nTiO₂ induced changes in intracellular composition and nutrient stoichiometry in primary producer — cyanobacteria

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Abstract

The widely and increasing use of nano-titanium dioxide (nTiO₂) has led to its release in the environment and concerns of consequent impact on aquatic eco-relevant biota. Previous studies indicated possible physiological changes (i.e., nitrogen storage) induced by nano-titanium dioxide (nTiO₂) exposure in algae, which will likely have ecological implications. This study investigated the short- (96 h) and long-term (21 days) ecotoxic impact of environmentally relevant nTiO₂ concentrations on the cellular biochemical pools and nutrient stoichiometry in the nitrogen-fixing cyanobacteria Anabaena variabilis. Changes in nutrient element ratios and cellular composition were analyzed using both chemical elemental analysis and Fourier Transform Infrared (FT-IR) spectroscopy. Chemical elemental analysis showed that exposure to nTiO₂ at varying dose concentrations and exposure duration led to statistically significant changes in intracellular C:N, C:P and N:P stoichiometries compared with those in the controls. In general, there seemed to be a decreasing trends of cellular C:N ratio and increase in the cellular C:P and N:P ratios with the increasing level of nTiO₂ exposure. Further FT-IR analysis results revealed both temporal and dose-dependent change patterns of major macromolecules, including protein, lipids, nucleic acids and carbohydrates, in A. variabilis upon nTiO₂ exposure. The relative ratio of amide II, lipids, nucleic acids and carbohydrates to the cellular protein content (quantified as amide I stretch) changed significantly within the initial 96 h of exposure and, both the magnitude of changes and levels of recovery seemed to be nTiO₂ dose-dependent. This study, for the first time, demonstrated that the intracellular composition and nutrient stoichiometry of cyanobacteria were affected by nTiO₂ exposure.

Keywords:
Nano-titanium dioxide
Ecotoxicity
Anabaena variabilis
FT-IR
Nutrients

HIGHLIGHTS

• Exposure to nTiO₂ induced changes in cell composition and intracellular nutrient element ratios.
• nTiO₂ exposure led to both temporal and dose-dependent change patterns of major macromolecules
• Physiological cellular changes in Cyanobacteria suggest ecological implications of nTiO₂ exposure

GRAPHICAL ABSTRACT

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stoichiometry changes could be induced by long-term and short-term exposures to nTiO2 to primary producers, which may have ecological implications for interspecies equilibriums and community dynamics in aquatic ecosystems.

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1. Introduction

Progress of nanotechnology poses an urgent need for fundamental understanding of the potential environmental impacts of engineered nanomaterials (NMs). Nanosize titanium dioxide (nTiO2) has a wide range of applications because of its excellent electrical properties and optical performances (Chen et al., 2009). Its presence in wastewater effluents has been anticipated (Mueller and Nowack, 2008), recently evidenced (Kiser et al., 2009) and it will likely impact aquatic biota and eco-relevant organisms. In particular, the toxic effect of nTiO2 on algal species and on ecosystems is still largely unexplored.

Previous literature have reported geno- and cyto-toxic effect of nTiO2 on human cell lines and bacteria (Wang et al., 2007; Gou et al., 2010; Xu et al., 2009), indicating that DNA damage (Jha et al., 2008), membrane disruption (Wiesner and Bottero, 2007) and protein oxidation via reactive oxygen species formation (Neal, 2008) are the major mechanisms of toxicity involved. Recent studies investigated nTiO2 impact on algal ecosystems, showing deleterious effects on algal growth (Lin et al., 2012; Ji et al., 2011) and photosynthetic and nitrogen-fixing activities (Ji et al., 2011; Miller et al., 2010; Cherchi and Gu, 2010; Arooja et al., 2009; Sharma, 2009; Wang et al., 2008). However, studies on long-term impact on cellular physiological changes at environmentally relevant low exposure concentrations have been scarce. Our previous study revealed that cell topology, mechanical properties and intracellular structures of cyanobacteria were compromised after exposure to nTiO2 (Cherchi et al., 2011). Particularly, the dose-dependent accumulation of the nitrogen storage polymer (cyanophycin grana, CGPs) in the cyanobacteria _Anabaena variabilis_ in response to nTiO2 treatments was demonstrated, which indicated that the intracellular nutrient metabolism and stoichiometry may be potentially modified by the exposure to nTiO2 (Cherchi and Gu, 2010). The implications of C: P and N:P variations are well-documented, although not fully elucidated, known to impair biologically mediated flows of energy and nutrients in ecosystems and modify organisms' sensitivity to external nutrients (Elser, 2006).

To further investigate the toxicity impact of nTiO2 to essential macromolecular cell components linked to specific cell functions and metabolism and consequently to the intracellular nutrient stoichiometry, in the present study, we evaluated the effects of nTiO2 exposure at sub-lethal environmental relevant concentrations on the cellular allocation of macromolecules (nucleic acids, proteins, lipids, carbohydrates) in the cyanobacterium _A. variabilis_. Cyanobacteria are of significant biogeochemical importance due to their contribution to primary productivity and their ability to tolerate adverse and fluctuating environmental stressors by implementing unique metabolic strategies (Apte et al., 1998). Microalgae are known for their ability to carry a dramatic reorganization of internal macromolecules and therefore, of their nutrient status, when overcoming abiotic stress conditions (Patel et al., 2008; Giordano et al., 2001). Previous studies have reported such responses in algal ecosystems exposed to toxicants, such as the decrease in protein content in the algae _Micrasterias hardyi_ after contact with active pharmaceutical ingredients (Patel et al., 2008) and the decrease in energy storage products (i.e., carbohydrates) in the brown algae _Padina tetrastromatica_ under stress induced by cadmium (D’Souza et al., 2008). Alterations in the relative presence of carbohydrate, phosphoryl, hydroxyl, and amine functional groups will generate variations not only at the single cell level (i.e., homeostatic regulation processes) but also at larger scale with modifications on cell growth, fitness, interspecies relationship, trophic interactions and food web dynamics (Levin, 2009).

In this study, a quantitative elemental analysis was performed to demonstrate nTiO2-induced deviations of intracellular nutrient stoichiometry from those of cells in control (untreated) conditions. In addition, cellular composition fingerprinting obtained with the analysis of spectral signatures using FT-IR, as well as chemometric methods, revealed high-resolution temporal change patterns of major biochemical pools and chemical markers upon short- (96 h) and long-term (21 days) exposure to different doses of nTiO2. The results provided important insights into the impact of nTiO2 exposure on intracellular composition and stoichiometry and revealed potential ecological implications of long-term NM exposure.

2. Experimental methods

2.1. NM preparation and characterization

Nano-TiO2 anatase (nTiO2, primary size 10 nm, NanoStructured & Amorphous Materials, Houston, Texas, USA) was prepared in a modified Mes-Volvox medium (Cherchi et al., 2011) and then dispersed via sonication in a High energy Cup-sonicator (Fisher scientific, Inc.), at ~90 Watt power for 20 min. Bovine Serum Albumin (1% BSA) was added in the stock solution (10 g-nTiO2/L) to enhance the dispersion for uniform dosing (Pal et al., 2011). Physical and chemical characterization of nTiO2 was detailed in previous studies (Cherchi and Gu, 2010; Bello et al., 2008) and is also shown in Table 1. Detailed physical and chemical characterization of the nTiO2 used in this study, including aggregate size distribution, metal impurities, surface charge, zeta potential, organic and elemental carbon etc., was conducted and reported by Bello et al. Primary size nTiO2 from 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 NM dispersion in the culture media (single crystal). The polydispersity index (Pdi) after dispersion in culture media was found to be 0.479. A specific surface area of 274.2 m² g⁻¹ was measured and the X-ray diffraction showed the presence of small amount of both anatase and rutile soluble extracts in the nTiO2 anatase used in this study (Bello et al.). Transmission electron microscopy was used to observe nTiO2 behavior during ecotoxicological tests of exposed _Anabaena_ cells. From the analysis of 115 aggregates observed across samples exposed for to 24 and 96 h to 1 mg/L to 150 mg/L, nTiO2 agglomerates of average 425.0 nm ± 275.5 nm (longest dimension) were observed (Stable 1).

2.2. Culture conditions and ecotoxicological tests

_A. variabilis_ strain (UTEX #1444), also referred to as _Trichormus variabilis_, was cultured in a modified Mes-Volvox media, with conditions described in our previous work (Cherchi and Gu, 2010). For exposure tests, 500 mL batch reactors of initial chlorophyll a concentration of 200 µg/L were dosed with different nTiO2 concentrations, ranging from 0 mg-nTiO2/L (control sample) to 1 mg-nTiO2/L, and incubated for 21 days under a 12 h light/12 h dark regime to mimic natural environmental conditions. The light source used 1:1 ratio of 34 W cool white and 40 W gro-lux fluorescent bulbs (Sylvania, Danvers) of wavelength output >400 nm, which yields a low PAR value of 35 µmol photon/m²-s. Batches were continuously mixed (300 rpm) and aeration was continuously provided via 0.2 µm filtered compressed air to deliver air in the reactors’ headspace at a rate of 5 mL/min. Chlorophyll a has often served as indicator for algal growth assessment.
and CO2, H2O and N2 were detected by thermal conductivity
measured using a CE-440 elemental analyzer (Exeter Analytical, Inc., Chelmsford, MA). Combustion was obtained at 950 °C in the presence of interferences of BSA introduced from nTiO2 stock solution preparation were measured to be negligible (lower than detection limit). All protein measurements were performed in triplicates.

2.3. Chemical elemental analysis of intracellular macronutrients

An independent experiment was performed to determine the intracellular elemental nutrients in A. variabilis’ cells exposed to nTiO2 concentrations of 1, 10 and 100 µg/L and 1 and 10 mg/L for 96 hour and 21 day exposures. Exposure condition was similar to those ecotox assays described previously and all tests were run in triplicate batches. Elemental carbon and nitrogen analysis was performed on per-washed and overnight freeze-dried samples containing 1–3 mg of biomass, and measured using a CE-440 elemental analyzer (Exeter Analytical, Inc., Chelmsford, MA). Combustion was obtained at 950 °C in the presence of ultrapure oxygen and CO2, H2O and N2 detected by thermal conductivity. Spectra were collected using a Perkin Elmer Spectrum One (Kevley Technologies, Chesterland, USA), desiccated under vacuum besieing

2.4. FT-IR spectroscopy analysis of cell components

Aliquots of sample (20 mL) were periodically withdrawn and cells were fixed in Lugol’s iodine solution (1 µL/mL) followed by resuspension in deionized water. An aliquot (4 µL) of the cell suspension was transferred onto a MirrIR low-e re-ectance microscopic slides (Kevley Technologies, Chesterland, USA), desiccated under vacuum besieing

3. Results and discussions

3.1. Nano-TiO2 exposure impact on intracellular element stoichiometry

To test the hypothesis that the nTiO2 exposure may induce cellular changes in composition and macromolecule pools, we evaluated the intracellular nutrient element balances and stoichiometry of A. variabilis upon nTiO2 exposure at 96 h and 21 days with varying dose concentrations ranging from 1 µg-nTiO2/L to 10 mg-nTiO2/L. Elemental quantitative analysis (Table 1) shows impact of nTiO2 exposure on intracellular nutrient stoichiometry (C:N, C:P, N:P) at varying dose concentrations (including untreated controls), and with different exposure time lengths (96 h versus 21 days). In the controls without nTiO2 exposure, the cellular C:N ratio showed a 5.6% decrease after 96 h of culture, and a 4.5% increase after 21 day exposure. However, both C:P and N:P ratios in the controls showed slight increase compared to initial measurements at time zero. This biochemical variability at various phases of growth has been previously reported on various aquatic species, including green algae (Sigee et al., 2007; Liang et al., 2006), and likely linked to changes in the extent of carbon and nitrogen fixation of cells when approaching late exponential growth conditions (Mulholland and Capone, 2001). Note that the exposure of A. variabilis cultures to nTiO2 at environmentally relevant low concentrations (1–1000 µg/L) did not lead to any observable growth inhibition (based on chlorophyll a) with short-term exposure (96 h) (data not shown); however, growth inhibition of 6.5 ± 0.7% (p = 0.03) was detected with long-term exposure (21 days) at 1 mg/L (Fig. 1). This is consistent with our previous report of the CT (concentration (C) and exposure time (T))-dependent toxicity of nTiO2 (Cherchi and Gu, 2010).

Exposure to nTiO2 at varying dose concentrations and exposure durations led to statistically significant changes in intracellular C:N:P

<table>
<thead>
<tr>
<th>Time − 0</th>
<th>C:N</th>
<th>C:P</th>
<th>N:P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.4 ± 1.4</td>
<td>143.3 ± 1.61</td>
<td>19.9 ± 0.4</td>
</tr>
<tr>
<td>10 µg/L</td>
<td>7.5 ± 0.1</td>
<td>153.3 ± 19.2</td>
<td>20.7 ± 2.6</td>
</tr>
<tr>
<td>Time − 96 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.9 ± 0.1</td>
<td>292.6 ± 17.0</td>
<td>42.1 ± 2.8</td>
</tr>
<tr>
<td>1 µg/L</td>
<td>7.0 ± 0.5</td>
<td>235.0 ± 79.3</td>
<td>32.0 ± 6.6</td>
</tr>
<tr>
<td>10 µg/L</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>100 µg/L</td>
<td>6.5 ± 0.3</td>
<td>421.2 ± 50.6*</td>
<td>64.2 ± 4.9*</td>
</tr>
<tr>
<td>1 mg/L</td>
<td>6.3 ± 0.2*</td>
<td>347.4 ± 31.7</td>
<td>55.4 ± 6.5*</td>
</tr>
<tr>
<td>10 mg/L</td>
<td>7.4 ± 0.3*</td>
<td>327.8 ± 10.9</td>
<td>43.5 ± 0.1</td>
</tr>
<tr>
<td>Time − 21 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.7 ± 0.4</td>
<td>250.6 ± 17.1</td>
<td>30.2 ± 4.5</td>
</tr>
<tr>
<td>1 µg/L</td>
<td>7.1 ± 0.1</td>
<td>367.7 ± 32.8*</td>
<td>51.5 ± 4.2*</td>
</tr>
<tr>
<td>10 µg/L</td>
<td>6.5 ± 0.4*</td>
<td>195.3 ± 31.8</td>
<td>30.2 ± 6.4</td>
</tr>
<tr>
<td>100 µg/L</td>
<td>7.3 ± 0.3</td>
<td>294.1 ± 43.2</td>
<td>40.4 ± 7.9</td>
</tr>
<tr>
<td>1 mg/L</td>
<td>7.3 ± 0.3</td>
<td>303.9 ± 50.6</td>
<td>42.3 ± 6.7</td>
</tr>
<tr>
<td>10 mg/L</td>
<td>6.1 ± 0.7*</td>
<td>328.1 ± 38.4*</td>
<td>53.9 ± 6.4*</td>
</tr>
</tbody>
</table>

* Statistically significant values based on Student’s t-test at p < 0.05.

FT-IR spectra were imported into Matlab v. 7.8.0 (R2009a), where surface fitting and data analysis were carried out using the PLS toolbox (Eigenvector Technologies, Manson, USA). To reduce the potential bias associated with the spectra baseline, baseline correction was performed and spectra were pre-processed using the maximum normalization algorithm which normalizes to the most intense frequency in the spectrum (amide I). For the analysis, first derivatives of the corrected spectra were computed using the Savitsky and Golay algorithm with an 11 point window and a third order polynomial fitting. Data were then mean centered before being subjected to the PLS toolbox for principal component analysis (PCA). Principal components (PCs) were calculated and PCA score plots were used to visualize any clustering of the samples. Loading plots were used to determine the spectral region that contributed the most to the variance in the dataset.

Table 1

Elemental intracellular ratios of C:N, C:P and N:P (on a % dry mass basis) for A. variabilis exposed to nTiO2 concentrations (1, 10, 100 µg/L and 1, 10 mg/L) for 96 h and 21 days. Average and standard deviations are of 3 independent experiments.

2.5. Principal component analysis

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stochiometry compared with those in the controls (Table 1). Potential interferences of nTiO2 on chemical elemental analysis were excluded, since the C:N, C:P and N:P ratios of the control were consistent and comparable to those at 10 mg-nTiO2/L at initial time zero. Both short-term exposure and long-term exposure to nTiO2 led to statistically significant changes in the cellular C:N, C:P and N:P ratios. Trends of elemental ratios are found to be dose-specific, indicating that different doses can trigger different biochemical responses and cell behaviors at various exposure time lengths. In general, there seemed to be decreasing trends of cellular C:N ratio and increase in the cellular C:P and N:P ratios with the increasing level of nTiO2 exposure. For example, at 21 day-exposure study, exposed cells mostly exhibited a general decrease in C:N, as much as 21% compared to the control, and concurrent increase in C: P and N:P ratios, by up to 31% and 77% respectively, for exposure at 10 mg-nTiO2/L (p < 0.05).

These results clearly demonstrate that nTiO2 exposure induces alterations in the intracellular C:P and N:P ratios in comparison to controls without nTiO2 exposure. The implications of C:P and N:P variations upon conditions of stress are well-documented, although not fully elucidated, and certainly known to impair biologically mediated flows of energy and nutrients in ecosystems and modify organisms’ sensitivity to external nutrients, thus ecological nutrients cycling (Elser, 2006). Algal species composition regulates cell growth and affects food webs at all levels, therefore influencing their ability to meet their reproductive and nutritional requirements (Gilbert, 2012). For example, previous study has suggested that lower molar C:N ratio (<10) of the microalgae Rhodomonas sp. resulted in a lower egg production in the copepod Acartia sp. (often used as bio-indicators) than those fed with microalgae with higher cellular C:N ratio of 10–15 (Augustin and Boersma, 2006). Others have shown that algae with both phosphorus and nitrogen limitation may alter the thickness of their cell wall, making them more resistant to zooplankton digestion than algae with balanced nutrient conditions (Schwarzenberger et al., 2013). In addition, production of toxins in algae, which are known to impair higher trophic organisms, for example, often occurs when disproportion of internal nutrient stoichiometry exists (Gilbert, 2012). Based on these observations, toxicity responses of primary producers to nTiO2 exposure, such as change in intracellular stoichiometry, carbohydrate and lipid relative composition (shown in this study), as well as the increase in N storage cyanophycin and alterations of cell membrane thickness as reported in our previous studies (Cherchi and Gu, 2010; Cherchi et al., 2011) may likely lead to changes in species competition, and consequent implications and impact on aquatic ecosystems.

3.2. Short- and long-term changes in cellular proteins upon nTiO2 exposure

The measured changes in the intracellular elemental nutrient ratios in algal cells motivate further investigation into the underlying causal factors and mechanisms. Increase in proteins has been suggested by abiotic stresses, as the increase in proteins (i.e., stress response proteins) has been reported to be part of the stress response mechanism within cells (Fernandes et al., 1993).

To gain further insights into the effects of nTiO2 exposure at sublethal environmentally relevant concentrations on the cellular allocation of macromolecules (nucleic acids, proteins, lipids, carbohydrates) in the cyanobacteria A. variabilis and consequently the intracellular element stoichiometric ratios, we further applied FT-IR to monitor the physiological state of A. variabilis cells and the results revealed structural changes and reallocation of intracellular pools in response to nTiO2 exposure at various concentrations. Fig. 2 shows the example of temporal changes in the FT-IR spectra of the culture exposed to 1000 µg-nTiO2/L compared to the control with no NM exposure. Two protein signatures, namely amide I and amide II, are interpreted from the most prominent stretching at 1650 cm⁻¹ (C=O stretch), typical of α-helical and parallel β-sheets of random coiled protein structures, and the in-plane N–H bending of amides (1540 cm⁻¹), respectively. Antisymmetric C–H markers at 2920 cm⁻¹ and 2956 cm⁻¹ and symmetric stretching at 2852 cm⁻¹ and 2876 cm⁻¹ are typical of lipids and fatty acids. Insoluble glucose polymers and polysaccharides (e.g., glycogen) exhibit a series of absorption bands due to C–O stretching and C–O–C deformations at 1150 cm⁻¹ and 1032 cm⁻¹, respectively. Nucleic acids and phosphorylated molecules have functional groups with absorption bands in the same region of the carbohydrate spectrum, with major asymmetric and symmetric vibrations associated at 1078 cm⁻¹ and 1240 cm⁻¹, representing the asymmetric PO2⁻ stretch of DNA/RNA backbones, phosphorylated proteins and polysaccharide storage products (Pal et al., 2011). All spectra were normalized to the strongest amide I band and the ratios indicated certain carbon balance within the cell as suggested by Sige et al. (2007).

A mean centered principal component analysis (PCA) on derivatized spectra was employed to classify A. variabilis response as a function of nTiO2 dose and to examine differences between spectra as a function of exposure time length. The score plots (Fig. 2) project the spectral data onto two principal components (PC1 vs. PC2) and help visualizing

### Table 2

| p-Value (t-test) of statistical significance for the changes in protein/chlorophyll a ratio of exposed samples compared to the control for different exposure time lengths. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1 µg/L | 10 µg/L | 100 µg/L | 1000 µg/L |
| Over 21 day exposure | 0.14 | 0.00 | 0.02 | 0.02 |
| Over 96 hour exposure | 0.23 | 0.02 | 0.04 | 0.06 |
the degree of separation among the conditions tested. Loading plots (Fig. 3) help to reveal the specific regions of the spectrum (frequencies) that contribute to the variation within the set of data.

The score plots of the PCA performed on both 96 hour and 21 day exposure times show a clear shifting of the mean PC scores associated to different doses of nTiO2 treatment in respect with the mean PC score of the unexposed class, and the separation is distinctive for each exposure time analyzed. Particularly, in the short term (96 h), samples treated with 1, 10 and 100 µg-nTiO2/L show some degree of separation from the control along PC1, with the 100 µg-nTiO2/L class diverging towards the positive PC2, whereas the variation caused by the highest exposure concentration (1000 µg-nTiO2/L) only extends along the PC2. The loading plots showed that the separation observed for cells exposed to 1 and 10 µg-nTiO2/L can potentially be attributed to differences in the symmetric stretches of methyl and methylene groups in fatty acids and methyl groups of lipids (2876 cm⁻¹) and to phosphodiester backbone of nucleic acids (νas P=O). In addition, differences in intracellular carbohydrate components (ν C–O–C, ν C–O) contribute to the shift in the 100 µg-nTiO2/L class. Positive PC1 and PC2 scores for the 1000 µg-nTiO2/L class suggest that, in the short period, this exposure concentration induces an array of effects in A. variabilis cell, which include changes in symmetric and asymmetric stretches of lipids (2852 cm⁻¹, 2920 cm⁻¹ and 2956 cm⁻¹), polysaccharides (ν C=O), nucleic acids (ν P=O) and amide (II) groups associated to proteins (ν N–H, ν C–N).

In the long term exposure scenario (21 days), the classification obtained was different from that observed after only 96 h. Interestingly the sample exposed to the lowest concentration (1 µg-nTiO2/L) seems to be the farthest from the control, possibly due to changes in protein stretches (δ N–H, ν C–N and ν C=O) and symmetric CH₂ and CH₃ groups associated to lipids. A very similar long-term response was instead obtained in cells exposed to nTiO2 concentrations ranging from 10 to 1000 µg/L, where the visible stretching along the negative PC1 again reflects a more comprehensive biochemical damage of cytoplasmic components, such as nucleic acids (ν P=O) and polysaccharides (ν C–O–C, ν C–O), and membrane characteristic groups (ν CH₃, ν CH₂, νas CH₂ and νas CH₃ of lipids).

From the results of principal component analysis, it is evident the ability of nTiO2 to target A. variabilis' cellular components and induce a set of molecular modifications in a dose-dependent manner as results of dose-dependent toxicity mechanism and effects. Temporally dynamic

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**Fig. 2.** Score plots on PC1 and PC2 from principal component analysis performed over the region (3000–2700 cm⁻¹; 1800–750 cm⁻¹) of FT-IR spectra obtained from samples exposed to nTiO2 concentrations ranging from 1 to 1000 µg/L for 96 h (a) and 21 days (b). Each point represents the mean PC score for each treatment calculated on 120–160 spectra (60–80 spectra for each), and error bars are the standard error of the mean along the principal component axes.

**Fig. 3.** Loading plots on PC1 vs. PC2 from principal component analysis performed over the region (3000–2700 cm⁻¹; 1800–750 cm⁻¹) of 6000 FT-IR spectra obtained from samples exposed to nTiO2 concentrations ranging from 1 to 1000 µg/L for 96 h (a) and 21 days (b). In red: proteins; in blue: lipids; in green: polysaccharides; in pink: nucleic acids. Details on band assignment are presented in STable 3.
and nTiO₂ concentration-dependent changes in the major cellular macromolecules, as indicated by the FT-IR fingerprints, were observed and they are further discussed in more details in the following sections.

3.4. Changes in cellular carbohydrates during nTiO₂ exposure

Dose-dependent temporal trends of carbohydrates/amide I ratios (Fig. 4) showed clear differences (p < 0.06) in the ratio value between those treated with nTiO₂ dose higher than 10 µg/L and untreated controls. The reduction in the carbohydrate/amide I ratio was most pronounced (>32%) with the highest nTiO₂ dose at 1000 µg/L during the first 96 hour exposure. Temporal variation of the carbohydrate/amide I ratio values was observed in all samples, including the control with no exposure, indicating that transitional and temporal cellular changes occur at initial exposure and self-recovering or adjustment to more stable conditions is achieved after a longer period of time (>10 days).

Except for a few exclusions, the average carbohydrate/amide I ratio found in this study is in the range of those reported in Dean et al. (0.25 to 0.82) (Dean et al., 2007). The carbon/protein ratios are sensitive indicators of algal chemical composition and rates of physiological processes, and provide insights into the adaptive response in the allocation of cell resources after exposure to pollutants or to generic conditions of stress (Geider, 1987). Dynamic reallocations of intracellular carbon into polysaccharides during regular growth of cyanobacteria (Carr and Whitten, 1982) and fluctuations after cell transfer to fresh media (Dean et al., 2008) have been observed before. These results showed consistently lower carbohydrate/amide I ratios in those treated with nTiO₂ (10–1000 µg-nTiO₂/L) than the control, suggesting likely lower carbohydrate (energy) content and/or storage capacity of cells under nTiO₂ stress than the controls. Phenomena of carbon re-allocation have been reported in a previous study where cyanobacteria cells subjected to environmental perturbation (i.e., P limitation) and explained as a cell physiological need to survive stress (Sige et al., 2007).

3.5. Changes in nucleic acids during nTiO₂ exposure

Two IR vibrations at 1078 cm⁻¹ (v P=O) and 1240 cm⁻¹ (v₁ and v₃ of P=O) wavenumbers were assigned to nucleic acids, representing the symmetric stretching and asymmetric stretching (P=O) of the phosphodiester backbones of nucleic acids (DNA and RNA), respectively. The characteristic functional group of nucleic acids at 1078 cm⁻¹ (v P=O) was normalized to amide I (1078 cm⁻¹/amide I) and it showed dose-dependent changes upon nTiO₂ exposure with progressively decreasing values as dose concentration increased (Fig. 5a). Throughout the testing period, the average internal ratio of the (1078 cm⁻¹) to amide I was consistently lower in the cultures exposed to nTiO₂ at >10 µg/L than the control. The second identified asymmetric P=O functional group at 1240 cm⁻¹ of phosphodiester backbones of nucleic acids did not show dose-dependent relations for most of the time points analyzed (data not shown). This suggests that functional groups of the same macromolecule (e.g., DNA) may respond differently to nTiO₂ action.

Variations of the symmetric and asymmetric P=O vibrational modes and the fluctuation in their ratios (1240 cm⁻¹/1078 cm⁻¹; Fig. 5b) may reveal important insights into the potential mechanisms of the toxicant action. The alteration of the 1240 cm⁻¹/1078 cm⁻¹ ratio has been previously reported in bacteria after exposure to ascorbic acid and linked to free radical generation (Melin et al., 2001). Our results may indicate that a similar effect is exerted by nTiO₂, which has been reported by us and others to generate reactive oxygen species and induce oxidative damage in cyanobacteria (Cherchi et al., 2011). In addition, shifts or intensity fluctuations of these stretching are often linked to the recognized binding potential of the phosphodiester bond with pollutants (DSouza et al.; Li et al., 2010). Alterations in P=O modes are also believed to reflect changes in RNA cellular content (Chen et al., 2006), thus influencing important mechanisms of protein synthesis sustaining organisms’ reproduction and growth (Gillooly et al., 2005). In addition, impairment of RNA allocation and cellular P content will more broadly impact the biogenesis of ribosomes, significant repository of P in ecosystems and intracellular element proportions, particularly associated to P-rich biomolecules (Li et al., 2010; Else et al., 2003). In the long-term, this will have the potential to affect organisms’ growth rate and fitness (Levin, 2009), and thus also biological productivity.
3.6. Changes in protein structure during nTiO2 exposure

FT-IR allowed a finer-resolution examination of the potential protein pool structure and compositional changes. Fig. 6 shows the temporal changes in amide II/amide I ratio in all treatments, during both short and long term exposures. The highest nTiO2 concentrations applied (1000 μg·nTiO2/L) caused a significant increase ($p < 0.01$) of this ratio compared to the control by 0.4% (at day 1) to 16% (day 2), with an average of 10.6% increase during the other exposure times analyzed. In general, the relative increase of amide II with respect to amide I intensities indicates that conformational modifications in protein folding and unfolding, possible surface protein denaturation and changes in membrane protein secondary structure, are likely induced by nTiO2 treatment during the initial exposure (Parikh and Chorover, 2006). Modifications of protein backbone conformation, such as secondary structure profiles and α-helix to β-sheet ratios may have important implications in cyanobacteria metabolism and response to stress, as well as protein internal utilization and availability (Yu, 2006).

3.7. Changes in lipids upon exposure to nTiO2

Similar to the observation for carbohydrates over long-term exposure, although a temporal variation in the lipid/amide I ratio was observed for the first 96 h, a more conservative average value of the lipid/amide I ratio was obtained after 10 days for both treated and untreated cultures (data not shown). The final average ratio after 10 days seemed to decrease with increasing nTiO2 doses, from 0.63 ± 0.01 in the control and 0.56 ± 0.04 and 0.42 ± 0.04 in the 1 and 10 μg/L exposures ($p < 0.04$), to 0.41 ± 0.02 and 0.44 ± 0.05 ($p < 0.003$) for treatments at 100 and 1000 μg·nTiO2/L, respectively.

The comparison of the trends of lipid/amide I and carbohydrate/amide I ratios did not show any consistency or correlation, indicating that the dynamic trends are mostly due to variations in carbohydrate or lipids rather than modifications in the protein content alone. This is because if protein changes were responsible for the observed trend of the ratios of lipids/amide I and carbohydrates/amide I overtime, they would then correlate as suggested by Dean et al. (2008). These results suggest that nTiO2 promotes changes in carbon allocation, decreasing both carbohydrate and lipid ratios. Alterations in the relative abundance of lipids caused by nTiO2 exposure might also have contributed to A. variabilis structural changes (Cherchi et al., 2011). Changes in the asymmetrical –CH₂ vibrational frequencies caused by NM exposure were reported in recent studies, where Gram-positive and negative bacteria exposed to fullerenes showed dramatic changes in the conformational order of the membrane acyl chains (Fang et al., 2007).

4. Conclusions

In summary, chemical elemental analysis demonstrated nTiO2-induced changes in cellular nutrient stoichiometry in A. variabilis, and further application of FT-IR analysis revealed cellular structure and composition (macromolecular ratios) alterations as results of nTiO2 exposure at environmentally-relevant low doses (dose below those causing observable growth inhibition) on A. variabilis. The results indicated changes in intracellular stoichiometry of nutrients, such as in the decrease of C:N and concurrent increase in C:P and N:P ratios, and the PCA analysis based on FT-IR spectrum suggests possible potential reallocation of carbon among macromolecules and, particularly, from storage of C-rich products into proteins. The results demonstrated the pioneering application of FT-IR to physiological nanotoxicity investigations to reveal subtle intracellular effects such as the modifications in the intracellular pools of proteins and RNA-associated functional groups, energy storage products (i.e., carbohydrates and lipids), as well as the depository of genetic information (i.e., DNA). These findings imply an important warning that there might be long-term changes in the intracellular composition of ecologically relevant organisms at very low and environmentally relevant nTiO2 concentrations (i.e., 1–100 μg·nTiO2/L) and are interesting starting point for further studies. Primary producers' composition, in fact, affects food webs at all levels influencing their ability to thrive and to meet their nutritional requirements as previously discussed. This confirms the importance of understanding the effect of NMs on intracellular modifications of functionally key macromolecules, which can reflect changes at a larger scale involving community structures and dynamics in ecological systems.

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Appendix A. Supplementary data

SFig. 1 shows the growth inhibition of A. variabilis as a function of nTiO2 dose concentrations for 21 day exposure. SFig. 2 shows time-dependent matrix plots of FT-IR spectra collected during A. variabilis growth. STable 1 summarizes the physical and chemical characterization of nTiO2. STable 2 shows the correlation parameters of chlorophyll a and protein measurement with cell count of A. variabilis exposed to different nTiO2 concentrations for 13 day exposure. STable 3 lists the FT-IR frequency band assignments for A. variabilis. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.scitotenv.2015.01.037.

References


