporting goods, tires, clothing, tooth paste, sunscreens (EPA, 2009), cosmetics (Muller et al., 2002), electronics (Kachynski et al., 2008), solar energy production (Jiang et al., 2002),
renewable energy (Pavasupree et al., 2006; Wei et al., 2008), waste disposal (Shan, 2009), environmental remediation (Zhang, 2003; Joo, 2006) and medicine (Sosonovik et al., 2008, Corchero et al., 2009). Despite the global increase in the production and utilization of nanoparticles little is known regarding their ultimate fate, accumulation or transfer through food webs (Klaine et al., 2008; Handy et al., 2008). Development and exploitation of these novel materials have vastly outpaced the understanding of the toxicological mechanisms and the environmental fates thus raising concerns about the safety of the nanotech industry (Whatmore, 2006). Titanium dioxide (TiO\textsubscript{2}), for example, is one of the most utilized nanoparticles (NP) in the world, yet there is very little data concerning the toxic effects of TiO\textsubscript{2} nanoparticles (TiO\textsubscript{2}-NP) (Menard, 2011). It is more than likely that despite current safeguards that the industrial wastes of waste management, remediation, electronics, and cosmetics will inevitably be deposited in aquatic systems (Moore, 2006).

\(\text{TiO}_2\) is one of the most commonly used nanoparticles (Menard, 2010). TiO\textsubscript{2} is naturally occurring in three crystal formations: brookite, rutile and anatase. Rutile is the most stable in bulk particles, while anatase is more stable at particle sizes between 2nm-14nm (Naicker et al., 2005; Chen 2009). At temperatures around 1,123K, brookite and anatase conformations are capable of transforming into the rutile conformation. Unlike the bulk form of TiO\textsubscript{2}, the physical and chemical properties of nano-TiO\textsubscript{2} change with the decrease in particle size (Donaldson and Tran, 2002; Oberdoster, 2001). For example, the bond length of the Ti-O bond in bulk TiO\textsubscript{2} is 0.196nm in a tetrahedral unit cell. At particle diameters below 20nm the Ti-O bond length is 0.179nm (Sclafani, 1996) and adopts a hexa-coordinated unit cell (Rajh, 2003), increasing both the affinity of
these uncoordinated defects to ortho-substituted enediol ligands and the overall surface reactivity of the nanoparticle.

Rutile TiO$_2$ (Figure 2a) is currently the most abundant form of TiO$_2$ in nature (EPA, 2009) and the most stable conformation. Rutile TiO$_2$ is widely used as a white pigment in paints, papers, inks, plastics, milk products, sunscreens, UV absorption, (Mueller, 2008) and cosmetics (Gurr, et al., 2005). Anatase TiO$_2$ (Figure 2b) is the most reactive and demonstrates both catalytic and photocatalytic behavior. The

![Figure 2. (A) Rutile crystal structure  (B) Anatase crystal structure American Mineralogist Crystal Structure Database (2003)](image)

photocatalytic properties are unique to anatase and lead to the prolific use of anatase TiO$_2$ in self-cleaning surfaces, light-emitting diodes, solar cells, disinfectants, water treatment, and sunscreens (EPA, 2009).

TiO$_2$ is a naturally occurring ore that must be refined by either the sulfate process or the chloride process to extract the TiO$_2$ (Reck, 1999). The pure forms of rutile and anatase particles are then subject to numerous methods of processing, such as sol-gel, sol, hydrothermal, solvo-thermal, physical vapor deposition, chemical vapor deposition, and electro-deposition to produce purified nanoparticles (Chen, 2009). Precise and careful manipulation of the temperatures and reagents of these processes can be
utilized to dictate the crystal formation produced through refinement as well as producing TiO$_2$ nanorods, nanowires, and ultrafine nanoparticles.

Titanium dioxide was one of the first nanoparticles to be utilized commercially (Menard, 2011). Production of TiO$_2$ is estimated at 5000 metric tons per year for the years 2006-2010, and 10,000 metric tons for 2011 and 2014 (UNEP, 2007). TiO$_2$ production is projected to increase to 2.5 million metric tons by 2025 (Robichaud et al., 2009).

Environmental Concentrations of TiO$_2$

Very little data exists concerning environmental concentrations of nano-TiO$_2$ (Griffit et al., 2008). Kaegi et al. (2008) demonstrated surface paint runoff concentrations of nano-TiO$_2$ 600ppb. Kiser et al. (2009) observed concentrations of 100-3000ppb in raw sewage and 5-15ppb in wastewater effluent. Gottschalk et al. (2009) and Mueller and Nowack (2008) have developed models to predict the distribution of TiO$_2$-NP into environmental compartments (Table 1). Gottschalk et al. (2009) used probabilistic material flow analysis based on inputs garnered from empirical data and extrapolation to determine the flow of commonly used nanoparticles through environmental compartments. These models showed that TiO$_2$ concentrations would reach such high concentrations in sewage effluent that it would merit further research to evaluate the risk of TiO$_2$-NP. The model also showed that when compared to nano-zinc, nano-silver, carbon nanotubes and fullerenes, TiO$_2$ showed the highest degree of concentration in the sewage effluent and sewage treated soils (Gottschalk et al., 2009). These results of sewage effluent concentration were echoed in a similar modeling
experiment performed by Mueller and Nowack (2008) using the same nanoparticles and environmental compartments.

<table>
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<tr>
<th>Compartment</th>
<th>Predicted Concentration United States</th>
<th>Environmental Concentration Switzerland</th>
<th>Concentration Europe</th>
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<tbody>
<tr>
<td>Water</td>
<td>0.002-0.01µg/L</td>
<td>0.7-0.16µg/L</td>
<td>0.012-0.057µg/L</td>
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<tr>
<td>Soil has been added</td>
<td></td>
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<tr>
<td>Sludge Treated Soil</td>
<td>34.5-170µg/kg</td>
<td>----------------------------------------</td>
<td>70.6-310µg/kg</td>
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<td>Sediment</td>
<td>44-251µg/kg</td>
<td>426-2383µg/kg</td>
<td>273-1409µg/kg</td>
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<td>Air</td>
<td>0.005µg/m³</td>
<td>0.002-0.042µg/m³</td>
<td>0.005µg/m³</td>
</tr>
<tr>
<td>Sewage Plant Sludge</td>
<td>1.37-6.7 mg/kg</td>
<td>3.5-16.3 mg/kg</td>
<td>2.5-10.8 mg/kg</td>
</tr>
<tr>
<td>Sewage Plant Effluent</td>
<td>107-523 µg/L</td>
<td>172-802 µg/L</td>
<td>100-433 µg/L</td>
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</table>

Table 1. Modeled Concentrations TiO$_{2}$-NPreleased into environmental compartments in different countries. (Mueller and Nowack, 2008; Gottschalk et al., 2009; Menard, 2010)

Due to the relative stability of both crystalline formations, it is likely that nano-TiO$_{2}$ will aggregate and settle out into sediments and adsorb to particulate matter in the water column (Boxall et al., 2007). Factors such as the presence of humic acids (Pettibone et al., 2008; Domingos et al., 2009), pH and ionic properties of the water body (Navarro et al., 2008; Sharma et al., 2009) will affect the degree and rate of aggregation of nano- TiO$_{2}$. These sediment and particulate bound particles are then capable of reacting with sediment dwelling organisms and filter feeders (Farre et al., 2009). A study by Ferry et al. (2009) was able to demonstrate that NPs could pass from the water into the aquatic food web. Nano TiO$_{2}$ was found to not only pass into the food web, but to accumulate in the primary consumer Daphnia magna (Zhu et al., 2010).

Another metallic NP, quantum dots, was shown to transfer from Selenastrum capricornutum to Ceriodaphnia dubia (Bouldin et al., 2008), indicating that trophic transfer and bioaccumulation of nanoparticles was a possibility. Aggregation of
nanoparticles could cause suspended particles to settle out of solution (Fortner, 2005; Phenrat et al., 2007), thus reducing their bioavailability. The aggregation behavior is based upon the physiochemical properties of the particle itself: geometry (Pal et al., 2007), particle size (Morones et al., 2007) electrostatic repulsion (Phenrat et al., 2007), crystal structure (Tiede et al., 2007), nature of surface coatings (Warheit et al., 2007) Aggregation behavior is further impacted by the ionic strength, composition, availability of natural organic matter (NOM) (Fabrega et al., 2011), pH (French et al., 2009), temperature and the concentration of the nanomaterial during exposure (Phenrat et al., 2007; Dunphy et al., 2004).

Silver nanoparticles (Ag-NP) may shed some light as to the environmental fate and behaviors of other metallic nanoparticles in aquatic systems. It has been demonstrated that increased levels of NOM reduced bioavailability and thus the toxicity of Ag-NP (Gao et al., 2009). Liu and Hurt (2010) demonstrated that the humic and fulvic acids present in NOM were able to reduce the release of Ag-NP through particle aggregation. Increased aggregation would naturally lead to a reduced water column bioavailability as the NP solubility decreased. However, the increase aggregation behavior of the colloidal NP would increase the likelihood of the NP to sorb to organic matter (Chen and Elimelch, 2007). As the particles aggregate and settle out in solution, the nanoparticles were then show be taken up by mussels, oysters (Ward and Kach, 2009), and snails (Croteau et al., 2010) which bioaccumulated the Ag NP through ingestion. It is presumed that these organisms feed primarily upon media in which the Ag-NP may accumulate (Fabrega et al., 2011; Battin et al., 2009) such as surface
nanofilm produced by interactions between Ag-NP and NOM. Similar mechanisms of humic and fulvic acid interaction has been shown with TiO$_2$-NP (Lin, 2009).

**TiO$_2$-NP Toxicity**

TiO$_2$ is one of the most tested nanoparticles (Cattaneo et al., 2009; Kahru and Dubourguier, 2010) due to its prolific use in many industries. Recent research has demonstrated that nanoparticles including TiO$_2$–NP, may accumulate within the cytoplasm (Sakai et al., 1994; Wamer, 1997, Park et al., 2008; Singh et al., 2007). They likely enter through some cellular transport mechanism, such as clathrin mediated transport, phagocytosis, scavenger receptors, and caveolae (Geiser et al., 2005). As with many other engineered nanoparticles, nano-TiO$_2$ has demonstrated the ability to generate reactive oxygen species (Reeves et al., 2008). Reactive oxygen species (ROS) can be generated by the redox cycling and catalytic chemistry of the surface defects of the particle itself. These ROS are capable of damaging proteins, lipids and DNA leading to necrosis or apoptosis (Valko et al., 2004). Additionally, the crystal plane of the nano crystal itself is able to interact with molecular oxygen to produce active electronic configurations such as superoxide radicals, which then initiate a cascade of free radical reactions. In addition to inducing a state of oxidative stress, TiO$_2$ is also capable of causing membrane destabilization through lipid peroxidation (Esterbauer et al., 1992; Riley, 1994), signaling perturbation (Sies et al., 1986; Riley, 1994), DNA damage (Riley, 1994; Sycheva et al., 2011, Trouiller et al., 2011), and the formation of granulomas (Hamilton, 2009)

Significant research has implied that the surface defect present in the anatase conformation results in increased toxicity when compared to the rutile structure.
Inhalation effects of rutile nanocrystals were observed to have an LC$_{50}$ 550ppm in lung epidermal cells (Sayes et al., 2006). In a similar experiment, exposure via oral gavage showed an increase in DNA strand breakage at concentrations of 40ppm -1000ppm of 160nm anatase TiO$_2$–NP in mice (Trouiller et al., 2012). Anatase TiO$_2$ has also been shown to be toxic to aquatic organisms. Federici et al. (2007) demonstrated oxidative stress was present in rainbow trout gills, liver and intestinal epithelium at concentrations ranging from 0.1-1ppm anatase TiO$_2$. Additional studies by Patterson et al. (2011) on developing japanese medaka (Oryzias latipes) showed that anatase TiO$_2$-NP induced early hatching with perturbed behavior and significantly reduced hatching rates in exposure treatments at 10ppm. Palaniappan (2011) was able to demonstrate that exposure to 10ppm anatase TiO$_2$-NP resulted in significant alterations in the biochemical constituents of brain tissues in Danio rerio. Acute toxicity was demonstrated in Caenorhabditis elegans significantly increased ROS generation, reduced growth and behavioral perturbation by Roh et al. (2012) at 50ppm using 4nm-10nm anatase TiO$_2$-NP. In algal toxicological studies, Blaise et al. did not note any acute toxic effect of TiO$_2$-NP, while studies by Aruoja et al. (2009) show growth inhibition at an EC$_{50}$ of 5.83ppm in S. caprincornatum. This is supported by the work of Hartmann et al. (2010) on S. caprincornatum where growth was inhibited EC$_{50}$ levels of 241ppm for the formulation mixture of 67.2% anatase, 32.8% unknown crystal, and 71ppm for the formulation mixture 72.6% anatase, 18.4% rutile, 9% unknown.

The term phototoxicity is used to explain the phenomenon by which the toxicity of a chemical is enhanced during the co-exposure to light energy, usually within the ultraviolet wavelengths. (Landrum,1987). This chemical may be any chemical that
reacts or responds to high energy photons and is capable of transferring that energy into chemical or biological systems (Diamond 2003). Plants have incorporated phototoxic molecules into self defense mechanisms that prevent ingestion (Ivie, 1978) or fungal colonization (Scheel, 1963). Anthropogenic chemicals such as polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals (Tetracycline) and pesticides (erythrosine B) (Spikes, 1969) have all demonstrated phototoxic mechanisms.

The photocatalytic properties of the anatase nanocrystal is often utilized in industries to photodegrade wastes and pollutants (Ma et al., 2012), as a disinfectant (Kuhn et al., 2003), and shows promise as a tool to kill tumor cells (Fujishima et al., 2000). When the anatase TiO$_2$ absorbs a photon of light that possesses higher energy than the band gap of TiO$_2$ an electron is elevated from the valence band to the conduction band, resulting in an electron hole. The interaction of anatase TiO$_2$ with high energy photons is capable of producing electron hole pairs that form free radicals from the splitting of the covalent bonds in water molecules (Ma et al., 2012). This photodecomposition process often produces ROS that are responsible for the photocatalytic properties inherent in the anatase structure. This action paired with the increase surface area to mass ratio of nanocrystals allows for an increased rate of photocatalytic activity than would otherwise be found in bulk TiO$_2$ (Donaldson and Tran, 2002; Oberdoster, 2001, Ma et al., 2012).

Early work exploring the phototoxic effects of anatase TiO$_2$ and UV light showed significant growth reduction at an EC50s of 44ppm in *Desmodesmus subspicatus* and no toxic effects in UV negative controls at 50ppm (Hund-Rinke and Simon, 2006). LC$_{50}$ values of 2.42ppm (with UV) and 155 ppm (without UV) were reported for medaka (Ma
et al., 2012; Brennan et al., 2012). Huang et al. (1999) examined the bactericidal effects of anatase TiO$_2$ and reported mortality at concentrations of 100ppm in the presence of UV. Further work by Maness determined the mechanism of toxicity to be lipid peroxidation and a decrease in cellular function of *Escherichia coli*. This work was supported in the experiments of Matsunuga et al. (1994), which examined *E. coli* mortality and the cellular leakage of β-D galactosidase to extracellular fluids, an indicator of cellular membrane breakdown. A phototoxic effect was demonstrated in *Artemia salina* and *Chattonella Antigua* at 1ppt anatase TiO$_2$ under UV exposure in work performed by Matsuo (2001). Bar-Ilan (et al. 2013) showed an LC$_{50}$ of ~8ppb of anatase TiO$_2$ in developing *Danio rerio* co-exposed to UV. In addition to mortality, Bar-Ilan also noted survivors of the UV/Tio$_2$ exposure showed significant growth malformations when compared to UV-negative controls. Ma et al. (2012) reported an LC$_{50}$ of 29.8ppb under simulated solar radiation (SSR) and a calculated LC$_{50}$ of 500ppm in UV negative controls in *D. magna*. The SSR for this experiment was measured at 1700µW/cm$^2$, which is approximately 25% of the total solar radiation on a sunny day. To date, no study examining TiO$_2$ phototoxicity under natural sunlight with full spectrum conditions has been conducted.

**Goals and Hypothesis**

The phototoxic effects of anatase TiO$_2$ and natural sunlight has not been adequately studied. Previous experiments have examined a relatively narrow set of UV conditions and have not represented natural sunlight. Such information must be reported in order to provide more accurate data for TiO$_2$ phototoxicity in ecological risk assessments. This study seeks to examine the phototoxic effects of TiO$_2$ upon a
grazing primary consumer using aqueous and uptake exposure methodologies. In this study, I (1) assess the uptake of anatase TiO$_2$-NP via two exposure methods (dietary v. waterborne) in *D. magna*, (2) determine whether toxicity is affected by exposure method, and (3) compare the photoenhanced toxicity of rutile and anatase crystal structure under varying natural sunlight conditions.

Hypothesis 1:

The uptake of TiO$_2$ NP by *D. magna* will be significant via both dietary and aqueous exposure.

Rationale:

The interactions of water chemistry and the TiO$_2$-NP will favor particle aggregation that will increase the likelihood of uptake. The pH ranges of the anterior and posterior gut tracts of *D. magna* are 6.0-6.8 and 6.6-7.2, respectively. The zero point charge of TiO$_2$-NP is between 6 > pH > 7. In these conditions, the TiO$_2$-NP are at zero charge and non-polar. With their small size and non-polar charge, the particles theoretically are able to cross the gut membrane into the tissues of the organism.

Hypothesis 2:

The aqueous exposures of TiO$_2$ NP will demonstrate a higher degree of mortality under phototoxic conditions than the dietary uptake of similar suspensions of TiO$_2$ NP.

Rationale:

Previous experiments by Diamond et al. (2012) have suggested that aqueous suspensions of TiO$_2$ NP will demonstrate a greater toxic effect than the body burden of TiO$_2$ NP within the organism due to the increase in reactive surface area on the outside of the organism as opposed to the gut-tract accumulation within the organism.

Hypothesis 3:
Pure mixtures of the anatase crystalline structure will demonstrate a higher degree of photo-enhanced toxicity than mixed preparations containing higher percentages of rutile.

Rationale:

The surface defect responsible for the surface reactivity of the anatase crystal structure is absent in the rutile crystal form, rendering the rutile form relatively inert in the presence of UV.
CHAPTER 2
PHOTOTOXIC EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES ON DAPHNIA MAGNA UNDER NATURAL SUNLIGHT

Manufactured nano-scale particles are a global market garnering in excess of $10 billion worth of investments for research and development (Harper, 2011). Of this, titanium dioxide nanoparticles (TiO$_2$ NP) are one of the most utilized nanoparticles in the global industry, being utilized in renewable energy (Pavasupree et al., 2006), cosmetics and paints (Mueller and Nowack, 2008), waste disposal (Weisner, 2007), environmental remediation (Zhang, 2003) and self-sterilizing surface coatings (Lopez, 2002). The current usage of TiO$_2$ NP is 10,000 metric tons (UNEP, 2013) and is projected to increase to 2.5 million metric tons by the year 2025 (Robichaud et al., 2009). The industrial use of is dependent upon the crystalline conformation of the TiO$_2$ NP. Rutile is utilized primarily as a pigment in paints, sunscreens, and cosmetics due to its absorbance of ultraviolet (UV) radiation (Mueller and Nowack, 2008). Anatase, the more reactive form of TiO$_2$, is used in many industrial and consumer processes that capitalize on its unique form of photo-reactivity. This photo-reactivity allows anatase to cleave water molecules and produce reactive oxygen species (ROS) that allow it to be used in environmental remediation (Zhang, 2003), solar energy production (Jiang et al., 2002), waste disposal (Weisner, 2007), and self-sterilizing surfaces (Lopez, 2002). However, the rapid development and deployment of these technologies has vastly outpaced our understanding of the environmental fate and toxicity of these particles, which leads to concern about the safety of the nanotech industry as a whole (Whatmore, 2006). With its global use and high degree of industrial application, it is inevitable that all forms of TiO$_2$ NP will end up within aquatic ecosystems (Moore, 2004).
TiO$_2$ is a naturally occurring and can be refined from ore or synthetically produced. By controlling the chemical substrates, reaction temperatures and cooling temperatures, industries are capable of determining the dominant crystal confirmation of the end product (Chen, 2009). The most common forms of TiO$_2$ are rutile, brookite and anatase (Reyes-Coronado et al., 2008). Of the three, anatase is the most reactive, owing its reactivity to a unique surface defect in the crystalline structure. It is this surface defect that is believed to be to be responsible for the understood mechanism of toxicity, reactive oxygen species (ROS) generation. The mechanism for this generation of ROS is well understood, with the defect allowing for the generation of an electron-hole pair, allowing anatase TiO$_2$ to interact with oxygen and water to produce hydroxyl, hydrogen and superoxide radicals (Reeves et al., 2008; Ma et al., 2012). This ROS production is increased when anatase is exposed to high energy radiation (UV, X-ray) as it increases the generation of electron-hole pairs which paired with the surface area of the NPs, exponentially increasing the production of ROS. These ROS can then react with organic substrates and initiate damaging chain reactions such as lipid peroxidation that lead to the destabilization of cellular membranes (Esterbauer et al., 1988; Riley, 1994). Elevated ROS are capable of damaging cellular proteins (Wolffe et al., 1986), forming DNA adducts and strand breakage (Riley, 1994, Trouiller et al., 2011), and slowly exhausting cellular resources leading to cell death (Valko et al., 2004).

Despite being one of the most studied NP on the market today, anatase TiO$_2$ NP has been observed to exhibit a wide range of LC$_{50}$ ranging from 5.83ppm in *P. subcapita* (Aruoja et al., 2009) to 241ppm in *P. subcapita* (Hartmann et al., 2010) and 10ppm in *D. magna* (Kim et al., 2010) to 20,000ppm in *D. magna* (Heinlaan et al.,
This is due in part to the large variability in the characterization of the TiO$_2$ NPs themselves, dispersion methods, and exposure protocols. Many toxicology studies have focused on the toxic effects associated with particle size of the nanocrystal. More recently, toxicity has been linked to the surface reactivity of the nanocrystal itself (Warheit et al., 2007). Surface reactivity is of particular importance in regard to the phenomenon of photo induced toxicity.

Photo induced toxicity is a phenomenon in which the toxicity of a given compound is amplified in the presence of light, most often from the UV spectrum (Diamond, 2013; Santamaria, 1964). Early studies exploring the phototoxic effects of co-exposure to anatase TiO$_2$ and UV light reported growth inhibition at 44ppm using Desmodesmus subspicatus while no toxic effects were reported at similar concentrations without UV light (Hund-Rinke and Simon, 2006). Diamond et al. (2012) reported a 48hr LC$_{50}$ of 29.8ppb in Daphnia magna when organisms were co-exposed to TiO$_2$ NP and UV, while UV negative controls (TiO$_2$ only) had calculated LC$_{50}$ of 500ppm. Diamond et al. (2012) reported a 96hr LC$_{50}$ of 2.19ppm in O. latipes co-exposed to UV and anatase TiO$_2$ NP with a UV negative control LC50 of 155ppm. The studies by Diamond showed significantly lower LC$_{50}$ of anatase TiO$_2$ NP at simulated solar radiation of output 1700µW/cm$^2$, which the authors estimated to be 25% of natural solar radiation on a sunny day. To date, there has been little to no research looking at the phototoxic effects of anatase TiO$_2$ NP under full spectrum solar radiation (natural sunlight).

This study was designed to examine the effects of natural solar radiation on the photoenhanced toxicity of anatase TiO$_2$ NP. Body burdens of anatase TiO$_2$ NP were
determined in 48hr uptake/depuration studies and quantified using inductively coupled mass spectrometry (ICP-MS) in order to more accurately compare concentrations of waterborne TiO$_2$ and body burdens present in test organisms. Phototoxicity in organisms with body burden TiO$_2$ vs. waterborne TiO$_2$ was compared to examine different exposure pathways. Phototoxic effects of anatase TiO$_2$ NP were compared to that of a mixture of anatase and rutile TiO$_2$ under natural solar radiation to compare effects of crystal structure.
CHAPTER 3

METHODS AND MATERIALS.

Organism Culture

*Daphnia magna* were obtained from existing cultures at the University of North Texas. Organisms were cultured according to standard US EPA methods (600/4-90). Organisms were cultured in the reconstituted hard water (RHW) (4800mg NaHCO$_3$, 3000mg MgSO$_4$, 3,000mg CaSO$_4$·2H$_2$O, 200mg KCl dissolved in 50L Milli-q H$_2$O stock) and at 26°C. *Daphnia* were fed a mixture of *S. capricornutum* and yeast:cerophyl:tetramin (YTC) that was purchased through Aquatic Ecosystems (Apopka, Fl) at a 3:1 ratio of YTC to algae and kept at a light:dark cycle of 16:8 hours.

TiO$_2$-NP Preparation

Anatase TiO$_2$ was purchased through Fisher Scientific (Acros Organics Titanium(IV) dioxide, 98%+ anatase powder, CAS:12463-67-7). Titanium dioxide was mixed with RHW and sonicated using a Fisher Scientific Sonic Dismembrator (model 500) at 60% intensity for fifteen minutes with thirty second pulses on/off cycles. Suspensions were allowed to cool to room temperature before dilution for organism exposures. Mixed ratio 70:30 (anatase:rutile) TiO$_2$ was purchased through Sigma-Aldrich(CAS: 13463-67-7).

Characterization of TiO$_2$-NP

Suspensions of TiO$_2$ were prepared from stocks of 98% anatase and 70:30 anatase:rutile suspension were prepared at concentrations of 2ppb, 20ppb, 200ppb, 2ppm, 20ppm, and 200ppm. These suspensions were placed in 250mL amber vials and shipped to Clemson University for analysis of zeta potentials and mean particle
diameter and particle size/distribution. TiO$_2$-NP suspensions were brought to a pH of 3.6 by adding three drops of .1M HCl and sonicated to ensure that particles were separated prior to analysis. Particle size and distribution was analyzed on a Wyatt QELS and a Brookhaven 90 plus particle size analyzer. Two mixes of TiO$_2$-NP were analyzed: a 90:10 (anatase:rutile) and 70:30 (anatase:rutile).

**Uptake of TiO$_2$-NP by *D. magna***

Adult *D. magna* were placed in 250mL glass crystallization dishes (15 adults per dish) containing one of three test suspensions (0, 20ppm, 200ppm) with five replicates per concentration per time point, total 35 replicates per concentration. Throughout the experiment, five dishes of each concentration were collected at time points of 0, 1, 8, 24 hours to determine the rate of uptake. At 24 hours, *D. magna* were removed from the test suspension and placed in control RHW, without TiO$_2$, and sampled at 25, 32 and 48 hours for depuration. At each sampling point, *D. magna* were collected and frozen (-80°C). The *D. magna* were then lyophilized (Labconco 7753027) and dry weights were measured on a microbalance (Mettler H51AR). Samples were then digested using 143µL 70% metals free nitric acid (HNO$_3$) and 60µL 30% hydrogen peroxide and microwaved at 30% power on a Magic-chef microwave. After samples were then diluted back up to 5mL yielding a suspension with an acid content of <2% so that it could be directly injected onto a Varian 820 ICP-MS set to detect the titanium ion (47.99amu). Values were compared to a nine point standard curve to determine concentration.

Uptake data was analyzed by two-way analysis of variance (ANOVA) in Microsoft excel followed by a Tukey’s post hoc test. TiO$_2$-NP uptake was the dependent variable and time and TiO$_2$-NP concentrations were the independent variable.
ICP-MS settings:

<table>
<thead>
<tr>
<th>Flow Parameters (L/min)</th>
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<th>Torch Alignment (mm)</th>
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</tr>
<tr>
<td>Second Extraction Lens</td>
<td>-174</td>
<td>Sampler Flow</td>
</tr>
</tbody>
</table>

Table 2. ICP-MS protocol used for the detection of Ti (47) under high attenuation.

Stability Testing of TiO$_2$-NP

Anatase TiO$_2$-NP were suspended in RHW at experimental concentrations (Control, 20ppm and 200ppm) and analyzed for stability in aqueous solution at a pH of 7.8, 356µs. 250mL of test suspension were placed in 300mL beakers and sampled every twenty minutes for three hours. Samples were drawn using a 5mL pipette, drawing off 5mL of the suspension at each time point. The samples were taken at the same depth at every sampling, using the 1.5mL mark as a guideline for four centimeters. Samples were then diluted into 50mL samples so that they could be directly injected onto the ICP-MS in order to determine the concentration of Ti (47.99) ions in the water column. A nine point standard curve was created to determined concentration/counts using TiO$_2$-NP suspended in RHW. Counts were then used to
back calculate the concentration suspended in order to demonstrate a representation of concentration over time to determine the rate of sedimentation of anatase TiO$_2$-NP.

Stability data was analyzed by a two-way Analysis of variance (ANOVA) in Microsoft excel followed by a Tukey’s post hoc test. Mean water column concentration was the dependent variable and the anatase TiO2-NP initial exposure concentrations and time were the independent variables.

Phototoxic Mortality

All photo-enhanced toxicity bioassays were run in the following manner with variation in exposure pathway/concentration. *D. magna* neonates (<48hr) were harvested from brood boards and placed into 250mL glass crystallizing dishes containing test suspensions which were then split among three experimental groups; a UV-10% intensity, UV-50% intensity and UV-100% intensity in a full factorial design. Natural sunlight was used as the source of UV radiation for outdoor exposures in Denton, Texas (33.2147° N, 97.1328° W, 642 ft elevation) during the daytime hours. UV opaque (SACROP3. 125x48000X96.000CEP) and transparent (SACRUVT. 187x63.000X88.000CP) film was obtained from Professional Plastics (Fullerton, CA) and used to control the UV exposure in temperature controlled water baths. UV exposure measurements were evaluated using the A PUV 2500 Biospherical radiometer (Biospherical Instruments, San Diego, CA). Crystallization dishes were suspended in a 100 gallon water bath on a recirculation system in order to maintain an average temperature of ~25°C +/- 2°C throughout the UV exposure. Phototoxic mortality was analyzed by two-way analysis of variance (ANOVA) in Microsoft excel followed by a Tukey’s post hoc test. Mean survival was the dependent
variable and anatase TiO$_2$-NP concentration and UV concentration were the independent variables. LC$_{50}$s were calculated using TRAP-TOX statistical program provided by the Environmental Protection Agency (EPA).

**Body Burden versus Waterborne Exposure Pathways**

Ten *D. magna* neonates (<48hr) were placed in 250mL glass crystallization with five replicates per exposure pathway/concentration per UV exposure. One set of dishes contained waterborne TiO$_2$-NP (control, 20ppm, and 200ppm). Organisms in these dishes were placed in the UV exposure system with waterborne TiO$_2$-NP. A second set of dishes contained waterborne TiO$_2$-NP (control, 20ppm, 200ppm) were also prepared in which organisms were exposed to waterborne TiO$_2$-NP for one hour and then transferred to control water before being placed in the UV exposure system. Organisms were counted every two hours for mortality, which was determined by immobility of the organism.

Phototoxic mortality was analyzed by two-way analysis of variance (ANOVA) in Microsoft excel followed by a Tukey’s post hoc test. Mean survival was the dependent variable and anatase TiO$_2$-NP concentration and UV concentrations were the independent variables.

**Comparison of Anatase Phototoxicity and Rutile Photo-enhanced Toxicity**

Using the same outdoor exposure system as the phototoxic mortality experiment, test organisms were suspended in exposure concentrations (control, 2ppb, 20ppb, 200ppb, 2ppm, of three TiO$_2$-NP suspensions: 99:1 (anatase:rutile) TiO$_2$-NP, 85:15 (anatase:rutile) TiO$_2$-NP, 70:30 (anatase:rutile) TiO$_2$-NP. As with the phototoxic mortality experiment, samples were split among three experimental groups; a UV-10%
intensity, UV-50% intensity and UV-100% intensity in a full factorial design. All organisms were then exposed to eight hours of natural sunlight in Denton, Texas from 10am-6pm. Organisms were counted every two hours for mortality, being determined by the immobility of the organism.
It was shown that TiO$_2$-NP concentration in the water column reduced by 50% after 1.5 hours and by another 50% after two and a half hours. Over the period of three hours, anatase TiO$_2$-NP could visibly be seen settling out of solution and accumulating at the bottom of the 300mL beaker in a thin layer of white powder. In the 20ppm dishes, approximately ~2% of the TiO$_2$-NP settled out of solution by the end of the two and a half hour period ($p = 0.03$) when compared to time zero. In the 200ppm dishes, a much higher rate of loss was observed with ~75% settling out of solution over two and a half hours when compared to time zero ($p < 0.01$).
Analysis of the zeta potentials of the experimental suspensions determined that the higher concentration resulted in a lower zeta potential, which increased the likelihood of agglomeration of the NP, thus increasing the diameter of the NP. In samples where the anatase concentration was higher, there was an increase in zeta potential and a reduction in particle size.

Comparison of Anatase Phototoxicity and Rutile Phototoxicity

<table>
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<th>20.00ppm</th>
<th>200.00ppm</th>
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<td></td>
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<tr>
<td>99 anatase: 1 rutile</td>
<td>47.75</td>
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<td>24.40</td>
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<tr>
<td>70 anatase: 30 rutile</td>
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<td>36.45</td>
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<td>Zeta Potential (mV)</td>
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<td></td>
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<tr>
<td>99 anatase: 1 rutile</td>
<td>-16.77</td>
<td>-23.51</td>
<td>-19.79</td>
<td>-20.78</td>
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</table>

Table 3. Mean particle diameter and mean zeta potential of TiO$_2$-NP suspensions at experimental suspension concentrations.
Anatase TiO$_2$-NP demonstrated a significantly greater degree of phototoxicity than rutile TiO$_2$-NP ($p < 0.01$). The highest degree of mortality (25%) was noted in the rutile concentrations occurred at 200ppm. LC$_{50}$’s for both formations were calculated using EPA TRAP tox software. The rutile LC50 after eight hours of 100% UVR exposure was 282ppm (177-296ppm), which was higher than the calculated LC50 of the anatase structure under the same exposure calculated at 180ppb (43-467ppb).

Daphnia Anatase TiO$_2$-NP Uptake

![Graph showing Daphnia Anatase TiO$_2$-NP Uptake](image)

Figure 5. Mean concentration of anatase TiO$_2$-NP (±SD) in Daphnia magna exposed to TiO$_2$-NP for 24 hours followed by a 24 hour depuration period.
Body burdens of TiO$_2$-NP in *D. magna* increased significantly during the first hour of exposure compared to controls ($p < 0.01$). It was noted that the TiO$_2$-NP agglomerated and settled out of solution after two hours and was observed to coat the bottom of the crystallization dishes. Body burdens in test organisms were at their peak just prior to this period (1-2 hour), when the highest concentration of TiO$_2$-NP was suspended in the water column. The degree of adsorption was such that it was visible with the naked eye as test organisms became coated with TiO$_2$-NP. Through the uptake portion of the exposure, *Daphnia* exposed to TiO$_2$-NP were noted to have distinctly outlined gut tracts laced with TiO$_2$-NP when compared to controls.

The TiO$_2$-NP suspensions (20ppm and 200ppm) and their measured body burdens did not significantly vary from each other ($p = 0.38$). When the depuration began at the 24 hour mark, there was a noticed increase in body burden of TiO$_2$-NP measured at 150ppb. This trend was not seen in the 200ppm exposure, which declined steadily over both the uptake exposure and the depuration period. There was no mortality noted in any of the exposures during this experiment.
Mortality was both UV and TiO$_2$ concentration dependent following exposure to waterborne TiO$_2$-NP ($p = 0.01$; two way ANOVA). Toxicity was highest in the waterborne 200ppm concentration, with 100% mortality in the 50 and 100% UV treatments and 50% mortality even in the 10% UV treatment. Waterborne exposures demonstrated significantly greater toxicity ($p < 0.01$) at a given TiO$_2$ concentration/UV intensity compared to their dietary exposure counterparts. Survival for both 20ppm and 200ppm body burden organisms was $\geq 80\%$ in all UV exposures.
Because nearly all toxicity was observed in the waterborne TiO$_2$-NP exposures, a second dose-response experiment was conducted using a wider range of waterborne TiO$_2$-NP concentrations. Mortality was both UV and TiO$_2$ concentration dependent following exposure to waterborne TiO$_2$-NP ($p < 0.01$). Toxicity was greatest in the 200ppm concentration, with 100% mortality in the UV-50 and UV-100 treatments and 10% in the UV-10 treatments. Duration of UV exposure significantly impacted mortality.
with a four hour LC$_{50}$ of 1.2ppm (95% CL .210-247ppm) and an eight hour LC$_{50}$ of 180ppb (95% CL 43-467ppb) TiO$_2$-NP ($p < 0.01$).

Figure 8. Reciprocity model for UVR/anatase TiO$_2$-NP over four hours (A) and eight hours (B) for D. magna.

exposure and the waterborne concentration of anatase TiO$_2$-NP. In the 100% UVR treatment at 200ppm anatase TiO2-NP there was 100% mortality, whereas in the 10% UVR treatment at 200ppm there was 20% mortality.
CHAPTER 5
DISCUSSION

Results of these studies demonstrates that anatase TiO$_2$-NP exerted an acute toxic effect (mortality) after four hours of natural UVR exposure, which is a significantly reduced time of exposure when compared to non-UV exposure work with $D$. magna. Work done by Wiench et al. (2009) showed an EC$_{50}$ of 100ppm, Heinlann et al. (2008) report an LC$_{50}$ of ~20,000ppm, and Kim et al. (2010) with an LC$_{50}$ of >10ppm. This study supported findings by Diamond et al. (2012) which showed the photo enhanced toxic effect of anatase TiO$_2$-NP reporting a 48 hour LC$_{50}$ of 29.8ppb after 8 hours of UVR. Work done in this study dealt with higher concentrations of anatase TiO$_2$-NP and increased concentrations of UVR, which are responsible for the reduced time of toxicity seen in our results.

Stability Testing of TiO$_2$-NP

Peak body burden concentrations were measured at time points at which the waterborne TiO$_2$-NP were also at their highest concentration. As the waterborne concentration decreased, the body burden of TiO$_2$-NP did as well. Suspensions of 20ppm and 200ppm fall within the sewage plant effluent concentrations (107-802ppm) modeled by Gottschalk et al. (2009) and Mueller and Nowack (2008), implying that it may be likely that aquatic organisms will be exposed to similar concentrations in aquatic ecosystems. Toxicity studies by Lover and Klaper (2006) and Hund-Rinke and Simon (2006) are lacking in stability information of their suspended TiO$_2$-NP thus making it difficult to compare results directly to this study. Stability and sedimentation rates are
crucial in determining the period as to which the organisms are exposed to waterborne TiO$_2$-NP.

Characterization of the particles supports the stability data as it shows that the zeta potential decreases as the concentration of 99:1 (anatase:rutile). The increased hydrophobicity favors agglomeration behavior and increases the likelihood of the TiO$_2$-NP settling out of solution. Characterization data demonstrates that as the TiO$_2$.NP concentration in the water column decreases there is an increase in zeta potential, reducing the hydrophobicity, which would lead to a greater stability of suspensions and prolonged concentrations of TiO$_2$.NP within the water column itself.

Comparison of Anatase Phototoxicity and Rutile Phototoxicity

Due to the surface defect found in its crystal structure, anatase TiO$_2$-NP demonstrated higher degrees of phototoxicity when compared to the 30% rutile mix. The photocatalytic reaction between anatase TiO$_2$-NP was capable of increasing ROS generation to concentrations high enough to induce acute toxic effects in the *D. magna*. This ROS generation was likely absent in the rutile mix due to the absence of the surface defect. Additionally, the rutile formulation is often exploit for its UV absorbent properties, which may have been capable of attenuating the UVR within the samples, further reducing the ROS generation and thus the toxic effect.

Daphnia Anatase TiO$_2$-NP Uptake

We observed a rapid increase in daphnid TiO$_2$ body burden followed by a decline after two hours. This is likely the result of increased particle aggregation of the TiO$_2$-NP themselves that led to increase sedimentation rates, and reduced the bioavailability of TiO$_2$-NP to the organisms. This is supported by the results of the stability experiment,
which also demonstrated a rapid loss of TiO$_2$-NP out of the water column. At the beginning of the depuration period there is a noticeable increase in concentration of TiO$_2$-NP in the body burdens of the test organisms. During the depuration phase, the test organisms were fed fresh cultures of algae, which resulted in the excretion of TiO$_2$-NP present in the gut tract. The depuration of TiO$_2$-NP from *D. magna* into the surrounding environment occurred to such a degree that waterborne concentration of TiO$_2$-NP in these depuration chambers was measured between 200-900ppb and allowed the organism to re-take up the suspended TiO$_2$-NP.

Experiments performed here were single exposures in still water, allowing organisms a brief time frame to be exposed to suspended TiO$_2$-NP in the water column before it settled out of solution. In real world exposures, the exposure is likely to be periodic or constant. Additionally, many aquatic environments are turbulent, which will increase the time TiO$_2$-NP will be suspended in the water column. These conditions would allow organisms, such as *D. magna*, to be continually exposed to TiO$_2$-NP.

Previous work by Zhu et al. (2010) has demonstrated the ability for TiO$_2$-NP to be tropically transferred from *D. magna* to *Danio rerio*. Further research is needed to examine the waterborne uptake of TiO$_2$-NP in *D. rerio* to determine the ability of TiO$_2$-NP to bioaccumulate through the food web. However, the results of this study demonstrate that body burdens of anatase TiO$_2$-NP demonstrate little to no phototoxic effect within organisms.

**Phototoxic Effects of Waterborne TiO$_2$ and TiO$_2$ Body Burdens**

This experiment demonstrated significantly higher phototoxicity of waterborne TiO$_2$-NP suspensions to *D. magna* compared to body burden TiO$_2$. Any TiO$_2$-NP bound
within the tissues of the daphnid are encapsulated by the carapace of the organism containing the pigment melanin, which is reported to protect organisms from damage done by UV exposure (Hessen, et al., 1999). It is possible that the melanin present in the carapace of the organism was capable of blocking enough UVR that it reduced the production of ROS within the tissues of the organism to a sub-lethal level. Furthermore, body burden TiO$_2$ would have less opportunity with which to react with water, reducing the photocatalytic production of the highly reactive hydroxyl radical.

After determining that aqueous TiO$_2$-NP exerted a greater toxic effect than TiO$_2$-NP body burdens, dose response curves were made using a variety of concentrations of waterborne TiO$_2$-NP. LC$_{50}$ were calculated to be 180ppb at eight hours of 100% UVR and 1.2ppm at four hours 100% UVR. These values are orders of magnitude lower than toxicity values reported by Wiench et al. (2009), Heinlann et al. (2008) and Kim et al. (2010). All of these studies were done in the absence of UV irradiation, thus demonstrating the photoenhanced toxicity mechanism. Studies by Diamond et al. (2012) demonstrate a lower 48 hour LC$_{50}$ (29.8ppb) after eight hours of UVR demonstrating the photoenhanced toxicity of anatase TiO$_2$-NP under simulated solar radiation (SSR). The Diamond study exposed organisms to seven TiO2 (86:14, antase:rutile, $z_p$=16.5) suspensions (5, 15, 25, 50, 75, 100, 250, 500 ppb) and exposed to four hours of UVR (1700µW/cm$^2$/s) every 24 hours for 48 hours. Experimental solutions we changed out after the first 24 hour period. This study exposed organisms to higher concentrations of suspended (99:1, anatase:rutile) TiO$_2$-NP (200ppb, 2ppm, 20ppm, 200ppm) and higher concentrations of UVR (2700µW/cm$^2$/s) allowing for a four hour LC$_{50}$ (1.2ppm) and an eight hour LC$_{50}$ (128ppb) to be calculated. Our exposure
protocol differed from Diamond et al. due to the fact that we had one suspension of TiO$_2$-NP exposed without interruption over an 8 hour period to higher intensity UVR. As organisms were subject to higher concentrations of TiO$_2$-NP and UVR for a longer period of time, we saw LC$_{50}$s earlier than in the Diamond study.

The work done in this experiment also demonstrates a consistent, reciprocal relationship between the intensity of UV exposure, concentration of TiO$_2$-NP and time of UV exposure. This demonstration of reciprocity in the dose response phototoxicity is missing in the work of Hunde-Rinke and Simon (2006) and Diamond et al. (2012). The data gathered in this experiment was able to show significant differences (p<0.01) between 100%, 50% and control samples in the same concentrations of TiO$_2$-NP at the same time, demonstrating that the variation in UV intensity has a direct impact on the phototoxic effect seen in the organism. When the UV intensity remains constant, we see significantly different degrees of toxicity across doses varying by orders of magnitude. Lastly, as you increase both the TiO$_2$-NP concentration and the UV exposure, there is also a significant increase in the phototoxic effect with rates of 80-100% mortality in highest exposure concentrations.

The polycyclic hydrocarbon, anthracene, has been extensively studied for its photoenhanced toxicity. Without UVR, anthracene had been show to exhibit no acutely toxic effect (immobilization) in *daphnia* (Herbes et al., 1976). When Herbes at al. co-exposed with natural UVR, anthracene showed acute toxic effects (immobilization) at 18.9ppb after thirty minutes. Additional research demonstrated that PAHs exert acute phototoxicity upon algae (Cody et al., 1984, Gala and Giesy, 1994), amphipods (Ankley et al. 1994), mosquito larvae (Kagan and Kagan, 1986), and bluegill sunfish (Oris and
Giesy, 1985). Oris and Geisy went on to also demonstrate that not only was the concentration of PAHs/UVR important, but the time of UV exposure was also a determining factor in PAH toxicity (1986). Both PAHs and TiO$_2$ present themselves as wide spread environmental contaminants that are both capable of exhibiting acute toxicity that is not seen in the absence of UV exposure.
CHAPTER 6

CONCLUSIONS

In these experiments, it was shown that aqueous suspensions of TiO$_2$ NP were more phototoxic than TiO$_2$ NP found in body burdens in _D. magna_. Furthermore, these experiments have demonstrated effect levels at or below concentrations presented in the current TiO$_2$-NP environmental fate models. Depending on the physiochemical properties of the water and the effects upon UV attenuation, it is likely that aquatic organisms will be co-exposed to anatase TiO$_2$-NP and natural UVR. This mechanism is likely most relevant to alpine aquatic, estuarine, and shallow fresh water ecosystems where UV penetration is likely highest.

TiO$_2$ NP has demonstrated acute mechanisms of toxicity due to the ROS generation when co-exposed with UVR. As nanoparticles, they also possess low particle diameters and molecular weights. Taken together, these properties of TiO$_2$ NP bear many similarities to another class environmental contaminant, PAHs. Both compounds possess the capability for large scale environmental exposure, low molecular weights and a mechanism of phototoxicity that greatly increases the acute toxic effect of the compound in aquatic environments exposed to UVR. Further research would be necessary to compare the phototoxicity of both compounds to determine if PAHs could be used as a template with which how to address TiO$_2$ NP contamination. Due to the lipophilic nature and lipid peroxidation during ROS generation, body burdens are an accurate predictor of PAH toxicity. TiO$_2$-NP however, waterborne concentration is a better predictor of toxicity when compared to body burdens.
Uptake data has demonstrated that *D. magna* are not only capable of building up body burdens of TiO$_2$.NP, they demonstrate the potential to re-suspend TiO$_2$.NP in solution at ppb concentrations. Stability data showed that water column concentrations of TiO$_2$.NP dropped drastically after two hours of exposure, which is when the peak body burden of TiO$_2$.NP was measured. In a more natural setting, such as a flowing river, the TiO$_2$.NP would be less likely to settle out of solution and remain suspended for longer periods of time. While the NPs themselves are non-polar in nature, but research has demonstrated the ability for NP to be transported into the cytoplasm, which could lead to an accumulation in tissue under constant exposure. Further researcher is needed to determine a body burden model for a constant flow through exposure and how this would relate to work by Zhu et al. and how this body burden would be transferred through the aquatic food web.

TiO$_2$.NP uptake is directly proportional to the amount of NP suspended in the water column at the time of contamination. Thus far, models have accounted for industrial runoff, accidental exposure and sewage plant operation as mechanisms of exposure. One likely mechanism of constant exposure and reexposure might be the drying and sale of solid sewage sludge as organic fertilizer. These fertilizers are applied in commercial and personal irrigation settings. In this small environment, terrestrial plants and organisms may become exposed to TiO$_2$.NP in a phototoxic manner. If not, the possibility exists by which agricultural runoff may function as a constant source of TiO$_2$.NP in aquatic ecosystems that could possibly increase concentrations of TiO$_2$.NP in the water column. Previous research with PAHs has shown that the phototoxic effect during co-exposures to different compounds of PAH were additive in nature and not
synergistic. When examining TiO$_2$ NP concentrations in large bodies of water such as rivers, lakes, estuaries, and Shallow Ocean environments it would be pertinent to determine the nature of phototoxicity when organisms were exposed to TiO$_2$ NP, PAH, and UVR as it is likely that this will occur due to the widespread nature of environmental contaminants.

It was observed during the exposure that the organisms demonstrated photo-avoiding behavior. Organisms in control dishes would seek the ‘shadiest’ part of the exposure dish and descent to the lowest part of the dish itself. TiO$_2$-NP exposed organisms also relocated to the bottom of the dish, but they did not attempt to seek out the shaded areas of the crystallization dish. Many of them organisms remained resting, covered in the TiO$_2$-NP. Due to the surface area of the NP themselves, the waterborne exposures were exposed to a greater bio-reactive surface area (Nel et al., 2006) than their uptake and control counterparts. This increased surface area would increase the interface between the oxygen rich water and the TiO$_2$-NP exponentially increasing the organism’s exposure to ROS, which may be responsible for the increased toxicity seen in waterborne concentrations. One explanation for the reduced photo-avoidance is the fact that the light sensing abilities of the daphnid’s eye were reduced due to ROS damage from exposure to TiO$_2$-NP. Further research could be done examining if TiO$_2$-NP organisms demonstrate a reduced ability to daphnid to sense incoming light in order to avoid it.


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