

## ABSTRACT

Effects of Titanium Dioxide Nanoparticles on Bacteria Community Composition Under Snail Grazing Pressure

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In 2013, the King and Matson labs at Baylor University conducted an outdoor stream mesocosm study at the Baylor Experimental Aquatic Research (BEAR) facility using wastewater treatment plant effluent and P25 titanium dioxide nanoparticles (TiO<sub>2</sub>NPs). A study of the effects of snail grazing and TiO<sub>2</sub>NPs on the algal and bacterial components of the periphyton in the top pools showed that bacterial cell densities were unaffected by the presence of the TiO<sub>2</sub>NPs, but the algal cell densities were affected in the low dose streams. In order to explore the mechanisms driving the trends observed, an indoor stream microcosm (Baby BEAR) was conducted. Two species of snails were used, and the periphyton was exposed to similar concentrations of TiO<sub>2</sub> nanoparticles that were used in the outdoor mesocosm study. 16S rRNA sequencing was used to identify 17 bacterial orders that increased in the treated streams relative to the controls.

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EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES ON BACTERIA  
COMMUNITY COMPOSITION UNDER SNAIL GRAZING PRESSURE

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## CHAPTER ONE

### Introduction

Wastewater treatment plant effluent is often prevalent in streams, especially those that are periodically exposed to drought. With the recent and continuing growth of nanotechnology, engineered nanomaterials have been produced and incorporated into a variety of products and processes in a number of industries; as a result, industrial and manufacturing discharges can lead to an increase in the amount of nanoparticles that find their way into the environment (Robichaud et al. 2009). The release of nanoparticles into the environment ultimately leads to their residence in natural waters, where their fate and potentially hazardous effects are not yet well understood (Weinberg et al. 2011; Farre et al. 2011).

Nanoparticles have several unique properties that differentiate them from their bulk forms, including small size, a high surface area to volume ratio and their ability to interact with biological membranes (Clemente et al. 2012 and Navarro et al. 2008). Because of this, nanoparticles are being increasingly utilized to produce a wide variety of products. The proliferation of materials and products as a result of continued developments in nanotechnology is expected to increase the amount of engineered nanoparticles released into the environment, where their unique properties could have a negative impact. Titanium dioxide nanoparticles (TiO<sub>2</sub>NPs) are one of the most abundantly produced nanoparticles in the world due to its photoreactivity, high refractive index, and transparency (Kulacki et al. 2012). It is used in the production of paints, coatings, sunscreens, as well as many other products.

Illumination of TiO<sub>2</sub>NPs with ultraviolet light results in ultra-band gap excitation that triggers the production of reactive oxygen species, which are powerful oxidizing agents that can damage various cell components including the DNA of the cell (Binh et al. 2014). As a result, there is growing concern that TiO<sub>2</sub>NPs will have a negative impact on many organisms in aquatic systems (Binh et al. 2014).

Because of the direct association between industrial discharges and the transport and fate of TiO<sub>2</sub>NPs, freshwater ecosystems are the main aquatic reservoirs for TiO<sub>2</sub>NPs. While effluent from such discharges may only contain a small portion of the total amount of TiO<sub>2</sub>NPs released into the wastewater treatment process, they do have the potential to negatively impact freshwater ecosystems (Kiser et al. 2009). Periphyton, a community of algae, bacteria, and fungi attached on hard substrates in streams, often contributes to the base of the food web in stream ecosystems (Tlili et al. 2011). As a result, this complex assemblage of organisms bioaccumulates nutrients and contaminants from the water column (Hill et al. 2010).

Herbivores such as snails are an important link between the base of the food web and higher trophic levels (Wallace and Webster 1996). In addition to affecting lower trophic levels through their grazing activity, snails can impact higher trophic levels by accumulating toxicants through their diet. One 72-hour study showed that snails accrued high concentrations of TiO<sub>2</sub>NPs as a result of dietary exposure to periphyton that contained high levels of contaminant (Kulacki et al. 2012). Snails can have a significant impact on periphyton biomass and community structure by grazing on the periphyton and exposing less tolerant cells within the periphyton matrix to contaminants such as TiO<sub>2</sub>NPs (Feminella and Hawkins 1995; Munoz et al. 2001). This affects the ability of the periphyton to retain and transport nutrients and other substances from the water

column (Sabater et al. 2002). Thus, increased snail grazing activity could lead to the decrease in periphyton biomass and a lower uptake of nutrients, while decreased snail grazing activity could result in an increase in the uptake of nutrients and other substances such as toxicants. Thus, the exposure of periphyton to grazing as well as TiO<sub>2</sub>NPs represent two distinct environmental stressors that can have negative effects within stream ecosystems.

An outdoor stream dosing study with TiO<sub>2</sub>NPs was conducted in 2013, part of which examined the interactions between periphyton and TiO<sub>2</sub>NPs under snail grazing pressure. Approximately one week before the beginning of the study, two hundred snails of genus *Elimia* were placed in each of the top pools of twelve outdoor stream mesocosms. It was expected that the low dose streams would result in a slight decrease in bacterial cell densities as compared to the controls, and that the high dose treatment would result in a much larger decrease in bacterial cell densities as compared to the controls. However, by day 27 of the study the low dose streams had significantly reduced bacterial cell densities compared to both the controls and high dose streams, which suggests that high doses of TiO<sub>2</sub>NPs may somehow be inhibiting snail grazing activity.

The purpose of the present study is to further investigate the mechanisms responsible for the trends observed in the previous study by conducting an indoor stream mesocosm study and analyzing changes in bacterial mortality as well as changes in bacteria community composition. Recent studies investigating the bacterial community response to TiO<sub>2</sub>NPs indicate that aquatic bacterial communities can vary significantly in response to TiO<sub>2</sub>NP exposure, which alters the composition of the bacterial community (Binh et al. 2014). This can result in changes in bacterial diversity as concentrations of TiO<sub>2</sub>NPs increase, since the less tolerant bacteria are more susceptible to the cytotoxic

effects of TiO<sub>2</sub>NPs. Thus, I hypothesize that TiO<sub>2</sub>NP exposure and snail grazing will alter the bacterial community structure due to the selection for more tolerant bacterial species.

## CHAPTER TWO

### Methods

For this study twenty-four indoor stream channels were assembled. Each channel was 10 cm deep, 9 cm wide, and 1 m long. The channels were supplied with EPA moderately hard synthetic water and lined with unglazed porcelain tiles, which were seeded with periphyton and stocked with 25 snails of the genus *Physa* and 35 snails of the genus *Elimia*. The water was maintained at a temperature of 23°C and a depth of 5 cm. Water was continuously recirculated throughout the course of the experiment. Three concentration groups were used, including a control, low, and high concentration group. The control group contained no titanium dioxide nanoparticles, the low concentration group contained a titanium dioxide nanoparticle concentration of 50 ppb ( $\mu\text{g L}^{-1}$ ), and the high concentration group contained a titanium dioxide nanoparticle concentration of 500 ppb ( $\mu\text{g L}^{-1}$ ). Each of the concentrations was renewed weekly with a 50% water replacement. PowerVeg T5 lights were used to cover the active range of titanium dioxide (245-380 nm) and photosynthetically active radiation (400-700 nm). A broad spectrum spectrophotometer was used to measure light intensity.

Periphyton samples were collected on days 0, 7, 14, and 28. Two strips of 3 tiles were collected at days 0 and 7, and three strips of tiles were collected at days 14 and 28 from randomly selected sections of the streams and placed in Whirl-pak storage bags, and immediately stored in a -80°C freezer until DNA extractions could be performed. The frozen tile strips were later partially thawed for DNA extractions. The DNA was extracted from the periphyton samples using the PowerBiofilm DNA Isolation Kit

(MoBio Laboratories). The DNA samples were sent to the Duke University Marine Lab for PCR and library prep and then to the Duke Sequencing Facility for 16S rRNA sequencing, using the protocol by Yung et al. (2016) with 30 cycles and Econo Taq DNA Polymerase. The forward primers used were SA501, SA502, SA503, SA504, SA505, SA506, SA507, and SA508. The reverse primers used were SA701, SA702, SA703, SA704, SA705, SA706, SA707, SA708, SA709, SA710, SA711, and SA712. Sequencing depth was between 725 and 615,569 reads. Analysis of community composition and community change based on the 16S rRNA sequences from day 28 of the study was performed using the software R.

The “metaMDS” function in the “vegan” package of R was used to complete nonmetric multidimensional scaling (NMDS) on the bacteria community data. The points were graphed using the “ggplot” package (Figure 1). A permanova test for differences in bacteria community composition was performed with the “adonis” function in the package “vegan”. The function “vegdist” was used to calculate the dissimilarity indices, and the Bray-Curtis dissimilarity was used to calculate the index. The function “betadisper” was used to test the data for overdispersion using the homogeneity of multivariate dispersions. ANOVA was used to compare the control and treatments. The multivariate generalized linear model or “manyglm” function in the “mvabund” package of R was used to model the bacterial taxonomy data, and to fit the bacterial count data to a generalized linear model with a negative binomial function. The model was evaluated using the plots of the fitted values of the model versus the residuals and the results of the test for overdispersion. Univariate tests for each bacterial order were obtained by performing ANOVA on the model summary in order to determine which orders contributed to the generalized linear model.

## CHAPTER THREE

### Results

Samples from three of the streams, two from the high dose streams and one from the control streams, could not be amplified, possibly due to low biomass. Ordination of the taxonomy data (stress = 0.162) demonstrated that there were differences in the bacterial community composition (permanova, Table 1;  $p = 0.004$ ). The taxonomy data was fit to the multivariate generalized linear model with a negative binomial distribution function (Table 2;  $\text{manyglm } p = 0.000999$ ). Seventeen bacterial orders that contributed significantly to the model fit were identified (Table 3).

	Degrees of freedom	Sum of squares	Mean Squares	F model	R2	p-value
Treatment	2	0.21512	0.107561	2.22078	0.19699	0.004
Residuals	18	0.87692	0.048718		0.80301	
Total	20	1.09204	1		1.00000	

**Table 1. Output of permanova analysis (with 999 permutations performed) “Adonis” function in R. Treatment was the predictor variable and bacterial order was the response variable, with distances between the streams calculated using the Bray-Curtis dissimilarity.**

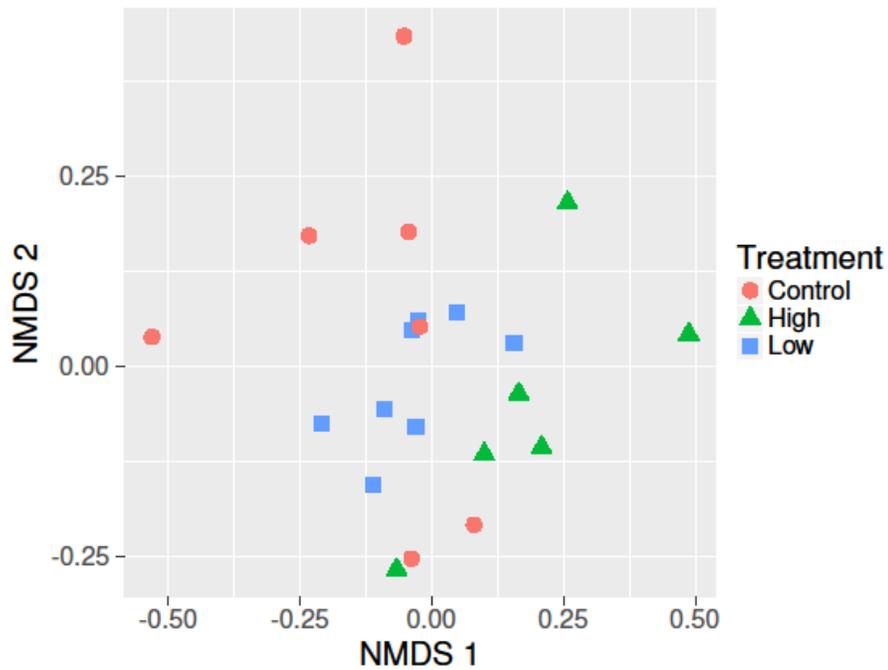


Figure 1.

Table 2. Output summary of the multivariate generalized linear model (manyglm in the mvabund package in R) with a negative binomial distribution. The p-value was determined using 1000 resampling iterations using probability integral transform (PIT) residual.

	Wald-test value	p-value
High dose treatment	19.02	0.001998
Low dose treatment	13.37	0.131868
Test Statistic: 237.55, p-value = 0.000999		

**Table 3. Individual taxa that were significantly affected by the treatment from the multivariate GLM.**

<b>Bacteria order</b>	<b>p-value</b>	<b>Direction of change</b>
Acidomicrobiales	0.01	Increased in high dose
Anaerolineales	0.008	Increased in low and high dose
Bifidobacteriales	0.002	Increased in high dose
Brocadiales	0.02	Increased in high dose
Burkholderiales	0.001	Increased in high dose
Caldilineales	0.013	Increased in low and high dose
Chromatiales	0.022	Increased in high dose
Chroococcales	0.035	Increased in high dose
Legionellales	0.043	Increased in high dose
Nitrospirales	0.022	Increased in low and high dose
Oscillatoriales	0.025	Increased in high dose
Planctomycetales	0.008	Increased in low dose
Pseudanabaenales	0.001	Increased in high dose
Puniceococcales	0.034	Increased in high dose
Thermanaerobacterales	0.005	Increased in high dose
Thiobacterales	0.047	Increased in low dose
Xanthomonadales	0.018	Increased in low dose

## CHAPTER FOUR

### Discussion

The results show that there was a significant difference in bacterial community composition in the low and high dose streams as compared to the control, indicating that the presence of TiO<sub>2</sub> NPs has a significant impact on bacterial community composition. Seventeen orders were identified that had significantly increased in the treated streams relative to the control. A few of these orders, including Chroococcales, Oscillatoriales, and Pseudobaenales, belong to Phylum Cyanobacteria. Cyanobacteria is the largest and most diverse group of photosynthetic bacteria, and is particularly successful in establishing symbiotic relationships with other organisms such as protozoa and fungi (Prescott, Harley, and Klein 2004). This is noteworthy because protozoa and fungi are components of periphyton, indicating that the orders belonging to this phylum may have thrived as a result of an established relationship with the periphyton matrix. In addition, species of Oscillatoriales have been found to be dominant in heavily polluted streams and are indicative of degraded environments (Abed et al. 2002 and Pinedo et al. 2007).

Several of the orders identified belong to Phylum Proteobacteria, which contains bacteria that vary considerably in morphology, physiology, and metabolism (Prescott, Harley, and Klein 2004). In addition, several studies have suggested that Proteobacteria are dominant in metal-contaminated sediments (Gillan et al. 2005, Hemme et al. 2010, and Roosa et al. 2014). Orders Burkholderiales, Chromatiales, Legionellales, and Xanthomonadales belong to Phylum Proteobacteria, which is divided into five classes.

Order Burkholderiales belongs to Class Betaproteobacteria, and is diverse phenotypically, metabolically, and ecologically (Garrity et al. 2015). It contains aerobic and facultative anaerobic chemoorganotrophs, obligate and facultative chemolithotrophs, nitrogen-fixing organisms, and plant, animal, and human pathogens (Garrity et al. 2015). Members of Burkholderiales are microaerophilic and tolerant to oxidative stress, and some species, such as *Burkholderia metalliresistents* and *Herminiimonas arsenicoxydans*, have been shown to be metal-resistant, which could explain why Order Burkholderiales increased in the treated streams despite exposure to TiO<sub>2</sub>NPs (Doi et al. 2014, Guo et al. 2015, and Muller et al. 2006).

Orders Chromatales, Legionellales, and Xanthomonadales belong to the class Gammaproteobacteria. Order Chromatales contains purple sulfur bacteria, which oxidize sulfide during anoxygenic photosynthesis and are typically found in anoxic zones of lakes (Schneider et al. 2013). Members of Chromatales are microaerophilic, anaerobic, phytopathogenic, and phototrophic (Bertrand et al. 2011). Order Legionellales contains the well-known intracellular pathogen *Legionella*, which is found naturally in freshwater environments and can grow in contaminated man-made water systems (“Legionella: Causes,” 2016). Legionellales organisms have also been shown to increase in association with Microcystis blooms (Huabing et al. 2011.) Xanthomonadales is one of the largest phytopathogenic bacterial groups and contains many human pathogens, but has no known molecular or biochemical characteristic (Cutino-Jiminez et al. 2010 and Naushad and Gupta 2013).

Two of the orders identified belong to the Phylum Actinobacteria, including Bifidobacteriales and Acidimicrobiales. Actinobacteria have been shown to correlate inversely with abundance of certain Cyanobacteria that are responsible for sustained

damage to freshwater ecosystems, suggesting that they could act as ecological sentinels (Ghai et al. 2014). Orders Planctomycetales and Brocadiales belong to Phylum Planctomycetes, which contains the annamox bacteria. Annamox organisms perform anaerobic ammonium oxidation, which is the reduction of ammonia to nitrate. One study found that, while there is limited evidence for the presence of annamox bacteria in freshwater ecosystems, annamox bacteria contributed significantly to nitrogen loss in a eutrophic lake, which suggests that the concentration of nitrate is fundamental to the development of annamox bacterial populations (Hu et al. 2011).

Order Nitrospirales, belonging to Phylum Nitrospira, is also involved in the nitrogen cycle. Nitrospirales organisms are nitrite oxidizers, and *Nitrospira*-like nitrite oxidizers have been found to be dominant in activated sludge of industrial wastewater treatment plants (Dionisi et al. 2002 and Juretschko et al. 1998). In addition, Nitrospirales members have been found to increase with depth in lake sediments, suggesting their involvement in iron reduction (Bar-Or et al. 2014.) The increase in annamox organisms as well as Nitrospirales organisms as a result of TiO<sub>2</sub>NP exposure suggests an increase in ammonia and nitrite available for nitrogen cycling.

The results demonstrate that the presence of TiO<sub>2</sub>NPs as well as snail-grazing pressure causes a shift in the composition of the microbial community. While previous studies have suggested that TiO<sub>2</sub>NPs impact microbial community composition (Battin et al. 2009 and Binh et al. 2014), this study identified seventeen bacterial orders that increased in response to the presence of TiO<sub>2</sub>NPs. Increases in these orders, including orders belonging to Cyanobacteria, Proteobacteria, and Nitrospirales, suggest that the presence of TiO<sub>2</sub>NPs causes a shift toward orders with organisms that are pollution-resistant, capable of establishing symbiotic relationships with components of the

periphyton matrix, metal-resistant, and metal-reducing. There were no significant decreases for any of the orders in the low and high dose streams, but it is possible that each of the non-significant decreases in the low and high dose streams taken together could have constituted a major difference. In addition, certain orders such as Rhodobacterales, Lactobacillales, and Flavobacteriales appeared to decrease in abundance in the high dose, but high variability prevented the GLM from recognizing significant differences. Thus, while our results suggest that the presence of TiO<sub>2</sub>NPs in addition to grazing pressure select for certain taxa, the high variability present in the bacterial endpoints indicates that there is still much to be done in characterizing the changes in bacterial community composition in response to TiO<sub>2</sub>NPs, and the impact of those changes in the environment.

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