

Titanium Dioxide (TiO₂) nanoparticles more strongly affect bacteria compared to algae in stream ecosystems

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Titanium dioxide (TiO₂) is a novel nano-particulate contaminant found in surface waters. Nano-TiO₂ is commonly used in numerous pharmaceutical and personal care products ranging from make-up to pill casings, and is an additive in food and household products. Despite the commercialized use of TiO₂, its increased presence in surface waters, and toxic effects on stream organisms, little information exists on how nano-TiO₂ affects stream ecosystems as a whole. We examined the effect of various concentrations (0.5 - 3 mg/L) of nano-TiO₂ on stream ecosystems by measuring the response of algal and microbial communities to acute (12 hr) and chronic (22 day) exposures. We measured gross primary production (GPP), community respiration (CR), and chlorophyll *a* (chl *a*) concentrations on intact algae from a local stream. We expected metabolic function of both algal and microbial components of the benthic biofilm to decline with exposure due to sensitivities to metal oxides. However, we found exposure to any of the concentrations of nano-TiO₂ tested caused CR to decrease compared to controls, but, GPP either increased or stayed the same as our controls. We found algal chl *a* concentrations to increase in the high exposure treatment. Since nano-TiO₂ had a negative effect on the microbes, we hypothesized that either autotrophs were released from microbial competition and increased chl *a* production, or that shading from TiO₂ particles may have caused increased chl *a* production. Additional studies investigating the effects of higher concentrations and longer exposure times to these compounds are warranted.

Keywords: aquatic; autotroph; contaminant; environmental; heterotroph; toxicology; nano-particles

Although advances in technology have allowed the developed world to keep pace with ever changing consumer, agricultural, and health demands, they have also introduced new sources and types of pollution. Such compounds that are associated with manufacturing often make their way into aquatic systems due to inefficient removal at wastewater treatment plants, storm runoff, or point sources, but the exact fate of these compounds are unknown (Daughton and Ternes 1999). Nanoparticles were developed to improve properties of a wide variety of products and services, such as pharmaceutical and personal care products that range from make-up to pill casings, additives in food, clothing, and household products (Arora et al. 2012). Once materials are created, nanoparticles can be discharged into aquatic systems by household waste, sewer, and surface flows (Rogers 1996, Benn and Westerhoff 2008). Nanoparticles are small (10-100 nm) and widely used in a range of consumer and industrial products (Lebedev 2013). Because of their nano-scale, these materials have a high surface area to volume ratio and are dramatically more reactive compared to larger bulk materials. For example, studies show that nanoparticles are more reactive and toxic because they can easily cross cell walls and accumulate in tissues (Hristozov and Malsch 2009). This rapidly expanding field of nanotechnology coupled with the persistence of particles in aquatic systems and high detection limits pose potential risks to biological ecosystems. According to Consumer Products Inventory, 1,814 nano-enabled consumer products were identified in the commercial market as of March 2015. This current listing of nano-enabled products has shown a thirty fold increase from the original listing of 54 products in 2005 (Vance et al. 2015).

Wastewater effluent has been found to contain anywhere from less than 5µg up to 15 µg of nano-TiO₂ per liter after treatment (Kiser et al. 2009). Although this number may seem insignificant, these levels have been found across a range of treatment plants, which means that nano-TiO₂ is entering waterways from a large number of different sources.

Furthermore, influent levels of nano-TiO₂ to treatment plants were found to range between 181 to 1233 µg/L (Westerhoff 2011). An average of 98.3% of incoming titanium is removed during treatment, however, not all wastewater makes it through treatment plants (Westerhoff 2011). In major cities such as New York, water surges during large storms necessitate that some wastewater bypasses treatment and is expelled directly into waterways using combined sewer overflows (CSOs) in order to avoid overloading the treatment plants (Homme 2011). Nano-TiO₂ also enters waterways more directly since it is a major component in a variety of commonly used products ranging from paints, sock and clothing fabrics and cosmetics. When people or watercraft enter waterways it is possible for TiO₂ to enter surface waters directly without undergoing treatment (Gottschalk 2009). Combining these input factors, concentrations of TiO₂ in the environment have the potential to increase in the coming years. Furthermore, TiO₂ levels can be expected to accumulate over time as more and more particles continually enter aquatic systems.

Nano-TiO₂ has been shown to have toxic effects on all levels of aquatic food webs, including fish (Federici et al. 2007), algae (Kulacki et al. 2012), and other organisms (Warheit et al. 2007). Nano-TiO₂ was shown to result in species-specific alterations to maximum potential growth rates of a number of different algal species examined (Kulacki et al. 2012). When rainbow trout were exposed to various concentrations of nano-TiO₂ a number of effects were observed including loss of position holding in the water column, which indicates fatigue and abnormal buoyancy control. Important sub-lethal effects were also found in trout including organ pathologies, biochemical disturbances, and respiratory distress (Federici et al. 2007). Overall, these findings indicate that while not lethal, nano-TiO₂ may alter a number of physiological aspects of aquatic organisms, which demonstrates a significant toxic response. The effects of nanoparticles on cells are very dependent on the type and size

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of the nanoparticle, and the health implications of most nanoparticles are still relatively unknown in humans. Factors such as size, chemical composition, and shape all have a great influence on the health implications of nanoparticles. One reason nanoparticles may be extremely harmful to the health of living organisms is the ease with which small nanoparticles can pass through cell membranes, where they have the potential to disrupt normal cellular functions (Andreescu et al. 2011). Studies have shown that nanoparticles could actively engage and intervene with cellular and molecular processes that are essential for the regulation of a cell such as cell activity and create toxic effects that lag behind exposure time (Panariti et al. 2012).

Although several studies target the biological toxicity of nanoparticles, few studies have been conducted that investigate the effects of nanoparticles on the environment and basal food-web organisms such as algae. Algae are primary producers that are a combination of both autotrophs and heterotrophs and regulate the nutrient dynamics of nitrogen, phosphorous, and carbon in stream water (Bechtold et al. 2013). In addition, because algae is at the base of the food web, any disturbance that affects algae will have wide reaching effects on invertebrates and fish. The goal of this study is to investigate the short-term and long-term effects of nano-TiO₂ on stream benthic algal communities in order to evaluate ecosystem response to this compound. We tested the response of algae to nano-TiO₂ on diffusing substrates (DS), and the response of algae to nano-TiO₂ added to the water column in chambers. We hypothesized that all exposure types will decrease photosynthesis and respiration of algae due to the ability of nano-TiO₂ to permeate through cell membranes and disrupt cellular activity. We either incubated algae *in-situ* on diffusing substrates in the stream, or in chambers (18 days) in the greenhouse. We measured change in dissolved oxygen, and/or chlorophyll *a* content. These experiments allowed for investigation of the potential acute and chronic effects over a range of nano-TiO₂ exposure levels.

Methods

We first tested how algae will respond to nano-TiO₂ exposure by deploying I.) diffusing substrates (DS) into the stream to allow for *in-situ* growth on exposed substrates, we then completed a chamber experiment in the greenhouse investigating how intact algae will respond to II.) long and III.) short-term TiO₂ exposure times. This design was to allow for natural growth of algae *in-stream* under exposed conditions, and then to test the response of intact algae to nano-TiO₂ in the water column. Both of these scenarios are likely to occur since wastewater treatment plants often have pulsed discharge of waters into stream systems, both algae with new growth, or intact algae would be exposed to these compounds. Our stream site Fishing Creek, is located in Mill Hall, PA, and is 24 miles downstream from a wastewater treatment plant and remains relatively undisturbed.

Experimental Design

I. *In-stream Exposure Experiment (diffusing substrates)*

We used diffusing substrates (DS) bioassays to determine the effect of nano-TiO₂ on algal and microbial growth as well as survival and function, since the substrates started out with

no growth prior to being placed in the stream. DS are constructed to slowly release different concentrations of nano-TiO₂ nanoparticles over time and to allow for algal accrual on the substrate attached to it.

Bioassays were constructed using small (120mL) polycarbonate plastic containers (US Plastics Corp) with hinged lids. We drilled 2.3cm diameter holes through the lids, and labeled them by etching treatment names into the cups. Our treatments were adapted from Gibeau and Miller's (1989) construction of similar diffusing substrates. We included three treatments: control treatment, a low (0.50mg/L) nano-TiO₂ treatment, and a high (3mg/L) nano-TiO₂ treatment; each treatment consisted of ten replicates either having fritted glass or sponge discs attached to the hole in the lid. Fritted glass discs target the autotrophic component of biofilm, while the cellulose sponges act as a carbon source such as leaves or organic matter in the stream, and target the heterotrophic component. We amended 2% agar with 0.25mg (0.50mg/L) or 1.5mg (3mg/L) for the two treatments. Solutions were stirred vigorously with a stir bar to ensure even distribution of the nano-TiO₂ and proper mixing of the agarose, and poured into labeled DS cups, cooled and either fitted with a fritted glass disk or sponge, then deployed into the stream attached to angle iron with zip ties. Bioassays were attached to the streambed of Fishing Creek in a sunny area, and left for 22 days.

After 22 days we retrieved the DS bioassays. We then removed the fritted glass disks and sponges from the bioassays and placed them into a glass chamber, filled with creek water and sealed so that no bubbles were inside the chambers. Initial dissolved oxygen was recorded and at 30 minute intervals. There was not enough creek water to fill all of the chambers, so tap water was added twice throughout to supplement the creek water. After each tap water addition we recorded the dissolved oxygen level. Once all of the chambers were filled, we incubated them under natural sunlight conditions for approximately two hours, after which we removed the lid and took a dissolved oxygen (DO) readings in mg/L and % saturated. The DO sensor was mounted such that readings were always taken at approximately 1 inch deep into the water.

Once all the DO readings were taken, we refilled jars with creek water, recorded initial DO, and incubated chambers under dark conditions for 2 hours in order to stop photosynthesis and allow for respiration to occur. After 2 hours, the lids were again removed, and the dissolved oxygen readings and time were taken and recorded. Glass substrates were collected in aluminum foil and placed in the freezer for chl *a* analysis.

After freezing for two days, the glass disks were removed from the aluminum foil and placed into small glass beakers. We extracted chl *a* from the glass substrates only (targeting the autotrophs) in 10ml of 71% isopropyl alcohol, placed in the refrigerator overnight to allow the chlorophyll *a* to fully extract into the alcohol and read on the spectrophotometer for absorbance levels at 664, and 750 nm before and after acidification to determine chl *a* concentrations (Hauer 2006) per area of substrate. Data was averaged in order to determine the effects of each treatment.

II. *Short-term Chamber Experiment*

We completed two chamber experiments in the greenhouse investigating how intact algae will respond to long and short-term TiO₂ exposure times. This design was to allow

to test the response of intact algae to various concentrations of TiO₂ in the water column. We collected rocks with intact algae from Fishing Creek, placed them in chambers with creek water streamside and brought them back to the greenhouse for incubations. Our treatments included 10 control replicates with no nano-TiO₂ added, and 5 controls with organic matter added (leaves) to provide a substrate for microbes. Ten replicates were constructed for low concentrations concentration (0.5mg/L), high concentration (3mg/L), and 5 replicates with Low and High concentrations plus organic matter added. We also had 6 chambers as blanks which contained only creekwater with no rocks or treatments added.

Substrates were then incubated in the chambers for 2 hr in the light and 2 hrs in the dark to obtain change in dissolved oxygen measurements in order to determine metabolism, similar to what was described above in the stream experiment. After the incubations, algae was scrubbed from the rocks using a toothbrush. The algal slurry water was collected, filtered, wrapped in foil, and placed in a freezer for chl *a* analysis.

After all of the algae was collected, the frozen filter papers were placed in small beakers and submerged in 10 mL of 71% isopropyl alcohol according to the same methods above in the stream study. We determined the surface area of the rocks by taking a picture of the individual rocks next to a ruler, and using the ImageJ (<http://imagej.nih.gov>) program to determine the surface area. We calculated changes chlorophyll concentration per area of substrate, and averaged the replicates for each treatment.

II. Long-term Chamber Experiment

The long-term chamber experiment tested the effects of exposure to higher concentrations of nano-TiO₂ on rock algae for longer time periods. Treatments included: control, 50mg/L, 100mg/L, 200mg/L, and 300mg/L nano-TiO₂ concentrations, each replicated five times. Rocks with attached algae were collected from Fishing Creek and placed immediately into chambers with the correct nano-TiO₂ dosage. In addition, we collected rocks from the stream and analyzed them for a baseline concentrations of chl *a* before treatments.

Chambers were placed on a shaker table under natural and supplemented with artificial full spectrum lights for 18 days. However, on day seven, chambers were opened and algae was sub-sampled from each rock by scrubbing an area of 1.11 cm² using a homemade periphyton sampler. The chambers were then re-sealed and placed back on the shaker for the remaining time. The sub-sampler was made from a standard 10 mL syringe that was cut and glued to a rubber ring. The rubber sampler acted to create a seal on the rock while water was placed in the syringe and a modified toothbrush was inserted into the syringe so only the specified area of the rock was scrubbed. The water in the syringe was then pipetted out of the periphyton sampler and filtered, and frozen until chlorophyll *a* analysis could be determined.

At the end of the 18-day incubation, we removed the chambers from the shaker table and scrubbed the entire rock surface area as previously described. The algae from each rock was filtered and frozen until chlorophyll *a* analysis. Chlorophyll *a* was then extracted in 71% isopropyl alcohol overnight and read on a spectrophotometer at 750 and 664 nm before and after acidification. We determined rock area by taking a picture of the individual rocks next to a ruler, and using the ImageJ (<http://imagej.nih.gov>) program to determine the

surface area and calculated averages of response variables for each treatment.

Statistical Analysis

To test whether chl *a*, gross primary production, and community respiration in bioassay and chamber experiments were significantly affected by nano-TiO₂ exposure, we used a two-way analysis of variance (ANOVA) to test the effects of nano-TiO₂ on each response metric (GPP, R, or chl *a*) and separately substrate type (glass, sponge). We compared the response variables (GPP, R, chl *a*) to controls using T-tests. Statistical analyses were performed using SYSTAT 12 (Systat Software, Richmond, California, USA) with a level of statistical significance at alpha 0.05. We tested for normality ($P > 0.05$) using a Kolmogorov-Smirnov (Lilliefors) test.

Results

We found that across a range of exposure concentrations, nano-TiO₂ can negatively affect some ecosystem processes both with long-term and short-term exposure times.

In-stream Exposure Experiment (diffusing substrates)

Our diffusing substrate bioassays were deployed in stream for 22 days. We found no difference ($p = 0.14$) in biofilm chlorophyll *a* concentration between low (0.25 mg/L) nano-TiO₂ exposure and control (Figure 1), but at high exposure levels (1.5 mg/L), chlorophyll *a* increased compared to controls ($p = 0.05$). Community respiration (CR) was significantly negatively affected by exposure to both low ($p < 0.001$) and high doses ($p < 0.001$) of nano-TiO₂, while GPP was only affected at low exposure ($p = 0.003$) but not at high levels ($p = 0.379$) compared to controls (Figure 2).

Short-term Chamber Experiment

Stream substrates with intact biofilm were placed in chambers for 24 hours with different exposure levels. We found that gross primary production nor CR was affected by the addition of any level of TiO₂ ($p > 0.05$), but that community respiration (CR), although CR declined although not significantly (Figure 3). Chlorophyll *a* was not affected in this short time of exposure ($p > 0.05$).

Long-term Chamber Experiment

Stream substrate with intact biofilm was then placed in chambers for 18 days and exposed to a gradient of nano-TiO₂ concentrations. We found differences between treatments compared to controls (overall ANOVA, $p = 0.06$, Figure 4). Specifically, we found chl *a* concentrations at the lowest level (50 mg/L) did not change compared to controls ($p = 0.10$). However, at 100 mg/L, 200 mg/L, and 300 mg/L concentrations we found chl *a* to be significantly different than the control (all $p < 0.03$, Figure 4). There was a 128% increase in chlorophyll *a* at exposure to 100 mg/L nano-TiO₂ compared to controls, but then chl *a* declined at 200 mg/L and 300 mg/L exposure levels, however, concentrations were still 101% and 75% higher than controls.

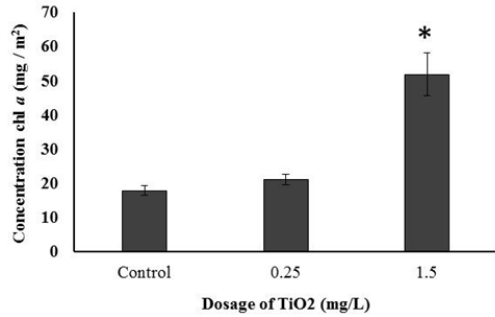


Figure 1: Benthic biofilm chlorophyll *a* increased when exposed to higher concentrations of nano-TiO₂, but were unaffected at lower concentrations on diffusing substrates deployed in-stream.

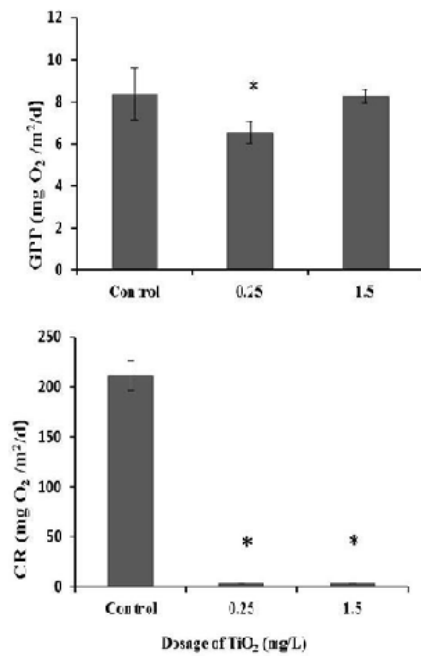


Figure 2: In-stream response of gross primary production (GPP) and community respiration (CR) of benthic biofilm to nano-TiO₂ at low (0.25 mg/L) and high concentrations (1.25 mg/L) over a 22 day deployment of diffusing substrates.

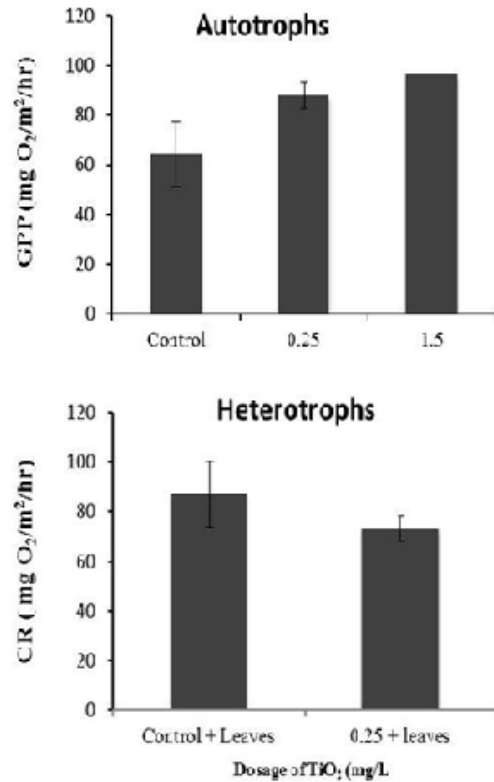


Figure 3. Short-term response (24 hr) of intact stream biofilm found on rocks. Gross primary production (GPP) and community respiration (CR) responded differently to the addition of nano-TiO₂ in chambers.

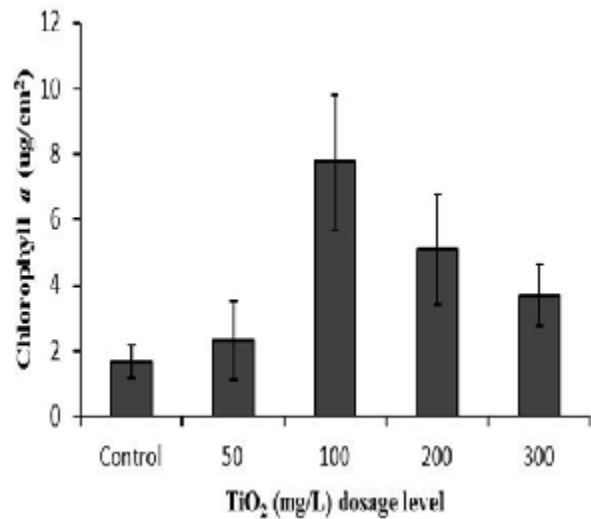


Figure 4: Chronic exposure of biofilm to a range of nano-TiO₂ concentrations in chamber experiments over 18 day exposure time.

Discussion

Exposure to nano-TiO₂ was found to have a more

pronounced effect on the response of heterotrophs compared to autotrophs. We found that long-term exposure to nano-TiO₂ increased benthic chl *a* compared to controls, particularly at higher concentrations. We also found metabolism to be more sensitive than chl *a* as a response measure to both short-term and long-term nano-TiO₂ exposure.

Our findings report that GPP slightly increased in response to even low doses of nano-TiO₂. This result was surprising as nanoparticles have been shown to be toxic to a number of aquatic organisms. We speculate, that the increase in algal production (GPP) may arise from algal compensation to the decline in bacteria in the biofilm community. Bacterial groups are likely more sensitive to exposure to nanoparticles than algal groups, and loss of bacteria may act to release algae from its competitive ties within the biofilm, causing an increase in chl *a* production. Previous studies have shown that nanomaterials can have antibacterial effects and can bind to the bacteria cell membranes causing a change in cellular activity and function (Aslani et al. 2014, Tong et al. 2013). Studies have found that nano-TiO₂ can be species specific in bacteria and act as a selective agent and decrease the diversity within bacterial communities (Binh et al. 2014). In addition, the autotrophic component of biofilm is namely comprised of diatoms, which have a hard silica shell surrounding membranes (Conley 1997), these shells may act as a stronger barrier to the entry of nanoparticles compared to bacteria. Although, nano-TiO₂ has been shown to inhibit the growth of freshwater algae but can vary with species type (Cardinale et al. 2012). Our findings suggest that the increased response of GPP from the autotrophs after exposure to nano-TiO₂ may be due to both an algal tolerance and a bacterial/fungal sensitivity to exposure and thus a release of algae from competition with heterotrophs.

We found GPP and CR to be less affected by short-term compared to long-term exposure times. The short-term experiments were 12 hrs long, and this may have not been enough time to change biotic activity or for the nano-TiO₂ to react with the biofilm community. However, both GPP, CR and chl *a* responded to long-term exposure to nano-TiO₂.

Algal chl *a* increased after exposure to a wide range of concentrations to nano-TiO₂, and then declined at 200 mg/L (Figure 4). We hypothesized that the reason for the initial increase in chlorophyll was due to shading caused by nanoparticles resulting in increased chlorophyll production. We attribute this response to the overcompensation of chlorophyll production by the algal cells to increased shading caused by the higher levels of nano-TiO₂ coating the biofilms. When algal cells are shaded, algae increases cellular chlorophyll in response to the low light conditions. Because this effect was not observed with the significantly higher concentrations, it may be that the nanoparticles overwhelmed the algae, causing them to die off, or stop producing chlorophyll all together since they were receiving very minimal light. The decline in chl *a* at high concentrations may signal a threshold of sensitivity, where high levels became toxic to or blocked out all light reaching algal cells. An investigation conducted by Kulacki and Cardinale (2012) suggested that TiO₂ nanoparticles could increase the maximum biomass of certain species of diatoms, green algae, and cyanobacteria. They suggested that the increase in maximum biomass may be due to competitive interactions between algae and microbes for limiting nutrients within the environment. They also found that nano-TiO₂ had little consistent affect on algal growth rates,

some species in the experiment responded positively to the increasing nano-TiO₂ concentration, while other species responded negatively. Yet, other studies have reported that TiO₂ nanoparticles inhibit growth of green algae (Cardinale et al. 2012). Our findings suggest that the response of algal biomass is dependant on concentration of nano-TiO₂ in the water.

Variation between results between previous research and our experiments may be due to the size and type of particles used which can affect the reactivity of the particles with cell membranes and thus alter how strongly it may influence microorganisms response. Additionally, TiO₂ is photoactive in the presence of UV radiation, reactive oxygen species (ROS) which have the capability to break down natural organic matter and free nutrients that could encourage the growth of algae and bacteria (Tong et al. 2013).

Previous studies have also conducted experiments to assess the toxicity of nano-TiO₂ on bacterial communities in the environment. Nano-TiO₂ was shown to have a significant effect on the relative abundance of viable bacterial cells in communities found in areas previously exposed and not exposed to nano-TiO₂. Certain bacterial phyla were a great deal more sensitive to the concentrations of nano-TiO₂ than other bacterial phyla suggesting that bacteria that were most resilient in recovery after exposure, which may be due to prior exposure to pollutants from areas such as wastewater treatment plants (Binh et al. 2014). These studies also suggested that the exposure to this and other toxins can select for tolerant species, and would decrease the diversity and alter the bacterial composition in the various communities. Since the bacterial composition was unknown in this study, we do not know if the concentrations of nano-TiO₂ affected bacterial or algal diversity; however, the results from this study do clearly demonstrate a decrease in microbial activity, which does not rule out the possibility of a decreased bacterial diversity.

This study suggests that increased concentrations of nano-TiO₂ may be beneficial to autotrophs, while also having more deleterious effects on bacteria. Investigation into the ecological effects of novel contaminants, like nanoparticles, are crucial to understanding how new technologies may influence ecosystem level processes that may alter food-webs, public safety, and stream health.

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