Environ. Sci. Technol. **2010,** *44,* 8302–8307

Impact of Titanium Dioxide Nanomaterials on Nitrogen Fixation Rate and Intracellular Nitrogen Storage in *Anabaena variabilis*

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Received May 14, 2010. Revised manuscript received August 8, 2010. Accepted August 13, 2010.

This study comprehensively investigated the impact of titanium dioxide nanomaterials ($nTiO_2$) exposure on cell growth, nitrogen fixation activity, and nitrogen storage dynamics in the primary producer cyanobacteria *Anabaena variabilis* at various dose concentrations and exposure time lengths. The results indicated that both growth rate (EC_{50}-96 h of 0.62 mgTiO_2/L) and nitrogen fixation activity (EC_{50}-96 h of 0.4 mgTiO_2/L) were inhibited by $nTiO_2$ exposure. The Hom's law (C^nT^m) was used as inactivation model to predict the concentration- and time-dependent inhibition of growth and nitrogen fixation activity. The kinetic parameters determined suggested that the time of exposure has a greater influence than the $nTiO_2$ concentration in toxicity. We observed, for the first time, that $nTiO_2$ induced a dose (concentration and time)-dependent increase in both the occurrence and intracellular levels of the nitrogen-rich cyanophycin grana proteins (CGPs). The results implied that CGPs may play an important role in the stress response mechanisms of $nTiO_2$ exposure and can serve as a toxicity assessment endpoint indicator. This study demonstrated that nitrogen-fixing activity could be hampered by the release of $nTiO_2$ in aquatic environments; therefore it potentially impacts important biogeochemical processes, such as carbon and nitrogen cycling.

Introduction

Since the early 1900s, titanium dioxide (TiO_2) has been widely used in numerous consumer and industrial applications, particularly in coatings and pigments. Recently, the nanotechnology industry has incorporated titanium dioxide nanomaterials ($nTiO_2$) in a larger variety of commercial and biomedical applications, mainly exploiting its photocatalytic properties (*1*−*3*). It is anticipated that the increased use of $nTiO_2$ will result in its release into aquatic environments. A recent study by Kiser et al. reported effluent concentrations from wastewater treatment processes of 5−15 μgTi/L (*4*), which is consistent with the predicted environmental concentrations of titania nanomaterials (0.7−16 μg/L) based on worldwide production volumes in typical Swiss environmental scenarios (*5*).

Currently, limited information is available on the potential impact of nanomaterials (NMs) on aquatic ecological systems and on primary producers such as algae. A limited number of studies have demonstrated that NMs such as nAg, TiO_2, ZnO, and quantum dots nanoparticles exhibit toxic effects on algal growth, algal photosynthetic activity, and nutrients uptake (*1, 6*−*9*). Most previous studies used green algae (i.e., *Selenastrum capricornutum*) as model algal species. The potential impact of NMs on nitrogen-fixing algae such as the cyanobacteria *Anabaena variabilis*, especially on its nitrogen fixation activity and nitrogen metabolism has not been investigated.

Cyanobacteria perform oxygenic photosynthesis and play an important role in primary production and nitrogen cycling with their ability to fix atmospheric dinitrogen into ammonia, a bioavailable form of nitrogen source for various organisms (*10*). Cyanobacteria have been previously used as a model algae for evaluating environmental stresses (*11*), due to their phylogenetic relationship with plants' chloroplasts and their historical ecological tolerance that contributed to their survival in a wide range of hostile environments (*12*). The metabolic strategies used by cyanobacteria to tolerate adverse and fluctuating conditions through physiological adaptation are unique and widely reported (*13*−*15*); these strategies involve physiological, morphological, and ecological modifications (*16, 17*). In addition, the accumulation and degradation of cyanobacterial intracellular inclusions with reserve functions has been previously reported under conditions of starvation or in exposure of stressors. In particular, alterations in stores of nitrogen (cyanophycin) (*14*), carbon and energy (polyglucose, poly-f3-hydroxybutyrate) (*18*), polyphosphate granules (*15*), and polyhedral bodies (*13*) have been previously reported in various cyanobacterial species when the cells are exposed to heavy metals or other altered unfavorable growth conditions. These variations in cellular substructure reflect the alteration in the internal biochemical equilibriums of blue-green algae in response to stress.

In this study, we investigated the effect of $nTiO_2$ exposure on the growth rate, N-fixing activity, and intracellular nitrogen-storage structures in cyanobacteria *A. variabilis*. Quantitative inhibition effects of $nTiO_2$ on cell growth rate and nitrogen fixation rate were systematically evaluated at various concentrations and exposure time length. Additionally, a morphometric analysis allowed the quantification of intrastructural changes in response to the toxicant. In particular, the impact on temporal and spatial accumulation on the cyanophycin grana proteins (CGPs), a functionally relevant biomolecule in *A. variabilis* cells involved in nitrogen storage and consumption, was assessed, providing insights into the possible alteration of nitrogen metabolic pathways in algae upon $nTiO_2$ exposure.

Experimental Methods

NMs Preparation and Characterization. Nano-TiO_2 anatase ($nTiO_2$; NanoStructured & Amorphous Materials, Houston, TX) was prepared in culture Mes-Volvox medium and then dispersed before use. Dispersion was facilitated with the addition of crude Bovine Serum Albumin (1% BSA) and sonication in a high energy cup-sonicator (Fisher scientific, Inc.), at ∼90 W power for 20 min. Primary size of TiO_2 nanoparticles from the manufacturer was 10 nm (outer diameter) and the average size of NM aggregates of 192 ± 0.8 nm was determined through dynamic light scattering (Zetasizer Nano ZS90, Malvern Instruments Ltd.) after NMs dispersion in the culture media (single crystal). The polydisperisty index (PdI) after dispersion in culture media was found to be 0.479. Detailed physical and chemical characterization of the $nTiO_2$ used in this study, including aggregate size distribution, metal impurities, surface charge, zeta potential, organic and elemental carbon, etc., was conducted

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10.1021/es101658p
Published on Web 09/20/2010

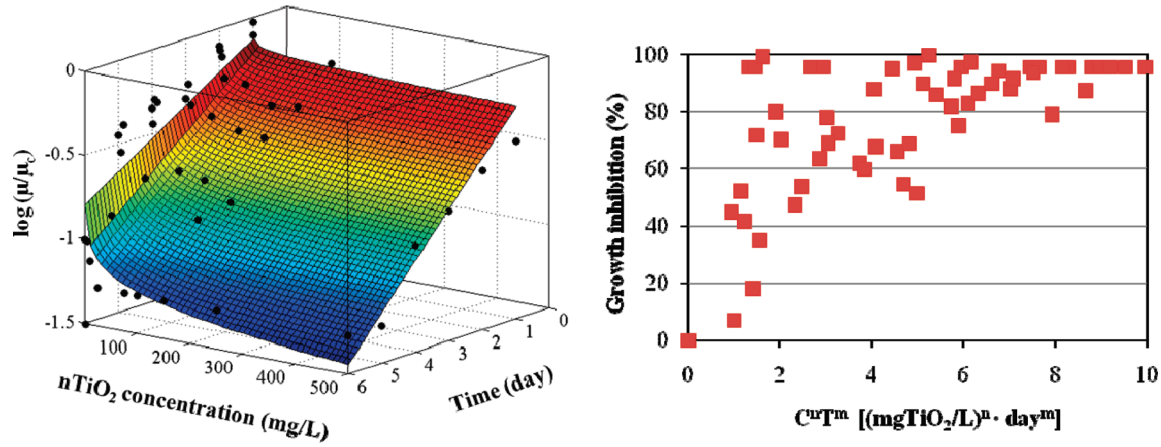

FIGURE 1. Concentration · Time ($C \cdot T$)-dependent growth inhibition of *Anabaena variabilis* cells exposed to $nTiO_2$ at various concentrations and different exposure time lengths (left). Log (μ/μ_c) represents the logarithm of the ratio of the growth rate of exposed cultures to control (unexposed). Data fitted in a generalized Hom's inactivation law to show the percentage of *A. variabilis* growth inhibition as a function of C^nT^m (right). (Matlab surface fitting tool was used for data interpolation and kinetic parameters estimation).

and reported by our collaborator Bello et al. (*19*). A specific surface area (SSA) of 274.2 $m^2\ g^{-1}$ was measured and the X-ray diffraction showed the presence of small amounts of both anatase and rutile soluble extracts in the $nTiO_2$ anatase used in this study (*19*).

Culture Conditions and Ecotoxicological Tests. *Anabaena variabilis* strain (UTEX 1444) was axenically cultured at 20 °C in a nitrogen-free Mes-Volvox media. Cells were cultured in 1-L chemostats with 0.15 d^{-1} dilution rate, and incubated under a 12 h light/12 h dark regime using 1:1 ratio of 34 W cool white and 40 W Sylvania gro-lux fluorescent bulbs (Sylvania, Danver). Chemostats were continuously mixed and aerated (compressed air was filtered via 0.2 μm and purged at a rate of 5 mL/min) and algal concentration was maintained at 1.0 g/L of chlorophyll *a*. Growth inhibition tests based on chlorophyll *a* measurements were performed according to the standard protocol designed for the freshwater indicator green algae (*20*). Briefly, 75-mL test volumes of initial chlorophyll *a* concentration of 500 mg/L were subjected to different $nTiO_2$ concentrations, from 0 $mgTiO_2/L$ (control sample) to 500 $mgTiO_2/L$, and incubated for 6 days under the same culturing conditions. Chlorophyll *a* data for each test condition were calculated as the average of 3 duplicate samples taken at any given time point. Aliquots of cells were periodically collected and processed for nitrogen fixation rate analysis, according to the method described by Pratte (*21*). Samples were also subject to transmission electron microscopy imaging to observe *A. variabilis'* intracellular changes.

Nitrogen Fixation Activity via Acetylene Reduction Assay. To establish whether *A. variabilis* exposure to $nTiO_2$ inhibits its N_2 fixation ability, *nitrogenase* activity was measured in the cultures prepared for growth inhibition test using acetylene reduction assay (ARA), according to the method described in Pratte et al. (*21*). Briefly, 3 mL of cultures, taken at different time points, were added to 10-mL gastight serum bottles followed by acetylene (≥99.5% purity Medtech, Medford) addition to the headspace to obtain a concentration of 12.5% v/v C_2H_2. Vials were incubated and shaken (350 rpm) for 8 h and then the reaction was stopped by the addition of 300 μL of 2N NaOH. The evolved ethylene concentration was measured using an SRI 8610C gas chromatograph with FID equipped with a Restek Corp. ShinCarbonST 80/100 2 m packed column using helium as the carrier gas at 20 psi. The detection limit for ethylene was 15 ppm with an injection volume of 100 μL. Results from ecotoxicological tests were fitted into the Hom's inactivation model (*22*) for the kinetic parameters (k, n, and m) determination. Matlab v. 7.8.0 (R2009a) was used for surface fitting and model parameters estimation.

Observation of Intracellular Cyanophycin Grana Proteins via Transmission Electron Microscopy (TEM). Temporal high-resolution TEM imaging was employed to observe intracellular structural changes in *Anabaena variabilis* upon exposure to $nTiO_2$. Cells were periodically collected from cultures subjected to growth inhibition tests, harvested, and fixed for 1.5 h at 4 °C in Karnovsky's fixative. Specimens were then washed twice in 0.1 M cocodylate buffer and embedded in 2% agarose for beads preparations. Post-fixation was completed in 2 h in 1% osmium tetroxide followed by two rinsing steps in 0.1 M cocodylate buffer. A sequential dehydration series of beads in 30, 50, 70, 85, 95, and 100% ethanol was then followed by a gradual replacement of ethanol with Spurr's resin before completing infiltration and embedding in capsules. Capsules were placed in an oven and polymerized at 60 °C for 24 h. Sample blocks were then trimmed and ultrathin sections (80 nm) were obtained using a Diatome diamond knife with a Reichart Ultracut E ultramicrotome. Ultrathin sections collected on 200-mesh copper grids were stained with 5% uranyl acetate and Reynold's lead citrate and observed on a JEOL JEM-1010 transmission electron microscope (JEOL Ltd. Tokyo, Japan) operated at 70 kV. Digital images were captured using an XR-41B bottom-mount CCD camera system (AMT Corp., Danvers, MA). NMs particles size as well as intracellular biomolecules' dimensions were analyzed with the software Image J 1.43q (http://rsbweb.nih.gov/ij/). A range of 46−67 cells per sample were analyzed to obtain statistical confidence.

Results and Discussion

Effect of $nTiO_2$ Exposure on Growth of Anabaena variabilis. The effects of $nTiO_2$ exposure on the growth rates of *A. variabilis* was evaluated for various $nTiO_2$ concentrations ranging from 0.5 to 500 mg/L, and for various exposure lengths, ranging from 24 h to 6 days. At concentrations above 250 mg/L, greater than 90% inhibition was observed even with the shortest exposure time of 24 h. The same percentage of inhibition was also observed with 6 days exposure with $nTiO_2$ concentrations as low as 0.5 mg/L. These results indicated that the inhibition on *A. variabilis* growth depends on both $nTiO_2$ concentration and exposure time; therefore, the $C^n \cdot T^m$ (e.g., Hom's law) concept was applied to refer to the toxicity effect (Figure 1). The Hom's function is a generalization of the pseudo-first-order Chick−Watson's law

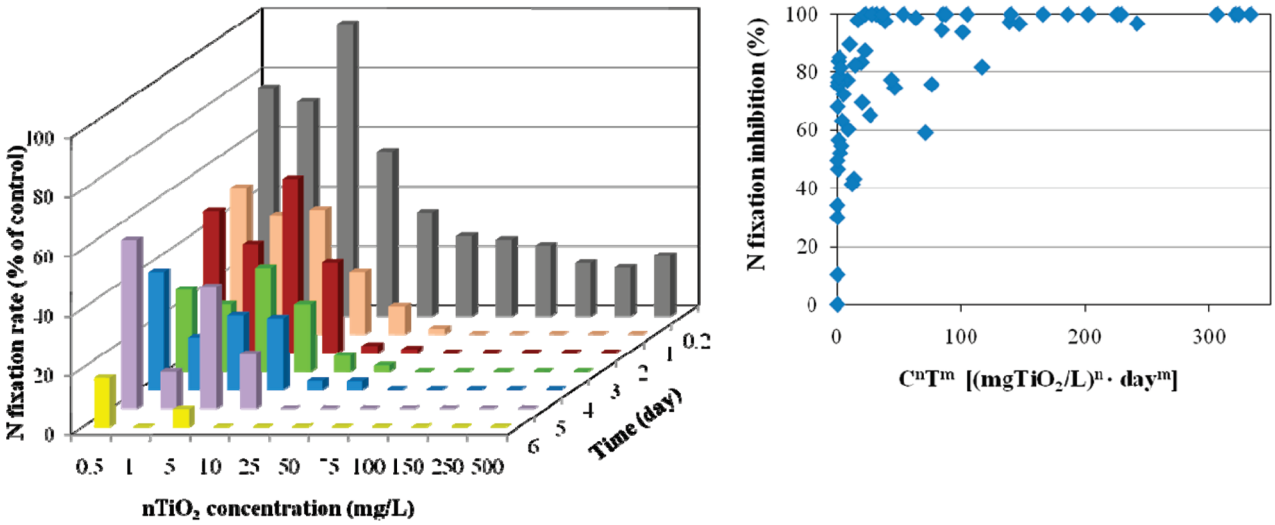

FIGURE 2. Inhibition of specific nitrogen fixation ability of *Anabaena variabilis* by $nTiO_2$ at different concentrations and varying exposure time lengths. Nitrogen fixation rate is expressed as the percentage of the specific ethylene production rate measured with respect to the one of the control sample without $nTiO_2$ exposure (left). Nitrogen fixation inhibition (%) as a function of C^nT^m (upper right).

typically used to model bacterial inactivation in disinfection processes in water and wastewater (*22*). The nonlinearity of the function for concentration (C) and time (T) requires the estimation of the model parameters n and m respectively linked to both independent variables ($C^n \cdot T^m$). The kinetic constant k value is expected to be microorganism and nanomaterial specific as well controlled by the experimental conditions used. To our knowledge, this is the first time the Hom's model was applied to understand the role of $nTiO_2$ concentration and exposure time in nanoecotoxicology. CT-dependent antimicrobial effects of TiO_2 have been reported by several studies that examined the photocatalytic disinfection potential of TiO_2 (coupled with ultraviolet irradiation) on *E. coli* in drinking water treatment processes (*23, 24*). Ng and his collegues reported a first-order rate constant k of photocatalytic nanoarray TiO_2 of 0.064 min^{-1} obtained under constant irradiation conditions in a simplified first-order Chick−Watson disinfection model (*25*). In this study's experimental results, the constants of k, m, and n were found to be 0.14 (95% CI: 0.08584, 0.1872), 1.01 (95% CI: 0.7979, 1.23), and 0.09 (95% CI: 0.04966, 0.1249), respectively, demonstrating that under these experimental conditions, the exposure time length has a greater influence than the $nTiO_2$ concentration in inhibiting *A. variabilis* growth. The model fits with a goodness of 0.7403 (R^2) and 0.2132 (RMSE). These results suggest that even at very low concentrations, extended exposure time length in aquatic ecosystems beyond the regulatory endpoints (e.g., 96 h) need to be considered in assessing NMs impact in environmental ecosystems.

The effects on *A. variabilis* growth rate at various concentrations and exposure time length allowed the determination of the half maximal effective concentration (EC_{50}) as a function of exposure time. The EC_{50} value decreased significantly from 13.98 $mgTiO_2$/L at 24 h to 0.15 $mgTiO_2$/L after 6 days of exposure. EC_{50}-96 h is usually used as the regulatory endpoint for *Selenastrum capricornutum* chronic toxicity assessment. In this study, the EC_{50}-96 h was determined to be 0.62 mg/L (95% confidence interval of 0.6−0.677 $mgTiO_2$/L). This value is lower than those endpoints (EC_{50}-72 h) previously reported in literature on the green algae *Pseudokirchneriella subcapitata* (5.83 mgTi/L) (*1*) and *Desmodemus subspicatus* (44 $mgTiO_2$/L) (*26*) exposed to TiO_2 nanomaterials.

It is known that toxicity is organism-specific (*27*), which may explain the difference in EC_{50} obtained. However, the variations in the culturing procedure and $nTiO_2$ preparation protocols might affect the results obtained among different laboratories. Nevertheless, the higher sensitivity to toxicity shown by *A. variabilis* makes this organism suitable for toxicity assessment by $nTiO_2$ exposure. This finding is consistent with the recognized importance of identifying appropriate sensitive test organisms for specific stressors (*27*).

Impact of $nTiO_2$ Exposure on Nitrogen Fixation Activity of *Anabaena variabilis*. To our knowledge, no systematic studies have been performed to understand if algal nitrogen fixing activity and related functions are prone to be compromised after NMs exposure. Figure 2 shows the toxicity effects of $nTiO_2$ on the nitrogen fixing activity of the cyanobacteria *A. variabilis* at different concentrations. Impact on nitrogen fixation activity was monitored through the ability of the oxygen labile *nitrogenase* enzyme, which is expressed under diazotrophic conditions in heterocyst cells (*21*), to reduce acetylene into ethylene. $nTiO_2$ concentrations higher than 10 mg/L led to greater than the 50% nitrogen fixation inhibition after a short exposure time of 24 h. Nitrogen fixing activity was completely inhibited at a $nTiO_2$ concentration of 75 $mgTiO_2$/L after 24 h exposure and at 1 $mgTiO_2$/L after 6 days exposure, indicating that the inhibition effect of $nTiO_2$ on *nitrogenase* enzyme activity rates is also $C^n \cdot T^m$-dependent, depending on both $nTiO_2$ doses and exposure time. It is unclear why, between day 4 and day 6, the culture exposed to 5 mg/L slightly recovered its nitrogen fixing activity before dropping again to be below 10% of the activity of the control sample. The results presented in Figure 2 were also fitted into a Hom-type model (*22*) to quantify the N fixation inhibition kinetics of *A. variabilis* when interacting with $nTiO_2$. Values of n and m parameters were found to be 0.72 (95% confidence bounds: 0.332, 1.107) and 1.93 (95% confidence bounds: 0.8492, 3.009), respectively, suggesting that under the experimental conditions, the time of exposure also has a greater effect than the $nTiO_2$ concentration in inactivating the *A. variabilis*' ability to fix nitrogen, although the magnitude of inactivation is less when compared to the inhibition effect on growth as previously discussed. A value of the inactivation coefficient k of 0.04681 (95% confidence bounds: −0.02905, 0.19227) was determined with R^2 of 0.7482 and root-mean-square error of 0.2712.

The difference in the shape of % inhibition versus C^nT^m curves for growth inhibition and for nitrogen fixation inhibition, as shown in Figures 1 and 2, suggested that the latter was not an indirect result from the former. The

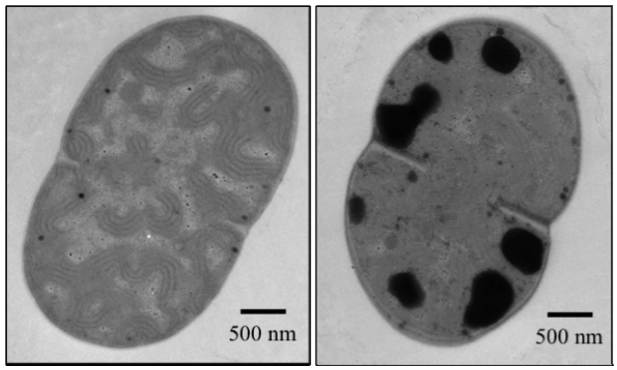

FIGURE 3. Observed increase in cyanophycin granules size in *Anabaena variabilis* cells after exposure to $nTiO_2$. Cell without CGPs (left); cell showing the 16.4% of sectional area occupied by CPGs after 96 h exposure to 150 mg/L $nTiO_2$ (right).

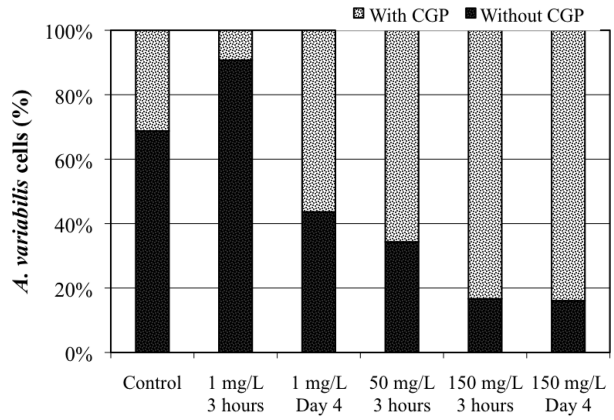

FIGURE 4. $nTiO_2$ induced an increase in the occurrence of CGPs in *Anabaena variabilis* cells, as indicated by the distribution of CGPs-containing and non CGPs-containing cells in samples exposed to $nTiO_2$ at different concentrations and with different exposure times. Numbers on top of the columns refer to the total number of cells analyzed for that specific sample.

correlation of N fixation inhibition and growth inhibition data at given $C \cdot T$ values was further evaluated (Figure S1, Supporting Information) and the results indicate that the N fixation inhibition was not solely caused by the inhibition of cell growth of *A. variabilis* and there were likely other metabolic/toxicity mechanisms playing roles in the N fixation inhibition observed. The inhibition data at various concentrations and exposure time allowed the determination of the EC_{50} values as a function of time (Figure S2, Supporting Information). The 24 and 96 h inhibitory EC_{50} for nitrogen fixation was found to be 1.16 and 0.4 $mgTiO_2/L$, respectively. The EC_{50}-96 h obtained based on N fixation inhibition was lower than that determined based on cell growth inhibition, suggesting again that N fixation maybe a more sensitive toxicity endpoint indicator than growth rate.

Observation of Intracellular Cyanophycin Grana Protein (CGP) Changes in Response to $nTiO_2$. The ability of cyanobacteria to adjust their structure (i.e., polyphosphate bodies, envelop thickness, etc.) and functions in response to environmental changes or stress has been widely documented (*17, 28*). In this study, time-sequential TEM observations revealed variations in the dynamics of nitrogen storage of *A. variabilis* caused by the exposure to $nTiO_2$ at various concentrations and exposure times, with the increase in the occurrence and size of intracellular cyanophycin granules also referred to as cyanophycin grana protein (CGP) (Figure 3). Recognition of CGPs in cyanobacterial cells was facilitated by their characteristic morphology, shape, and peripheral location within the cells and for the typical contrast they acquire after the staining procedure (*29*). These biomolecules are high molecular weight nitrogen-rich storage polymers, mainly composed of aspartic acid and arginine, and they are nonribosomally synthesized typically in blue-green algae (*14*). A morphometric analysis was performed on CGPs-containing cells to quantify the differences in the abundance of N-rich storage polymer between the exposed and unexposed samples.

Figures 4 and 5 show that the increase in both the occurrence (percentage of cells that contained CGPs) and size (relative surface area of CGPs to cell total area) of the CGPs depended on both $nTiO_2$ concentration and exposure time duration. Less than 30% of the cells analyzed contained CGPs in the control culture with no $nTiO_2$ exposure (at 96 h) (Figure 4). In exposure to a high concentration of $nTiO_2$ at 150 mg/L, more than 80% of cells were found to contain CGPs granules. Increase in the intracellular levels of CGPs (as relative surface area) was further quantified and the data were fitted based on log-normal distributions (Figure 5). In the control, the average percentage of the area occupied by these CGPs granules relative to the cell sectional area was found to be 0.87%. This background level was consistent with the value (0.9%) previously reported by Lawry and Simon for the same strain of *Anabaena* under regular growth conditions (*30*). The distribution curves for the sample exposed to low $nTiO_2$ concentration (1 mg/L) or with short exposure time (3 h) overlapped with that obtained for the control (with average relative area of 0.8%). However, distributions characteristic of all other samples were shifted toward higher values of CGPs relative surface area, ranging from 0.5% to 16.4% of the cells surface (Figure 5a) in the populations that were either exposed for longer time (96 h) for all tested concentrations ranging from 1 to 150 mg/L or at a higher $nTiO_2$ concentration (50 and 150 mg/L) for all exposures time lengths studied (3−96 h). For example, after 96 h of exposure at 1 mg/L, the occurrence and size of CGPs increased by 1.8 and 3.8 fold, respectively, compared to the control sample without any exposure. The dose-dependent increase in CGPs to $nTiO_2$ exposure suggests that this molecule and its function might be involved in $nTiO_2$-induced cell response mechanisms. Therefore, it is potentially a good indicator for $nTiO_2$ exposure, and it can be applied as a possible toxicity assessment endpoint for cyanobacteria. However, the specificity of this indicator to $nTiO_2$ and other toxicants requires further investigation.

Potential Role of CGPs in Algal Response to $nTiO_2$ Toxicity. CGPs have a dynamic role in nitrogen metabolism and storage in nitrogen-fixing cyanobacteria such as *A. variabilis* and *Cyanothece sp.*, similar to the role of phycobilisomes that serve as a major reservoir for N in the nonfixing strain *Synechococcus sp.* during stressed (i.e., nutrient (N)-limiting) conditions (*31*). CGPs are essential in separating the processes of nitrogen fixation and nitrogen utilization and enabling cells to overcome nitrogen shortage (*32*) because they allow cells to store and then degrade and constantly distribute limited amounts of nitrogen in the form of proteins to the cell (*16*). *Anabaena* species tend to accumulate cyanophycin grana in the polar plugs, which are typical structures located at the connecting neck between the heterocyst and the vegetative cell, during nonexponential growth conditions (*33*). The dynamics of formation of cyanophycin granules was also observed by Mackerras et al. (*16*) with *Anabaena cylindrica* under nitrogen-deprived environments. In contrast, Rachilin et al. showed that CGPs could also rapidly degrade in *Anabaena flos aquae* under exposure to zinc (*13*) as a possible detoxifying mechanism to accommodate the cell's need to increase the mobilization of proteins for cations sequestration. These observations suggest that it is possible that in this study, elevated cyanophycin granules formation is associated with stress

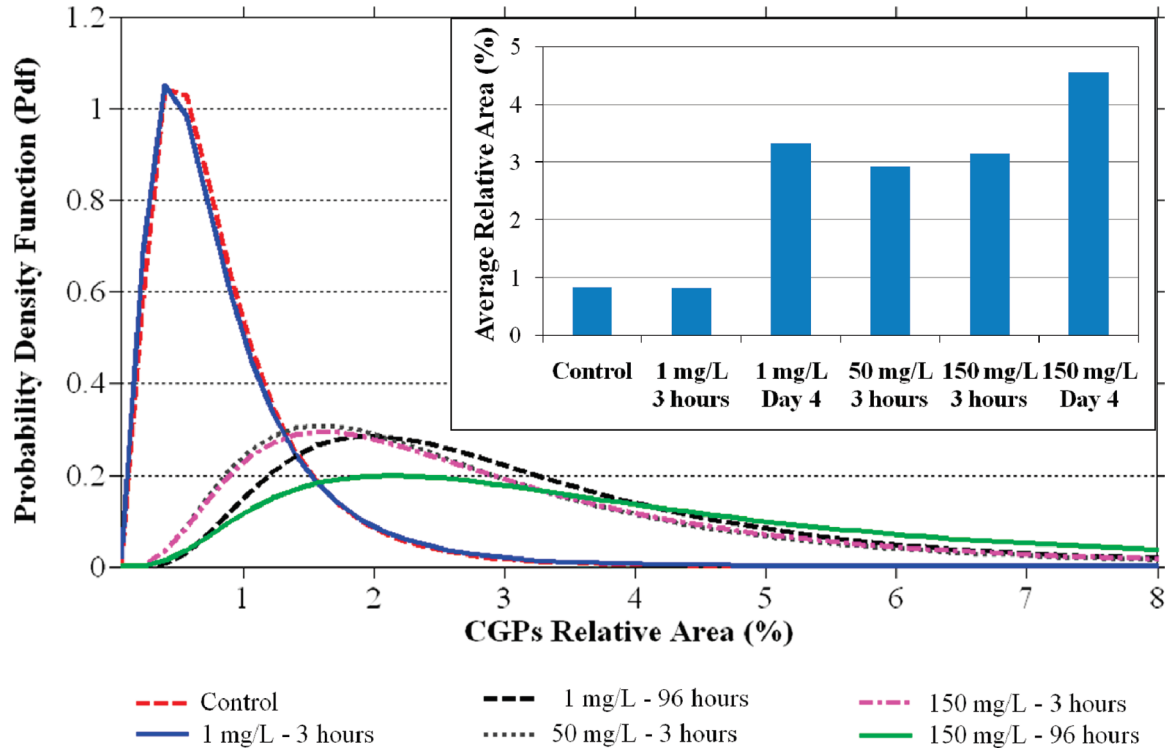

FIGURE 5. Lognormal distributions of intracellular levels of CGPs in CGPs-containing *Anabaena variabilis* cells for samples exposed to different concentrations of $nTiO_2$ and different exposure time lengths. The upper right figure shows the average relative area of intracellular CGPs with respect to cell total area for cells exposed to different concentrations of $nTiO_2$ and different exposure time lengths.

conditions caused by $nTiO_2$ exposure. The results show that this granule is promptly responsive to the toxicity induced by the $nTiO_2$ and it is quantitatively dependent on both exposure concentration and times; therefore it is likely that this intracellular molecule plays an active role in the stress response mechanisms on $nTiO_2$. In addition, its rapid formation upon exposure suggests that it can be immediately induced rather than an accumulative long-term effect.

To understand the transient accumulation of CGPs in *A. variabilis,* more fundamental knowledge is required on the dynamics of nitrogen and carbon fixation products' (C and N) transfer between vegetative and heterocysts cells in diazotrophic cyanobacteria. Recent studies have given important insights on carbon and nitrogen synchronization under regular growth conditions (*31, 34*); however, their equilibriums under environmental stress are still widely unexplored and may lead to various interpretations of phenomena. Based on previous studies (*35*), CGPs do not immediately store fixed nitrogen; rather, their synthesis results from internal conversion of proteins previously provided by heterocyst cells to the rest of the filament with mechanisms that are still not yet understood (*36*). Therefore, one possible hypothesis that supports the dynamics of CGPs observed in this study is that the cell modifies the redistribution of nutrients and increases the diversion of nitrogen into storage products for long-term survival and/or decreases the N usage under stress conditions caused by $nTiO_2$. Another possible scenario that may justify the elevated formation of CGPs in stressed cells involves the binding of $nTiO_2$ with intracellular peptides (*37*), which may lead to alteration of the functions of the proteinaceous cellular machinery. Internalization of $nTiO_2$ nanomaterials has been observed in our other study (unpublished) and therefore contact with intracellular peptides is likely. In addition, binding of $nTiO_2$ with phosphate species in aqueous solution (*38*) may have limited P availability for metabolic needs and stimulated the accumulation of CGPs, as also previously observed in cyanobacteria subject to P starvation conditions (*35*). Lastly, the observed accumulation of CGPs in vegetative cells exposed to $nTiO_2$ might also be correlated with the inhibition of the enzymatic activity (*cyanophycinase* and *peptidase*) responsible for cyanophycin degradation, or with the inhibition (or simply production rate reduction) of the cellular protein synthesis, due to lower metabolic nitrogen required. On the other hand, it is also possible that the cell under the presence of the toxicant increases the activity of the cyanophycin-synthesizing enzyme, named *cyanophycin synthetase*, to prepare the cell for long-term survival. Further and more detailed investigation of the metabolism of these granules in response to $nTiO_2$ and other NMs is therefore warranted.

In summary, this study, for the first time, quantitatively assessed the impact of $nTiO_2$ on cell growth and nitrogen-fixing activity of *A. variabilis* and revealed the possible involvement of intracellular CGPs granules in the stress response mechanism to $nTiO_2$ exposure. Changes in the cyanophycin grana protein accumulation confirm that exposure to NMs can affect patterns of nitrogen metabolism and potentially other key functional biomolecules in algae. The CT-dependent inhibition effect implies that extended exposure time can lead to severe impacts even at very low concentrations. For example, the 90% growth inhibition would be predicted at a very low concentration of 0.7 μg/L (lower end of the range predicted in Mueller et al. study (*5*)) with much longer exposure time of >13 days. The results provided evidence that the release of $nTiO_2$ in aquatic environments will impact the ecological system and its carbon and nitrogen cycling.

Acknowledgments

This study was funded by Northeastern University RSDF award and National Science Foundation Nanoscale Science and Engineering Center (NSEC) for High-rate Nanomanufacturing (0425826). We are grateful to Professor Dhimiter Bello and his student Anoop Pal (Department of Work

Environment, School of Health and Environment, University of Massachusetts Lowell, Lowell, MA) for DLS analysis. We thank William Fowle from the Center for Electron Microscopy at Northeastern University (Boston, MA) for his assistance with the TEM imaging.

Supporting Information Available

Figure S1 shows the correlation of growth inhibition and N fixation inhibition values at a given CT for *A. variabilis* exposed to different concentrations of $nTiO_2$ and different exposure times. Figure S2 shows the time-dependent EC_{50} values obtained based on nitrogen fixation inhibition of *A. variabilis* cells exposed to concentrations of $nTiO_2$ ranging from 0−500 mg/L and exposure time ranging from 3 h to 6 days. This material is available free of charge via the Internet at http://pubs.acs.org.

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