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Environmental Toxicology and Chemistry, Vol. 31, No. 10, pp. 2414–2422, 2012

Printed in the USA
DOI: 10.1002/etc.1962

HOW DO STREAM ORGANISMS RESPOND TO, AND INFLUENCE, THE CONCENTRATION OF TITANIUM DIOXIDE NANOPARTICLES? A MESOCOSM STUDY WITH ALGAE AND HERBIVORES

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(*Submitted* 14 *March* 2012; *Returned for Revision* 30 *April* 2012; *Accepted* 22 *June* 2012)

Abstract—The biologically active properties of many nanomaterials, coupled with their rapidly expanding production and use, has generated concern that certain types of nanoparticles could have unintended impacts when released into natural ecosystems. In the present study, the authors report the results of an experiment in which they grew three common species of stream algae as monocultures and together as polycultures in the biofilms of stream mesocosms that were exposed to 0, 0.1, or 1.0 ppm nanoparticle titanium dioxide ($nTiO_2$). The $nTiO_2$ did not alter the growth trajectory of any algal biofilm over 10+ generations. However, Ti accrual in biofilms not only differed among the algal species but was also higher in polycultures than in the average monoculture. Variation in accrual among species compositions was readily predicted by differences in the total biomass achieved by the different biofilms. When biofilms were fed to the herbivorous snail *Physa acuta* at the end of the experiment, initial concentrations of $nTiO_2$ did not alter short-term rates of herbivory. However, because of differences in palatability among the algae, biofilm composition influenced the amount of $nTiO_2$ that accumulated in the herbivore tissue. The results have important implications for understanding how efficiently $nTiO_2$ is removed from surface waters and the potential transfer of nanomaterials to higher trophic levels. Environ. Toxicol. Chem. 2012;31:2414–2422.

Keywords—Nanoparticle TiO_2 Metal oxide Stream *Physa acuta*

INTRODUCTION

Nanotechnology—the ability to manufacture and control the physical behavior of particles on the scale of less than 100 nm—has opened the door to a new industrial and medical revolution. The production of engineered nanoparticles has increased exponentially over the past decade, and the number of consumer products that now use them is proliferating at a rate of nearly one per day [1]. Most of these products take advantage of the unique physical properties of engineered nanoparticles—their high surface area to volume ratios that enhance reactivity, their abnormally high strength to mass ratios, and their ability to interact with biological membranes [2,3]. But many of the properties that make engineered nanoparticles so promising from a technological perspective also have the potential to exert adverse impacts on biological systems [4–6]. The potential for adverse biological effects has prompted several governments to proactively fund research on the health and environmental impacts of nanomaterials [7,8]. In addition, the field of ecotoxicology has moved rapidly to expand its traditional set of models to include new behaviors and modes of toxicity that are unique to nanoparticles [9,10].

Here, we report the results of a laboratory study that examined how stream algae and herbivores simultaneously respond to, as well as influence, exposure to titanium dioxide nanoparticles ($nTiO_2$) in the benthic habitats of a large set of stream mesocosms. This is one of the most abundantly produced nanoparticles in the world, with annual production estimates in the tens of thousands of tons per year in the United States alone [10,11]. It has increasingly been used in the production of solar cells, paints, glass coatings, and personal healthcare products (e.g., sunscreen and cosmetics) [12] to name a few. The widening uses of $nTiO_2$ are primarily driven by its photoactivity, high refractive index, and transparency in the nanoparticulate form. The amount of $nTiO_2$ being released into the environment is not yet clear, but some models predict that Ti concentrations in freshwater habitats of developed nations have reached 0.7 to 16 μg/L [10], with Ti in urban runoff being as high as 600 μg/L [13]. Such concentrations are still well below levels of exposure that have been shown to be toxic to certain types of microbes [14,15] and to pelagic algae [16,17]. However, by comparison, little work has examined the impact of $nTiO_2$ on benthic ecosystems such as those in streams. Benthic habitats have the potential to be particularly prone to high exposure because most metal oxide nanoparticles have high sedimentation rates [18].

To examine how benthic organisms simultaneously respond to and influence $nTiO_2$ in streams, we manipulated the composition of species of freshwater algae growing in the biofilms of 81 recirculating stream channels at the University of California–Santa Barbara's freshwater flume facility (Fig. 1). Prior studies in this facility have focused on how the composition of biofilms influences the productivity of streams and their potential to remove nutrient pollutants from water [19]. In this facility, we established streams with five types of biofilms: algae-free controls with no focal algal species (i.e., prokaryotes only), monocultures of each of three common species of stream

All Supplemental Data may be found in the online version of this article.
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Published online 30 July 2012 in Wiley Online Library (wileyonlinelibrary.com).

Fig. 1. Experimental flumes used in the present study. (**A**) We manipulated the presence or absence of three types of common stream algae in a set of 81 recirculating laboratory flumes and then exposed the developing biofilms to three initial concentrations of titanium dioxide (TiO_2) nanoparticles (0, 0.1, or 1.0 ppm). (**B**) Over the course of 32 d, or approximately 10 generations of cell division, we sampled biofilms from "tape-strips" that were placed on the polyvinyl chloride growth surfaces in each flume. This allowed us to collect time series of biofilm biomass without destructive sampling. (**C, D**) After 32 d of growth, 23 individual *Physa acuta*, a common freshwater snail, were added to flumes and allowed to feed for 72 h. **C** and **D** give one example of algal biomass before and after snail addition.

algae (the Chlorophycean green algae *Stigeoclonium tenue*, *Scenedesmus quadricauda*, and the diatom *Synedra ulna*), and polycultures where all three algal species were grown together. Five to seven replicate streams of each biofilm were exposed to 0.1 and 1.0 ppm $nTiO_2$, and there was an $nTiO_2$-free control; $nTiO_2$ was added to the stream water in nanoparticulate form at the beginning of the experiment. We then tracked the accumulation of biomass in the biofilms for approximately 10 generations of cell division. Once biofilms neared a steady state, we determined final biomasses, measured Ti accumulation in the benthos, and fed the biofilms to a common herbivore (*Physa acuta*) to assess the potential for trophic transfer of Ti. As we will show, exposure to TiO_2 nanoparticles had little, if any, impact on the benthic organisms. Yet, the composition of algal species that comprised the stream biofilms played an important role in controlling the accumulation and fate of Ti in the benthos.

METHODS

Focal species

Our experiment manipulated three species of freshwater algae, *S. ulna*, *S. quadricauda*, and *S. tenue*, and one species of freshwater snail, *P. acuta*. *Synedra ulna* is a relatively large diatom (Bacillariophyceae) that grows either as a unicellular form or as rosette colonies [20]. This species is widespread, found in approximately 65% of all streams and rivers in North America [19]. *Scenedesmus quadricauda* is a Chlorophyceaen green algae that typically grows as a four-celled colony in both benthic and pelagic habitats [21] and is found in approximately 20% of all streams [19]. *Stigeoclonium tenue*, also a Chlorophyceaen green algae, grows as filamentous mats [22] and is found in 12% of streams [19]. When present, *S. tenue* often dominates the biomass of benthic biofilms. The populations of algae used for the present study were obtained from the Culture Collection of Algae at the University of Texas (http://www.sbs.utexas.edu/utex/) and maintained in nonaxenic laboratory cultures for approximately three months prior to the start of the experiment.

Physa acuta is a common snail found in streams and ponds throughout Asia, Australia, Europe, Africa, North America, and South America [23]. This species is opportunistic, sometimes characterized as an omnivorous feeder on benthic biofilms [24]. The population of *Physa* used for the present experiment was collected from San Ysidro Creek near Santa Barbara, California, and maintained in laboratory culture for approximately 10 generations in spring water at 20°C before use in the study.

Experimental mesocosms

The experiment was performed in 81 recirculating "flumes" (see Fig. 1A), each of which was 0.5 m long × 0.1 m wide × 0.1 m deep. Flow in each flume was controlled by a 7-cm-diameter propeller that was driven by a direct current motor attached to a TechPower HY3020E 3-amp voltage regulator. Similar flumes have been widely used for laboratory studies of freshwater algae and invertebrates [25]. Flumes are by no means a realistic depiction of streams, and we will discuss some of their limitations below. However, they have certain features that make them a useful model of select features of stream ecosystems. For example, they allow for highly replicated experiments, the accumulation of large population sizes (millions of cells of algae), and species that can be grown for many generations to a steady state. These are experimental features that would be difficult, if not impossible, to achieve in natural streams.

Within these flumes, biofilms were grown on polyvinyl chloride (PVC) substrates that were angled vertically in the working section to generate spatial variation in near-bed velocities ranging from <2 to 55 cm s^{-1} (velocities were measured by dissolution of gypsum pellets calibrated to free stream velocity using a 16-MHz Sontek Micro Acoustic Doppler Velocimeter).

This flow variation mimics one form of spatial heterogeneity that is known to allow algal species to coexist in natural streams [20,26,27], and we have previously shown that such variation is necessary for the three species used in this study to coexist [19]. To a 200-cm^2 working section of the PVC growth substrates, we affixed white duct tape that was cut into 23×0.5-cm strips running up and down the length of the flow ramp (Fig. 1B). Algae readily grew on the surface of the tape strips, which provided a convenient means of sampling on different dates to characterize algal biomass and population sizes without having to destructively sample entire streams (which would have increased the number of flumes required 10-fold).

Lighting above the 200-cm^2 working section of each stream was provided by a Coralife T5 Aquarium light fixture containing two 9-watt, 10 K daylight spectrum fluorescent lamps. The full light spectrum of these lamps was measured with an Ocean Optics Jaz light meter and is shown in the Supplemental Data, Figure S1. These lamps emit over the entire visible spectrum, with irradiance peaks at 550 and 610 nm, and in the upper UV spectrum at low intensities (360–400 nm, intensities of $>0.2\ \mu W\ cm^{-2}$ were apparent). Because Ti can be a photocatalyst when exposed to UV [28], and photocatalysis underlies many of the presumed effects that Ti has on organisms, we will revisit the relevance of the UV spectrum later in the discussion.

Fluid media

Prior to the start of the experiment, streams were sterilized with 30% H_2O_2 to degrade organic matter and 10% bleach to kill microorganisms and then repeatedly rinsed with UV-sterilized E-pure water until no residual bleach could be detected using residual chlorine test strips (Indigo Instruments; 33815-10). Streams were then filled with 9.5 L of UV-sterilized E-pure water that was augmented with 4 L of sterile soil–water extract medium (SWM), a widely used growth medium for culturing algae made by steeping greenhouse potting soil for 24 h in UV-sterilized E-pure water to leach micro- and macronutrients that are required for growth [29]. One drawback of SWM is that it is not a "synthetic" medium where the concentrations of all elements are controlled directly by the researcher. However, it does have several advantages. First, nearly all species of algae grow readily in SWM. Most synthetic media are formulated to maximize the growth of a single species or taxonomic group, and they rarely work well across a variety of taxa. Second, SWM mimics many physical and chemical aspects of real stream water that have the potential to influence the physical aggregation of $nTiO_2$, including pH, conductivity, and concentrations of natural organic matter. To ensure repeatability of our study, we conducted a companion study three months prior to this experiment [18] in which we performed a detailed characterization of the physical and chemical properties of soil extract medium to examine how its properties influence the aggregation and sedimentation rates of $nTiO_2$ particles. Aside from referring readers to this additional publication, we summarize information on key physical parameters that control $nTiO_2$ behavior in solution and give the data that would be needed to replicate the time series of key physical parameters of the present study (Supplementary Data, Tables S1 and S2).

Experimental design and setup

Five types of biofilms were randomly assigned to each of the 81 experimental streams: algae-free controls, inoculated only with soil extract medium; monocultures of each of the three types of algae; and polycultures containing all three algal species grown together. These treatments obviously do not mimic real biofilms, which are far more complex and diverse than what our treatments represent in the present study. But our goal here was not to mimic the complex reality of natural streams. Rather, our goal was to examine how algal biofilm composition impacts the accumulation of $nTiO_2$, and the simplest way to do this and generate unambiguous results is to grow a small number of species alone and together.

Each of the five biofilm types was crossed with three levels of $nTiO_2$: $nTiO_2$-free controls or a concentration of 0.1 or 1.0 ppm. Each combination of biofilm composition and $nTiO_2$ concentration was replicated in five streams, with the exception of the polycultures, which were replicated in seven streams. At the start of the experiment, species assigned to a stream were inoculated according to a replacement series (i.e., substitutive) experimental design in which a sum total of 1.65×10^5 cells were added to the water of each stream irrespective of the treatment. This total was divided among three inoculation dates (days 1, 3, and 9) to ensure successful establishment of populations.

The TiO_2 nanoparticles were added to the stream channels on day 1 of the experiment. The $nTiO_2$ was acquired from Evonik Degussa (4168063098). We previously published a full characterization of the physical properties of the batch of material used in the present experiment [18], which was composed of 82% anatase/18% rutile (98% pure) with a mean size of 27 nm (±SD 4). Stock dispersions were produced by adding 1,000 mg of TiO_2 nanoparticles to 1.0 L of E-pure water. The stock solution was sonicated for 30 min, after which inoculations of 1,287 μl (0.1 ppm) or 12,873 μl (1 ppm) were released into the running water of the appropriate streams using a pipette.

Measurements

We monitored the concentration of Ti in the water column as well as the size distribution of particles in the water column of the streams every 2 to 4 d over the course of the experiment in replicates 1 through 3 of each treatment. Water samples (50 ml) were collected from a well-mixed portion of the midchannel of each flume using a sterile syringe placed through a sampling septa (see Fig. 1A). Titanium concentrations were measured on a Thermo iCAP 6300 inductively coupled plasma atomic emission spectrometer in the Materials Research Laboratory at the University of California–Santa Barbara. Particle sizes were determined via dynamic light scattering (BI-200SM; Brookhaven Instruments). Because the dynamic light-scattering methods used to measure particle size do not allow us to distinguish abiotic from biotic material (e.g., algal cells that may have sloughed off and entered the water column), we also report a second, associated measure: the size of the particles giving the peak signal. This second measure is likely to be more representative of the size of $nTiO_2$ after aggregation because $nTiO_2$ aggregates would greatly outnumber algal cells in the water column at any given time.

We also monitored the biomass of algae every 2 to 4 d over the course of the experiment using sterile forceps to remove one randomly selected tape strip from each flume. Algae were brushed from the tape strip into SWM, after which the sample was homogenized. A 1.5-ml subsample was collected and preserved in 2% formalin and later used to determine algal population sizes by counting cells on a hemacytometer under 400× magnification on an Olympus X41 compound microscope. Population sizes were multiplied by the average cellular biovolume of each species, which was determined by measuring the dimensions of approximately 20 individuals and fitting these to known geometric shapes [30]. Biovolumes were converted to

species-specific biomass assuming a specific gravity of 1.0, and then the biomass was summed across all species in a flume to estimate the total biomass of algae in the stream benthos.

Immediately after the final time-series sampling of algae on day 32, we added 23 reproductively mature *P. acuta* that had a mean body mass of 4.7 mg per individual (± SD 1.8) to three (or four) replicates for each biofilm composition and $nTiO_2$ concentration of the algal monocultures (or polycultures). Snails were allowed to feed on the biofilms for 72 h, after which they were re-collected from each flume (average recovery was 75%). Snails were placed in spring water for 24 h to ensure that undigested algal material had cleared the digestive tract. Following this, snail tissue was dissected from the shells, dried at 60°C, and weighed. Tissue was then digested in aqua regia, and Ti concentrations in the digested samples were measured using inductively coupled plasma atomic emission spectrometry.

After removing snails, all flumes were destructively sampled to measure final biofilm biomass and Ti accrual in the biofilms. All material remaining was scraped from the tape strips and homogenized in a blender. As these were recirculating flumes and we could not sample any surfaces in the return pipe underneath the flume, mass balance calculations on Ti were not possible. One subsample was collected and used to measure the species-specific biomass of the algae as described previously. This measure allowed us to calculate the rates of herbivory as the proportional change in mass during the feeding period ($\ln[\text{biofilm mass}]_{\text{initial}} - \ln[\text{biofilm mass}]_{\text{final}}$). A second subsample was dried at 60°C for 24 h, after which the final dry mass of the biofilm was determined. A final subsample was dried as above and then digested in aqua regia. Titanium concentrations in the digested samples were measured using inductively coupled plasma atomic emission spectrometry.

Data analyses

To determine whether initial concentrations of $nTiO_2$ influenced the development of stream biofilms, we used repeated measures mixed model analyses of variance (ANOVA) with an autoregressive covariance matrix to model the biomass of each biofilm as a function of the initial concentration of $nTiO_2$, the day of the experiment, and the $nTiO_2 \times$ time interaction. Any significant interaction was taken as evidence that $nTiO_2$ alters the growth trajectory of algal biomass. General linear models were then used to assess how the final biofilm biomass, final concentration of Ti in the biofilm, rates of herbivory, and Ti in snail tissue varied as a function of the initial concentrations of $nTiO_2$ and biofilm composition. All analyses were performed using SAS Version 9.1.3 (SAS Institute).

RESULTS

Titanium time series

Temporal analyses of Ti in stream water samples suggest, there was a rapid, exponential decline in the concentration of suspended Ti. For streams that were initially inoculated with concentrations of 0.1 ppm $nTiO_2$, concentrations of Ti in the water declined to the same ambient levels measured in $nTiO_2$-free control streams after just 4 d from the start of the experiment (see Fig. 2A: note horizontal lines that show the 95% confidence interval for $nTiO_2$-free control streams). In streams that were inoculated with higher concentrations of 1.0 ppm $nTiO_2$, levels of Ti remained higher than in $nTiO_2$-free control streams for the duration of the experiment (Fig. 2B). Final water column concentrations on day 32 were 0.026 ppm Ti compared to background levels in the $nTiO_2$-free controls of 0.015 ppm.

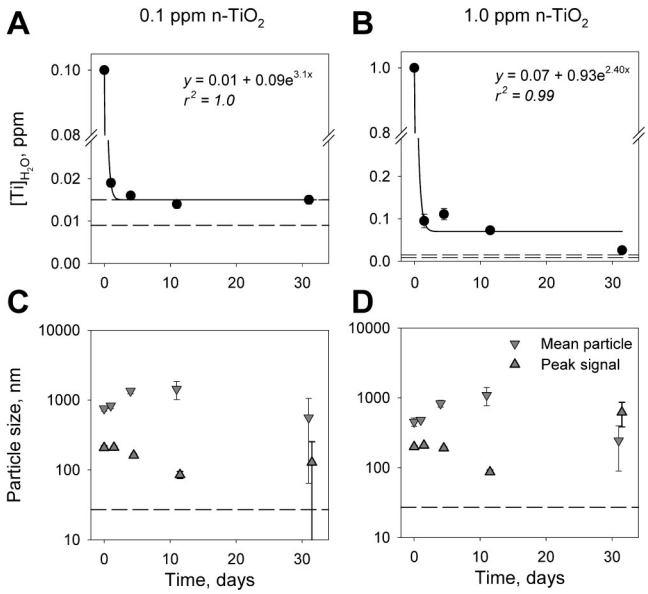

Fig. 2. Time series showing the concentration of titanium measured in stream water (**A**, **B**) and the size of particles in the water (**C**, **D**). Two measures of particle size, mean, and peak signal, are shown in **C** and **D**. Each data point represents the mean ± SEM of all flumes that were initially exposed to 0.1 (left) or 1.0 (right) ppm nanoparticle titanium dioxide ($nTiO_2$). The horizontal dashed lines in **A** and **B** give the upper and lower bounds of the 95% confidence interval for ambient levels of titanium measured in control streams that were not exposed to $nTiO_2$. The horizontal lines in **C** and **D** show the original particle size of added material, which was 27 nm.

Particle sizes measured in water samples suggest that Ti nanoparticles quickly aggregated into larger particles after being added to stream water. While the mean initial size of $nTiO_2$ particles was 27 nm, the mean size of waterborne particles after 24 h was 700 to 1,000 nm, which remained relatively constant over the course of the experiment (Fig. 2C and D). The mean particle size may be an overestimate of actual Ti particle size because it may include measurements of algal cells that are much larger and cannot be distinguished from abiotic material in the light-scattering process. A measure that is probably more representative of Ti is the size of the most abundant particle in suspension. This measure suggests that particle sizes averaged 160 to 250 nm over the course of the experiment (Fig. 2C and D), a value that is nearly identical to the equilibrium particle sizes measured by Keller et al. [18] in sterile SWM that had no organisms. Taken collectively, these results suggest that $nTiO_2$ rapidly aggregated and was removed from the water column during the first 24 h. Continuous exposure to particles that were approximately 200 nm in size was likely to have occurred only at the highest initial Ti concentration.

Impacts of $nTiO_2$ on biofilm growth

We found no evidence that the initial presence of $nTiO_2$ in stream water influenced the growth or biomass of algal biofilms (Fig. 3). Over the 32-d duration of the experiment, algal biomass increased between one and four orders of magnitude (Fig. 3A–D), with most biofilms reaching a steady state by the end of the experiment (with the exception of the slow-growing *Scenedesmus*). Yet, repeated measures ANOVA revealed no significant $nTiO_2 \times$ time interaction for any of the biofilm compositions (Figs. 3A–D and Table 1), indicating that growth trajectories for

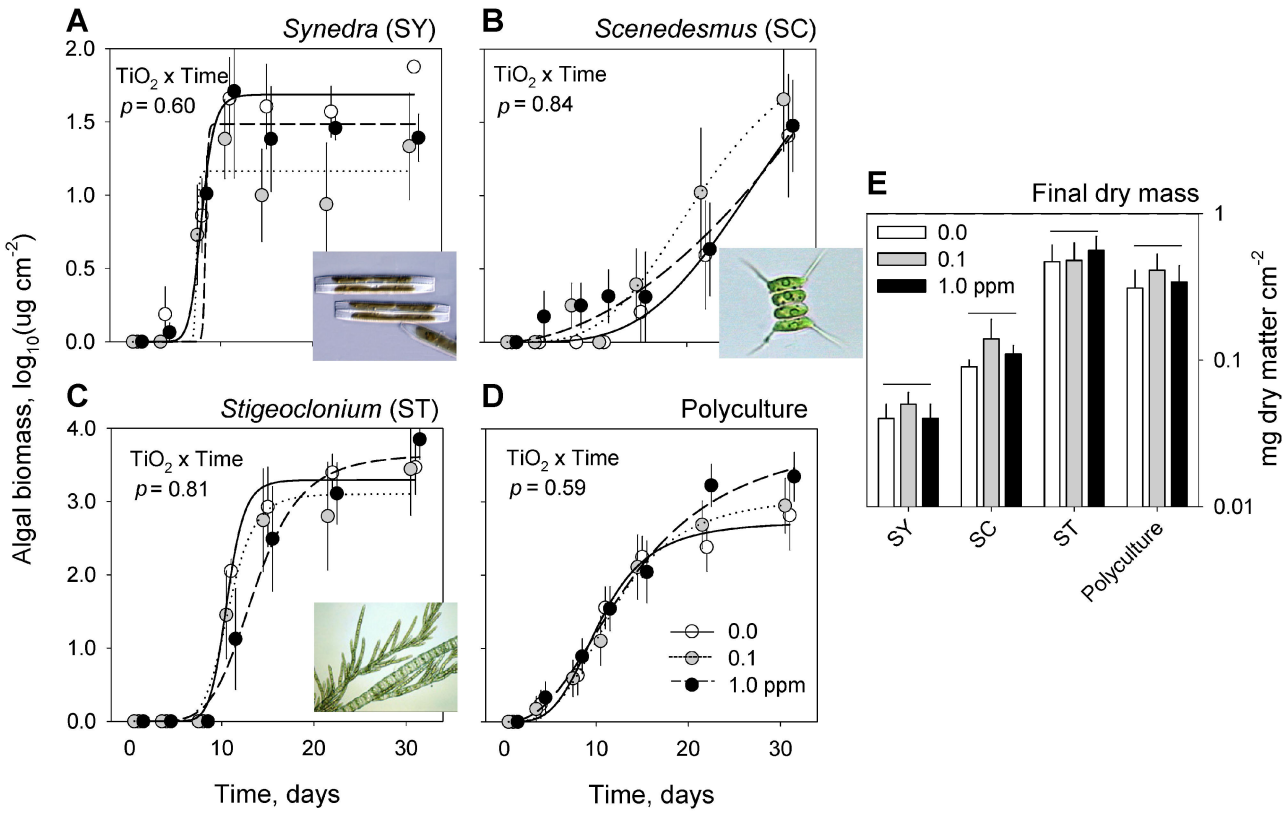

Fig. 3. Response of stream biofilms to the addition of nanoparticle titanium dioxide ($nTiO_2$). Panels **A–D** show that $nTiO_2$ had no impact on the growth dynamics of any species grown in monoculture or on species grown together in polyculture. Best-fitting logistic curves are plotted to illustrate growth trends, and corresponding results of repeated measures ANOVAs are given (also see Table 1). Panel **E** shows that $nTiO_2$ had no impact on the final dry mass of any biofilm (see Table 2). All data are means ± standard error mean. Lines connect treatments that are not significantly different as measured by post hoc Fisher's least significant difference tests ($p > 0.10$).

streams exposed to $nTiO_2$ could not be distinguished from those of $nTiO_2$-free control streams.

Destructive sampling of the streams on the final day of the experiment to collect all material further revealed no significant effect of $nTiO_2$ on the dry mass of any algal species grown in monoculture (Fig. 3E and Table 2, model A) and no effect on the final dry mass of algal polycultures (Fig. 3E and Table 2,

Table 1. Results of repeated measures, mixed model ANOVAs used to assess treatment effects

Model/dependent variable[a]	*df*	*F*	Pr > *F*
A. Biomass of *Synedra* $\log_{10}$ (μg cm^{-2})			
Initial $nTiO_2$ (ppm)	2	0.14	0.87
Time (d)	1	37.70	<0.01
$nTiO_2$ × time		0.52	0.60
B. Biomass of *Scenedesmus* $\log_{10}$ (μg cm^{-2})			
Initial $nTiO_2$ (ppm)	2	0.05	0.95
Time (d)	1	80.72	<0.01
$nTiO_2$ × time	2	0.17	0.84
C. Biomass of *Stigeoclonium* $\log_{10}$ (μg cm^{-2})			
Initial $nTiO_2$ (ppm)	2	0.18	0.84
Time (d)	1	102.01	<0.01
$nTiO_2$ × time	2	0.21	0.81
D. Biomass of three-species polyculture $\log_{10}$ (μg cm^{-2})			
Initial $nTiO_2$ (ppm)	2	0.05	0.95
Time (d)	1	150.26	<0.01
$nTiO_2$ × time	2	0.53	0.59

[a] The ANOVAs assessed the effects of initial $nTiO_2$ concentration, time, and their interaction on the biomass and growth of *Synedra* (model A), *Scenedesmus* (model B), and *Stigeoclonium* (model C) monocultures and a polyculture consisting of all three species (model D).

df = degrees of freedom; $nTiO_2$ = nanoparticle titanium dioxide.

Table 2. Results of general linear models and maximum likelihood methods used to assess treatment effects

Model/dependent variable[a]	*df*	*F*	Pr > *F*
A. Final biofilm biomass (mg dry mass cm^{-2})			
Initial $nTiO_2$ (ppm)	2	0.19	0.83
Taxa (SY, SC, vs ST)	4	19.92	<0.01
Initial $nTiO_2$ × taxa	8	0.19	0.99
B. Final biofilm biomass (mg dry mass cm^{-2})			
Initial $nTiO_2$ (ppm)	2	0.11	0.90
Richness (0, 1, vs 3 species)	2	7.02	<0.01
Initial $nTiO_2$ × richness	4	0.14	0.96
C. Mass-specific concentration of Ti in biofilm ($\log_{10}$ [μg Ti mg^{-1} dry mass])			
Initial $nTiO_2$ (ppm)	1	101.62	<0.01
Taxa (SY, SC, vs ST)	2	16.79	<0.01
Initial $nTiO_2$ × taxa	2	0.38	0.69
D. Mass-specific concentration of Ti in biofilm ($\log_{10}$ [μg Ti mg^{-1} dry mass])			
Initial $nTiO_2$ (ppm)	1	63.36	<0.01
Richness (0, 1, vs 3 species)	2	2.51	0.09
Initial $nTiO_2$ × richness	2	0.24	0.79
E. Areal concentration of Ti in biofilm ($\log_{10}$ [μg Ti cm^{-2}])			
Initial $nTiO_2$ (ppm)	1	108.95	<0.01
Taxa (SY, SC, vs ST)	2	3.49	0.05
Initial $nTiO_2$ × taxa	2	0.28	0.75
F. Areal concentration of Ti in biofilm ($\log_{10}$ [μg Ti cm^{-2}])			
Initial $nTiO_2$ (ppm)	1	87.01	<0.01
Richness (0, 1, vs 3 species)	2	10.82	<0.01
Initial $nTiO_2$ × richness	2	1.18	0.32

[a] Models A and B show how final biofilm biomass varied as a function of initial $nTiO_2$ concentrations in a stream, algal species, and mono- versus polyculture. Models C and D show how these same factors influenced the final concentrations of Ti that had accumulated in the biofilms per unit mass, whereas models E and F give accumulation per unit stream area.

df = numerator degrees of freedom; $nTiO_2$ = nanoparticle titanium dioxide; Ti = titanium; SY = *Synedra ulna*; SC = *Scenedesmus quadricauda*; ST = *Stigeoclonium tenue*.

model B). Within polycultures, the final relative biomass of species was also independent of $nTiO_2$ concentrations, with *Stigeoclonium* dominating 82 to 97% of total cell biovolumes across all streams irrespective of initial Ti concentrations. Taken collectively, these results suggest that $nTiO_2$ had no effect on the growth, biomass, or relative abundance of any algal species.

Variation in Ti accrual among biofilms

The amount of Ti measured in biofilms at the end of the experiment was significantly elevated above that in control streams that had not been exposed to $nTiO_2$ (see Fig. 4: note that all means lie above the horizontal lines showing the 95% confidence intervals for $nTiO_2$-free control streams). Thus, while we cannot say whether $nTiO_2$ was attached to cell walls, taken up into the algal cells, or simply within the biofilm matrix, there was clear evidence of accumulation of $nTiO_2$ particles in all biofilms. Not surprisingly, the amount of Ti that accumulated in the biofilm was proportional to the initial amount of $nTiO_2$ that was added to the stream water (Fig. 4A and B). Concentrations of Ti in stream biofilms initially exposed to 0.1 ppm $nTiO_2$ averaged 0.013 $\mu g\,cm^{-2}$, which was just above background concentrations of Ti measured in the algal biofilms of control streams that were not exposed to $nTiO_2$ (Fig. 4A, dashed horizontal lines). Concentrations of Ti in stream biofilms initially exposed to 1.0 ppm $nTiO_2$ averaged an order of magnitude higher at 0.129 $\mu g\,cm^{-2}$.

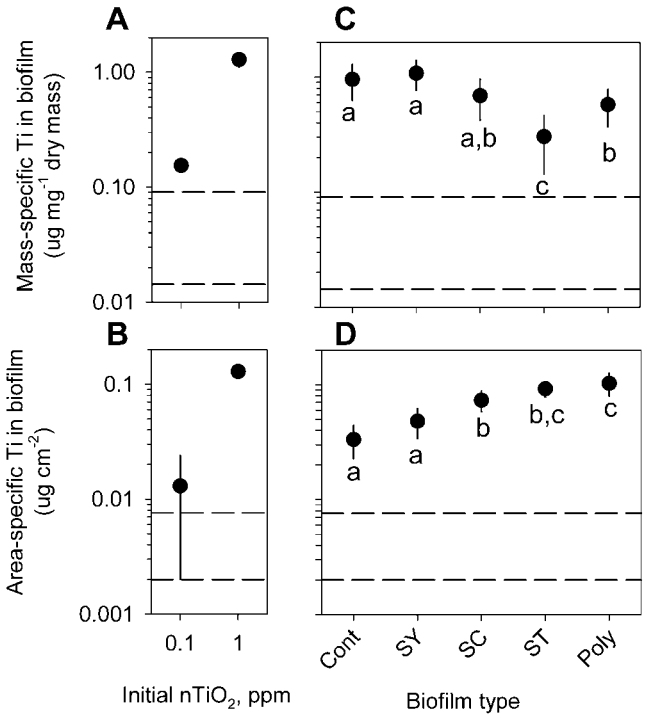

Fig. 4. Variation in the accumulation of titanium (Ti) among biofilms. After 32 d of growth, final concentrations of Ti in the biofilm were compared for initial levels of nanoparticle titanium dioxide ($nTiO_2$) (**A**,**B**) and among different biofilm compositions (**C**,**D**). Panels **A** and **C** standardize concentrations by biofilm biomass, whereas panels **B** and **D** standardize by benthic area. Because there were no significant interactions between $nTiO_2$ and biofilm composition, data for 0.1 and 1.0 ppm $nTiO_2$ are combined for **C** and **D**. All bars represent means ± standard error mean. Horizontal dashed lines give the upper and lower bounds of the 95% confidence interval for mean Ti measured in the biofilms of control streams that were not exposed to $nTiO_2$. Letters in **C** and **D** correspond to significant differences among biofilm types as measured by post hoc Fisher's least significant difference tests ($p \leq 0.10$). Cont = algae-free control, SY = *Synedra ulna*, SC = *Scenedesmus quadricauda*, ST = *Stigeoclonium tenue*, Poly = polyculture. Corresponding statistical analyses are given in Table 2.

The amount of Ti accumulation in benthic habitats was influenced by the composition of the biofilms (Table 2, models C and D, and Fig. 4C and D), and these compositional differences were maintained across both Ti concentrations (note the lack of any $nTiO_2$ × biofilm composition interaction, Table 2, models C and D). On a mass-specific basis, control streams with no algae and monocultures of *Synedra* had the highest levels of accumulation. This trend was primarily driven by the low overall biomass attained by each of these biofilms (see Fig. 3E). On an area-specific basis, >80% of all variation in final benthic Ti concentration could be explained by a simple model that included initial $nTiO_2$ and final biofilm dry mass as explanatory terms ($\log_{10}[\mu g\ Ti\ cm^{-2}] = [(1.44 + 0.90) \times \text{initial ppm}] + [0.47\ (\log10(\text{dry mass}\ cm^{-2})]$ ($p < 0.01$, $r^2 = 0.82$). The diatom *Synedra*, which achieved the lowest overall biomass of any biofilm (Fig. 3E), had levels of Ti accumulation that did not differ from algae-free control streams. In contrast, the colonial green algae *Scenedesmus* and the filamentous green algae *Stigeoclonium* both achieved significantly higher levels of biomass (Fig. 3E), translating to higher accumulation of Ti (Fig. 4D). Accumulation of Ti in the biofilms of the three-species polycultures was significantly greater than accumulation in the average monoculture ($p < 0.01$). However, there was no significant difference between the concentration of Ti in polycultures and that in *Stigeoclonium*, which was the dominant species in polyculture and which accumulated the greatest amount of Ti in monoculture.

Herbivory and Ti accrual in snails

On average, *Physa* consumed 13% of all the final biofilm dry mass over the 72-h feeding period at the end of the experiment. The initial concentration of $nTiO_2$ in the streams did not influence the amount of herbivory (Fig. 5A) ($p = 0.93$). However, there were significant differences in the amount of consumption among different biofilms (Fig. 5C).

Snails consuming biofilms exposed to 1.0 ppm $nTiO_2$ exhibited significantly higher accumulation of Ti ($p < 0.05$) into their tissue than those feeding on $nTiO_2$-free controls, whereas snails consuming biofilms exposed to 0.1 ppm $nTiO_2$ had tissue concentrations that were not different from $nTiO_2$-free controls (Fig. 5B). Differences in herbivory among biofilms may help to explain differences in the accumulation of Ti in tissue of snails consuming biofilms exposed to 1.0 ppm $nTiO_2$ (Fig. 5D). *Physa* did not appear to consume any significant amount of biomass in the algae-free control streams or stream biofilms composed of *Scenedesmus* (Fig. 5C). Probably as a result, *Physa* that fed on these two biofilms showed little to no evidence of Ti enrichment relative to snails that fed in $nTiO_2$-free control streams (see Fig. 5D, compare data points to horizontal dashed lines that give the 95% confidence interval for $nTiO_2$-free control streams).

In contrast, large fractions of the biofilm biomass were consumed in streams containing other algal species compositions. For example, herbivory approached 30% of the total mass of streams containing *Stigeoclonium* and roughly 15% of the mass of the three-species algal polyculture, which was also dominated by *Stigeoclonium* (Fig. 5C). Snails in both of these streams showed significant enrichment in Ti tissue concentrations (Fig. 5D). Although the diatom *Synedra* achieved the lowest biomass of any biofilm, herbivory in streams containing *Synedra* was exceptionally high, with more than half of all

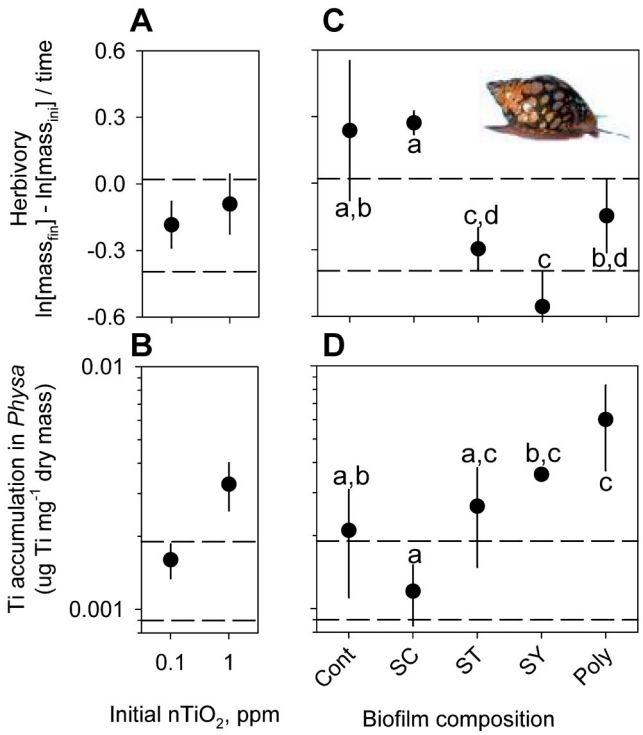

Fig. 5. Herbivory and titanium (Ti) accumulation in *Physa acuta*. After 32 d of growth, 23 adult *Physa* were added to each experimental flume and allowed to feed on biofilms for 72 h. Panels **A** and **C** show the change in biofilm dry mass during the feeding period. Because there were no significant interactions between nanoparticle titanium dioxide ($nTiO_2$) concentration and biofilm composition, data for these two treatments are combined in **C**. After the feeding period, snails were removed from flumes, placed in spring water for 24 h to clear their guts, and then removed from their shells and digested. Panel **B** shows tissue concentrations of Ti in snails that consumed 0.1 and 1.0 ppm $nTiO_2$-exposed biofilms. Panel **D** shows tissue concentrations of Ti in snails consuming the different biofilm types exposed to 1.0 ppm $nTiO_2$ as only this level was different from $nTiO_2$-free controls. All data are means ± standard error mean. Horizontal dashed lines represent the upper and lower bounds of the 95% confidence interval for measurements taken on snails feeding on the biofilms of streams that were not exposed to $nTiO_2$. Letters in **C** and **D** correspond to significant differences among biofilm types as measured by post hoc Fisher's least significant difference tests ($p \leq 0.10$). Cont = algae-free control, SY = *Synedra ulna*, SC = *Scenedesmus quadricauda*, ST = *Stigeoclonium tenue*, Poly = polyculture. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

biomass consumed (Fig. 5C). Perhaps as a result, Ti accumulation in *Physa* that fed on *Synedra* was also high (Fig. 5D).

DISCUSSION

In the present study, we examined how benthic algae and herbivores respond to and influence the concentration of $nTiO_2$ in the benthic habitat of stream mesocosms. We found that $nTiO_2$ had little, if any, impact on benthic algae and herbivores. The growth and final biomass of the three species of benthic algae used in the study were all independent of the concentrations of $nTiO_2$ added to streams. No evidence was found that $nTiO_2$ altered the short-term rates of herbivory by *P. acuta*, which is a common herbivore in streams. For streams that were exposed to the lowest initial concentrations of $nTiO_2$ (0.1 ppm), the lack of any response may not be surprising because: (1) the amount of Ti in the water rapidly approached background concentrations in $nTiO_2$-free control streams and (2) concentrations of Ti in the biofilms at the end of the experiment were barely above background concentrations in the biofilms of $nTiO_2$-free control streams. Thus, it is possible that exposures at the lowest $nTiO_2$ concentrations were minimal or nonexistent.

But the lack of a response of the organisms to $nTiO_2$ at the higher concentration (1.0 ppm) cannot be dismissed as a lack of exposure. At this concentration, levels of Ti in the water column remained elevated for the entire 32-d duration of the experiment. In addition, we documented significant accumulation of Ti in every biofilm measured. In spite of this, the organisms showed no response for any variable measured. Previous studies have shown negative impacts of $nTiO_2$ on aquatic organisms, including both algae and herbivores [reviewed in 31], in both standard toxicity tests [16,32–34] and mesocosm work [15]. However, these studies have typically used, and seen their negative impacts at, environmentally unrealistic concentrations of $nTiO_2$. In the present study, we chose our test concentrations to be environmentally relevant [13] and did not detect significant effects on algal biofilms. Furthermore, many of the aforementioned studies on algae revealed no observed effect concentrations similar to ours, including 1.64 [16], 6.25 [32], 10 [33], and 0.89 to 1.2 [34] ppm $nTiO_2$. Thus, at the concentrations of $nTiO_2$ expected to be present in certain natural aquatic environments ($\leq$0.6 ppm) [10,13], we may not see significant effects on the growth of many algal taxa.

When negative effects on algal growth rates are seen, they are usually attributed to the photocatalytic properties of TiO_2, which can facilitate redox reactions that disrupt cell membranes [35,36] and/or interfere with photosynthesis [31,37]. A second group of studies have suggested that $nTiO_2$ may actually stimulate the growth of certain types of plants, which is thought to reflect the potential for Ti to enhance electron transport in ways that facilitate photosynthesis [38,39]. While $nTiO_2$ had no discernible effect on the algae used in the present study, it is unclear whether this would remain true under higher light intensities, such as those that occur under natural outdoor conditions. The mechanisms of toxicity proposed in past studies generally require that electrons on the surface of Ti become excited by UV light at approximately 380 nm, which can then stimulate electron transport and/or produce reactive oxygen species [28]. The fluorescent lamps used to grow algae in the present study, which are typical of T5 lamps used for algal culturing and mesocosm work, emit 0.2 to 0.4 $\mu W\,cm^{-2}$ at 360 to 380 nm (see Supplemental Data, Fig. S1). Light intensities in this range have been shown to stimulate photocatalytic activity of Ti [28], see Fig. 6. However, because electron excitation at low UV intensity is less frequent, any signs of a biological effect may be negligible, may take longer to detect, or can potentially be offset by tissue repair or compensatory growth. Therefore, we caution against using results of laboratory-based studies performed with artificial lighting to predict the response of ecosystems exposed to more natural lighting until further work can either confirm or refute results such as those reported in the present study.

Although $nTiO_2$ had no discernible effect on the benthic organisms, the organisms themselves had a large effect on the accumulation and fate of Ti in the benthic habitat. Indeed, both the accumulation of Ti in the biofilms and the transfer of Ti to herbivores depended on the composition of algal species in the stream biofilm. Most accumulation of Ti in the biofilms was proportional to biomass such that species having the greatest growth in monoculture accumulated the largest amounts of Ti. Polycultures comprised of all three species growing together attained higher biomass and more Ti than

the typical (i.e., average) monoculture; however, the biomass of the polyculture was dominated by the single most productive species (*Stigeoclonium*), which is perhaps why the polyculture accumulated no more Ti than *Stigeoclonium* did when grown alone. These findings parallel the results of another body of research that has examined how species composition of primary producers influences the uptake of nitrogen, a nutrient pollutant of global concern [40]. Over the last 20 years, 59 experiments have quantified how the richness of plants and algae influence concentrations of inorganic nitrogen in soil or water [41]. Of these, 86% have shown that N concentrations decrease as biodiversity increases (by an average 48%). Roughly half of this effect is driven by the fact that diverse communities are more likely to contain, and become dominated by, highly productive species [41]. Collectively, these results suggest not only that species vary in their ability to sequester certain types of potential pollutants from the environment but that diverse communities are more likely to contain those species which have the greatest rates of sequestration.

The accumulation of Ti at higher trophic levels was also influenced by algal species composition and appeared to be a joint function of the amount of Ti that had accumulated in the biofilm and the palatability of the specific algal taxa. The green algae *Stigeoclonium* achieved the highest biomass and greatest Ti of any monoculture (Fig. 4D). *Stigeoclonium* was also moderately edible to physid snails, which may have led to the moderate accumulation of Ti in the herbivores. In contrast, the diatom *Synedra* achieved a comparably low biomass among algae species and had only small amounts of Ti in the biofilm on a per-area basis (Fig. 4D). However, because *Physa* consumed the vast majority of *Synedra* biofilms (Fig. 5C), accumulation in herbivores was relatively high (Fig. 5D). Similar results have been seen in estuarine mesocosms, where biofilms were shown to accumulate gold nanoparticles but trophic transfer to snail herbivores was minimal [42]. These studies suggest that transfer of $nTiO_2$ from primary producers to consumers is not likely to be directly proportional to accumulation in the biofilm but is also influenced by compositional differences that regulate herbivore feeding preference.

One caveat to bear in mind when interpreting the results of the present study is that the stream mesocosms used for this experiment are an obvious oversimplification of natural streams. Natural streams tend to contain dozens to hundreds of species that are growing in a spatially complex and temporally variable environment. Like all caricatures of nature, mesocosms cannot possibly mimic all of the potential physical or biological variation that might control the biological impacts of a focal material, and interpretation of our results should be clearly articulated within this constraint. But when interpreted within their intended context, mesocosm studies are an important component of a broader research agenda. Because adding $nTiO_2$ to real streams would typically be impractical or unethical, it is perfectly reasonable to begin examining the ecological impacts of a novel material in simplified model systems. What we have learned from the simple model system used here is that any attempt to accurately model and predict the fate of Ti nanoparticles in streams, and perhaps other habitats, will need to explicitly consider the composition of species that dominate the benthic habitat.

SUPPLEMENTAL DATA

Tables S1-S2.
Fig. S1. (287 KB DOC)

Acknowledgement—Funds for the present research were provided by the U.S. National Science Foundation and Environmental Protection Agency under Cooperative Agreement NSF-EF0830117 to the University of California's Center for the Environmental Implications of Nanomaterials. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation or the Environmental Protection Agency.

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