Ecotoxicity and Environmental Implications of Nano Titanium Dioxide Revealed Through Primary Producers Surrogates – Cyanobacteria

A Dissertation Presented

by

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To

The Department of Civil and Environmental Engineering

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the field of

Civil and Environmental Engineering

Northeastern University

Boston, Massachusetts

(Dissertation completed in August 2012)
Abstract

Over the last decade, the rapid progress in nanoscience has led to the proliferation of new engineered nanomaterials (NMs), with global market worth estimated as US$1 trillion by 2015. Despite the potential exposure scenarios, and evidenced risk for human health and the environment, current national legislations and laws that regulate nanotechnology are lacking, partially due to insufficient toxicological knowledge available. Most of the existing nanotoxicological studies have focused on human impact and occupational safety, particularly on those routes with higher likelihood of exposure (i.e., inhalation, ingestion, and contact). The understanding of the fate and transport of NMs in the environment and of their ecological implications is in its infancy. Fundamental research on ecologically-relevant organisms, such as prokaryotic and eukaryotic primary producers, has been rather scarce.

A limited number of nanoeffecticity studies have demonstrated the potential impact of NMs exposure on freshwater microorganisms (i.e., algae), showing mostly acute toxic effects, rather than chronic responses. Conventional regulatory toxicological methods rely on phenotypic endpoint and are performed at higher NMs doses than those environmentally relevant and expected in natural systems, thus possibly not reflecting realistic exposure scenarios. As a result, sub-cellular and molecular changes induced by sub-cytotoxic dose levels over long-term exposure to the test organism have hardly been revealed. Ecological system and population-level evaluations of NMs ecotoxicity across trophic levels are in great need in order to predict the potential larger-scale impacts.

This study aims to fill in the above mentioned knowledge gaps and intends to develop and apply a comprehensive, mechanism- and biochemical-based approach for a holistic
investigation of NM-exposure on aquatic primary producers to reveal not only the phenotypic
damages with acute exposure but also subtle cellular adaptation with chronic exposure. In
addition to conventional toxicological approaches, modern molecular biology techniques (i.e.,
RT-PCR) and advanced analytical techniques, such as high-resolution microscopy (i.e., TEM,
AFM) and molecular spectroscopy (i.e., RAMAN, FT-IR), are applied to reveal detailed cellular
structure and composition changes in exposure to NMs. We choose nano titanium dioxide
(nTiO₂) because it is one of the most widely applied NMs and its presence in environment has
already been evidenced in WWTP effluents. Nitrogen-fixing cyanobacteria, *Anabaena sp.*, are
employed as representative primary producers, due to their essential biogeochemical role in food
webs as main carbon and nitrogen flux providers and their ability to implement unique metabolic
strategies to overcome environmental stresses. In addition, the impact on their health and
function may rebound on the health of higher trophic communities, since they govern ecological
equilibriums (chemical and biological) and maintain ecosystems nutrients balance and flow
through the extent of their nitrogen fixation and nutrients translocations to higher trophic
members.

We first evaluate the toxicity of nTiO₂ on *Anabaena sp.* cells using conventional
population-level endpoints including inhibition of growth and nitrogen fixation activity. We
propose and demonstrate the application of a Hom’s-type dose-response model for predicting
nanocytotoxicity at different nTiO₂ doses and exposure time. The model reveals the importance
of exposure time in nTiO₂ toxicity and suggests that long-term exposure at even very low doses
(predicted or measured exposure scenarios) can lead to similar damages as the acute exposure to
higher doses. Measurements of reactive oxygen species (ROS) show a dose-dependent
extracellular ROS production but low and consistent intracellular ROS levels, indicating that
oxidative stress and cell outer-membrane damage is likely the main initiating toxic action and mechanism. Using TEM and AFM, as well as quantitative images analysis and mechanic computations, we show and quantify the cell surface structure damage, cell surface morphological changes, as well as cell mechanical properties changes after nTiO$_2$ exposure. The measured increase in surface roughness and cytoplasmic turgor pressure (i.e., cell spring constant) suggests the potential collapse of membranes’ layers and changes in water afflux in the membrane folded compartments, possibly as a result of modified proteins structure and folding configurations mediated by ROS. High-resolution TEM images and the confocal feature of Raman imaging shows evidence, for the first time, of the internalization of nTiO$_2$ particles through the multilayered membrane of cyanobacterial cells, possibly occurring as a consequence of membrane damage of compromised or dead cells since cyanobacteria, as any prokaryotes, are believed not to have specific internalization mechanisms for nanoparticles, and colloidal particles. Nevertheless, their intracellular presence warrants on the potential nTiO$_2$ translocation to higher trophic members, thus possible biomagnification along the food web chain.

The morphometric analysis on TEM sections allows the identification of an array of intracellular structure modifications, including various functionally-relevant macromolecules and cell compartments. Particularly, the results show, for the first time, that nTiO$_2$ induces a concentration- and time-dependent increase in both the occurrence and intracellular levels of a functionally-relevant nitrogen-rich biomolecule, named cyanophycin grana protein (CGP), thus showing altered dynamics of nitrogen storage in exposed cells. Additionally, nTiO$_2$ exposure leads to observable alterations in the radial arrangement of thylakoidal membranes, primary sites of photosynthetic reactions in cyanobacteria. These findings suggest that patterns of nitrogen and
carbon metabolism, and potentially other key functional biomolecules in algae can be altered by the nTiO2 exposure.

The observed increase in intracellular nitrogen storage polymer (CGPs) and the inhibitory effects on nitrogen fixation rates suggests that nitrogen metabolism is sensitive and likely impacted by nTiO2 exposure. To further reveal the fundamental understanding of the nTiO2-induced effects on cellular nitrogen metabolism and status, transcriptional level changes of key biomarker genes in the global regulation of the cell N status and in N assimilation- and N storage-specific pathways is examined using real time RT-PCR for different nTiO2 doses and at both light and dark cycle. Key intracellular nitrogen metabolites (aminoacids) and the total proteins content are also monitored along with gene expression trends, to reveal additional toxicity responses involving nitrogen metabolism and the dynamics of nitrogen accumulation. The results show that nTiO2 inhibit cell N-fixing ability in a time- and concentration-dependent fashion and interrupts the intracellular nitrogen assimilation and storage balance. The results reveal that nTiO2 leads to the immediate protein increase in cell upon exposure, likely related to homeostasis and cell stress response. Nano-TiO2 causes unbalance in C/N equilibriums, which is evidenced by the increased levels of N-rich aminoacids (aspartic acid and arginine) and CGPs polymers, as well as transcriptional changes in enzymes involved in nitrogen fixation, assimilation, and in the storage polymer-CGPs-formation and degradation.

Furthermore, transcriptional profiles of N metabolism biomarker genes exhibits clear circadian patterns with more distinctive dose-dependent toxic impact in light cycle, whereas nTiO2-dose-independent pattern at dark. During night, when the influence of nTiO2 photocatalytic effects, thus of oxidative stress, is minimal or absent, the toxicity is not as severe as in light, and the energetic conditions of the cell (i.e., ATP levels) seem to govern the cellular
responses. These indicate that complicated sub-cellular responses of cyanobacteria in exposure to nTiO2 are associated with not only the external stressor but also light, internal circadian metabolism regulation and energy (ATP) levels.

The alteration of N metabolism and stimulated intracellular N storage polymer formation implies potential cellular biochemical pool and nutrient stoichiometry changes. We then perform cellular composition fingerprinting via Fourier Transform Infrared (FT-IR) and classify spectral signatures using principal component analysis (PCA) to reveal and quantify any intracellular compositional changes and global spectral responses as a function of nTiO2 dose for both short-term and long-term exposure. The analysis of Fourier Transform Infrared (FT-IR) spectral signatures reveals dynamic temporal and dose-dependent change patterns of major macromolecules, including protein, lipids, nucleic acids and carbohydrates, in Anabaena sp. upon nTiO2 exposure at low and environmentally-relevant concentrations. The relative ratio of amide II, lipids, nucleic acids and carbohydrates to the cellular protein content (quantified as the amide I intensity) change significantly within the initial 96 hours of exposure and, both the magnitude of changes and levels of recovery seem to be nTiO2 dose-dependent. Anabaena sp. cells self-recover back to a more conservative steady state over a longer period of 21 days, however, the relative intracellular macromolecule composition ratios seem to differ from that in the control and influenced by the dose concentrations applied. Principal component analysis on over 6000 FT-IR spectra under various treatment conditions shows clusters of data associated with different dosed nTiO2 concentrations and exposure time and the main biochemical contributors to the data variation.

Long-term and conservative changes of cell biochemical composition suggest the possible redistribution and reallocation of nutrients (i.e., carbon) among different
macromolecules and a limited carbon (energy) content and cell storage capacity induced by nTiO₂ exposure. Parallel cellular elemental analysis of cells exposed to nTiO₂ at different doses and exposure time shows clear and statistically significant changes in the cellular C:N:P ratios, consistently with FT-IR analysis results. This shows that nTiO₂ exposure to primary producers such as cyanobacteria can lead not only to population-level effects on one species but, more importantly, also to profoundly impact on the entire ecological aquatic system via sub-cellular cell composition and nutrients ratio changes. The macronutrients stoichiometry (C:N:P) of cyanobacteria, in fact, controls the nutritional diet of higher trophic levels organisms that feed on lower phytoplanktonic species. Nutrients ratios in cyanobacteria influence the dominance or survival of phytoplanktonic communities, thus, more broadly regulate the growth rates and reproduction of predators. This, in turn, strongly governs interspecies relationships and communities structures. Additionally, phytoplankton stoichiometry not only affects biological trophic dynamics but strongly influences the chemical nutrients inventory and water quality.

In summary, this study for the first time, conducted a comprehensive and fundamental investigation of both acute and chronic effects of nTiO₂ exposure to representative primary producer N-fixing algae. The results filled in the knowledge gap in providing toxicological information on NMs impact on aquatic primary producers by integrating typical phenotypic endpoints with sub-cellular and metabolic responses of model cyanobacteria to nTiO₂-induced toxicity. Of most importance, the innovative toxicity assessment approach allowed us to reveal, for the first time, the adaptation effects with cellular composition and nutrients stoichiometry changes in response to long-term exposure to nTiO₂ even at very low sub-cytotoxic dose levels that, at larger scale, might impact interspecies interactions and food web dynamics within complex ecological systems. Ultimately, long-term ecotoxic nTiO₂ effects on the
macromolecules assemblage linked to specific cell functions and on intracellular nutrients stoichiometry anticipate the possible impairment of biologically mediated flows of energy and nutrients in ecosystems.

The techniques and toxicity assessment approach presented in this study can be widely applied for the ecotoxicity assessment of other NMs, as well as for the evaluation of the adaptability of aquatic organism such as primary producers to various environmental stimuli, such as other emerging anthropogenic pollutants of concern.
Acknowledgments

When I left my lovely Sardinia six years ago I had not imagined that I was going to challenge myself dedicating my time and soul to this doctoral degree. It has been a long journey of joy and sacrifice, during which I have met many people to whom I owe my gratitude.

I would like to express my deepest gratitude to my advisor, Professor April Z. Gu for being an excellent example for me as a knowledgeable professor and successful woman. I warmly appreciate her enthusiasm, energy and contagious stimulation toward research and her supportive and encouraging attitude. I especially appreciate her trust and the freedom she gave me to pursue this independent work.

I am really grateful to my committee members Professor Akram Alshawabkeh, Professor Ferdinand L. Hellweger and Professor Philip Larese-Casanova for generously offering their time throughout the review of this document and their academic support through all these years.

I would like to thank the Department of Civil and Environmental Engineering at Northeastern University, particularly Professor Jerome F. Hajjar, Professor Tom Sheahan and Professor Peter Furth for providing me with the support needed through my Ph.D. I want to express my deepest appreciation to our lab director Dr. David Whelpley and Mr. Mike McNeil. Their kindness and assistance will always be cherished by me.

I gratefully acknowledge the funding sources that made my Ph.D. work possible, particularly the department of Civil and Environmental Engineering, the National Science Foundation (award # 0926284 and award# 0953633), the CHN seed grant from the Nanoscale Science and Engineering Centers Program of the National Science Foundation.
This work would not have been completed without the collaboration of various laboratories. I owe a great deal of gratitude to William Fowle from the Center for Electron Microscopy, Professor Max Diem from the Dept. of Chemistry & Chemical Biology, Professor Kai-tak Wan from the Dept. of Mechanical & Industrial Engineering at Northeastern University and Professor Dhimiter Bello from the Dept. of Work Environment at UMass Lowell. A special thanks to all the Ph.D. students and staff in their research groups who assisted me in my work.

I would also like to acknowledge my former advisors, Professor Alessandra Carucci and Professor Aldo Muntoni, whose classrooms were truly enjoyable and inspired me to pursue my doctoral studies.

My time at Northeastern was made enjoyable by many friends, which have become an important part of my life. I am particularly grateful for the time spent with my dearest friends Nehreen Majed and Indrani Ghosh and to our memorable journey together. And to my best friend Kevin McGarvey, who has always been next to me during the happiest and hardest moments, constantly pushing me to “keep my head up” in every situation. I especially thank Annalisa Onnis-Hayden for providing her support, protection and friendship, rescuing me many times, since my very first day I landed in Boston.

I have been fortunate to come across many other good friends along the way, the surrogate family while being far from home. My special thanks go to Alice and Raffaella for being able to constantly share the happier and crazier memories throughout this journey and for helping me overcoming the latest tough times with their unforgettable emotional support. And I never felt alone when thinking about my closest friends Elena, Lucia and Donatella, the sweetest memories of my lovely Sardinia.
And finally, none of this would have been possible without the love and support of my family, babbo, mamma and my brother Francesco, to whom this dissertation is dedicated. They have been a constant source of love, encouragement and strength through all these years. Grazie.

Lastly, I would like to dedicate this work to all new beginnings in my life for which I had aspirations since long time, and hope to cherish it until infinite time.

Carla Cherchi
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August, 2012
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Chapter 1

Introduction and Objectives

1.1 Background and Motivations

Over the last decade, the progress in nanoscience has rapidly led to the proliferation of new engineered nanomaterials (NMs), with global market worth estimated as US$1 trillion by 2015 [1]. NMs have been described as materials having a small feature dimension in the range of 1-100 nanometers and display fascinating physicochemical [2], thermal, electrical and mechanical properties that arise from their small size [3, 4], unique if compared to the bulk counterparts of the same composition. Thus, they are highly desirable for applications within the industrial and commercial sectors, and have the potential to revolutionize technological, health care and medical fields [5]. For example, from a mere environmental perspective, the increased levels of NMs production have great potential to contribute to the reduction of human footprint on the environment, with the development of cleaner and more efficient technologies in remediation of pollutants (i.e., chloro-organics), water treatment, sensor for environmental monitoring, and alternative energy (i.e., solar applications). It is anticipated that NMs and their by-products will unavoidably enter the environment proportional to their larger scale production and use [6]. This has led to the rising public concerns of the possible risks posed by NMs to human and environment. Despite the potential exposure scenarios, and the potential risk for human health and the environment, there are no current national legislations and laws that regulate nanotechnology [7]. Although, a few U.S. states (i.e., California and Massachusetts) have already pioneered policies addressing the environmental risk of nanotechnologies [8].
Thus far, information on the intentional or unintentional NMs release and the fate and transport in their life cycle is still very limited [9]. Limited data exists on the environmental concentrations of NMs due to the limited availability of methods able to detect and quantify trace concentrations of nanoparticles and distinguish them from the correspondent coarse counterparts in complex environments [10]. Recent probabilistic methods of exposure analysis estimated environmental concentrations of NMs in different matrixes and are reported in Table 1.1 [11]. A recent study by Kiser et al. reported effluent concentrations of titanium dioxide nanomaterials (nTiO_2) of 5-15 μgTi/L from wastewater treatment processes [12], which are consistent with the predicted nTiO_2 environmental concentrations (0.7-16 μg/L) based on worldwide production volumes in typical Swiss environmental scenarios [10].

Given the peculiarities of NMs and the acquired knowledge on the mobility of common pollutants in complex natural settings, the potential environmental fluxes of nanoparticles have been mere hypothesis [13]. Once entered the environment, via point or diffused source, NMs may be transferred through waterways (i.e., surface water bodies, groundwater, and water runoff) and be retained or break through soil particles and sediments [14]. Migration or retention in soils is influenced by particles’ affinity with media, and conditions favoring transport, such water chemistry and velocity, and physical trapping. Similarly, their fate in contaminated surfaces and groundwater are dictated by water parameters and components, and the presence of organic matter and electrolytes are known to play a significant role [15].

Interaction with these environmental matrixes also depend on particles’ physical-chemical properties and their surface chemistry, which will influence aggregation, degradation, sedimentation and transformation processes. Aggregation and disaggregation phenomena [6] are particularly governed by the charge at the NMs surface and by thickness of the surrounding
electric double layer, which may vary with pH, ionic strength and in presence of natural organic matter (NOM). This will in turn influence particles’ sedimentation and deposition, their adsorption onto other particles or on the surface of living organisms. The interaction with living systems is exploited in processes of biouptake, bioaccumulation and biotransformation, considered critical steps in the fate and translocation of nanoparticles among different trophic levels and across different domains.

**Table 1.1.** Measured and predicted environmental concentrations of NMs in typical exposure scenarios and environmental matrixes (surface water and sewage treatment plants effluents) [adapted from Kiser et al. (2009), Mueller et al. (2008) and Gottschalk et al. (2009)].

<table>
<thead>
<tr>
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<th>U.S.</th>
<th>Europe</th>
<th>Switzerland</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nTiO2</td>
<td>ZnO</td>
<td>nAg</td>
</tr>
<tr>
<td><strong>Surface water</strong></td>
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<td>*</td>
<td>0.002</td>
<td>0.001</td>
<td>0.116</td>
</tr>
<tr>
<td><strong>STP effluent</strong></td>
<td>1.75</td>
<td>0.3</td>
<td>21</td>
</tr>
<tr>
<td><strong>Surface water</strong></td>
<td></td>
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<tr>
<td><strong>Surface water</strong></td>
<td>0.7-16</td>
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* ug/L, Gottschalk et al. (2009)
*** ug/L, Mueller et al. (2008)
** ug/L, Ti, Kiser et al. (2009)
In the past years, increasing efforts have been made towards the understanding of the potential toxicity of NMs. The particles’ shape, atom arrangement and high surface reactivity of these nano-size materials (i.e., carbon-based nanomaterials) confer different properties and behaviors from their non-toxic bulk or elemental counterparts (i.e., carbon), thus imparting toxicity in various forms. Most of these studies have focused on human impact, occupational safety, and mainly on those routes with higher likelihood of exposure (i.e., inhalation, ingestion, and contact). Particularly, the cyto- and geno-toxicology of NMs on human health via the exposure route of respiratory system [6, 16] has been extensively explored.

In 2006, the term “Nanoecotoxicology” appeared for the first time in toxicological reports, but it was few years earlier than the field of nano-ecotoxicology had actually branched as independent discipline, focusing on the environmental hazards of nanomaterials and their sustainable development [17]. Today, the knowledge on the environmental implications of NMs on ecologically-relevant organisms and processes is rapidly growing, however, data are mostly restricted to species used for regulatory testing (bacteria, algae, vertebrates and invertebrates). A comprehensive toxicological information on NMs impact on other important terrestrial and aquatic species of ecological importance is lacking. In addition the underlying mechanisms of NMs toxicity to biological systems, and particularly to aquatic organisms, have not been fully elucidated.

Overall, toxicity mechanisms are shared among different cell lines and model organisms typical of toxicological assessments (i.e., human cell lines, bacteria, plants, etc.), although it should be clarified that their induced toxicity is specific to NMs type and properties, cell type, and also unique for given experimental conditions and settings.
Mechanisms of NMs toxicity in cells can be either chemical or physical, and in many cases they are interdependent [18]. The first recognized chemical mechanism is oxidative stress, consequent to an excessive production of reactive oxygen species (ROS) [19] and which might be enhanced if dissolution of metal ions or toxic materials from nanomaterials occurs. Other mechanisms, which can be dependent or independent by oxidative stress, include lipid peroxidation, direct and indirect genotoxicity and DNA damage, proteins’ alteration and oxidation [20], disturbance of signaling functions and of membrane transport activity. Physical alterations and damage, which can also be initiated by chemical mechanisms, generally occur at the membrane level [21], thus compromise membrane activity and the transport across, or intracellularly within specific cell compartments. Particles’ internalization is not a necessary condition for intracellular injuries to occur, however it is likely in those cells that have phagocytic and endocytic abilities. This has important consequences, especially for ecological species, since the possibility of nanoparticles translocation may lead to their biomagnification along the food web chain.

NMs exposures have been shown to cause severe cell damage and eventually cell death. Most toxicological studies have focused on acute exposure and, chronic and long-term responses, especially those under sub-cytotoxic dose levels, have hardly been explored. Thus, it is uncertain whether cells have the ability to adapt and recover their integrity and cellular functions compromised during exposure. Recent investigations have shown that higher plants are not as sensitive as lower trophic levels indicators (i.e., green algae) for nanotoxicity assessment [22-24] and often are found to show visible signs of recuperation after long term exposition, indicating only temporary effects [23]. Another challenge in nanoecotoxicological research is the
translation of laboratory results into in situ exposures [25], where the complexity will strongly influence the NMs fate and the dynamics of biological responses.

1.2 Motivation and Significance of this Study

Fundamental research on NMs toxicity to ecologically relevant organisms, such as prokaryotic and eukaryotic primary producers is scarce. As an indicator, the current (2012) Web of Science bibliometric search records that only about 3% of the studies on nanotoxicology focus on nanoeotoxicity. A limited number of previous studies have investigated the implications of NMs on freshwater indicator algae and microorganisms, mainly using conventional regulatory toxicological methods and relying on acute, rather than chronic responses [3]. Ecotoxicological studies of NMs that provide sub-cellular and molecular level information have hardly been reported [26]. Moreover, exposures studies have been performed at higher NMs doses than those environmentally relevant and expected levels in natural systems.

Despite the rising scientific efforts, investigations that elucidate the impact of NMs on lower aquatic trophic levels are still lacking. Particularly, cellular and molecular changes in response to long-term and chronic exposure to NMs yet need to be revealed. So far, the investigation of nanoeotoxicity is limited to a few species at certain trophic levels and they provide little knowledge on more chronic and ecological system-level impacts. Among the complex ecosystem members, primary producers have essential role in food webs, since they assimilate, incorporate, then translocate, nutrients (i.e., nitrogen), otherwise unavailable to higher trophic members. In addition, they are responsible for producing a large amount of oxygen and they regulate, thus mitigate atmospheric carbon levels and relieve from global warming effects.

Cyanobacteria (also known as blue-green algae) are one of the ancient classes of primary producers. Their activity account for 20–30% of Earth's photosynthetic productivity [27] and
play a significant role in nutrients cycling with their ability to access and fix atmospheric dinitrogen for metabolic purposes. Their ability to implement unique metabolic strategies and their physiological adaptation to tolerate adverse environmental conditions, and to carry a dramatic reorganization of internal macromolecules, make them good surrogates for evaluating environmental stresses. In addition, their phylogenetic relationship with plants’ chloroplasts and their structural similarities with other bacterial species (i.e., Gram-negative bacteria) make them good model organisms to help explain the potential nanotoxicity to other key ecological species. Moreover, as occupiers of the food webs base, they are candidates for initiating the trophic transfer of pollutants along the chain [28] and influence their own fitness, interspecies relationships, trophic interactions and food web dynamics, as well as those of other aquatic species of the ecosystems that they support [29, 30]. Also, the ecosystem stoichiometry and nutrients ratios in the environment are adjusted through the extent of their fixed nitrogen, and, in turn, the assimilated nitrogen is crucial for them to meet their metabolic requirements [31].

Therefore, a better understanding of the potential impact of NMs on representative primary producers, especially long-term effect at environmentally relevant low NM doses, is of great importance. Biochemical and structural changes induced in cells at sub-cytotoxic dose levels implies critical changes in cyanobacteria cells metabolism that might reflect larger scale adjustments on community structures and dynamics in ecological systems.

The present work aims to fill this knowledge gap in ecotoxicity of NMs to primary producers, using cyanobacteria as the representative organism, who plays important ecological role and whose functions may rebound on the health of higher trophic communities. We choose nTiO$_2$ because of the wide application of nTiO$_2$ in industrial processes and commercial products and, therefore, the high likelihood of its discharge into aquatic environments, as already
evidenced in WWTP effluents. Through the analysis of hypothetical environmental fluxes, and particularly in condition of slow water flows, nTiO$_2$ may reside at the air-water interface, where the likelihood of NMs interaction with buoyant cyanobacteria is expected to be high [32]. The methodology and a series of mechanism- and biochemical-based assessment tools developed or refined in this study can be further applied for ecotoxicity assessment of other NMs, as well as for the evaluation of the adaptability of primary producers to changing environmental conditions (i.e., nutrient availability), and their response to other anthropogenic pollutants of concern and emerging environmental stimuli.

1.3 Research Objectives and Overview

This study aims to assess the ecotoxicological effects of nano-titanium dioxide exposure on the cyanobacteria *Anabaena sp.* and the implications to its relevant ecological functions. Figure 1.1 shows the study overview that comprises a comprehensive investigation of both short-term and long-term impact of nTiO$_2$ exposure on cell growth and nitrogen fixation activity, cell integrity, cellular structure and intracellular composition changes, and cell potential to internalize NMs. Additionally, the impact on cell nitrogen metabolism, nitrogen storage and intracellular nutrients balance is assessed due to the essential contribution of the N-fixing function in the ecosystem nitrogen balance and flow, and the relayed effects of nutrient stoichiometry to higher members of aquatic food web chain.

In addition to conventional toxicological evaluation based on rather simple phenotypic endpoints (i.e., growth rates inhibition), more advanced molecular biology techniques (i.e., RT-PCR) and analytical methods such as very high-resolution microscopy (i.e., TEM, AFM) and
molecular spectroscopy (i.e., RAMAN, FT-IR) are applied to reveal molecular level high-resolution information regarding the cellular response and changes in exposure to nTiO₂.

**Figure 1.1:** Overview of the objectives and components of this study.

The specific objectives are:

1. Provide toxicological information on the cytotoxic impact of nTiO₂ on the cyanobacteria *Anabaena sp.* using population-level endpoints (inhibition of growth and of nitrogen fixation ability) and, develop a dose-response model for predicting nano-cytotoxicity at different nTiO₂ doses and exposure times.
2. Reveal the toxicity mechanisms of nTiO$_2$ exposure to cyanobacteria, via the examination of nTiO$_2$-induced oxidative stress and reactive oxygen species (ROS) production, as well as provide a systematic and quantitative evaluation of nTiO$_2$ impact on cell integrity, cell surface morphology, intracellular structure and mechanical properties of *Anabaena sp.* cells employing nano-scale high resolution imaging and computation analysis.

3. Investigate the potential internalization of nTiO$_2$ through the multilayered envelope of algal cells by means of confocal Raman microscopy and high-resolution TEM imaging.

4. Investigate the sub-cellular and molecular changes linked to cell nitrogen metabolism, by transcriptional profiling of key biomarkers involved in primary nitrogen regulation and metabolic pathways including global nitrogen regulation, nitrogen productivity (N fixation), N assimilation and N storage, along with parallel monitoring of intracellular N-rich metabolites and N-storage polymers at different nTiO$_2$ exposure conditions.

5. Investigate short- and long-term impact of environmentally-relevant sub-cytotoxic low nTiO$_2$ doses on the intracellular organization of biochemical pools, macromolecules and chemical markers associated to cell growth, cell structure and function using compositional Fourier Transform Infrared spectroscopy (FT-IR) fingerprints and chemometrics analysis.

6. Evaluate the changes in the intracellular macronutrients and element stoichiometry (C:N:P) via quantitative elemental analysis induced by sub-cytotoxic nTiO$_2$ doses during short- and long-term exposure.
1.4 Organization of the Thesis

The dissertation structure consists of seven chapters and the synopsis and content of each chapter is provided below:

- Chapter 1 provides the background and motivations of this study and describes the importance and relevance of gaining better fundamental understanding on the toxicity and mechanisms involved in NMs exposure to important ecological receptors, such as cyanobacteria.

- Chapter 2 reviews the current knowledge on the nano-ecotoxicology of engineered NMs on primary producers, with major focus on plants, algae and cyanobacteria species. The known and hypothesized underlying mechanisms of toxicity at the cell population- and molecular-level are here presented and discussed. The impact of NMs properties on their environmental behavior and fate is also presented with main focus on the known interactions with primary producers.

- Chapter 3 details the investigation on the time- and concentration-dependent impact of nTiO$_2$ exposure on cell growth and nitrogen fixation activity of the primary producer cyanobacteria *Anabaena sp.*. The use of the Hom’s inactivation concept as a predictive model to define nTiO$_2$ dose- and time-dependent relationships is presented and reveals the importance of nTiO$_2$ exposure time length in nanotoxicity (CT effect). The dynamics affecting the intracellular nitrogen storage are also shown through monitoring the accumulation of the cyanophycin grana protein (CGP), a N-rich intracellular polymer.

- Chapter 4 summarizes the investigation in the major toxicity mechanisms and toxic response induced in *Anabaena sp.* by nTiO$_2$ exposure, including oxidative stress and major cellular structural damages. The modifications induced on the intracellular structures, surface
morphology and cell mechanical properties are reported, with particular focus on the impairment of cell membrane configurations and roughness, and modifications of intracellular assemblies. The potential internalization of nTiO\textsubscript{2} through the multilayered envelope is observed and discussed.

- Chapter 5 provides details on the impairment of cellular metabolic pathways linked to the nitrogen status of cyanobacteria after treatment with sub-cytotoxic nTiO\textsubscript{2} doses. The section covers the changes in intracellular nitrogen metabolites (i.e., aminoacids) production and the expression of key marker genes encoding global regulatory pathways (\textit{ntcA}, \textit{glnB}) of cell nitrogen status, and pathway-specific genes involved in N fixation (\textit{nifH}, \textit{nifK}), N assimilation (\textit{glnA}, \textit{all2934}) and storage (\textit{cphA}, \textit{cphB}) during alternated cycles of light and dark.

- Chapter 6 describes the dramatic reorganization of internal macromolecules linked to specific cellular functions implemented by the model organism in response to the abiotic stress caused by short- and long-term exposure to low sub-lethal doses of nTiO\textsubscript{2}. Additionally, results of a quantitative elemental analysis are presented to demonstrate nTiO\textsubscript{2}-induced elemental ratios changes and intracellular nutrients stoichiometry alteration in exposed cells.

- Chapter 7 summarizes the conclusions and major contributions of this work and provides some recommendations for future studies.

1.5 References


Chapter 2
Ecotoxicity and Implications of Nanomaterials Exposure on Primary Producers

2.1 Overview
This chapter comprehensively summarizes the current state of knowledge on ecotoxicity and the environmental implications of nanomaterials (NMs) exposure to primary producers that includes higher plants, algae and cyanobacteria. First, the current understanding of fate and transport processes of NMs in the environment are summarized, with focus on those interactions and transformations involving or affecting primary producers. Second, the nano-ecotoxicological assessments and nanotoxicity mechanisms to relevant ecological primary producers-receptors are discussed. Then, the properties of NMs that have been recognized to contribute to the toxicity to primary producers and to environmental fate and transport processes are reviewed. Next, the repercussions of NMs exposure on phototrophic aquatic ecosystems are elucidated with discussion on the implications of NMs trophic transfer and NMs effect on ecosystems functions. This knowledge is fundamental and crucial to further nano-ecotoxicological research, since it sets the basics for the overall and system-scale understanding of the impact of NMs on trophic interactions and communities’ dynamics in ecosystems.

2.2 Classes of Manufactured Engineered Nanomaterials
Nanomaterials are generally categorized into different classes, which include metal- and metal-oxides NMs, carbon-based nanomaterials, quantum dots and nanopolymers-like dendrimers. The sources, applications, and key properties of each category are briefly summarized in the
following sections, which are relevant to the general understanding of their presence, fate and behaviors in the environment.

2.2.1 Metal-based and Metal Oxides Nanomaterials

Metal-based and metal oxides nanomaterials find applications in various fields [1, 2]. Based on production volumes, titanium dioxide and silver nanomaterials are the two fastest growing products in the nanotechnology industry [3], but numerous others have been also largely produced (ZnO, CeO2, SiO2, etc.).

Titanium Dioxide Nanomaterials (nTiO2). Nano-TiO2 is a naturally occurring mineral existing in three crystalline forms (rutile, anatase, and brookite) and in amorphous form. The two main crystal structures (anatase and rutile) differ in the distortion of each octahedron and in the assembly pattern of the octahedral chains. Due to the large energy gap (Eg =3.2 eV for anatase and 3.0 eV for rutile), nTiO2 is considered an excellent wide band-gap semiconductor. The increase in the band gap energy can enhance the redox potential of the valence band holes and the conduction band electrons, allowing photo-redox reactions. Various methods have been employed for nTiO2 production [4-7] and its intrinsic properties support a wide range of applications. The photocatalytic properties of nTiO2 have been employed in solar cells and photovoltaic devices due to its potential to absorb light into the visible light region and convert solar energy into electrical energy. The similar photoproperties have also been explored in environmental technologies to achieve the complete mineralization of organic pollutants from wastewater or groundwater [8]. The high brightness and high refractive index of nTiO2 encourages its use as a pigment in large variety of commercial applications, such paints, plastics, paper, foods and medicines. Nano-TiO2 has its most recognized applications in cosmeceutics (i.e., additive in sunscreen and skin lotions) due to its ultraviolet-blocking ability and the visible
transparency when in nanoparticulate form [9]. It is clear that nTiO₂ is expected to be released and largely present in the environment [3, 10] because of its wide application and extensive usage.

Silver Nanoparticles (nAg). For centuries, silver compounds have been used for their beneficial antimicrobial and photochemical properties in a variety of applications [11]. Recent developments in nanotechnology have uncovered key physicochemical properties of silver in its nanoscale size (<100 nm, nAg) conferring larger and more reactive surface area. Particularly, the high density of active facets, such as (111) in the truncated triangular silver nanoplates, has been found to strongly influence nAg reactivity, thus toxicity [12]. Traditional chemical methods are used for nAg production [13] and, in recent attempts, “green” means to synthetize nAg have also been proposed [14]. Thus, nAg is being widely included in textiles and cosmetics and has enhanced versatility of manipulation onto new products, such as electronics and household appliances [15]. Its antimicrobial effectiveness leads to a diversity of medical and pharmaceutical applications and water disinfection processes [13]. The potential presence and severe impact of nAg in the environment is warned [16], and recent studies have showed nAg discharge into wastewaters, from leachates of clothes washing activities [16].

Other Metal-oxide and Metal-based Nanomaterials (CeO₂, ZnO, Au, Fe, etc.). Cerium oxide nanoparticles (CeO₂) have been widely exploited in multiple applications, for their high refractivity and their known insulation, catalytic and electrolytic properties. Cerium’s high thermodynamic affinity for oxygen and sulfur, the unique useful absorption/excitation energy bands and its potential redox chemistry involving Ce(III)/Ce(IV) promote the use of CeO₂ in oxygen sensors [17], as a fuel additive to improve fuel efficiency and as a support material in three-way catalysts, due to its high oxygen storage capacity [18].
Zinc oxide nanomaterials (ZnO) exhibit semiconducting, optoelectric and piezoelectric dual properties and stands out for their higher exciton binding energy (60 meV). As for TiO$_2$ nanomaterials, the wide band gap (3.4 eV) makes them good candidates for many applications in electronics, ultraviolet (UV) light emitters, piezoelectric devices, chemical sensors and spin electronics. Gold nanoparticles are known for their stability, inertness and electronic, magnetic and optical properties and, zero-valent iron [Fe(0)] nanoparticles have gained attention for their potential use in contaminated groundwater remediation [19].

2.2.2 Carbon-based Nanomaterials

Carbon-based nanomaterials are currently considered one of the most fascinating nano-sized materials [20] and are manufactured in various forms, which include carbon black, single-walled and multi-walled carbon nanotubes (SWCNT, MWCNT), fullerene (a 60-carbon atom hollow sphere, also known as the buckyball), graphene, nanofibers, and so forth. Different production methods are used to synthetize carbon nanomaterials, such as fullerenes [21] and carbon nanotubes [22, 23] with distinct geometries.

The electronic and physical-chemical properties of carbon NMs and their specific applications are strongly influenced by the carbon’s structural conformation and, thus, their hybridization state (i.e., sp$^2$ hybridized carbon bonds) [24]. Fullerenes have excellent thermal and electrical properties and are known for their optical activity at the UV and visible wavelengths [24], thus it is used in photooxidative remediation processes of pollutants. Carbon nanotubes have distinctive electron-transport properties and their unique mechanical, thermal, photochemical features of SWCNTs [25] makes them ideal for a variety of new applications, including plastics, catalysts, electronics, sensors, etc. MWCNTs and fullerenes also found increased applications in the agriculture and food industry, increasing the existing concerns on
their safety with crops [26]. Lately, there has been an enormous interest in the use of graphene-based materials for chemical, electrochemical, and electrical energy storage, as in hydrogen storage systems, lithium batteries, and supercapacitors. Carbon-based nanomaterials are expected to severely impact the environment in view of the predicted worldwide yearly production volumes [27].

2.2.3 Quantum Dots

Quantum dots are small nanocrystal assembly consisting of an inner crystalline/metalloid core of semiconducting material (CdSe, CdTe, CdSeTe, ZnSe), which controls their optical properties, and of a surrounding shell (commonly silica) that protects the particle from oxidation and renders QDs bioavailable. QDs are inherently hydrophobic in nature; however, the functionalization of the shell using various capping ligand improves their solubility, durability and compatibility with specific targets.

Semiconductor Quantum Dots (QDs) have gained great attention for their promising applications in various fields, including medicine (in vivo biomedical imaging and targeted therapeutics), molecular biology and information technology [28], because of their unique electronic, magnetic, catalytic and tunable optical, properties [29]. Although their production volumes are not comparable with those of carbon- and metal-based NMs, QDs release in the environment may be detrimental not only because of the potentially severe damages that small QDs (1-10 nm) can pose to the biological receptors, but also for the possibility of internalization and bioaccumulation through various levels of the food web.
2.2.4 Dendrimers

Dendrimers are multifunctional polymers highly branched, star-shaped macromolecules used in many applications (sensors, electrodes, drug delivery) and fields, including biology, material sciences, catalysis, etc. Dendrimers are defined by three components: a central core, an interior dendritic structure and an exterior surface with functional surface groups, which affect their solubility and chelation ability. Their major advantage is to yield a very narrow size distribution and thus, provide more stability during manufacturing and applications. Their synthesis includes step-wise chemical methods, which allow for site-specific functionalization into the desired position of a dendritic scaffolds [30]. To date, there are no information or predictions made on the environmental presence of dendrimers, but their increasing applications will soon raise concerns on their potential toxicity and impact on ecological systems.

2.3 NMs Fate and Transport in Aquatic Environmental Systems

The fate and transport of nanomaterials in natural settings are strongly influenced by their intrinsic properties, the complexity of the ecosystem and their mutual interactions. Once released in the environment, NMs may be transferred through waterways (i.e., surface water bodies, groundwater, and water runoff) and be retained or break through soil particles and sediments [31]. Biotic and abiotic interactions of NMs with aquatic matrixes may lead to aggregation, degradation, dissolution, adsorption or other transformation processes which are dictated by surrounding environmental conditions (i.e., pH, natural organic matter, water chemistry, etc.). Consequently, NMs aggregation may lead to particles’ sedimentation and deposition, increasing the likelihood of interactions with soils (i.e., adsorption, physical trapping, etc.). Their presence in soil mats, or their migration through water flows favorites their interaction with both benthic
and aquatic species, increasing the likelihood of biouptake, bioaccumulation and biotransformation by different trophic levels species.

The following section summarizes the current understanding of fate and transport of NMs in the environment, with focus on those interactions and transformations involving or affecting primary producers.

2.3.1 NMs Aggregation and Disaggregation
Aggregation phenomena largely control NMs fate and behaviors [1], in both aquatic and terrestrial environments. When aggregation occurs, the increased NMs aggregate size enhances sedimentation and deposition into soils favoring their interaction with soil particles (i.e., adsorption, trapping, etc.), and limits NMs presence in aquatic suspension. The degree of aggregation or disaggregation strongly influences the availability of nanoparticles for uptake into aquatic and benthic biota [32], and the toxicity to environmental species, since larger aggregates show lower reactive surface/contact area than primary size particles and have different behaviors in aquatic and soil matrixes.

The aggregation and stability of particles in solution are mainly controlled by Van der Waal’s forces during particles Brownian motion and electrical and steric repulsion [33, 34] and it is promoted by their hydrophobicity or chemical bonding [35]. Ionic strength, pH conditions, ions and molecules in environmental matrixes can potentially influence NMs aggregation, aggregates size and distribution. Ionic strength and pH conditions in the environment influence the charge on NMs surface and the thickness of the surrounding electric double layer. As the pH of the aqueous system approaches the point of zero charge (PZC), the decrease in electrostatic repulsion between NMs of similar surface potential promotes a faster rate of aggregation [36,
At a given pH, increasing ionic strength may result in the reduction of the electric double layer thickness which surrounds the particles, hence decreases the electrostatic repulsion between two particles of same charge [38], with consequent increase in aggregation [36]. In addition, the increase in the concentration of divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)) in water and the presence of high-osmolarity fluids [39] strongly suppresses the electric double layer, and makes the Van der Waals attractive forces became predominant [33]. Particularly, this cationic influence may be related to the complexation of cations with negatively charged groups (i.e., COO\(^{-}\) and OH\(^{-}\)) and the consequent reduction of surface charge [37]. For marine environments, where seawater may facilitate NMs aggregation and precipitation [40], the increased aggregation due to high cations lead to an increased risk for benthic biota.

In complex environments, the presence of natural organic matters, or stabilizing agents, or the occurrence of environmental conditions that limit the electrostatic attraction among particles favor reverse phenomena of disaggregation [41]. In natural waters, the abundant presence of natural organic matter (NOM) (i.e., humic and fulvic acids) that originated primarily from plant and/or microbial residues, such as those from primary producers (i.e., DNA, protein, metabolites) [42], or from antrophogenic discharges (i.e., wastewater or agricultural inputs), can modify the surface charge of nanoparticles, either enhancing aggregation, or stabilizing nanoparticle suspensions through a combination of steric and electrostatic effects [43, 44]. It was reported that NMs are potentially stabilized in surface waters or wastewaters that typically contain NOM at concentrations around 4 mg/L and 5-10 mg/L, respectively [43]. However, the synergistic effect of both organic matter and other components (i.e., salts, microorganisms, etc.) often are reported to have counter-effects and alter the stabilizing tendency of NMs in water [45]. Additionally, surface charge neutralization and bridging mechanisms by the presence of humic
acids were documented and also found to enhance NMs aggregation, at particular pH conditions [43].

Plants exudates, which mainly consist of macromolecules such as sugars, proteins, phenolic acids and aminoacids [46] were shown to contribute to NMs (i.e., ZnO) stabilization in proximity of grass roots’ surface. On the other hand, phenolic-rich mucilage, naturally secreted by brown algae, contributes to aggregation acting as a complexing agents for nanoparticles [47].

2.3.2 Adhesion and Adsorption to Cells

When fate and transport processes favor NMs contact with aquatic or benthic living organisms, the likelihood of nanoparticles’ adhesion and adsorption to cells surfaces increases and this physical attachment of NMs on cellular membranes could pose a risk to aquatic organisms. The adsorption and attachment of NMs to the surface of living organisms directly result in other physical and toxic effects, such as oxidative stress, disruption of cell membrane, reduction of nutrient uptake and interruption of energy transduction. Other cellular activities, such as the ability of phytoplankton to remain suspended in water columns [48], or the reproductive success for species that reproduce by external fertilization [49] might also be compromised. For example, physical restraints caused by carbon black nanomaterials decrease sperm frequency of a marine algae with consequent impairment of seaweed embryo development [47].

Adhesion and adsorption processes are influenced by environmental factors, NMs intrinsic and surface properties, but also peculiarities of cellular membranes, such as the presence of surface functional groups. Among the environmental factors, major role is played by pH conditions, as shown in previous studies exposing P. subcapitata to nTiO₂ [50], but the degree of adsorption was also found to depend on the particles’ equilibrium concentrations.
Surface characteristics influence the likelihood of adsorption, particularly, nonspecific interactions (electrostatic, hydrophobic, and hydrogen bonding) between NMs and membranes’ surface groups. For example, the adsorption of CdSe/ZnS QDs to the surface of the green algae Chlamydomonas sp. was due to the binding between the carboxyl groups (-COOH) of QDs shells and the amines (-NH2) of surface polysaccharides and glycoproteins in membranes [51]. NMs that present typical surface functionalization, such as carbon nanotubes, are prone to undergo adsorption and have high affinity with algal cells surfaces, possibly through the formation of hydrogen bonds among algae and oxygen defects at the NMs surface [52].

2.3.3 NMs Dissolution and Release of Metal Ions

It is still unclear whether the toxicity associated to some metal-based nanomaterials, such as nAg or ZnO, is caused by the intrinsic nanosize feature of nanoparticles or by the speciation and consequent release of metal ions [53]. Release of metal ions from nanomaterial surface is not only influenced by environmental factors (i.e., higher dissolution at lower pH), but also by the intrinsic properties of nanomaterials. For example multilayered structures, as those found in CdTe/CdS quantum dots, were found to facilitate the release of metal ions (i.e., Cd2+) from the outer shell [54].

To date, the contribution of the dissolved metal ions on the toxicity to aquatic species is still not consistent among ecotoxicological studies. Some studies on freshwater algae showed comparable results in toxicity endpoints attributable to nano and bulk metal oxides (i.e., ZnO, NiO) to those associated to the related soluble salts, suggesting that the toxicity was attributable merely to dissolved metal rather than nanoparticles [32, 55]. The toxicity imparted by the release of metal ions may not only induce direct toxicity to cells, but it may indirectly affect cell growth and development by sequestering other macro- or micro-nutrients from the environment, as in
the limited uptake of manganese by plants in presence of free released Zn^{2+} [56], or simply by increasing free metal contents in water to levels that induce cytotoxic effects [57].

On the other hand, several examples reported in literature show that the toxicity exerted by nanomaterials to model plants, algae and bacteria is not fully explained by the release of dissolved metals, but other factors associated to the intrinsic nano-property play a role [55]. As an example, silver speciation was only partially found responsible for the toxicity of nAg to the green algae *C. reinhardtii*, with the possible uptake of Ag^{+} via a Cu (I)-transporters through the cell membranes [58], and imparting both growth and algal photosynthetic yield. Similarly, dissociation of free Zn^{2+} from ZnO treatment did not fully justify the inhibition of growth rates in four marine phytoplankton [56] and the inhibition of growth, seed germination and root growth in ryegrass [46, 59]. Conditions that favor the formation of nanoparticles’ aggregates, for example, can mitigate the role of metal ions in toxicity, reducing the average equilibrium solubility of the nanoparticles’ aggregates of decreased surface area, and providing kinetic hindrance to the diffusion process of ions [60].

In general, caution has to be taken in linking environmental responses to the sole nano-size of the NMs, especially for those containing metals, and further research is needed for a better understanding of the dissolution phenomena of metals from nanoparticles and their implications in ecological systems.

### 2.3.4 Environmental Transport and Fate of NMs

Understanding NMs transport in the environment is still a challenge and to date, no systematic studies have shown NMs presence and fluxes in real natural settings. Many environmental factors are known to modify nanomaterials’ behavior, thus their movement, sedimentation, persistence and transport in complex environments. As previously discussed, ionic strength, pH
conditions and the presence of complexing molecules, and biomolecules are important contributors to the process, but also physical-chemical processes (photo-, thermo- and hydrolytic conditions) are known to play a significant role [61].

Once released via manufacturing, use or disposal, NMs can widely migrate from the point of discharge or locally reside in limited “hot spots” [35], but generally with low residence time in the environment [62]. Nanoparticles can be transported following different routes in various domains, particularly in soil and water, and ultimately reach living organisms and predators that feed on them and on water components [63]. A recent experimental study showed the potential and complex routes undertaken by NMs in possible environmental scenarios [63] through water and soil, which culminated in the NMs transfer from predators (insects) to pray (plants).

Nanoparticles are prone to contaminate surface and groundwaters and their transport is facilitated by wind and water runoff [1]. For example, the widely application of TiO₂ nanomaterials in paints and cosmeceutics will most likely result in a significant presence of these NMs into urban runoff and domestic sewages, thus reach wastewater facilities before ending in soil or aquatic environments. On the other hand, they can eventually be directly discharged into water bodies, and, particularly, in conditions of slow water flows, nTiO₂ may reside at the air-water interface, and interact with buoyant aquatic species, such as cyanobacteria [64].

NMs behaviors in water (i.e., aggregation, degradation, sedimentation, transformation) are influenced by water chemistry and the presence of solutes (i.e., NOM), common monovalent and divalent electrolytes [65], nanoparticle charge (electrokinetic) properties, besides all other factors that influence their transport in soil and that are covered in previous and following sections. Yet, nanomaterials fate in WWTPs is largely unexplored, although previous studies on
metal-based nanomaterials have revealed that NMs adsorption into suspended activated biomass or biofilms [10], NMs aggregation and settling [66] allow their removal in wastewater treatment facilities and consequent disposal. Thus, when entering water treatments processes, nanomaterials such as nTiO$_2$ are expected to be mostly removed via sludge disposal and to be recirculated in soils amended with biosolids.

When in contact with soil, particles migrate differently depending on various conditions, such as media type, solution chemistry, pore water velocity and NMs physical-chemical properties. For example, it was shown that different types of surface functionalization may yield different transport behaviors in soils, due to the different affinity of surface groups with the porous media [61]. Soils, in fact, have various compositions (i.e., organic matter and metal content, pH, etc.) which will clearly govern these interactions. Physical trapping of particles might increase the retention of NMs in soil, possibly influenced by NMs size and shape and by the potential electrostatic interaction between particles and soil, which will contribute to NMs aggregation or their binding with other soil components [63].

Plant and other organisms’ uptake is also considered as a critical exposure and transport pathway for nanoparticles and, for plants, algae and cyanobacteria, the important mechanisms of uptake, biotransformation and potential translocation are widely discussed in the following sections.

2.3.5 Biouptake and Bioaccumulation

It is still uncertain whether intact nanomaterials are able to cross biological membranes of aquatic species and be potentially bioaccumulated in food web chains [54]. Most evidences of internalization of nanomaterials in plant and algal cells are based on X-ray and imaging (i.e.,
TEM) techniques, which do not reveal if the internalization actively occurs in live cells or after membrane disruption and cells inactivation.

Nanomaterials can enter plant cells and protoplasts by endocytosis [67], which is considered the major mechanism of entry [68]. Additionally, the binding with embedded carrier proteins, or passage through aquaporins and ion channels [44] are proposed, although, in plants, the process is not as efficient and as well understood as in animal systems. Penetration of nanomaterials through cell walls porosity is believed to happen if NMs particle size is smaller than the pores (5 to 20 nm) [69] or if the permeability of cells membranes increases by external actions (enlargement of pores or induction of new cell wall pores) [26]. Experimental evidences showed internalization of nanomaterials in plants via apoplastic passways or endocytosis [67, 70], followed by transport to epidermis and cortex (and root to shoot) via plasmodesmata [46] or via typical transmission routes of water and nutrients [71]. Nanoparticles are able to cross the stomatal openings or trichomes when in contact with leaf surfaces [26] and entry into plants’ roots by the effect of the osmotic pressure and capillary forces at the interface [59, 71] or via highly regulated symplastic paths [71]. A recent study has also demonstrated the ability of MWCNTs to cross plants cells envelopes via direct penetration [72], an alternative uptake pathway to endocytosis. In general, the uptake efficiency and translocation of nanoparticles vary differently with plant species, their reduction potential and the nature of nanomaterials [26].

The process of NMs internalization in algae and prokaryotic cyanobacteria still remains unclear. Like most high plants and bacteria, algae possess a semi-permeable cell wall outside their membrane, therefore the active biological uptake processes for NMs, typical of eukaryotes (i.e., endocytosis) are impeded or limited [51]. Likewise, cyanobacteria are believed not to have
internalization mechanisms for nanoparticles, as for supramolecular and colloidal particles. However, some hypothesis have been made to justify intracellular observations, which include non-specific diffusion (which only selects for <2 nm particles), nonspecific membrane damage and specific uptake [73]. Non-specific membrane damage, likely ROS-mediated, has possibly supported the real-time arrangement of new membranes’ channels of larger pore sizes, thus facilitate the NMs internalization through active transport via membrane pumps [74]. Specific uptake has been proven to support the internalization of adenine-conjugated 5 nm QDs in Gram-negative bacteria via purine-dependent mechanisms [73].

Although these evidences seem to suggest possible un-known or not well-understood mechanisms for NMs to transport into bacterial cells, most researchers believe that the presence of nanomaterials inside bacterial cytoplasm is still justified with diffusion processes after membrane disruption [62]. Additionally, it is still under debate whether intracellular uptake is a condition that truly causes toxicity [75].

2.3.6 Biodegradation and Biotransformation

Information regarding the process of biodegradation of NMs by primary producers and related biotransformation pathways is scarce. According to the potential of nanomaterials synthesis by plants and cyanobacteria [76] discussed in the following sections, their ability to transform engineered nanoparticles is expected [77]. Properties of engineered nanomaterials, such as their chemical composition and nature, will strongly affect their chemical degradability, biodegradability or persistence in ecosystems. Degradation processes can occur biotically or abiotically, depending on the contribution of biological entities in the process, and in both cases they are mainly driven by redox-type transformations.
Biotic transformations have been particularly demonstrated for metal-oxide NMs and particularly obtained via reductive or oxidative processes. For example, the study by Lopez-Moreno et al. [70] demonstrated the ability of soybeans roots to uptake and biotransform CeO$_2$ and ZnO nanomaterials via metal speciation. Cerium was found in the same oxidation state (IV) as in the CeO$_2$ nanoparticles, while Zn was in the oxidation state of Zn(II) but not present as ZnO NPs, demonstrating previous transformations on the root surface and later release of Zn ions in the media. Another study by Gong and colleagues reported the ability of the microalgae *Chlorella vulgaris* to reduce NiO nanoparticles to zero valence nickel, implying a possible role of soluble proteins in the transformation [78]. Traces of Ni$^{2+}$ in mesquite leaves also suggested the transformation of nickel-based nanomaterials, previously accumulated by the plants on the root surface [79]. Metals released by NMs via a biotic-mediated speciation might be available for uptake and thus, used for growth and development by cells [70].

Although not specifically controlled by primary producers, the biologically-mediated oxidation of fullerene was observed in the model system Cytocrome P450 [80], implying that also carbon-based nanomaterials can undergo these type of transformation. Additionally, fullerene was metabolized by rot basidiomycete fungi after uptake [81], and its fullerol carbon converted into lipid biomass. This demonstrates the possibility for microbes to decompose complex organic molecules and break down organic nanoparticles, such as fullerene. However, this is not believed to occur for metal-based nanomaterials.

Certain NMs are not only biotransformed, but are specifically designed to be abiotically modified in the environment as, for example in the application of iron (Fe0) nanoparticles for soils remediation or as reactants for the dechlorination of organic pollutants [82].
2.4 Nano-Ecotoxicological Assessment and Toxicity Mechanisms

Understanding the ecological toxicity of nanomaterials is important for both environmental integrity and human health. Compared to the extensive efforts on nanotoxicity to human, studies elucidating the potential mechanisms of NMs ecotoxicity on ecological members such as plants, algae and cyanobacteria have been limited [1]. In this section, the current knowledge on the population-level and of sub-cellular ecotoxicological information of NMs exposure is reviewed and discussed.

2.4.1 Population-level Endpoints for Nanotoxicological Assessment in Primary Producers

Currently, the environmental risk assessment of nanoparticles is based on toxicity test protocols adapted from established methodologies of conventional pollutants. Therefore, it is still under evaluation whether the endpoints used are meaningful to determine the safety and risk of nanomaterials, given their unique physical and chemical properties. For aquatic risk assessment, common population-level endpoints on target species (i.e., plants and algae) have been evaluated to establish the effects of NMs exposure.

Phytotoxicity assessment in higher plants was reviewed in the work by Lin and colleagues [59], which emphasized the use of seed germination, root elongation [59] and number of leaves [75] as rapid and simple acute endpoint indicators. Roots are generally considered the first symptomatic target tissues affected by exposure to pollutants [59]. During germination, the extent of seeds rootlets development after water uptake (emergence of root, >1 mm or >5 mm) is used as a representative endpoints.

The phytotoxicity of NMs was evaluated in different plants models, which include radish, rape, ryegrass, lettuce, corn, and cucumber and the results obtained were strongly dependent not
only on the type of treatment but also on plant species and developmental stage [26]. Results of previous literature and specific endpoints are summarized in Table 2.1.

Table 2.1. Selected NMs effects on primary producers and toxicity endpoints found in literature.

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>Test species</th>
<th>Endpoint effect</th>
<th>Endpoint value and effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnO</td>
<td>Soybean</td>
<td>Root elongation</td>
<td>-</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>60 μg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Lopez-Moreno (2010)</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>IC&lt;sub&gt;25&lt;/sub&gt; 1</td>
<td>1 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hall (2009)</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>IC&lt;sub&gt;25&lt;/sub&gt; 2</td>
<td>2 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hall (2009)</td>
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<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 241</td>
<td>241 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hartmann (2010)</td>
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<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 87</td>
<td>87 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td>Warheit (2007)</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 5.83</td>
<td>5.83 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Aruoja (2009)</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 71.1</td>
<td>71.1 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td>Hartmann (2010)</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>IC&lt;sub&gt;25&lt;/sub&gt; &gt;100</td>
<td>&gt;100 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Blaise (2008)</td>
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<tr>
<td></td>
<td><em>D. subspicatus</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 4.4</td>
<td>4.4 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hund-Rinke (2006)</td>
</tr>
<tr>
<td>TiO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Spinach</td>
<td>Growth</td>
<td></td>
<td>Improved</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>IC&lt;sub&gt;25&lt;/sub&gt; 1</td>
<td>1 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hall (2009)</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 241</td>
<td>241 mg/L</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Hartmann (2010)</td>
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<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 87</td>
<td>87 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Warheit (2007)</td>
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<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 5.83</td>
<td>5.83 mg/L</td>
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<td></td>
<td>Aruoja (2009)</td>
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<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 71.1</td>
<td>71.1 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hartmann (2010)</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>IC&lt;sub&gt;25&lt;/sub&gt; &gt;100</td>
<td>&gt;100 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td>Blaise (2008)</td>
</tr>
<tr>
<td></td>
<td><em>D. subspicatus</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 4.4</td>
<td>4.4 mg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hund-Rinke (2006)</td>
</tr>
<tr>
<td>CNT</td>
<td><em>C. vulgaris</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 1.8</td>
<td>1.8</td>
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<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; 20</td>
<td>20</td>
</tr>
<tr>
<td>nAg</td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; -96h 0.19</td>
<td>0.19 mg/L</td>
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<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; -8 days 500</td>
<td>500 μg/L</td>
</tr>
<tr>
<td></td>
<td><em>P. subcapitata</em></td>
<td>Growth</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; -96h 0.19</td>
<td>0.19 mg/L</td>
</tr>
<tr>
<td></td>
<td>Flax</td>
<td>Germination</td>
<td>-</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>El-Temsah (2010)</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Cabbage, carrot, lettuce</td>
<td>Growth</td>
<td>-</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Canas (2008)</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Tomato</td>
<td>Germination</td>
<td>-</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Khodakovskaya (2009)</td>
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</tbody>
</table>

Toxicity evaluations on algae and cyanobacteria are commonly reported in terms of population growth inhibition. Additionally, other endpoints such as sperm concentration and fertilization, body axis alignment, germination and rhizoid elongation quantification were determined to assess the effect on reproduction and early development in marine brown algae.
Growth inhibition was generally evaluated after 72 hours or 96 hours [55, 83] on freshwater model organisms (namely *Pseudokirchneriella subcapitata, Desmodesmus subspicatus* and *Chlamydomonas reinhardtii*) in OECD and US EPA algal growth inhibition test [84-86]. Additional studies have focused on other widespread phytoplanktonic groups, such as diatoms (Bacillariophyceae), green algae or chlorophytes (Chlorophyceae), and the prymnesiophytes (Prymnesiophyceae) [56]. Unicellular algae are convenient models for studying the toxicity of nanoparticles for their high contact surface area to volume ratios that increase their exposure to contaminants [87].

In overall, the nanotoxicological conclusions are not consistent and present great variations, as summarized in Table 2.1. At least partially, the great variability of the results can be attributed to the non-standardized NMs preparation, dosing protocols, testing conditions and varying organisms selected. These variations make it difficult to compare results across laboratories, or assess species-specific sensitivity to NMs [88].

### 2.4.2 Molecular Toxic Mechanisms

*Reactive Oxygen Species Production and Oxidative Stress*

Reactive oxygen species (ROS) are constantly produced as byproducts of aerobic metabolism during respiration and photosynthesis, via exogenous (extracellular) and endogenous (intracellular) processes [89]. ROS are commonly formed one electron step in oxygen reduction where the reduction of oxygen into water occurs via the electron transport chain [90], with superoxide ion and hydrogen peroxide as intermediates, according to the reaction:

\[
O_2 + e^- + 2H^+ \rightarrow H_2O_2 \rightarrow OH^- + OH \rightarrow 2H_2O
\]  

(1)
To avoid cytotoxic effects of ROS, during physiological steady state conditions, primary producers perform a complex of non-enzymatic and enzymatic detoxification and antioxidant mechanisms for ROS scavenging [91, 92]. However, biotic and abiotic environmental stresses (i.e., toxicants, drought, temperature and light intensity changes) can easily upset the equilibrium between production and scavenging of ROS. This will result in a rapid increase of intracellular oxygen species, which will ultimately overwhelm the antioxidant defense capacity of the cell [37] and its resiliency to other environmental stressors [93].

The formation of ROS has been proposed as one of the main antimicrobial mechanisms for cells exposed to nanomaterials [77] and as the primary mechanism inducing toxicity upon exposure to nTiO2 [94]. In particular, the photocatalytic properties of TiO2 strongly promote the production of highly oxidizing ROS. The electron hole pair, which forms in an electronically excited state, has the potential of reacting with H2O to form hydroxyl radicals to form (OH•) and ions (OH−), or with molecular oxygen to form O2•− ions. Particularly, OH•, with its relatively long lifetime of 10−7 s, is recognized to be extremely active in affecting biological molecules. Typical reactions were reported previously [62] and are presented below:

\[
\begin{align*}
\text{TiO}_2 + h\nu & \rightarrow \text{TiO}_2 (h^+ + e^-) \\
e^- + O_2 & \rightarrow O_2^- \\
O_2^- + 2H^+ + e^- & \rightarrow H_2O_2 \\
H_2O_2 + O_2^- & \rightarrow \cdot OH + OH^- + O_2 \\
H_2 + H_2O & \rightarrow \cdot OH + H^+
\end{align*}
\]

Although the redox active properties and the photocatalytic toxicity of nTiO2 are triggered by UV illumination, nTiO2 was found able of inducing oxidative stress also in the dark when dissolved oxygen is present [95]. The oxidative properties of nTiO2 and other nanomaterials (i.e.,
FeO, Fe₃O₄, CuO, CNTs) to biological systems, particularly algae, have been reported [87, 96, 97] and show to induce a series of cells alterations at both structural- and molecular- level and consequent defense (i.e., antioxidant) responses.

Free radicals are able to affect cellular components [77], such as double bonds in membrane phospholipids via lipid peroxidation, and denature DNA strands and proteins [40, 98] leading to the damage of cellular structure [62] and functions. In particular, impairment of enzymatic activity may lead to entire metabolic pathways failure, with severe consequences for the health of environmental biota. For example, the oxidation of polyunsaturated fatty acids components of cell membranes via lipid peroxidation has been recognized in primary producers [48, 62]. An induced lipid peroxidation [48, 97] and the accumulation of cellular glutathione, was exhibited in green algae, consequently reaching a certain degree of cell damage and oxidative stress [99].

In response to the increased intracellular ROS levels, algae can promote ROS diffusion from various organelles (thylakoids) to the cytosol [100], before the ultimate excretion across the plasma membranes or promote antioxidant stress by the activation of sod, cat, gp, and apx, key enzymes in antioxidative defense systems [101].

DNA Damage and Genotoxicity

The presence of genotoxic pollutants in the environment has important implications on the ecosystems’ health and integrity [96, 102]. Mutagenic contaminants are known to influence plants’ systems differently depending on plants and mutagen’s type, exposure conditions and environmental factors [103]. In vivo and in vitro studies have reported the potential of engineered nanomaterials to initiate a series of cellular genotoxic responses in human, animals
and bacterial cell lines [104]. Although in a limited number of reports, genotoxicity of nanomaterials was also proved in primary producers and mainly reveal the occurrence of genetic mutations and DNA damage. Nano-genotoxicity in plants caused DNA damage with mechanisms that were found to be NMs-specific. In general, nanomaterials cause DNA damage and the alteration of genetic stability in plants [70] through chromosomal aberrations and fragmentation via interphase micronuclei [105] and chromosomal breaks [106]. For example, SWCNT were found responsible for cytotoxicity via the endonucleolytic cleavage of DNA [107], whereas magnetic NMs were reported to induce the decrease of nucleic acid levels, chromosomal aberrations [108] in plant’s cells. Genotoxic effect are not always showed in plants cells exposed to NMs and, as an example, negligible shares of mitotic phases, frequencies of aberrations and abundance of micronuclei were reported under nTiO₂ treatment [109].

Currently, there are no definite mechanisms explaining the genotoxic potential of nanomaterials in plants, algae or cyanobacteria [96]. Leaching of metal ions and formation of superoxide radicals and lipid peroxidation, thus oxidative stress, are main recognized causes of the genotoxicity observed [70], although more studies are needed to corroborate these conclusions. Thus, more fundamental research, not only at the genomic, but also at the metabolic and proteomic level is required to make stronger conclusions on NMs genotoxic effect on primary producers.

2.4.3 Impact on Primary Producer Functions

Effect on Photosynthesis and Role of NMs Shading

Nanomaterials released in the environment have the potential to interact with photoautotrophic organisms, thus hamper key ecological processes, such as photosynthesis. To date, a limited number of studies have probed the effect of NMs on photorespiration and photosynthetic CO₂
fixation of higher plants, algae and cyanobacteria, and yet the mechanisms of NMs phytotoxicity have not been fully elucidated. In general, NMs exposure seems to affect photosynthesis mainly in lower species (i.e., algae and cyanobacteria), while higher plants mostly exhibit stimulation of primary productivity.

Nano-anatase TiO\textsubscript{2} was found to influence the incorporation of CO\textsubscript{2} into the biosphere in plants, promoting the activity of the ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) [9, 110] and its physical interaction with the chaperon Rubisco activase (Rubisco-R-A complex) [111]. In addition, nTiO\textsubscript{2} could improve light energy of Photosystem I (PSI) before transfer to Photosystem II (PSII) and stimulate the conversion from light energy to electron energy [112]. Enhancement of plant growth, water photolysis and oxygen evolution were the results of these stimulating effects.

On the other hand, lower species, such as model green algae (i.e., C. reinhardtii, P. subcapitata, C. vulgaris) were mainly inhibited in their photosynthetic ability when treated with metal-based and metal oxides nanomaterials (CuO, Fe\textsubscript{3}O\textsubscript{4}, nAg) [58, 87, 97] and QDs [51], while carbon nanotubes seems not to interfere with algal photosynthetic system [52]. Pathways linked to photosynthetic activities, thus primary productivity, in green algae were also slightly altered by NMs exposure, as showed in the expression patterns of marker genes (e.g., psa\textsubscript{A} and rbc\textsubscript{S}) and those of carotenoids biosynthesis (e.g., pds).

Light shading is often implicated in the reduced viability of phototrophic processes [52]. Recent studies questioned whether shading effects linked to the encapsulation of the cells by NMs are among the main mechanisms that contribute to the observed toxicity. Light is required in order for photosynthesis to occur, fueling a series of chemical reactions, where the conversion of light energy into chemical energy produces organic material to be utilized in cellular
biosynthesis and respiration [113]. Inhibition of cell growth by carbon nanotubes [52] and nickel oxide nanoparticles [78] or by nTiO₂ aggregates [37] was often associated with the reduction of light transmittance to the exposed algae. Moreover, developmental impairment of the photosynthetic apparatus of the fucoid embryo in brown algae and the inhibition of the polar axis alignment was the result of shading of zygotes, young embryos and of the polarizing light signal by carbon black nanomaterials [47]. On the contrary, dissenting literature have ruled out the contribution of shading effects to toxicity imparted by nano-anatase, ZnO and CuO on several microalgae [60, 83, 84]. These results suggest that general conclusions cannot be made, and potential shading effects are specific for NMs-algae interactions, experimental conditions and NMs properties.

**Impact on Nitrogen Metabolism**

Primary producers have a key role in preserving ecosystem health and in maintaining ecological nutrient balances. To date, it is still unknown whether the exposure to nanomaterials has the potential to compromise their function in nitrogen cycling and, thus, impair important ecological equilibriums. Understanding the implications of NMs exposure on nitrogen fixation, for example, is required since the process contributes to the global nitrogen economy of aquatic ecosystems, converts biologically unavailable nitrogen into a form (NH₃) that can be assimilated by biota, and accounts for 6-82% of nitrogen inputs to eutrophic lakes [135]. So far, the knowledge on the implication of NMs on cells nitrogen metabolisms acquired is fragmented and limited to specific classes of NMs (QDs and nTiO₂) targeting specific molecular pathways. A recent study demonstrated that the growth of bacteria involved in nitrogen cycling (i.e., autotrophs nitrifiers and heterotrophs denitrifiers) was affected by exposure to QDs and that
expression of genes involved in ammonia oxidation, denitrification and nitrogen fixation were stimulated [114].

Nitrogen metabolisms in higher plants was found to be stimulated in presence of TiO2 nanomaterials, which, under illuminating conditions, donates electrons to fuel the catalytic reduction of N2 to NH3 (nitrogen fixation). The activity of nitrate reductase was also promoted [115] and promoted a greater incorporation of nitrogen into the cell, into aminoacids and proteins [110]. This suggests that important regulatory pathways involved in the intracellular metabolism, assimilation and storage of nitrogen are potentially affected under exposure to nanomaterials, although the mechanisms involved are yet to be elucidated.

**Impact on Cell Physiology, Intracellular Structure and Functions**

Various cellular physiological events and structural components may be targeted by the exposure to contaminants [116], hence possibly by NMs treatment. The alteration and impairment of cellular membranes have been suggested as potential recognition mechanisms behind the antimicrobial activities exerted by NM exposure [77]. The main mechanism by which membranes are compromised may involve lipid peroxidation via ROS-mediated processes, as discussed in previous sections.

Surfaces alterations affecting cell functions were observed in plant and algal cells after exposure to metal- and carbon based nanomaterials. In particular, the reduction of cell pore diameter of maize root cell walls was observed after exposure to nTiO2 [36] and resulted in a reduced hydraulic conductivity and transpiration from leaves. Cortical cells were damaged after ZnO uptake in ryegrass [46] and detachment of plasma membranes were observed in rice plants exposed to MWCNTs [26]. Similar effects with plasma membrane disruption and cytosol leakage were reported in green algae after exposure to nickel oxide nanoparticles [78] and
increased cell wall permeability induced by ZnO was observed in Gram-negative bacteria [117], which are known to have similar structure of cyanobacterial envelopes. When both membrane integrity and intracellular components (i.e., genetic material, DNA) are compromised, leakage and release of intracellular components is possible [48].

Not only disruption, but also the reinforcement of membrane structures was reported in cells responding to NMs toxicity, with the initiation of a series of signaling cascades to enhance the production of molecules associated to cell thickening [26].

The action of NMs was also exerted in intracellular compartments, where NMs have the ability to bind with different organelles and interfere with metabolic processes at specific sites [26]. Disorders of thylakoidal lamella [78] and the decrease in chlorophyll a content [97] of the green algae *Chlorella vulgaris* were observed after exposure to metal oxide nanoparticles, implying the potential impairment of algal photosynthetic activity.

Recognizing the possibility of the uptake and internalization of NMs by living organisms, as previously described, the next question that arises is whether NMs are delivered and stored within specific cellular compartments, or bind to definite intracellular macromolecules. To date, only a limited number of observations showed intracellular accumulation of carbon- and metal-based NMs in plants, and particularly within vacuoles and cytoplasmic strands [118], and on the surface of root cell organelles and stem [119, 120].

### 2.4.4 Indirect Impact and Implications

**NMs Impact on Nutrients Availability and Gas Exchange**

In addition to direct effects on specific aquatic organisms, exposure to NMs may lead to secondary other outcomes that are prone to affect ecological systems. Of importance for primary
producers, are the decreases of nutrients bioavailability to cells and of the gas flows exchange between the cell and environment and vice versa.

The decrease in nutrients availability occurs via nutrients sequestration by nanomaterials or by impairing the uptake capability of the organism of external nutrients, due for example to structural damages. The high sorbent capacity of metal-based and carbonaceous NMs [24], has the potential to limit the availability of nutrients to primary producers, thus impair their uptake from surrounding environments. The reduction of dissolved macro (i.e., phosphorus) and micronutrients (i.e., zinc) due to the adsorption onto TiO₂ surface [121] was observed and suggested that possible partitioning of nutrients onto particles might limit the organisms nutritional requirements, in particular if the environment already suffers by nutrients limiting conditions. No effect on algae growth was observed after nutrients adsorption on MWCNT [122], although the specificity of the experimental conditions are likely to strongly influence the depletion of nutrients and their interactions with nanomaterial in a biologically active environment.

Structural modifications and the physical obstruction when in contact with nanomaterials can limit nutrients, gas and water inflows and outflows at the cell surface. Gas exchange through photosynthetic channels [26], transpiration rates and thermal balances in plants [123] and roots’ hydraulic conductivity [124] were reduced by NMs clogging of cells’ surfaces. Enhancement [26] or hindrance to the uptake of water [71] was reported in plants exposed to carbon-based nanomaterials with mechanisms unclear. To date, the studies focusing on the interactions between nutrients and nanomaterials in ecologically-rich environments are still limited, indeed desirable to elucidate the potential of NMs to limit the availability of nutrients to cells.
Synergistic/Antagonistic Interactions of NMs with Other Environmental Contaminants and Related Ecological Impacts

Interactions between engineered nanomaterials and other environmental contaminants are likely to occur in complex ecological systems. Synergistic/additive and antagonistic effects of nanomaterials with other organic or inorganic environmental contaminants were reported in a limited number of studies. Synergistic effects might be associated either with the increased bioavailability of the toxicant by the interaction with nanomaterials [49] or by the additive result of their mutual toxicities. In cases of antagonistic interactions the contaminants phytotoxicity is reduced [125, 126] or ceased [127], often because of the significant sorption of the tested chemicals onto NMs aggregates or other intrinsic chemical interactions.

The ability of NMs to sorb organic and inorganic pollutants in nature was reported to influence their bioavailability and toxicity [38]. Nanomaterials, such as nTiO₂ and C₆₀ fullerene also have the potential to adsorb and transport other pollutants, increase their uptake, enhance their mobility and, thus, retard their rate of deposition into sediment and soil [128]. These properties are also exploited for the current applications of NMs in the environmental bioremediation of soils and water. Moreover, the exposure of ecological receptors to multiple stressors might change their magnitude of impact and thus, modify the environmental behaviors of biological targets.

2.5 NMs properties that Impact Nanecotoxicity

Biological and chemical interactions of NMs are strongly influenced by their physical-chemical properties, which are known to be different from their bulk counterpart [129]. NMs characteristic parameters such as size, surface charge, surface function, aggregation etc. are directly or indirectly linked to their mobility, uptake and transport processes in the environment. Hence, it is
of importance to take these variables into consideration when determining their biological significance and their toxicity. The key parameters that have been recognized to influence their fate, transport and biocompatibility, therefore their implications in ecotoxicity are described in the following sections.

2.5.1 Specific Surface Area (SSA) and Particle Size

Particle size and specific surface area (SSA) are of importance when assessing NMs bioactivity and associated toxic effects. In particles with smaller size the total surface area per unit of nanoparticles’ mass (SSA) is high, and leads to increased reactivity, due to the greater proportion of surface atoms available to react with chemical and biological surroundings. Other properties such as solubility, conductivity, catalytic behaviors, and adsorption also change when varying particle size, thus changing their surface characteristic and properties [130, 131]. Typically, the mass concentration is used as dose metric to assess NMs toxicity, and a consistent correlation between particle size and toxicity levels has not yet been established among studies, especially in their implications to primary producers. Recently, it is believed that NMs specific surface area is a more appropriate parameter to use and shows good correlations with toxicity [88]. Particularly, it was reported that larger SSA well-correlated with the biological oxidative damage BOD (based on the ferric reducing ability of serum (FRAS) assay approach) induced by a large number of metal- and carbon-based nanomaterials, likely due to the greater availability of metals at the particles surface, which may, in turn, induce toxicity [132]. In addition it was hypothesized that particles of the same nature and shape, but of a different size, may display the same ecotoxicity when the dose is expressed as surface area [133].

Particles’ surface area is expected to play a role not only in cell toxicity but also in NMs fate and transport in the environment. SSA affects the dispersion properties of NMs through the
solution pH [134], and influences particles’ agglomeration, due to the greater collision frequency between particles. Additionally, it governs the diffusion rate of nanomaterials as well as the kinetics of cellular uptake [135], particularly in cells, where only particles of given sizes are able to cross cellular barriers.

The limited knowledge available on the role of these two parameters in toxicity is still scarce, thus results are often found to be inconsistent. For example, Hartmann et al. [49] compared the toxicity of two different sizes (<10 nm and 30 nm) of TiO$_2$ nanoparticles on *Pseudokirchneriella subcapitata* and did not find any clear relationship between the size and effect, while maximum levels of TiO$_2$ toxicity in *Pseudokirchneriella subcapitata* [48] and in *Desmodesmus subspicatus* [83] were obtained at specific particles size ranges in other studies. Thus, more research is needed to determine the actual implications of these two parameters on NMs toxicity on environmental species, also in view of the particles aggregation and disaggregation phenomena that surely take place in real environmental scenarios.

### 2.5.2 Elemental Composition and Cristallinity

Though nanomaterials may have the same composition, they may differ for their chemical or crystalline structure, which mostly refers to their three-dimensional atom arrangements. Cristallinity influences the thermal stability and internal energy of particles and other important parameters such as the pH$_{pzc}$, the maximum solubility (saturation concentration) and, in some cases, their photoreactivity [136]. Particle compositions and its purity also confer different physical and chemical properties to nanomaterials. In the work by Griffit et al. [55], it was reported that the chemical composition of metallic nanomaterials appears to be the most important factor in toxicity to species belonging to different trophic levels (i.e., filter feeders, primary producers, etc.). For example, the toxicity induced by the nTiO$_2$ crystal anatase was
higher than that of rutile, due to a greater generation of free radicals [60]. Crystallinity might also affect particles’ aggregation especially in those nanomaterials that present high degree of crystallinity, thus stronger Van der Waals interactions, such as MWCNTs [137]. The importance of crystallinity in the toxicity exerted to primary producers has not been deeply covered; therefore new research on this direction is warranted.

2.5.3 Shape and Aspect Ratio

It was reported that shape and aspect-ratios (ratio of length to width) of engineered nanomaterials strongly influence their toxicity in biological systems and the higher is the particles’ aspect ratio the higher is its associated toxicity. These parameters are controlled during NMs manufacturing and include spherical, tubular, planar, fiber-like shapes, etc.

A limited number of studies have focused on correlating the role of NMs shape with toxicity response in primary producers, and the mechanisms involved are not well-understood yet. Shape-dependent toxicity was involved in NMs uptake processes by mammalian cells [138] and in plants. Endocytosis is recognized to be the major mechanism of NMs biouptake in plants and it facilitates the uptake of spherical NMs more than rod-shaped or fiber-like NMs [138]. Rod-shape particles and, specifically, their high curvature ends are shown to generate the inhibition of engulfment with impairing the growing edges of the phagocytic cup and, thus, limit endocytosis [139]. Additionally, tubular NMs and fiber-particles with higher contact area are known to negatively interact with membranes and, for example, induce the block of ionic channels, thus hampering cells functions. Carbon nanofibers were found to block stomata pores, therefore might have the potential to limit gas flow and exchanges from leaves and stem epidermis of plants [138].
In algae and cyanobacteria, the effect of NMs shape and ratio has been poorly elucidated but may share some common mechanisms observed for gram-negative bacteria of similar membrane structure. Peng and colleagues [140] showed ZnO toxicity to marine diatoms dependent on particles’ morphology, particularly with rod-shapes NMs exerting higher levels of growth inhibition than spherical NMs. However, antibacterial activity of nAg on *E. coli* was more pronounced when using triangular nanoplates than rod-shaped or spherical particles [141]. Thus, it is likely that for algae and bacteria, for which mechanisms of NMs uptake require specific conditions [73, 74], a shape-dependent toxicity is potentially obtained through mechanical and physical interactions at the cell surface or by the increased surface reactivity characteristic of more irregular shapes.

### 2.5.4 Surface Properties

Surface properties of nanomaterials can control key processes regulating NMs fate and transport, such as agglomeration and adsorption, but also influence toxicity to living systems with their influence on intracellular uptake and interaction with cell components [142]. Surface properties often include surface charge, atomic coverage, number and type of functionalized groups.

Surface charge measures the particles potential to interact with surrounding ions or charged surfaces and it is often measured in terms of $\zeta$-potential. The control of surface charge and the manipulation of electrons at the surface allow the control of key NMs properties (i.e., photoluminescence, magnetism, etc.) and make NMs feasible for specific applications (biomedical for targeting specific cell components) [143]. The magnitude of the electrical charge and the thickness of the electric double layer can be modified by environmental factors (i.e., pH and ionic strength) [144] or by the nature and composition of the surrounding matrix. For example, the presence of cellular materials and bio-components (i.e., algae exudates, etc.) and
their non-specific adhesion to proteins [145] may shield the surface charge, enhance NMs aggregation processes and NMs hydrophobicity.

Alterations in the particles atomic structures, vacancies or other defect near and on the surface and specific functionalization can modify NMs surface properties and, therefore, govern their interaction with other particles and biological systems. The functionalization of nanomaterials’ surfaces and the use of coating agents were found to strongly dictate particles behavior and physical-chemical interactions (such as also biouptake) or their role in toxicity. For example, the presence of hydroxyl- or carboxyl- functional groups onto MWCNTs surface enhanced their stability in aqueous systems containing NOM [146] and decreased toxicity to filter-feeders. Additionally, it was reported that functionalized fullerene (i.e., carboxylated, hydroxylated, hydrogenated, etc.) can clearly decrease cell cytotoxic responses in respect of those induced by the unmodified counterparts [147] and shown to mitigate oxidative stress response in exposed cells [148], including aquatic organisms [149]. The lower available charge density characteristic of semi-metallic SWCNTs at the Fermi level shows different levels of toxicity than the metallic counterparts, because of their inherently distinct chemical reactivity [150].

Also, cationic side chains are believed to be more toxic than the negatively charged surfaces, likely due to their higher affinity with the negatively charged groups at the cell membranes (i.e., phospholipids and proteins) [138]. Functionalization was also found to enhance the delivery of MWCNTs via direct penetration through plants walls, as alternative to endocytic pathways of internalization [72].
2.6 Implication of NMs Exposure on Phototrophic Aquatic Ecosystems

2.6.1 Trophic Transfer and Bioamplification

Ecosystems equilibriums are based on a balanced flow of energy and nutrients from lower to higher trophic levels. In addition, contaminated soils and waters might experience the exchange of pollutants between primary producers (i.e., cyanobacteria, algae, plants) and secondary consumers (i.e., animals and ultimately humans), with bottom-up consequences and top-down effects. Bioamplification has been previously reported for some widely spread persistent xenobiotic contaminants such as PCBs, DDT, and mercury [151], and now expected for the newly emerging nanomaterials. Nanomaterials have no physiological significance for aquatic organisms, and are prone to cause toxic responses that might affect community composition, growth rates and metabolisms.

When released into the environment NMs can take different routes and, hence, enter the food web at various levels, bio-accumulate in microorganisms and primary producers, and translocate to consumers, hence to humans and ruminants [96], with eventual biomagnification. As reported in previous sections, the bioaccumulation at a specific point of the food nets might happened via internalization of the NMs into a specific receptor, via adhesion and adsorption onto a the organisms’ outer or internal surfaces. Intracellular presence and intracellular persistence of nanomaterials within organisms is often limited to the cells’ uptake competency, specifically for bacterial species that are lacking specific pathways [73], but also to the intrinsic properties of the nanoparticles (i.e., size, shape, surface functionalization, etc.). For example it was shown that QDs surface properties (byotinilated vs carboxylated) rather influence the extent of intracellular persistence than the degree of uptake [152].
The number of studies on this topic is not yet adequate to have strong conclusions on the potential bioaccumulation and biomagnification of nanomaterials in both lower and higher trophic levels [152]. However, a few studies have confirmed this translocation within subsequent generations of the same species, or among species of different trophic levels and with various feeding strategies, such as algae, daphnids, and invertebrates [55].

The first evidence of the translocation of C70 fullerenes to second generations of rice plants was reported by Lin et al. [71]. Bouldin and his colleagues [153] described the transport of quantum dots from the green algae *P. subcapitata* to *C. dubia* (water flea), whereas Morgalev et al. [154] showed that nTiO2 can be accumulated by algae and small crustaceans from zooplanktons, and ultimately potentially enter the food chain. Recent studies showed the trophic transfer dynamics of QDs nanomaterials in simplified invertebrate systems (bacterial/protozoa and bacteria-ciliate-rotifers) and conclude that, after bioaccumulation, trophic transfer of nanomaterials is possible, particularly from ciliates to rotifers [152] and from bacteria to protozoa [155]. Consequent biomagnification was also proved to make intact NMs within poisoned protozoa (of lower motility) available for higher food chain members [155]. Although these studies suggest that the specificity of trophic transfers are based on several factors including type of species, NMs properties and their mutual interactions, it is expected to have similar results in food web dynamics involving cyanobacteria, due to the similarity of their structure with that of gram-negative bacteria, but also for their significant role as dietary source of micronutrients for higher trophic levels.
2.6.2 Effects on Ecosystem Function

The release of anthropogenic materials and pollutants in aquatic environments has the potential to disrupt ecological balances and lead to adverse impacts on ecosystems integrity. Among the recent developments in nano-ecotoxicology, there is a growing interest in understanding the associations between the environmental presence of NMs and the impairment of ecosystems’ structure and function. Although several studies have targeted specific physiological or metabolic damages in various organisms, a global-scale assessment on a more comprehensive ecological impact is still missing. This is also impeded by the scarce knowledge on the fate and presence of NMs in surrounding environments. To date, few studies have reported NMs toxicity on aquatic and soil bacterial communities using advanced molecular methods [156, 157], however there are not reports on the sole implications of NMs exposure on complex communities dynamics of primary producers in real ecosystems.

Although it is expected that different environmental scenarios will govern the behaviors of NMs and NMs-species interactions, hypothesis on their potential ecological impact can be made based on previous laboratory results and NMs intrinsic properties and mode of action.

As previously discussed, many studies have shown inhibition of primary producers’ growth when exposed to different classes of nanomaterials at various concentrations. In addition to traditional growth inhibition assays, a more inclusive Dynamic Energy Budget (DEB) framework and model was used to connect rates of energy acquisition and expenditure supporting growth, maintenance, and reproduction in phytoplankton exposed to metal oxides nanomaterials [56]. Generally this decrease in organisms’ growth rates is expected to impair lower trophic levels, which appear to be more sensitive to NMs exposure than higher food web
members. As a consequence, this will cause ecological food imbalance among communities, thus shift communities dynamics and interspecies competitions.

Furthermore, nanomaterials might potentially be responsible of changes in the nutritional status of primary producers in freshwaters. The enhancement/reduction of primary productivity, thus carbon and nitrogen intake, and the alteration of uptake pathways involving other macro- and micro-nutrients, will certainly translate into changes of intracellular stoichiometry and composition. Additionally, limitation of nutrients availability due to their sequestration and binding with NMs might also hinder cellular uptake. This will, in turn, modify cells biochemical features, hence impair the dietary source and nutrition of higher trophic levels organisms that feed on lower phytoplanktonic species.

As an example, enhancement of phytoplankton cell death is known to have adverse impact on growth and reproduction of predators, for the limitation in primary products, but also alter water quality in increasing the levels of dissolved organic carbon and toxins [158]. Together, modifications of intracellular stoichiometry, organisms’ growth rates, and fitness will affect at a larger scale biological productivity, the dynamics of communities in food webs and the equilibriums of nutrient cycling [159].

In general it is still not clear if the effects that are often observed during short-term exposure to NMs in laboratory conditions truly reflect actual organisms’ responses. Also, we need to recognize that, typically, environmental strains show higher resistance than laboratory cultures and they are known to have more physiological plasticity and homeostatic ability in facing environmental perturbations and stressors. Thus, caution is needed in interpreting and generalizing these results.
2.7 Biosynthesis of NMs by Primary Producers

The biosynthesis of nanoparticles by living organisms has been recently proposed as a “greener”, ecofriendly and more sustainable alternative to the traditional physical and chemical method of NMs manufacturing [26]. Several phototrophic species, like plants and aquatic autotrophs, have found application in the synthesis of biocompatible metal-based nanostructures [160, 161], due to their reducing potential of converting inorganic metal ions into nano-sized particles. Yet, the mechanism of such nanoparticles synthesis and their possible translocation within plants is not fully understood. Nevertheless, recent findings show their promising applications for human benefit, such as the remediation of metal contaminated water.

Nucleation and synthesis of nanoparticles (i.e., ~50nm AuNPs and nAg) has been observed in plants, legumes and cyanobacteria, and occurs after the reduction of cationic metals into metallic nanoparticles (i.e., Au(III) ions into Au(0) and Ag⁺ to Ag0) in presence of specific electron donors [26, 76, 120, 162]. This conversion is often seen as a response mechanism of the organisms to the toxicity imparted by high accumulation of metals [163].

Nanoparticles’ biosynthesis was found to occur at both intracellular and extracellular levels and mediated by internal secondary metabolites as well as non-toxic biocompatible agents supplemented in solution [161]. The participation of extracellular phenolics, amine groups containing proteins, reducing agents (i.e., reductases) and other ligands (i.e., phosphorus, sulfur, iron) [26, 76, 160] as well as the importance of pH [161] in NMs synthesis has been documented. Functional groups in cell walls (i.e., carboxyl groups, phosphate and sulfonic groups) also played a key role in metal binding and reduction.

In industrial processes the use of leaf extracts, dead plants and dried algal cells and their binding with metal ions for extracellular NMs synthesis avoids the expensive and laborious
recovery of the intracellular particles, provides a better control over the NMs shape and size desired and facilitates the downstream processes and potential industrial scaling up [160, 161, 164].

2.8 References


Chapter 3

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

3.1 Abstract

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.
3.2 Introduction and Objectives

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 $\mu$gTi/L [4], which is consistent with the predicted environmental concentrations of titania nanomaterials (0.7-16 $\mu$g/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 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 nTiO2 exposure on the growth rate, N-fixing activity, and intracellular nitrogen-storage structures in the cyanobacteria A. variabilis. Quantitative inhibition effects of nTiO2 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 nTiO2 exposure.
3.3 Experimental Methodology

3.3.1 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 $\sim$90 W power for 20 min. Primary size of TiO$_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 and reported by our collaborator Bello et al. [19]. A specific surface area (SSA) of 274.2 m$^2$/g 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].

3.3.2 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 $\mu$m 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 \( n \text{TiO}_2 \) concentrations, from 0 mg\( \text{TiO}_2 \)/L (control sample) to 500 mg\( \text{TiO}_2 \)/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 et al. [21]. Samples were also subject to transmission electron microscopy imaging to observe \textit{A. variabilis’} intracellular changes.

### 3.3.3 Nitrogen Fixation Activity via Acetylene Reduction Assay

To establish whether \textit{A. variabilis} exposure to \( n \text{TiO}_2 \) inhibits its \( N_2 \) fixation ability, \textit{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 (\( \geq 99.5\% \) purity Medtech, Medford) addition to the headspace to obtain a concentration of 12.5\% \( \text{v/v} \) \( \text{C}_2\text{H}_2 \). Vials were incubated and shaken (350 rpm) for 8 hours and then the reaction was stopped by the addition of 300 \( \mu \text{L} \) 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 \( \mu \text{L} \). 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.
3.3.4 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 cacodylate buffer and embedded in 2% agarose for beads preparations. Post-fixation was completed in 2 hours in 1% osmium tetroxide followed by two rinsing steps in 0.1 M cacodylate 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 hours. 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.
3.4 Results and Discussion

3.4.1 Effect of nTiO₂ Exposure on Growth of Anabaena variabilis

The effects of nTiO₂ exposure on the growth rates of *A. variabilis* was evaluated for various nTiO₂ concentrations ranging from 0.5 to 500 mg/L, and for various exposure lengths, ranging from 24 hours to 6 days. At concentrations above 250 mg/L, greater than 90% inhibition was observed even with the shortest exposure time of 24 hours. The same percentage of inhibition was also observed with 6 days exposure with nTiO₂ concentrations as low as 0.5 mg/L. These results indicated that the inhibition on *A. variabilis* growth depends on both nTiO₂ concentration and exposure time; therefore, the $C^nT^m$ (e.g., Hom’s law) concept was applied to refer to the toxicity effect (Figure 3.1). The Hom’s function is a generalization of the pseudo-first-order Chick-Watson’s law 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 ($C^nT^m$). 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 nTiO₂ concentration and exposure time in nanoecotoxicology. CT-dependent antimicrobial effects of TiO₂ have been reported by several studies that examined the photocatalytic disinfection potential of TiO₂ (coupled with ultraviolet irradiation) on *E. coli* in drinking water treatment processes [23, 24]. Ng and his colleagues reported a first-order rate constant $k$ of photocatalytic nanoarray TiO₂ 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 nTiO₂ concentration in inhibiting *A. variabilis* growth. The model fits with a goodness of 0.7403 (R²) 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 (EC₅₀) as a function of exposure time. The EC₅₀ value decreased significantly from 13.98 mgTiO₂/L at 24 hours to 0.15 mgTiO₂/L after 6 days of exposure. EC₅₀-96 h is usually used as the regulatory endpoint for *Selenastrum capricornutum* chronic toxicity assessment. In this study, the EC₅₀-96 h was determined to be 0.62 mg/L (95% confidence interval of 0.600-0.677 mgTiO₂/L). This value is lower than those endpoints (EC₅₀-72 h) previously reported in literature on the green algae *Pseudokirchneriella subcapitata* (5.83 mgTi/L) [1] and *Desmodemus subspicatus* (44 mgTiO₂/L) [26] exposed to TiO₂ nanomaterials. It is known that toxicity is organism-specific [27], which may explain the difference in EC₅₀ obtained. However, the variations in the culturing procedure and nTiO₂ 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 nTiO₂ exposure. This finding is consistent with the recognized importance of identifying appropriate sensitive test organisms for specific stressors [27].
Figure 3.1. Concentration · Time (CT)-dependent growth inhibition of *Anabaena variabilis* cells exposed to nTiO₂ at various concentrations and different exposure time lengths (left). Log (μ/μc) 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 CₙTₘ (right). (Matlab surface fitting tool was used for data interpolation and kinetic parameters estimation).

3.4.2 Impact of nTiO₂ 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 3.2 shows the toxicity effects of nTiO₂ on the nitrogen fixing activity of the cyanobacteria *A. variabilis* at different concentrations. Impact on the 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. Nano-TiO₂ concentrations higher than 10 mg/L led to greater than the 50% nitrogen fixation inhibition after a short exposure time of 24 hours. Nitrogen fixing activity was completely inhibited at a nTiO₂ concentration of 75 mgTiO₂/L after 24 hours exposure and at 1 mgTiO₂/L after 6 days exposure,
indicating that the inhibition effect of nTiO$_2$ on nitrogenase enzyme activity rates is also C$^n$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$^n$T$^m$ curves for growth inhibition and for nitrogen fixation inhibition, as shown in Figures 3.1 and 3.2, suggested that the latter was not an indirect result from the former. The correlation of N fixation inhibition and growth inhibition data at given CT values was further evaluated (Figure A1, Appendix A) 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 A2, Appendix A). 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.

Figure 3.2. 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).

3.4.3 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 nTiO₂ 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 3.3). 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 non-ribosomally 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 3.4 and 3.5 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 nTiO₂ concentration and exposure time duration. Less than 30% of the cells analyzed contained CGPs in the control culture with no nTiO₂ exposure (at 96 hours) (Figure 3.4). In exposure to a high concentration of nTiO₂ at 150 mg/L, more than 80% of cells were found to contain CGPs granules.
Figure 3.3. 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).

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 3.5). 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 hours) 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 3.5a) in the populations that were either exposed for longer time (96 hours) 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 hours). For example, after 96 hours 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.

Figure 3.4. 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.

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.

Figure 3.5. 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.
3.4.4 Potential Role of CGPs in Algal Response to nTiO₂

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 non-fixing 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 non-exponential 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 this granule is promptly responsive to the toxicity induced by the nTiO₂ 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 nTiO₂. 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 nTiO₂. Another possible scenario that may justify the elevated formation of CGPs in stressed cells involves the binding of nTiO₂ with intracellular peptides [37], which may lead to alteration of the functions of the proteinaceous cellular machinery. Internalization of nTiO₂ nanomaterials has been observed in our other study (unpublished) and therefore contact with intracellular peptides is likely. In addition, binding of nTiO₂ 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 nTiO₂ 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 NMIs is therefore warranted.

3.5 Conclusions

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 NMIs 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 $\mu$g/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.

3.6 References


[31] Li, H.; Sherman, D. M.; Bao, S. L.; Sherman, L. A. Pattern of cyanophycin accumulation in 

[32] Carr, N. G. Nitrogen reserves and dynamic reservoirs in cyanobacteria. In *Biochemistry of 
the Algae and Cyanobacteria* (Annual Proceedings of the Phytochemical Society of Europe), 

[33] Gupta, M.; Carr, N. G. Enzymes activities related to cyanophycin metabolism in heterocysts 

K. H.; Capone, D. G. Carbon and nitrogen fixation and metabolite exchange in and between 

[35] Stevens, S. E.; Paone, D. A. M.; Balkwill D.L. Accumulation of cyanophycin granules as a 
719.

metabolism of cyanophycin, a bacterial nitrogen reserve polymer - Expression and 
mutational analysis of two *cyanophycin synthetase* and *cyanophycinase* gene clusters in the 
11582–11592.

[37] Chen, H. B.; Su, X. D.; Neoh, K. G.; Choe, W. S. Probing the interaction between peptides 
6852–6857.

[38] Connor, P. A.; McQuillan, A. J. Phosphate adsorption onto TiO$_2$ from aqueous solutions: 
Chapter 4

Impact of Nano Titanium Dioxide Exposure on Cellular Structure of *Anabaena variabilis* and Evidence of Internalization

4.1 Abstract

The present study investigated the impact of nano titanium dioxide (nTiO₂) exposure on the cellular structures of the nitrogen-fixing cyanobacteria *Anabaena variabilis*. Results of the present study showed that nTiO₂ exposure led to observable alteration in various intracellular structures and induced a series of recognized stress responses, including production of reactive oxygen species (ROS), appearance and increase in the abundance of membrane crystalline inclusions, membrane mucilage layer formation, opening of intrathylakoidal spaces, and internal plasma membrane disruption. The production of total ROS in *A. variabilis* cells increased with increasing nTiO₂ doses and exposure time, and the intracellular ROS contributed to only a small fraction (<10%) of the total ROS measured. The percentage of cells with loss of thylakoids and growth of membrane crystalline inclusions increased as the nTiO₂ dose and exposure time increased compared with controls, suggesting their possible roles in stress response to nTiO₂, as previously shown for metals. Algal cell surface morphology and mechanical properties were modified by nTiO₂ exposure, as indicated by the increase in cell surface roughness and shifts in cell spring constant determined by atomic force microscopy analysis. The change in cell surface structure and increase in the cellular turgor pressure likely resulted from the structural membrane damage mediated by the ROS production. Transmission electron microscopy (TEM) analysis of nTiO₂ aggregates size distribution seems to suggest possible disaggregation of nTiO₂ aggregates.
when in close contact with microbial cells, potentially as a result of biomolecules such as DNA excreted by organisms that may serve as a biodispersant. The present study also showed, for the first time, with both TEM and Raman imaging that internalization of nTiO₂ particles through multilayered membranes in algal cells is possible.

4.2 Introduction and Objectives

Progress in nanotechnology has raised concerns regarding the potential environmental impact of engineered nanomaterials (NMs). The increasing production rates of NMs and the utilization in various fields and commercial products are anticipated and will result in their release into aquatic habitats [1, 2]. Particularly, titanium dioxide nanomaterials (nTiO₂) are being incorporated in a wide range of promising applications, which include solar energy conversion [3], cosmeceutical production [4], and biocidal processes, such as drinking water treatment for pathogen removal [5], because of their unique nano- and photocatalytic properties. Recently, detectable concentrations (5–15 mgTi/L) of titanium nanomaterials from wastewater treatment processes were revealed [6], in agreement with the predictions of Mueller and Nowack (0.7–16 µg/L) based on worldwide production volumes in typical Swiss environmental scenarios [7].

Currently, most nanotoxicity studies have focused on the cyto- and genotoxicology of nTiO₂ in human health initiated by exposure through the respiratory system, and the potential environmental implications of nTiO₂ for other organisms have largely been unexplored [1, 8]. Fundamental research on the toxicity of nTiO₂ to ecologically relevant organisms, such as algae, bacteria, and fungi, is scarce [9]. The bioavailability and toxicity of nTiO₂ to algal ecosystems is of concern for the essential ecological role of prokaryotic and eukaryotic algae in primary productivity and aquatic food web chain equilibria [10]. A few studies have investigated the
impact of NMs on algal ecosystems [11–13] using conventional regulatory toxicological methods with freshwater indicator microorganisms (Selenastrum capricornutum, Desmodesmus subspicatus) [3, 14]. The results confirmed that exposure to nTiO$_2$ affects algal growth [3] and photosynthetic activity [12] and that abiotic parameters, such as particle size/aggregation and illumination, are key factors affecting nTiO$_2$ toxicity [15, 16]. The underlying toxicity mechanisms of nTiO$_2$ nanomaterials have been elucidated to some extent, and they include membrane disruption [17], protein oxidation via reactive oxygen species (ROS) formation [9], and possible DNA damage [18]. Furthermore, persistence and bioaccumulation of nTiO$_2$ in cells is mostly unknown, and this potentially presents a concern for possible introduction into the food web.

Thus far, there has been no report on the ecotoxicity of NMs on cyanobacteria (also called blue-green algae), which are prokaryotes of significant biogeochemical importance because of their global contribution to nitrogen and carbon atmospheric fixation [10]. The abundance and unique metabolic strategies used by cyanobacteria to tolerate adverse and fluctuating conditions often make cyanobacteria good model algae for evaluating environmental stresses [19]. In the present study, we, for the first time, investigated the impact of nTiO$_2$ exposure on the cellular structures of the N-fixing cyanobacteria Anabaena variabilis. The effects on cell growth, intracellular structure, and cell surface properties were evaluated, and changes in cellular membranes, as well as cell surface topological and mechanical properties, were revealed. The oxidative stress caused by nTiO$_2$ by means of ROS production analysis was then quantified. The results provided a systematic evaluation of the nTiO$_2$ toxicity to the N-fixing cyanobacteria Anabaena variabilis and provided insights into the inter-reaction of nTiO$_2$ with algal cells.
4.3 Experimental Methods

4.3.1 NMs Preparation and Characterization

Nano-TiO$_2$ anatase (nTiO$_2$; NanoStructured and Amorphous Materials) was prepared in culture Mes-Volvox medium in a stock concentration of 10 g/L, which contains 1% crude bovine serum albumin (BSA) as dispersant. An average size of NM aggregates of $192 \pm 0.8$ nm was determined through dynamic light scattering (Zetasizer Nano ZS90; Malvern Instruments) after nanomaterial dispersion in culture media (single crystal nTiO$_2$ primary size from manufacturer was 10 nm outer diameter). The stock solution was sonicated in a high-energy cup sonicator (Fisher Scientific) at 90W power for 20 min prior to tests. The polydispersity index (PdI) after dispersion in culture media was found to be 0.479. A specific surface area (SSA) of 274.2 m$^2$/g was previously reported for the nTiO$_2$ used in our study, by our collaborators Bello and colleagues [2]. Transition metals of the bulk material and other physical–chemical parameters (organic and elemental carbon, surface charge) determined for nTiO$_2$ suspension in phosphate-buffered saline were also reported by Bello et al. [2].

4.3.2 Culture Conditions and Ecotoxicological Tests

Anabaena variabilis strain (UTEX 1444) was axenically cultured at 20°C in a nitrogen-free modified Mes-Volvox medium containing 0.16 mM MgSO$_4$ · 7H$_2$O, 0.16 mM Na$_2$-glycerophosphate · 5H$_2$O, 0.67 mM KCl, 10 mM MES, 0.1 mM vitamin B12, 0.1 mM biotin vitamin solution, and trace metals. Cells were cultured in 1-L chemostats with 0.15 d$^{-1}$ dilution rate, incubated under a 12:12-h light:dark regime using a 1:1 ratio of 34-W cool white and 40-W Sylvania gro-lux fluorescent bulbs. Chemostats were continuously mixed and aerated (air was filtered via 0.2-mm filtered compressed air at a rate of 5 mL/min), and algal concentration was maintained at 1.0 g/L of chlorophyll $\alpha$. Cells from chemostats were used as starter for the stock
culture preparation needed for toxicity tests. Aliquots (75 ml) of cultures with initial chlorophyll a concentration of 500 mg/L were subjected to different nTiO2 concentrations (0–500 mg/L) and incubated for 96 h under the same conditions of culturing. Cells were periodically collected and prepared for various imaging analyses (atomic force microscopy [AFM], transmission electron microscopy [TEM], Raman; see below) to observe A. variabilis intracellular changes and nTiO2 distribution.

4.3.3 Reactive Oxygen Species Production: Oxidative Stress

Total ROS formation were determined according to the method described by Knuaert et al. [20] using the fluorogenic permeable probe 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen). The probe is first hydrolyzed to the nonfluorescent dichlorodihydrofluorescein (H2DCF) by cellular esterase before being transformed to the highly fluorescent dichlorofluorescein (DCF) in the presence of ROS and cellular peroxidases. Fluorescence associated with DCF was measured at certain time points using a Synergy™ HT Multi-Mode microplate reader (excitation filter 485 nm, emission filter 528 nm). Both total and intracellular ROS were analyzed with cells exposed to different concentrations of nTiO2 (0–200 mgTiO2/L) and different exposure times (0.5–2.5 hours). To differentiate intracellular ROS from measured total ROS, cells were spin down by centrifugation (2,000 g for 10 min), replaced in fresh dye-free medium, and then subjected to fluorescence measurements. The DCF fluorescence results were expressed in terms of H2O2 units, because hydrogen peroxide (30%; Fisher Scientific) was used as a standard for ROS measurements.

4.3.4 Cell Topology and Mechanical Properties Changes

Anabaena variabilis cells exposed to 50 mgTiO2/L for 24 hours and those from controls without nTiO2 exposure were dried by air onto the cleaved mica surfaces for cell surface characterization.
using AFM (Agilent 5500 Bio-AFM) analysis. Cell topography imaging and cell spring constant evaluation were obtained in contact mode at a low applied force of 0.2 N/m and scan rate of 1.04 s with rectangular nanoprobe cantilever of 0.05 N/m spring constant \((k)\). Gwiddion 2.12 software (Gwyddion 2.12; General Public License, http://www.gwyddion.net, 2009) was used to analyze topographic images of cells. Cell surface roughness parameters (average roughness, \(Ra\), and mean square roughness, \(Rq\)), for both \(A. variabilis\) cells exposed to nTiO\(_2\) and those in control with no exposure were determined based on information obtained for a total of 20 random \(A. variabilis\) cells. For each cell, 25 (300 nm\(^2\)) areas were selected at the center of the cell to avoid artifact resulting from edge effect to determine the average roughness and the root mean square roughness parameters. The cell spring constant \((K_{cell})\) was obtained from the slope of the linear portion of five deflection-piezo displacement curves determined per scanned cell, according to the method described by Francius et al. [21].

### 4.3.5 Intracellular Modifications and Spatial Distribution of Nanomaterials

High-resolution TEM imaging was used to observe intracellular structural changes in \(A. variabilis\) as well as the spatial distribution and fate of NMs agglomerates. Cells were collected and fixed for 1.5 h at 48°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) obtained with 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
with a JEOL JEM-1010 transmission electron microscope operated at 70 kV. Digital images
were captured with an XR-41B bottom mount CCD camera system (AMT). Nanomaterial
particles sizes were analyzed with the software Image J 1.43q (http://rsbweb.nih.gov/ij/).

4.3.6 Evaluation of nTiO$_2$ Fate through Raman Spectroscopy
In addition to TEM examination of NM presence, Raman microscopy was applied to identify and
confirm the presence of nTiO$_2$ particles inside and/or outside the algal cells. Raman spectral
images were acquired using a WITec model CRM 2000 confocal Raman microscope and a
water-immersion objective (x60/NA = 1.00, working distance = 2.0 mm). Excitation (~30mW at
488 nm) was provided by an air-cooled argon ion laser (Melles Griot). The exciting laser
radiation was coupled to a Zeiss microscope through a wavelength-specific single mode optical
fiber. The backscattered light was finally detected by a back-illuminated deep depletion, 1,024-
x128- pixel charge-coupled device camera operating at -82°C. Three of the ten cells (exposed to
10 mg/L nTiO$_2$) analyzed via Raman microscopy showed intracellular presence of nTiO$_2$. Fixed
samples were prepared on CaF$_2$ windows (Sigma-Aldrich) for imaging. The samples were placed
on a piezoelectrically driven microscope scanning stage and raster scanned through the laser
focus at 500-nm step size. Spectra were collected at a dwell time 250 msec. The Raman images
presented have an overlay of color planes resulting from biomatrices and nTiO$_2$ spectral
contributions, x, y resolution of approximately 3 nm and a repeatability of ±5 nm, and z
resolution of approximately 0.3 nm and ±2 nm repeatability. The continuous motion prevents
sample degradation at the focal point of the laser beam.
4.4 Results and Discussion

4.4.1 Reactive Oxygen Species Production after Exposure to nTiO₂

The formation of ROS has been proposed as the primary mechanism inducing toxicity in cells exposed to nTiO₂ [22]. Once formed, ROS have the ability to activate a chain of radicals that can affect cellular components [17]. In the present study, oxidative status of *A. variabilis* cells exposed to different concentrations of nTiO₂ was monitored through the widely used and established DCFH-DA assay in order to determine intracellular (endogenous) and extracellular (exogenous) ROS. Results of the present study (Figure 4.1a) showed a proportional production of ROS in *A. variabilis* cells with increasing nTiO₂ doses and exposure times under illuminating (growth) conditions. The increase in fluorescence of the dichlorofluorescein indicator over time measures the rate of total ROS production (Figure 4.1b), and the ROS production rates increased from approximately 190 to 340 nM H₂O₂/h as the dose nTiO₂ concentrations increased from 10 to 200 mg/L. The intracellular ROS production determined at various nTiO₂ doses, as shown in Appendix B, Figure B1, contributed to only a small fraction (<10%) of the total ROS measured.

Our results seem to be consistent with the study by Knauert and Knauer [20] on the green algae *P. subcapitata* exposed to Cu, in which they showed that more than 90% of the total ROS produced were found to be extracellular. These results indicate that either the primary toxic impact of nTiO₂ occurs at the membrane site, because the majority of ROS have been exogenously produced, or there might be transport of endogenous ROS (H₂O₂) to outside the cells, and the latter was reported through aquaporins in plants [20]. The dose-dependent ROS production confirms that nTiO₂ causes oxidative stress to *A. variabilis*, and the SoxRS regulatory machinery is recognized to play an important role in maintaining cellular viability, as previously indicated [18]. Although similar response mechanisms are characteristic of a broad spectrum of
microorganisms, the production of ROS and the generation of hydroxyl radical have been found to be microbe dependent [23]; therefore, this result is specific to cyanobacteria and nTiO₂ interactions.

**Figure 4.1:** Total reactive oxygen species (ROS) production (a) and total ROS production rate (b) in *Anabaena variabilis* samples exposed to nTiO₂ concentrations ranging from 0-200 mg/L for 2.5 hours.

### 4.4.2 Intracellular Modifications from nTiO₂ Exposure

Analysis of the ultrathin TEM sections allowed the identification of modifications in *A. variabilis* subcellular structure when exposed to nTiO₂. The cross-section of a typical control cell
of *A. variabilis* (vegetative cell) is shown in Figure 4.2a and clearly presents a typical radial arrangement of thylakoidal membranes, cellular sites of photosynthetic reactions, and various electron-dense or non-dense intracellular inclusions of different functions (lipid inclusions, cyanophycin granules, etc). Under N-deficient conditions, vegetative cells develop heterocysts, specialized cells lacking photosystem II exhibiting structural and functional features distinct from those of vegetative-type cells. Figure 4.2e shows an untreated heterocyst with characteristic multicomponent envelope providing anoxygenic protection to the N-fixing activity of *nitrogenase*.

The structure of the cyanobacterium exhibited changes after exposure to various nTiO₂ concentrations and exposure times. The opening of intrathylakoidal spaces (Fig. 4.2b) and the appearance of intracellular open spaces was induced at all nTiO₂ concentrations tested (1, 50, 150 mg/L) and at different exposure times (24–96 h), with likely consequent alteration of the internal integrity of the cell. There was generally an increase in the percentage of cells with loss of thylakoids in the samples exposed to nTiO₂ compared with controls; however, a consistent dose-dependent trend was not found (Fig. 4.3). The reduction of these proteinaceous compartments might possibly indicate the loss of cellular photosynthetic potential and carbon fixation ability of *A. variabilis*, limiting the availability of important nutrients for growth, as previously indicated for *A. variabilis* cells exposed to Cd [24] or other heavy metals inducing stress conditions [25]. In a previous study [26], we observed that the growth and the N-fixation ability of *A. variabilis* was inhibited by nTiO₂ exposure with resulting median effective concentration 96 h (EC₅₀-96 h) of 0.62 mg/L, and 0.4 mg/L, respectively. A possible imbalanced exchange or lack of nutrients between heterocysts and vegetative cell within the filament might have played a role in the toxicity effect.
The increase of crystals bound in intracellular membranes, also known as membrane-limited crystalline inclusions (Fig. 4.2c and 4.2d), was observed in cells exposed to various nTiO$_2$ concentrations and exposure durations. These inclusions exhibit a characteristic needle-like crystal structure that is usually found to be calcite, apatite, or hydroxyapatite [27]; their recognition is based on images from the literature and is facilitated by their characteristic morphology, shape, and location within the cells [27]. Compared with controls without any nTiO$_2$ exposure, there seemed to be an overall increase in the percentage of cells that showed crystalline inclusions. The relative abundance of cells with crystalline inclusions increased from 14% to as high as 27 to 57% in the samples exposed to nTiO$_2$ at various concentrations and exposure time lengths (Fig. 4.3).

The functionality of these inclusions is largely unknown because of limited observations of this phenomenon. Previous investigations [28] showed an increase in the number of these crystals after exposure of $A. variabilis$ and $Anabaena flos-aquae$ to zinc, indicating its possible role in stress response to metals. Further studies on specific role and formation of these membranes structures during cellular stress response to nTiO$_2$ exposure are warranted. Disruption of internal plasma membranes in heterocyst cells was also observed and is shown in Figure 4.2f. Such a phenomenon was found to be common among cyanobacterial and algal cells under different types of stress conditions, such as the presence of allelochemicals [29] or the exposure to heavy metals [25].
Figure 4.2. Electron micrographs showing the effects of nTiO\textsubscript{2} exposure on \textit{A. variabilis} cells. \textit{A. variabilis} vegetative cell from control sample (a) with typical thylakoidal membranes (arrow), cyanophycin grana proteins (CGPs), lipid inclusions (L). Opening of intrathylakoidal spaces in cell exposed to nTiO\textsubscript{2} (b). Membrane limited inclusions without crystals in control sample (c) and with crystal in cell 48 hours exposed to 50 mgTiO\textsubscript{2}/L (d). Typical heterocyst cell from control with polar nodule (PN) and thick envelope (e). Disruption of plasma membrane (arrow) in heterocyst after 24 hours exposure to 50 mgTiO\textsubscript{2}/L (f).
**Figure 4.3.** Percentage of cells presenting intrathylakoidal spaces openings relative to the total cells observed (blue) and percentage of cells that show crystalline inclusions within membrane-bound structure relative to the total cells scanned containing the membranes (red).

### 4.4.3 Impact of nTiO$_2$ Exposure

Structural and surface alterations induced in *A. variabilis* cells by the exposure of nTiO$_2$ were investigated and imaged via AFM. Several studies [30, 31] have considered the AFM imaging technique as a suitable tool for investigating biological systems at high resolution and at the nanoscale level. Results of the present study indicated that cells surface topography and mechanical properties were modified after exposure to nTiO$_2$. Figure 4.4 shows representative AFM images to demonstrate the visual changes in cell surface topology (smoothness) after exposure to 50 mg/L for 24 hours). As shown in Figure 4.4, the surface of unexposed cells (Fig. 4.4a, c, and e) appeared to be fairly smooth in comparison with those cells exposed to 50 mgTiO$_2$/L for 24 hours (Fig. 4.4b, d, and f). Quantitative cell surface roughness analysis was
conducted to confirm the changes in cell surface properties. Roughness values collected from 300-nm² areas of 20 untreated and exposed cells were fitted in log-normal distributions for comparison (Fig. 5a). The mean of roughness values (Ra) of exposed cells increased from 28.6 nm (measured in the control sample) to 45.9 nm, and the root mean square roughness parameter increased from 33.5 to 55.0 nm. Thus, the differences in roughness observed after the incubation of A. variabilis cells with nTiO₂ are possibly caused by morphological modifications at the cellular surface level or by nTiO₂ deposition onto cell membranes, as also shown previously in the TEM observations.

In the present study, AFM scanning also allowed us to probe the modifications occurring in the nanomechanical properties of A. variabilis cells after 24 hours of exposure to 50 mg/L nTiO₂. To provide quantitative information on cellular surface mechanical properties, the bacterial spring constant (K_{cell}) was calculated based on the correlation between the force applied to the samples by the AFM cantilever and the indentation depth obtained. Arnoldi et al. [32] reported that the bacterial spring constant is a parameter directly related to the inner turgor pressure of the cell. The difference between the inner and the outer osmotic pressures required for preserving cellular shapes in cyanobacteria is typically 0.8 atm [32]. Our results showed that exposure to nTiO₂ caused changes in the cells spring constant (K_{cell}) distribution of native cells towards higher x values (Fig. 4.5b). The mean of the log-normally fitted distribution of the spring constant (K_{cell}) increased from 0.094 N/m in untreated samples to 0.11 N/m after 24 hours of exposure (at 50 mgTiO₂/L concentration). These values are in the range (0.01–0.5 N/m) of those reported in the existing literature and related to bacterial cells [31].

One possible explanation for the increase in cellular turgor pressure as a result of exposure to nTiO₂ can be inferred from a previous study by Cerf [33] based on heat-treated
Gram-negative bacteria. Briefly, structural membrane changes may be explained by the mediation of ROS, which have the potential to modify protein structure [17], protein folding configuration, and periplasmic layer thickness at the cellular membrane level, which results in the collapse of membranes’ layers. This generates an increase of water efflux in the membrane-folded compartments and, consequently, the increase in the contrasting cytoplasmic turgor pressure.
Figure 4.4. Impact of nTiO₂ exposure on cell surface smoothness and topology. Comparison of atomic force microscopy results of control cell (top; a, c, e) and cell exposed to 50 mg/L of nTiO₂ for 24 hours (bottom; b, d, f). Cell topographies (left; a, b), bidimensional image gray-graded by height with sections identified (middle; c, d) and section profiles (right; e, f).
Figure 4.5. Changes in the cell surface roughness (a) and in the cellular spring constant (b) as a result of exposure to 50 mg TiO$_2$/L for 24 hours. The roughness data and cellular spring constant results were fitted in log-normal distributions and compared with the control.

4.4.4 Impact on Anabaena variabilis Cellular Membrane

Disruption, alteration, and impairment of cellular membrane have been suggested as potential recognition mechanisms behind the antimicrobial activities exerted by NM exposure [17]. Transmission electron micrographs of cells exposed to different concentrations of nTiO$_2$ (1, 50, 150 mg/L) showed considerable changes in cell membranes upon treatment. Some of the *A. variabilis* cells observed (Figure B2a and b, Appendix B) showed a lack of internal structural organization and compromised envelopes compared with the controls (Figure 4.2a and 4.2e). Thus, vegetative and heterocyst cells were both damaged to a similar extent, revealing that heterocyst cells with functionally relevant thicker envelopes are also susceptible to lysis damage by nTiO$_2$. Freely released empty walls (data not shown) and intracellular material (Figure B2c,
Appendix B) from cell leakage or lysis was observed in exposed samples, likely related to the nTiO₂ potential to disrupt and oxidize the multilayered wall of *A. variabilis* cells. Membrane damage by nTiO₂ exposure likely occurs either through puncturing when direct cell–nanoparticle interaction occurs or perhaps via nTiO₂ adsorption onto cell surfaces (Figure B2d and e, Appendix B). The main mechanism by which membranes are compromised may involve lipid peroxidation via ROS-mediated processes.

Prolonged (48–96 hours) exposure of *A. variabilis* cells to nTiO₂ apparently has induced other cellular defense mechanisms, such as an increase in the outer mucilage layer thickness (Figure 4.2f). This layer of protection, common in cyanobacteria, has not been found in the unexposed algal cells, but it was present in a few cells exposed for 96 hours to 1 and 50 mgTiO₂/L with variable thickness ranging from 250 nm to 300 nm. Such phenomena have been found to be common among cyanobacterial and algal cells under different types of stress conditions (i.e., exposure to heavy metals [25]). A previous study by Reynolds [34] showed that the thickness and texture of the cyanobacterial mucilage are responsive to environmental variations, sequestration and storage of nutrients in deprived environments, exclusion of toxic metals, and general adaptation to conditions of stress. Our preliminary results demonstrated that an increase in outer mucilage layer thickness may be one of the nTiO₂-induced stress responses for *A. variabilis* as well; however, further studies are needed to confirm this phenomenon.

**4.4.5 Evidence of Internalization of nTiO₂ in algal cells**

Literature on internalization of NMs by prokaryotic organisms is scarce, and it is thought that the possibility of transport of 100-nm-sized particles across 1-µm-sized prokaryotic cell membranes is likely only if the integrity of the cellular envelope has previously been compromised [17]. In the present study, we used combined Raman microscopy and TEM to observe the location and
possible presence of nTiO$_2$ inside *A. variabilis* cells. Raman spectroscopy is a well-established method for the investigation of nanoparticle properties [35] as well as for the characterization of biological samples, such as algae [36]. High-resolution Raman images (Figure 4.6) showed the spatial distribution of nTiO$_2$ inclusions inside individual cells in relation to the cellular organic matrix (C–H stretching region). Thus, simultaneous occurrence of Raman bands associated with nTiO$_2$ and C–H stretching demonstrates that the internalization of nanomaterials by *A. variabilis* cells is possible. nTiO$_2$-anatase appears with three major vibrations occurring at 400 cm$^{-1}$, 518 cm$^{-1}$ and 629 cm$^{-1}$, slightly shifted from the peaks identified by Robert and colleagues [37], most likely as a result of differences in NM particle size [35]. Major Raman peaks features at approximately 1,005 cm$^{-1}$, 1,155 cm$^{-1}$, and 1,525 cm$^{-1}$ have also been highlighted in Figure 4.6c and are associated with carotenoids, typical pigments in algae. The most pronounced Raman intensity, between 2,800 and 3,050 cm$^{-1}$, originates from C–H stretching vibrations of the organic molecules of the organism. The confocal feature and the high Z-axis resolution of our Raman analysis confirmed that the nTiO$_2$ was indeed present inside *A. variabilis* cells, rather than possibly on top of or beneath the algal cells.

To our knowledge, this is the first study showing the internalization of nTiO$_2$ by cyanobacteria algae cells, which is contrary to the previous hypothesis of inability of NMs to pass thick algal cell walls [13]. However, further studies are needed to understand fully the mechanisms involved in NMs transport through cell membranes at the nanoscale. It is not clear from the present study whether this NM internalization in the algal cells occurs after the cell damage or death via passive approach. At this stage, information is lacking on membrane pores’ size in living cells and their real-time changes. Research has been oriented to establish the mechanisms of membrane transport, with a major focus on multidrug resistance and antibiotic
treatments [38], but information is lacking on the mechanisms of NMs transport through cellular envelopes.

**Figure 4.6.** High-resolution Raman images and Raman spectra of nTiO$_2$ internalization in *Anabaena variabilis* cells. Microscopic images taken with a water immersion objective (60x) (a) indicating cell with inclusion (arrow). High resolution images reconstructed from Raman intensities reflecting the protein density within the cell in blue, and the nTiO$_2$ inclusion in red (arrow) (b). Spectra collected at the inclusion level showing characteristic nTiO$_2$ peaks (400 cm$^{-1}$, 518 cm$^{-1}$ and 629 cm$^{-1}$), carotenoids peaks (1005 cm$^{-1}$, 1155 cm$^{-1}$ and 1525 cm$^{-1}$) and C-H stretching vibrations (2800 cm$^{-1}$ and 3050 cm$^{-1}$).
4.4.6 Insights into the Impact of Biomolecules on NMs Aggregation

Time sequential TEM observations on algal cells exposed to different concentrations of nTiO₂ showed that, over the test period of 96 hours, agglomeration of nTiO₂ (10-nm primary particles) occurred and resulted in various sizes of aggregates (115 aggregates observed across all samples tested), with an average longest dimension of 435.0 ± 275.5 nm (Figure B3, Appendix B). Interestingly, the analysis of several TEM images showing NM aggregates surrounding algal cells seemed to suggest that NM aggregates could disrupt and release small NMs particles (10–20 nm) in very close proximity to algal cells (Figure B3, Appendix B). Previous studies indicated that DNA [39] or other biomolecules released from cells may act as a dispersant and could facilitate the disaggregation of NM aggregates, which may explain what was observed here via TEM analysis. Previous literature showed the diffusion of 5-nm quantum dots through membranes of *Escherichia coli* and *Bacillus subtilis* [40], and nanosilver particles smaller than 80 nm were proven to pass through pores in membranes of living *Pseudomonas aeruginosa* cells [38]. The latter NM passing size was determined to be 50 times greater than the size of conventional detergent and antibiotic with proven capability to permeate cells envelopes [38]. Our finding implies that, although the NMs are expected to aggregate in media, the primary single particle size for the NMs may be more important than the aggregates, because the latter is possibly dispersed by biomolecules excreted by organisms in the microenvironment when in close contact with microbial cells. This is consistent with the previous finding by Oberdörster [41], who found that the same-sized NM agglomerates originated from two different sized nTiO₂ primary particles and exhibited different levels of toxicity.
4.5 Conclusions

The understanding of NMs interactions with algal ecosystems is still in its infancy, and the present study, for the first time, systematically investigated the impact of nTiO$_2$ on the N-fixing cyanobacteria *Anabaena variabilis*. The observed impact and cyanobacteria–nTiO$_2$ interactions are summarized in Table 4.1. Our results showed that nTiO$_2$ exposure led to observable alteration in various intracellular structures and induced a series of recognized stress responses, including production of ROS, appearance of and increase in the abundance of membrane crystalline inclusions, membrane mucilage layer formation, opening of intrathylakoidal spaces, and internal plasma membrane disruption. Quantitative AFM analysis revealed that algal cells surface morphology and mechanical properties were modified, as indicated by the increase in cell surface roughness and shifts in cell spring constant upon nTiO$_2$ exposure. Our high-resolution sequential TEM image analysis seems to suggest possible disaggregation of nTiO$_2$ aggregates when in close contact with microbial cells, potentially as a result of biomolecules (e.g., DNA) excreted by organisms that may serve as biodispersant. The present study also showed evidence, for the first time, from both TEM and Raman imaging that the internalization of nTiO$_2$ particles through multilayered membranes in algal cells is possible; therefore, it may be transported along the ecological food web and ultimately impact important biogeochemical processes, such as the carbon and nitrogen cycle.
Table 4.1. Summary of impacts of nTiO$_2$ exposure on *Anabaena variabilis* morphology and intracellular structure observed at different nTiO$_2$ dosed concentrations and exposure durations

<table>
<thead>
<tr>
<th>Impact of nTiO$_2$ on <em>Anabaena variabilis</em> morphology and intracellular structure</th>
<th>nTiO$_2$ concentration and exposure time</th>
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<tbody>
<tr>
<td></td>
<td>1 mg/L</td>
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<tr>
<td>S$^a$</td>
<td>Membrane disruption in vegetative and heterocysts cells</td>
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<tr>
<td></td>
<td>Direct contact between nTiO$_2$ and cellular membranes</td>
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<tr>
<td></td>
<td>Increase of membrane mucilage with variable depth</td>
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<tr>
<td>I$^b$</td>
<td>Internal plasma membrane disruption in heterocysts cells</td>
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<td>Opening of intrathylakoidal spaces</td>
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<td>Release of intracellular material (biomolecules, etc)</td>
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<td>Appearing of membrane limited crystalline inclusions</td>
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<td>S/I</td>
<td>Membranes roughness increase, morphological changes</td>
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<td>Modification of cellular mechanical properties</td>
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<td>nTiO$_2$ diffusion through multilayered membrane</td>
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$^a$ Surface level; $^b$ Intracellular level
4.6 References


Anabaena flos-aquae (cyanophyceae) to cadmium. J. Arch. Environ. Contam. Toxicol.
1984, 13, 143–151.
[25] Surosz, W., Palinska, K.A. Effects of heavy-metal stress on cyanobacterium Anabaena flos-
[26] Cherchi, C.; Gu, A.Z. Impact of titanium dioxide nanomaterials on nitrogen fixation rate
and intracellular nitrogen storage in Anabaena variabilis. Environ. Sci. Technol. 2010, 44,
8302–8307.
[28] Rachlin, J.W.; Jensen, T.E.; Warkentine, B. Morphometric analysis of the response of
Anabaena flos-aquae and Anabaena variabilis (Cyanophyceae) to selected concentrations of
[29] Wu, J.T.; Chiang Y.R.; Huang W.Y.; Jane W.N. Cytotoxic effects of free fatty acids on
[31] Gaboriaud, F.; Dufrene, Y.F. Atomic force microscopy of microbial cells: Application to
nanomechanical properties, surface forces and molecular recognition forces. Colloids
Bacterial turgor pressure can be measured by atomic force microscopy. Phys. Rev. E 2000,
62, 1034–1044.
[33] Cerf, A.; Cau, J.C.; Vieu, C.; Dague, E. Nanomechanical properties of dead or alive single-
[34] Reynolds, C.S. Variability in the provision and function of mucilage in phytoplankton:
photosynthetic bacteria and algae by resonance Raman spectroscopy. Proceedings, 7th


Chapter 5

Nano-Titanium Dioxide Exposure Impact Nitrogen Storage and Metabolism in Cyanobacteria

5.1 Abstract

The widely and increasing use of nano-titanium dioxide (nTiO₂) have led to its release in the environment and it will likely impact aquatic eco-relevant biota. In this study we investigated the impact of sub-lethal concentrations of nTiO₂ on the nitrogen (N) metabolism of the primary producer, nitrogen-fixing cyanobacteria *Anabaena* PCC 7120, using transcriptional-level information of biomarker genes involved in global N regulation, N fixation-, N assimilation- and N storage-specific pathways. The results showed that both the circadian patterns of cyanobacterial metabolism and nTiO₂ intrinsic properties distinctively govern the toxicity response during light and dark exposure to nTiO₂. During illuminated conditions, the majority of genes linked to cellular nitrogen status exhibited a clear up-regulation when exposed to doses higher than 6-60 mg L⁻¹ h, whereas overall gene down-regulation was shown at the end of dark cycle, characterized by low cell metabolism and energy (ATP) levels. The nTiO₂ dose-dependent production of intracellular metabolites (aminoacids) involved in both the GS-GOGAT pathway of N assimilation and in the intracellular N storage pathway suggests that pathways involving aminoacids biosynthesis or degradation might be activated or repressed. This, possibly, contributes to the increase of newly synthesized proteins needed to carry out stress response mechanisms induced by nTiO₂ treatment. These findings suggest that the subcellular modifications observed and the affected genetic regulatory mechanisms of cyanobacteria under
environmental perturbations generated by nTiO₂ are likely to modify their intracellular C/N ratios, thus impact, at larger scale, ecological trophic interactions and food web dynamics within complex ecological systems.

5.2 Introduction and Objectives

Emerging water pollutants are responsible for a significant share of risk with respect to environmental and ecological integrity and human health [1]. The advancements in nanotechnology and application of engineered nanomaterials (NMs) have raised public concerns for their potential environmental and health impacts. Nano size titanium dioxide (nTiO₂) is among the most widely used NMs due to its excellent electrical properties and optical performances [2], and the projected production quantities are up to 7,800-38,000 tons/year [3, 4]. The increased manufacture will inherently result in increased environmental exposure [5]. Surface waters that receive WWTP effluents have shown detectable levels of nTiO₂ in the range of 5-15 μgTi/L [6], which is comparable to the predicted levels of 0.7-16 μg/L based on models of exposure scenarios [3, 4].

In the past years, increasing efforts have been made towards the understanding of the potential toxicity of NMs. Most of these studies have focused on human impact, occupational exposure, and mainly on the cyto- and geno-toxicology of NMs on those routes with higher likelihood of exposure (i.e., inhalation, ingestion, and contact) [7]. Today, the knowledge on the environmental implications of NMs on ecologically-relevant organisms and processes is rapidly growing, however data on aquatic species of ecological importance, such as primary producers (i.e., algae and plants) is lacking.
Perturbations on primary producers can potentially impact nutrient cycling and their essential role for maintaining food chain equilibriums in water ecosystems. Nano-TiO₂ interactions with photosynthetic and nitrogen-fixing species (plant, algae, and phytoplankton) also led to modifications of the organisms’ membrane integrity, mechanical properties, physiology [8-10] and metabolic functions [11-14]. Particularly, in our previous study, the observed dose-dependent accumulation of cyanophycin (a nitrogen-rich storage polymer) in *Anabaena sp.* after treatment with nTiO₂ has raised the question on whether cell nitrogen metabolism was more globally impacted [15].

In filamentous diazotrophic cyanobacteria species, such as *Anabaena sp.*, the fixation of dinitrogen is catalyzed by the enzyme *nitrogenase* within terminally differentiated heterocysts cells, providing nitrogen fixation products to neighboring vegetative cells and in turn receiving fixed carbon produced from their plant-type oxygenic photosynthesis. Hence, the importance of nitrogen/carbon coordination, intracellular nutrient balance and homeostatic acclimation is recognized for maintaining productivity and adapting to perceived stress in changing environments [16]. Cyanobacteria sense their intracellular nitrogen balance through the global PII and NtcA regulators, which relay signals to the gene expression machinery of main pathways associated with nitrogen and carbon metabolism, photosynthesis and stress responses [16-19]. The activity of NtcA, the transcriptional regulator synthetized by *ntcA* gene, is triggered by the accumulation of 2-oxoglutarate (2-OG), a known intermediate of the Krebs cycle which provides the carbon skeleton for nitrogen assimilation, and acts, along with glutamine, as important signaling molecules within the GS-GOGAT pathway [20].

In this study, we hypothesized that long-term exposure to sub-cytotoxic level of nTiO₂ can lead to potential alterations in the molecular activities controlling the nitrogen and carbon
metabolisms in cyanobacteria. The consequent changes in the level of intracellular metabolites perceived by the sensing systems as well as the initiation of a more economic usage of intracellular resources, might impact cells’ perception of extracellular nutrients availability, cellular nutrients uptake and the flux of energy between the organism and the surrounding environments [21]. We examined the regulatory networking linked to the N status in the cyanobacterium *Anabaena* PCC 7120 (hereafter *Anabaena sp.* ) under exposure to environmentally-relevant nTiO$_2$ concentrations. Temporal gene expression response patterns during light and dark cycle of key marker genes encoding global regulatory pathways (*ntcA, glnB*) were investigated. Additionally, we selected known candidate genes associated with N fixation (*nifH, nifK*), N assimilation (*glnA, all2934*) and N storage (*cphA, cphB*) to elucidate the effect of nTiO$_2$ on the global response, on the interplay between nitrogen and carbon metabolism, and on the modulation of N reserve inclusions in cyanobacteria upon nTiO$_2$ exposure. Intracellular nitrogen metabolites (i.e., aminoacids) and the total proteins content have also been monitored along with gene expression trends, to reveal additional toxicity responses influencing nitrogen metabolism and the dynamics of nitrogen accumulation. Because of the important role of nitrogen in cyanobacterial ecology, the molecular mechanisms regulating its metabolism in response to environmental changes (i.e., exposure to pollutants) will progress our understanding on the adaptability of cyanobacteria to changing environmental conditions with their cellular response to emerging environmental stimuli, such as nanomaterials.
5.3 Experimental Methods

5.3.1 NMs Preparation and Characterization

Nano-TiO$_2$ anatase (nTiO$_2$, <25 nm particle size, specific surface area 45-55 m$^2$/g, purity 99.7% on trace metal basis, Sigma-Aldrich) was prepared in a nitrogen-free BG-11 medium and then dispersed via sonication in a High energy Cup-sonicator (Brandson, Danbury, CT), at ~90Watt power for 15 minutes with 30 sec pulse [22]. Tween 20 (1‰) was added to enhance the dispersion for uniform dosing [23]. Particle size after dispersion in BG-11 media was measured via Dynamic Light Scattering analysis using a Malvern Nanosizer ZS90 and counted an average 689.8±102.5 nm. The polydispersity index after dispersion in culture media was found to be 0.549 and zeta potential reached -23.5±3.95 mV.

5.3.2 Culture Conditions and Ecotoxicological Tests

Anabaena PCC 7120 (UTEX #2576) was cultured in a nitrogen-free BG-11 medium, as described in our previous work [8]. For exposure tests, 1800 mL volumes of an initial chlorophyll $a$ concentration of 1.5 mg/L were incubated under a 12 h light/12 h dark regime to mimic natural environmental conditions. The light source consists of 1:1 ratio of 34 W cool white and 40 W gro-lux fluorescent bulbs (Sylvania, Danver) which yields a low PAR value of 35 $\mu$E m$^{-2}$·s$^{-1}$. The lamp wavelengths output > 400 nm is beyond the ultraviolet region (< 400 nm) known to promote the photocatalytic activity of the anatase crystalline form of nTiO$_2$ [24]. Batches were continuously mixed (300 rpm) and periodically aerated using compressed 0.2 µm filtered air. Cultures were exposed to different nTiO$_2$ concentrations, at 0 mgTiO$_2$/L (control sample with same media matrix), 0.01, 0.1, 1 and 10 mgTiO$_2$/L for a total of 24 hours and, samples for analysis were collected at specific time points during incubation at light- (0.25, 1.5, 3 and 6 hours) and dark-(12, 18, 24 hours) conditions (L-D). Exposure tests were performed in
duplicate batches and the results of one representative experiment is here shown. Analysis of each sample was performed in triplicates.

5.3.3 Chemical Analysis of Biomass and Cellular Components

Chlorophyll $a$ was measured after ethanol extraction by a Synergy HT fluorometer (BioTek, Winooski, VT) with excitation and emission at 440 nm and 670 nm, respectively. Biomass dry weight was also measured according to previous studies [25]. Total proteins were assayed colorimetrically on sonicated cells (75W, 60 s), with a bicinchoninic acid protein assay kit (Pierce Chemical Co. Rockford, IL) and standards prepared with bovine serum albumin (BSA). All measurements were performed in triplicates.

5.3.4 Aminoacids Quantification

Aminoacids content of algal cells were quantified according to the method by Bartolomeo et al. [26]. Briefly, 15 mL of cell cultures were centrifuged, lyophilized before aminoacids extraction with 0.5 mL boiling 6M HCl at 110°C for 24 hours. Extracted samples were centrifuged and hydrolyzed again to obtain dried residues, which were resuspended in 100 µL 0.1 M HCl. After a semi-automated derivatization with o-phthalaldehyde (OPA), samples were injected (5 µL) and analyzed on a Agilent 1260 Infinity Quaternary LC (Agilent Inc., Santa Clara, CA) using an Eclipse AAA (4.6x150mm, 5µm) column. Aminoacids separation was obtained at a flow rate of 2 mL/min with a programmed linear gradient program from 40 mM NaH$_2$PO$_4$ (pH = 7.8, 1.9 min) to acetonitrile/methanol/water (45:45:10 in v/v/v,16.3 min). The following aminoacids (retention times, min) were monitored: arginine (8.4), aspartic acid (1.2), glutamine (6.9), and glutamic acid (3.2). L-Norvaline (13.5) at 0.5 mM was used as internal standard for chromatographic spectra normalization. Calibration curves of the aminoacids of interest were
obtained by diluting the aminoacid standard mixture (Pierce Biotechnology, Rockford, IL) in the concentration range of 10-500 µmol/mL. Measurements were performed in triplicates.

5.3.5 Monitor Gene Expression Changes Using RT-qPCR Procedure

Total RNA was isolated from 100 mL of mid-exponential phase cultures of *Anabaena* PCC 7120 cultures, then precipitated with isopropanol, and washed with ethanol before final resuspension in *RNAs*e free water, according to Simms et al. [27]. The concentration and purity of the extracted RNA was determined spectrophotometrically at OD260 and OD280, using Take3 Micro-Volume Plates (Synergy HT Microplate Reader, Biotek, Winooski, VT). cDNA was synthesized using a Verso™ Reverse Transcriptase (Thermoscientific, Barrington, IL) with random hexamers, according to the manufacturer’s instructions.

RT-qPCR reactions were carried out in a Biorad iQ5 Thermocycler (Biorad, Hercules, CA). The amplification reactions were cycled as follow: 95°C for 3 min, 45 cycles at 94°C for 30 sec, an annealing temperature-primer specific (Table C1, Appendix C) for 30 sec, at 72°C for 30 sec, and final elongation at 72°C for 3 min. The *cphA* primer was design using the Primer-BLAST design tool from NCBI and confirmed by endpoint melting curve (Figure C1, Appendix C). All other primers used in these PCR reactions were obtained from literature and listed in Table C1 of Appendix C. The absence of unspecific products in PCR reactions was confirmed by the end-point melting curve analysis. PCR reactions were run in triplicates.

5.3.6 Data Analysis and Statistics

Data analyses for a relative quantification of gene expression were performed by the comparative CT (threshold cycle) method. Fold changes of gene expression were normalized using the housekeeping *rnpB* gene, the RNA concentration and the genomic 16S rDNA. Clustering
analysis and visualization were performed using the MeV (MultiExperiment Viewer) software version 4.8.1 (http://www.tm4.org/mev). Hierarchical clustering of gene trees and samples trees construction was performed employing Euclidean distance using an average linkage clustering. Statistical significance of data analyzed was assigned using a pairwise, 2-tailed, t-test at p<0.05.

5.4 Results and Discussion

5.4.1 Distinct N Metabolism Transcriptional Profiling Under Light and Dark Cycle

Figure 5.1 illustrates the linked pathways involved in nitrogen fixation, metabolism and storage in vegetative and heterocysts cells of *Anabaena sp.*. Nitrogen fixation is carried out in heterocyst, where the enzyme *nitrogenase* catalyzes the conversion of one mole of dinitrogen into two moles of ammonia. The energy (ATP) and reducing power (NADPH) required for the reaction are, respectively, provided by the PSI-mediated cyclic photophosphorylation and the oxidative pentose phosphate cycle (PCC) via the electron carrier ferredoxin (FdxH). The fixed ammonia is assimilated in the GS-GOGAT cycle, through the reaction with glutamate to form glutamine that is then sent to vegetative cells, where glutamine is transformed into glutamate along with 2-OG and was then recycled back into the heterocyst [28].
Eight genetic biomarkers indicative of the activity of above key N metabolism pathways were monitored under exposure to different concentrations of nTiO₂ for 24 hours (Table C1, Appendix C). Concentration-specific and time-dependent transcriptional patterns and magnitude of expression levels (fold-changes) are shown in Figure 5.2. Distinctive transcriptional effects were observed for dark and light cycles, likely due to the essential role of light in photosynthesis and metabolism [29], its direct interconnection with nitrogen metabolic network [30] and the recognized photo-induced toxicity of nTiO₂ [31]. During light exposure, hierarchical clustering
grouped similar expression profiles of all genes into three major clusters (cutoff distance 2.64) based on treatments concentration and exposure time lengths (Figure 5.2, left). Interestingly, the gene expression profiles reflecting immediate responses at 0.25 hours exposure clustered together disregard the dose concentrations, indicating similar sub-cellular responses at this short exposure time. All treatments with relatively lower doses, defined as CT (concentration x time (dose) < 0.15 mg L⁻¹h), except for one (1 mg/L at 1.5 hr) clustered together, with most of genes showing down-regulations. However, as dose increases to be higher than 6-60 mg L⁻¹h, the same genes exhibited clear and significant up-regulation. The dose-dependent distinctive clusters indicated that the sub-cellular responses are sensitive to both nTiO₂ concentration as well exposure time and, nitrogen metabolic activities respond quite differently depending on the dose.

In contrast, during dark conditions (Figure 5.2, right), the exposure time rather than dose seems to govern the separation, with treatments at the initial (12 hrs), intermediate (18 hrs) and final phases (24 hrs) of dark cycle clustered separately. While minimal expression levels were observed for the biomarker genes at the beginning of the dark stage, up-regulations in most genes were observed after 6 hours into the dark cycle (18 hrs) and they transit into mostly down-regulations at the end of dark cycle (24 hrs). Although the underlying reason for these distinguishable light-dark cycle-dependent nitrogen metabolism patterns are not fully understood, the results demonstrated complicated sub-cellular responses of *Anabaena* in exposure to nTiO₂ associated with not only external stressor but also light, as well as internal circadian metabolism regulation and energy (ATP) levels.
Figure 5.2. Temporal expression profiles (fold change) of genes describing *Anabaena* PCC 7120 nitrogen status in illuminated (left) and dark (right) conditions, after treatment with various nTiO$_2$ concentrations in the range 0.01-10 mg/L. Values represent log ratios. Color-graded log ratios of up-regulated response are highlighted in red, while green represents down-regulation. Numbers in parenthesis on the left refer to the applied dose and are expressed as CT (concentration x time) in mg L$^{-1}$h.

5.4.2 Changes in the Dynamics of Nitrogen Storage Under nTiO$_2$ Exposure

In aquatic environments cyanobacteria are known to implement unique metabolic strategies when “sensing” adverse conditions to their optimal growth. Cells are known to initiate a “forward planning” [32], adjusting the redistribution of internal resources, such as in the synthesis and accumulation of intracellular storage polymers. In our previous work, a dose-dependent accumulation of the nitrogen-rich cyanophycin grana protein (CGPs) in *Anabaena* sp. was observed during 96 hours treatment to nTiO$_2$ [15]. CGP is a copolymer of arginine and
aspartic acid, and it serves as a dynamic reservoir of newly fixed nitrogen before redistribution to vegetative cells, or to overcome periods of nutrient shortage [33]. Synthesis and degradation of CGPs are catalyzed by cyanophycin synthetase (encoded by \textit{cph}A \textit{cph}B) and cyanophycinase (encoded by \textit{cph}B), respectively.

Here, we monitored the transcription level of the CGPs synthesis and degradation, as well as the cellular arginine and aspartic acids concentration during the nTiO\textsubscript{2} exposure (Figure 5.3 and 5.4). At the beginning of exposure, there seems to be an immediate upregulation of CGPs synthesis and relatively no significant changes in CGPs degradation, suggesting the increase of reserve material as one of the first auto-protective reaction of the cells to nTiO\textsubscript{2} toxicity (Figure 5.3). However, with extended exposure, only higher nTiO\textsubscript{2} doses (10 mg/L) seemed to stimulate CGPs synthesis and degradation. After transition to dark growth cycle, the \textit{cph}A gene continued to be mostly up-regulated, while the \textit{cph}B gene was concurrently being for the majority down-regulated. Figure 5.4 shows pronounced changes in intracellular aminoacids pools of arginine and aspartic acid during nTiO\textsubscript{2} treatment compared to the control with no nTiO\textsubscript{2} exposure (p<0.001) for treatments at >0.1 mg/L. Similar to the patterns observed with glutamine and glutamate (Figure 5.6), both arginine and aspartic acid exhibited dose-dependent increase over time and highest levels of aminoacids accumulation occurred at L-D transition time points (12 and 24 hours).
Figure 5.3. Time-dependent levels of transcriptional expression fold changes of genes involved in nitrogen storage (cphA, top and cphB, bottom) of Anabaena PCC 7120 exposed to nTiO2 concentrations ranged from 0.01 to 10 mg/L at different times of light/dark regimens. Standard deviations are of PCR reactions.

The mechanisms regulating cyanophycin accumulation have not been fully elucidated [34], therefore, definite correlations between the rate of CGPs accumulation and cyanophycin synthetase activity are yet difficult to confirm. Nevertheless, the progressive increase of the intracellular metabolites that are essential for CGPs formation induced by nTiO2 treatment still clearly indicates its impact on cellular N storage processes. Arginine is the amino acid with the highest nitrogen content [35], and, catabolized by multiple pathways [36], it serves as a source for protein synthesis and a nitrogen buffer within cyanophycin [34]. Aspartic acid can be utilized
for the synthesis of methionine, lysine, and threonine [37] if needed in response to stress, or combined with 2-OG to produce glutamine, which can then be combined with ammonia to produce glutamate. In light of the high production of metabolites observed under nTiO$_2$ treatment, along with the down-regulation of $nif$, $glnA$ and $all2934$ genes at the end of dark phase, cells might have sensed conditions of nitrogen shortage, therefore activate alternative pathways to provide nitrogen for storage [38] or new protein synthesis [39]. In this regard, the increase in total proteins was observed in this study and found to be, for the most, both time- and nTiO$_2$ concentration-dependent, reaching as high as 50% increase (of the control) in the sample exposed to 10 mgTiO$_2$/L during dark phase, as showed in Figure C2, Appendix C. Thus, the over production of aminoacids might partially contribute to the synthesis of newly stress-induced proteins needed to carry out stress response mechanisms likely induced by nTiO$_2$ treatment, and previously reported in response to salinity, osmotic stresses [40] and exposure to metals [41].
Figure 5.4. Time-dependent increase of arginine (top) and aspartic acid (bottom) levels in respect of control in *Anabaena* PCC 7120 exposed to nTiO$_2$ concentrations ranged from 0.01 to 10 mg/L at different times of light/dark regimens.

5.4.3 Impact of nTiO$_2$ on the GS-GOGAT Nitrogen Assimilation

Vegetative and heterocystous cells of nitrogen-fixing cyanobacteria share the GS-GOGAT metabolic pathway (Figure 5.1) to achieve ammonia transfer and assimilation into cellular carbon skeleton [42]. *Glutamine synthetase (glnA)* and *glutaminase (all2934)* play essential role in this pathway. Figure 5.5 shows dose-dependent, yet distinctive patterns in gene expression changes for the light and dark growth cycle, respectively. Pairwise t-test on distinct nTiO$_2$
treatments during the entire 24 hours L-D period showed high similarities between the expression of both transcripts (p>0.27), which is likely related to their sequential interrelationship and mutual dependence within the GS-GOGAT pathway.

In light exposure, high nTiO2 concentrations induced increased upregulated levels of $glnA$ and $all2934$ (up to 26 and 27-fold changes at 6 hours to 10 mg/L, respectively). Upregulation of the $glnA$ transcript is modulated at both transcriptional and posttranscriptional level [43], in dependence on both carbon and nitrogen supply and their ratio. Particularly, conditions of nitrogen shortage warrant higher production of the nitrogen-starvation-signaling molecule 2-OG, in a positive feed-back mechanisms [44], thus promoting the expression of NtcA and PII-regulated genes. Therefore, the activation of gene activities involved in nitrogen fixation (i.e. $nifH$), presented in the following section, and nitrogen assimilation (i.e., $glnA$) are likely explained with the cell need to compensate for lower levels of intracellular NH$_3$ sensed, and likely associated with the decrease in nitrogen fixation activity observed in our previous study [15]. This is essential in Anabaena sp. in order to maintain carbon to nitrogen balance within the filament, therefore, its homeostatic state.

During light switches (12 and 24 hours), the exposure mostly causes genes underexpression, although at lower levels with nTiO$_2$ increase, indicating the tendency of high nTiO$_2$ concentrations to stimulate genes overexpression even in down-regulated conditions.
Figure 5.5. Concentration-dependent levels of expression of all2934 and glnA genes of *Anabaena* PCC 7120 exposed to nTiO$_2$ concentrations ranged from 0.01 to 10 mg/L at different times of light (left)-dark (right) cycle. Standard deviations are of PCR reactions.

The upregulation of *glnA* and *all2934* genes lead to the overall increase of key metabolites (i.e., glutamine and glutamate) in the GS-GOGAT reactions pathway (Figure 5.6). In overall, with few exceptions at 0.01 mg/L exposure, nTiO$_2$ led to increased levels of glutamate than those observed in the control in a dose-dependent manner during light cycle. The intracellular content of glutamine, in the first 3 hours of exposure, was nearly similar to those in the control (p>0.05), however significantly increase (p<0.02) was observed after longer exposure. The overproduction of intracellular metabolites of glutamine metabolism observed at
24 hours exposure might have potentially caused the downregulation of \( glnA \) and \( all2934 \) genes through mechanisms of cumulative feedback inhibition [45], in order to maintain cells homeostatic equilibrium in conditions of external perturbations [42].

Glutamine is univocally produced by the GS-catalyzed addition of ammonia to glutamate, while different pathways exists for glutamate formation, including the reaction GOGAT-catalyzed from glutamine, the catalysis of glutamate dehydrogenase from ammonia and 2-OG, the loss of carbon and nitrogen atoms from the degradation of other aminoacids, and the transfer of an amino group to 2-OG via transamination. Additionally, of the two molecules of glutamate produced during GS-GOGAT (Figure 5.1), only one is re-utilized for glutamine production, while the other is expended in vegetative cells for net amino acid synthesis. For example, it was previously reported that in conditions of abiotic stress, such the exposure to metals, plants and algae are known to over-synthetize a number of diverse metabolites, such as proline, which primarily derives from glutamate [46]. Thus, the involvement of glutamate in other pathways might justify the different trends and levels of glutamine and glutamate observed, although both sequential key players of equal importance in the nitrogen assimilation pathway.

The increased level of GS-GOGAT aminoacids, players in the assimilation of ammonia into carbon skeleton, clearly demonstrated that alterations in C/N equilibriums take place in samples treated with nTiO\(_2\) if compared to controls, thus with possible impairment of intracellular nutrients balance and homeostatic stability.
5.4.4 Responses of Global Nitrogen Metabolism Regulators to nTiO$_2$

The regulation of intracellular carbon and nitrogen balance is crucial for cyanobacteria to meet their metabolic requirements under regular growth conditions and to enable survival during environmental perturbations [37]. The global regulator, NtcA protein, integrates signals from carbon and nitrogen metabolisms and, thus mediates the nitrogen status of the cell with the control of a large number of targets genes [44]. The binding of NtcA protein to its target

**Figure 5.6.** Time-dependent increase of glutamate (top) and glutamine (bottom) levels in respect of control in *Anabaena* PCC 7120 exposed to nTiO$_2$ concentrations ranged from 0.01 to 10 mg/L at different times of light/dark regimens.

The regulation of intracellular carbon and nitrogen balance is crucial for cyanobacteria to meet their metabolic requirements under regular growth conditions and to enable survival during environmental perturbations [37]. The global regulator, NtcA protein, integrates signals from carbon and nitrogen metabolisms and, thus mediates the nitrogen status of the cell with the control of a large number of targets genes [44]. The binding of NtcA protein to its target...
promoters is triggered by the intracellular levels of 2-OG, the carbon skeleton involved in the assimilation of nitrogen. The signal transducer PII protein (encoded by the \textit{glnB} gene) also coordinates C/N ratios binding with 2-OG and via phosphorylation. The protein NtcA and the PII regulator are functionally interdependent, since they are mechanistically linked by the transcriptional coactivator PipX protein [47], the \textit{glnB} gene is transcriptionally activated by NtcA and the PII protein is required for the activation of NtcA-regulated genes [48].

Figure 5.7 shows dose-dependent altered gene expression patterns of these two global nitrogen regulators compared to control with no nTiO$_2$ exposure. At higher dose of 10 mg/L, significant transcriptional changes as high as 20-fold (\textit{ntcA}) and 7-fold (\textit{glnB}) was exhibited at 6 hours exposure in light cycle, and 5.5- and 5-fold changes at 18 hours during dark. These results clearly demonstrated the impact of nTiO$_2$ exposure on subcellular nitrogen regulations. Recently, the knowledge of the role of NtcA was broadened to the regulation of genes involved in carbon metabolism, photosynthesis, and stress responses [49]. This indicates that other factors, rather than only nitrogen perturbations, can affect \textit{ntcA} and \textit{glnB} expression.

Previous literature has also demonstrated the importance of maintaining reducing internal conditions and an optimal redox status to favorite the DNA-binding capacity of NtcA to target promoters in \textit{Anabaena} sp. [50]. Thus, the expression patterns observed during dark and light cycles are possibly affected by the unbalanced oxidative/reducing conditions caused by intracellular reactive oxygen species formation upon nTiO$_2$ treatment (reported in our previous work) [8], as well as the potential changes in the levels of reducing power linked to the alteration of cell photosynthetic, nitrogen fixation potential [15] and electron transport [51].

Note that the changes in gene regulators do not fully reflect the changes at translational and post-translational level. Nevertheless, our results evidenced the alteration in the
transcriptional response of the global nitrogen regulator genes (*ntcA* and *glnB*) by the exposure to sub-lethal environmentally-relevant concentrations of nTiO$_2$.

**Figure 5.7.** Concentration-dependent alteration in the fold change expression (relative to control) of *ntcA* and *glnB* genes of *Anabaena* PCC 7120, after exposure to nTiO$_2$ concentrations ranged from 0.01 to 10 mg/L at different time points during the 24 hours light/dark cycle.

5.4.5 Impact of nTiO$_2$ on Nitrogen Fixation-related Transcriptional Activity

The two protein-system complex of *nitrogenase* catalyzes the fixation of dinitrogen through the activity of approximately 20 different *nif* genes [52]. Among all, the *nifH* gene encodes for
*dinitrogenase reductase*, which is the obligate electron donor to *dinitrogenase* during the *nitrogenase* turnover, whereas *nifK* encodes for the β subunit of *dinitrogenase*.

Exposure to nTiO₂ induced changes on the *nif* genes expression during both light and dark cycle (Figure 5.8). Alterations in the gene expression levels of both *nifH* and *nifK* genes were observed and they seemed to exhibit some dose-dependence, particularly in the light cycle, but surely dose-specific trends. Nano-TiO₂ at higher doses (10 mg/L) clearly led to significant response of *nif* genes, indicating that the nitrogen fixation pathway likely was impacted by nTiO₂ exposure, which in turn may affect the C/N balance, as previously mentioned. During dark, the impact on *nif* gene activities appears not to be dose-dependent, but dose-specific, with major up-regulation during the middle of dark cycle (18 hrs). Figure 5.2 also evidenced that in both transcripts the majority of genes down-regulation is limited to 1.5, 12 and 24 hours exposure after nanomaterials dosing, which correspond to conditions close to L-D and D-L switches. Differences in N fixation activities between light and dark cycles are expected since the expression and activity of *nitrogenase* require a high portion of the cellular energy pool [53] and are tightly controlled by reducing power (NADPH), ATP supply that are light-dependent. Lower nitrogen fixation rates are known to occur during dark conditions when Photosystem I-mediated photophosphorylation is lacking, and ATP is only provided by the oxidative phosphorylation.

Our previous study showed a decrease in *nitrogenase* activity and nitrogen fixation inhibition in *Anabaena variabilis* at comparable and higher dose of nTiO₂ as in this study. The definite link between *nif* genes, *nitrogenase* and nitrogen fixation is hard to draw because control and regulation of nitrogen fixation in cyanobacteria occurs at transcriptional, post-transcriptional and translational level, as previously reported [54]. Additionally, after synthesis the *nitrogenase*
components are not immediately competent for nitrogen fixation, and they acquire catalytic competency afterwards by the actions of a number of nif and non-nif gene products [55].

The metabolic liaison of the obligate electrons donor role of the Fe protein dinitrogenase reductase (nifH) with the MoFe protein component of dinitrogenase (nifK), was not evident for nTiO₂ concentrations in the range of 10-1000 µg/L (Pearson coefficients <0.52), showing that low nTiO₂ doses have the potential to hamper the relative nifH and nifK transcription, thus the structural stability of nitrogenase subunits. Additionally, if nTiO₂ affects the efficiency of their translation, the decoupled accumulations of these two genes may represent the cell potential to re-balance the relative levels of their transcript and re-optimize their functional linkage. For example, different levels of nifH and nifK mRNA were accumulated in A. vinelandii reaching steady-state nitrogen fixing conditions, to maintain high Fe protein-to-MoFe protein ratios required for an optimal nitrogenase catalytic activity [56]. On the other hand, high correlation (Pearson coefficient >0.75), between the two genes in their temporal L-D responses at higher doses (10 mg/L) was observed when the toxicity effect dominates suggesting their concomitant up- and down-regulation to toxicity.
Figure 5.8. Time-dependent levels of transcriptional expression fold changes of genes involved in nitrogen fixation (*nifH* and *nifK*) of *Anabaena* PCC 7120 exposed to nTiO$_2$ concentrations ranged from 0.01 to 10 mg/L at different times of light/dark cycle. Standard deviations are of PCR reactions.

5.5 Conclusions

Thus far, the report of molecular and genetic response to NMs exposure of ecological relevant organisms and, particularly of photoautotrophic species, has been scarce. The present work evidenced nTiO$_2$ potential to perturb multiple cellular pathways involved in the nitrogen status of nitrogen-fixing organisms and to alter their complex gene regulation systems. Both the circadian and fluctuating patterns of cyanobacterial metabolism and nTiO$_2$ intrinsic properties distinctively govern the toxicity response during light and dark exposure to nTiO$_2$. 
From our results, cell response to nTiO\textsubscript{2}-induced stress requires increased levels of proteins, also reflected in the aminoacids increase observed. Additionally, in consequence of a potential decreased nitrogen fixation activity, cell might have sensed lower intracellular levels of ammonia, thus conditions of limited availability of nitrogen. Immediately, cell activates *nitrogenase* to provide more nitrogen for assimilation, with consequent activation of genes involved in the GS-GOGAT, and the upregulation is mostly enhanced by increased nTiO\textsubscript{2} dose concentrations. In parallel to the immediate requirement for proteins, the cell seems to prepare for upcoming periods of nutrient shortage and stress, with accumulating nitrogen in form of storage products, particularly at night, when the lower metabolic requirements for proteins and nTiO\textsubscript{2} toxicity facilitate nitrogen diversion into cyanophycin grana proteins. These specific responses are clearly modulated by the expression of both global nitrogen regulators, implying that the signaling network linked not only to cell nitrogen status, but also more broadly, to carbon and nitrogen balance and metabolisms are affected under nTiO\textsubscript{2} exposure.

Ecosystems functions are controlled by flows of matter and energy, whose equilibrium is prone to be altered by anthropogenic materials and pollutants released in the environment. The findings of this study warn the potential of nanomaterials to affect ecological nutrients balances and metabolic capabilities in organisms, such as cyanobacteria, which significantly contribute to the biologically-available nitrogen on earth. This will broadly lead to adverse impacts on ecosystems stoichiometry, fitness and organisms’ growth rates and indirectly affect biological productivity and the dynamics of communities in food webs.

5.6 References


Chapter 6
Nano-Titanium Dioxide Exposure Induced Intracellular Biochemical Composition Changes in Cyanobacteria

6.1 Abstract
This study investigated the short- (96 hours) and long-term (21 days) ecotoxic impact of nano-titanium dioxide (nTiO₂) exposure on the cellular biochemical pools in the nitrogen-fixing cyanobacteria *Anabaena variabilis*. The analysis of Fourier Transform Infrared (FT-IR) spectral signatures revealed dynamic temporal and dose-dependent change patterns of major macromolecules, including protein, lipids, nucleic acids and carbohydrates, in *A. variabilis* upon nTiO₂ exposure at concentrations ranging from 1 to 1000 µg/L. 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 hours of exposure and, both the magnitude of changes and levels of recovery seemed to be nTiO₂ dose-dependent. *A. variabilis* cells self-recovered back to a more conservative steady state over a longer period of 21 days, however, the relative intracellular macromolecule composition ratios and cell stoichiometry (C:N, C:P and N:P) seemed to differ from that in the control and influenced by the dose concentrations applied. Principal component analysis on over 6000 FT-IR spectra under various treatment conditions showed clusters of data associated with different dosed nTiO₂ concentrations and exposure time and the main biochemical contributors to the data variation. This study, for the first time, provides insights into the intracellular metabolic strategies implemented by cyanobacteria under environmental perturbations generated by nTiO₂ exposure. The results of the intracellular composition changes induced by long-term exposure to nTiO₂ may have important ecological
implications in terms of the influence on nutrients uptake and storage, since the alteration of specie stoichiometry has effects on the ecological nutrient distribution, trophic interactions and communities’ structures.

6.2 Introduction and Objectives

The rapid progress of nanotechnology poses an urgent need for fundamental understanding of the potential environmental impacts of engineered nanomaterials (NMs). Nanosize titanium dioxide (nTiO$_2$) has shown great potential for a wide range of applications because of its excellent electrical properties and optical performances [1]. Its presence in wastewater effluents has been anticipated [2], recently evidenced [3] and it will likely impact aquatic biota and eco-relevant organisms. In particular, the toxic effect of nTiO$_2$ on algal species and on ecosystems is still largely unexplored.

Previous literature have reported geno- and cyto-toxic effect of nTiO$_2$ on human cells lines and bacteria [4-6], indicating that DNA damage [7], membrane disruption [8] and protein oxidation via reactive oxygen species formation [9] are the major mechanisms of toxicity involved. A limited number of studies investigated nTiO$_2$ impacts on algal ecosystems, showing deleterious effects on algal growth, photosynthetic and nitrogen-fixing activity [10-14]. It was revealed that cell topology, mechanical properties and intracellular structures of cyanobacteria were compromised after exposure to nTiO$_2$ [15]. Particularly, the dose-dependent accumulation of the nitrogen storage polymer (cyanophycin grana proteins, CGPs) in the cyanobacteria _A. variabilis_ in response to nTiO$_2$ treatments was demonstrated, indicating that the normal patterns of nitrogen metabolism may be potentially modified by the exposure to nTiO$_2$ [16].
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 [17, 18]. 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 [17, 18] and the decrease in energy storage products (i.e., carbohydrates) in the brown algae *Padina tetrastromatica* under stress induced by cadmium [19]. Alterations in the relative presence of carboxyl, 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 cells growth, fitness, interspecies relationship, trophic interactions and food web dynamics [20]. We hypothesize that the exposure to nTiO₂ alters the intracellular assemblage of essential macromolecular components linked to specific cell functions. Therefore, in this study, we evaluated the impact of nTiO₂ exposure at sub-lethal environmentally relevant concentrations on the cellular allocation of macromolecules (nucleic acids, proteins, lipids, carbohydrates) in the cyanobacteria *A. variabilis*. Cyanobacteria, are of significant biogeochemical importance due to their contribution to primary productivity and their ability to tolerate adverse and fluctuating environmental stresses by implementing unique metabolic strategies [21]. 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 hours) or long-term (21 days) exposure to different doses of nTiO₂. In addition, a quantitative elemental analysis was performed to demonstrate nTiO₂-induced deviations of intracellular nutrients stoichiometry from those of cells in control untreated conditions.
The results provided important insights into the impact of nTiO₂ exposure on intracellular biochemical pools and, revealed metabolic changes and strategies implemented by cyanobacteria under environmental perturbations generated by NMs exposure.

6.3 Experimental Methods

6.3.1 NMs 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 [22] and then dispersed via sonication in a High energy Cup-sonicator (Fisher scientific, Inc.), at approximately 90Watt power for 20 minutes. Bovine Serum Albumin (1% BSA) was added to enhance the dispersion for uniform dosing. Physical and chemical characterization of nTiO₂ was detailed in previous studies [11, 23] and is also shown in Table D1, Appendix D.

6.3.2 Culture Conditions and Ecotoxicological Tests
The Anabaena variabilis strain (UTEX #1444) was cultured in a modified Mes-Volvox media, with conditions described in our previous work [16]. For exposure tests, 500 mL volumes of initial chlorophyll a concentration of 200 µg/L were subjected to different nTiO₂ concentrations, ranging from 0 mgTiO₂/L (control sample) to 1 mgTiO₂/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, Danver) of wavelengths output > 400 nm, which yields a low PAR value of 35 µE/m²·s. Batches were continuously mixed (300 rpm) and periodically aerated using compressed 0.2 µm filtered air. Chlorophyll a measurements of growth showed exponential growth over duration of the experiment (21 days) for all treated and untreated cultures (data not shown). Batch tests were performed in duplicates.
and all measurements were conducted in triplicates. Growth inhibition tests based on chlorophyll $a$ measurements were performed according to standard protocols [24]. Results from our previous investigation (Table D2, Appendix D) showed the chlorophyll $a$ had better correlation with cell count than protein measurements and therefore is employed as a surrogate parameter to assess $A. variabilis$’ growth.

6.3.3 Non-spectroscopic analysis of cells components

During the 21-day exposure assay, samples were periodically taken and analyzed to determine the chlorophyll $a$, protein content of the cultures. Chlorophyll $a$ was measured by fluorescence after ethanol extraction by a Synergy HT fluorometer (BioTek, Winooski, VT) with excitation and emission at 440 nm and 670 nm, respectively. Total proteins were assayed colorimetrically on sonicated cells (75W, 60 s), with a bicinchoninic acid protein assay kit (Pierce Chemical Co. Rockford, IL) and standards prepared with bovine serum albumin (BSA). For all cell components above, each sample was analyzed in triplicates.

6.3.4 FT-IR spectroscopy analysis of cells 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 MirrIR low-e reflectance microscopic slides (Kevley Technologies, Chesterland, USA), and desiccated under vacuum before analysis. Spectra were collected using a Perkin Elmer Spectrum One Fourier Transform Infrared (FT-IR) spectrometer bench coupled to a Spotlight 300/400 IR microscope, fitted with a liquid $\text{N}_2$ cooled mercury-cadmium-tellurium detector (PerkinElmer Inc., Shelton, CT, USA). Absorbance spectra were collected in point-scan mode at a spectral resolution of 4 cm$^{-1}$ with 20-64 scans co-added and averaged on 25 µm$^2$ randomly selected areas of deposed cells. At least 60 to 80 spectra [25, 26]
in the wavenumber region between 750 and 4000 cm\(^{-1}\) were recorded for each sample, yielding a total of >6000 spectra analyzed. Band assignment is based on previous studies [18, 27, 28] and is summarized in Table D3, Appendix D.

### 6.3.5 Principal Component Analysis

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.

### 6.3.6 Elemental analysis of intracellular macronutrients

An independent experiment was performed to determine the intracellular elemental nutrients in *A. variabilis*’ cells exposed to nTiO\(_2\) concentrations of 1, 10, 100 µg/L and 1, 10 mg/L for 96 hours and 21 days exposure. Exposure condition was similar to those ecotoxicity assay described previously and all tests were run in triplicate batches. Elemental carbon and nitrogen analysis was performed on pre-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 presence of ultrapure oxygen and CO\(_2\), H\(_2\)O, N\(_2\) were
detected with thermal conductivity. Final concentrations of C, H, and N in the samples were expressed as percentage of the elements per unit mass of the freeze-dried samples. Intracellular levels of phosphorus were determined on pre-washed and oxalate-treated cells [29], following acid hydrolysis by the persulfate digestion method and measured via spectrophotometric analysis (Shimadzu, Columbia, MD), according to previous methods [30]. Elemental analysis of P was performed with triplicate samples. Pairwise Student t-test was used to assess statistical significance of the obtained results.

6.4 Results and Discussion

6.4.1 Short- and long-term changes of macromolecules in A. variabilis with nTiO₂ exposure

Exposure to nTiO₂ at environmentally relevant low concentrations (1-1000 µg/L) [2, 3] did not lead to any observable growth inhibition (based on chlorophyll α) with short-term exposure (96 hours) (data not shown); however, growth inhibition of 0.4±0.2% to 6.5±0.7% was detected with long-term exposure (21 days) (Figure D1, Appendix D). This is consistent with our previous report of the both dose and exposure time-dependent (CⁿTᵐ⁻) toxicity of nTiO₂ [16]. Growth profiles showed exponential trends in all cultures tested and stationary conditions were not reached during the 21 days assay.

Monitoring of the cell protein content showed interesting temporal patterns that seemed to be dose-dependent (Figure 6.1). A significant increase in the protein concentration was observed at 3 hours exposure at the highest dose concentration of 1000 µg/L, exhibiting a 8-fold higher proteins level (52.4±1.6 mg/L) than both control (6.4±0.1 mg/L) and other treatments with lower doses (5.9-8.7 mg/L). The induced high cellular protein level then decreased in the
following 4 days followed by an increase again in the long-term period, similarly to all the other treatments including control.

**Figure 6.1.** Total protein concentrations measured in *A. variabilis* cells after exposure to different nTiO$_2$ concentrations ranging from 0 to 1000 µg/L, during short term (96 hours) and long term (21 days) exposure.

The alterations in cellular proteins are also reflected by the changes in the protein/chlorophyll $a$ ratios over time (Figure D2, Appendix D). The ratio for the control remained relatively consistent during the entire test period of 21 days (average of 80±10, similar to previous reported values (10-80) for *Anabaena cylindrica* and *Anabaena doliolum*) [31, 32], however statistically significant ($p<0.06$) increase of protein to chlorophyll $a$ ratio were observed for dose concentrations higher than 100 µgTiO$_2$/L (Table D4, Appendix D). Changes in the ratio have been suggested to be linked to perturbations in the relative content of the two molecular
classes, especially related to protein synthesis and structures caused by abiotic stresses, such as iron starvation [33] and exposure to salinity [32].

6.4.2 Dose-dependent changes in cellular structure in A. variabilis upon nTiO$_2$ exposure

FT-IR allowed monitoring of the acute and chronic physiological state of A. variabilis cells and the results revealed structural changes and reallocation of intracellular pools in response to nTiO$_2$ exposure at various concentrations. Figure 6.2 shows the temporal changes in the FT-IR spectra of the culture exposed to 1000 μgTiO$_2$/L compared to the control with no NMs exposure. Two protein signatures, namely amide I and 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 (i.e., glycogen) exhibit a series of absorption bands due to C–O stretching and C–O–C deformations at 1150 and 1032 cm$^{-1}$, respectively. Nucleic acids and phosphorylated molecules have functional groups with absorption bands in the same region of the carbohydrates spectrum, with major asymmetric and symmetric vibrations associated at 1078 cm$^{-1}$ and 1240 cm$^{-1}$, representing the asymmetric PO$_2^-$ stretch of DNA/RNA backbones, phosphorylated proteins and polyphosphate storage products [27]. All spectra were normalized to the strongest amide I band and the ratios indicated certain carbon balance within the cell as suggested by Sigee et al. [34].
Figure 6.2. Time-dependent matrix plots of FT-IR spectra collected during *A. variabilis* growth in the control (a) and culture exposed to 1000 ugTiO$_2$/L (b). Spectra were baseline corrected and normalized to the amide I band.
6.4.3 Temporal and dose-dependent classification of cellular response to nTiO₂

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 function of exposure time. The scores plots (Figure 6.3) project the spectral data on two principal components (PC1 vs PC2) and help visualizing the degree of separation among the conditions tested. Loading plots (Figure 6.4) help revealing the specific regions of the spectrum (frequencies) that contribute to the variation within the set of data.

The scores plots of the PCA performed on both 96 hours and 21 days 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 hours), samples treated with 1, 10 and 100 µgTiO₂/L show some degree of separation from the control along PC1, with the 100 µgTiO₂/L class diverging towards the positive PC2, whereas the variation caused by the highest exposure concentration (1000 µgTiO₂/L) only extends along the PC2. The loading plots (Figure 6.4) showed that the separation observed for cells exposed to 1 and 10 µgTiO₂/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 (νₘₚ P=O). In addition to the previous, differences in intracellular carbohydrates components (ν C-O-C, ν C-O) contribute to the shift in the 100 µgTiO₂/L class. A positive PC1 and PC2 score for the 1000 µgTiO₂/L class suggests 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-C), nucleic acids (ν P=O) and amide (II) groups associated to proteins (δ N-H, ν C-N).
In the long term exposure scenario (21 days), the classification obtained was certainly different from that observed after only 96 hours. Interestingly the sample exposed to the lowest concentration (1 µgTiO₂/L) seems to be the farthest from the control, possibly due to changes in proteins 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 nucleic acids (ν P=O) and polysaccharides (ν C-O-C, ν C-O), and membranes characteristic groups (ν CH₃, ν CH₂, vas CH₂ and vas CH₃ of lipids).

**Figure 6.3.** Scores 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 hours (a) and 21 days (b). Each point represents the mean PC score for each treatment, and error bars are the standard error of the mean along the principal component axis.
From the results of the principal component analysis, it is evident the ability of nTiO$_2$ to target *A. variabilis*’ cellular components and induce a set of molecular modifications in a dose-dependent manner. Temporally dynamic and nTiO$_2$ concentration-dependent changes in the major cellular macromolecules, as indicated by the FT-IR fingerprints, were observed and they are further discussed in the following sections.

**Figure 6.4.** Loading plots on PC1 vs PC2 from principal component analysis performed over the region (3000–2700; 1800-750 cm$^{-1}$) of FT-IR spectra obtained from samples exposed to nTiO$_2$ concentrations ranging from 1 to 1000 µg/L for 96 hours (a) and 21 days (b).

6.4.4 Changes in carbohydrates during nTiO$_2$ exposure

Dose-dependent temporal trends of carbohydrates/amide I ratios (Figure 6.5) showed clear differences ($p<0.06$) in the ratio value between those treated with nTiO$_2$ dose higher than 10 µgTiO$_2$/L and untreated controls. The reduction in the carbohydrate/amide I ratio was most pronounced (>32 %) with the highest nTiO$_2$ dose at 1000 µg/L during the first 96 hours 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).

![Graph showing dose-dependent changes in Carbohydrates/Amide I ratio for different nTiO2 concentrations.](image)

**Figure 6.5.** Dose-dependent changes in Carbohydrates/Amide I ratio determined from 1032 cm$^{-1}$ and 1650 cm$^{-1}$ FT-IR vibrations intensities for *A. variabilis* cells after exposure to different nTiO$_2$ concentrations ranging from 0 to 1000 µg/L, during short term (96 hours) and long term (21 days) exposure.

Except for a few exclusions, the average carbohydrate/amide I ratio found in this study are in the range of those reported in Dean et al. (0.25 to 0.82) [35]. The carbon/protein ratios are sensitive indicators of algal chemical composition and rates of physiological processes, and provides insights into the adaptive response in the allocation of cell resources after exposure to pollutants or to generic conditions of stress [36]. Dynamic reallocations of intracellular carbon into polysaccharides during regular growth of cyanobacteria [37] and fluctuations after cell transfer to fresh media [26] has been observed before. These results showed consistently lower carbohydrates/amide I ratios in those treated with nTiO$_2$ (10-1000 µgTiO$_2$/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 were subjected to environmental perturbation (i.e., P limitation) and explained as a cell physiological need to survive stress [34].

6.4.5 Changes in nucleic acids during nTiO₂ exposure

Two IR vibrations at 1078 cm⁻¹ and 1240 cm⁻¹ wavenumbers were assigned to nucleic acids and monitored during exposure. The characteristic functional group (v P=O) related to nucleic acids at 1078 cm⁻¹, and amide I-normalized, showed dose-dependent changes upon nTiO₂ exposure (Figure 6.6a). Throughout the testing period, the average internal ratio of the symmetric phosphodiester stretching (1078 cm⁻¹) calculated at different time points were consistently lower in the cultures exposed to nTiO₂ at >10 µg/L than the control. There seemed to be a dose-dependent pattern with a progressively decreasing ratio as dose concentration increased. 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 the majority of time points analyzed (data not shown), suggesting that nTiO₂ mode of actions may only target specific functional groups of the same macromolecule in a different manner and extents, irrespective of the applied dose.
Figure 6.6. Dose-dependent changes in average nucleic acids (1078 cm\(^{-1}\))/Amide I ratio (a) and average nucleic acids ratios (1078 cm\(^{-1}\)/1240 cm\(^{-1}\)) (b) determined from FT-IR vibrations intensities for \textit{A. variabilis} cells after exposure to different nTiO\(_2\) concentrations ranging from 0 to 1000 µg/L, during short term (96 hours) and long term (21 days) exposure (a).
Based on previous literature [19, 38-40], the variations of the symmetric and asymmetric P=O vibration modes and the fluctuation in their ratios (1240 cm\(^{-1}\)/1078 cm\(^{-1}\), Figure 6.6b), may reveal important insights into the potential mechanisms of nTiO\(_2\) action. 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 [38]. Thus a similar effect is expected by chemicals, such as nTiO\(_2\), that have the potential to generate reactive oxygen species and induce oxidative damage in cyanobacteria [22]. In addition, shifts or intensities fluctuations of these stretching are often linked to the recognized binding potential of the phosphodiester bond with pollutants [19] and indeed, the binding potential of nTiO\(_2\) with oxygen or phosphorous atoms of nucleotides, and nitrogen atoms of base pairs in DNA previously reported [41] may result in an altered secondary structure of DNA molecules. Alterations in P=O modes are also believed to reflect changes in RNA cellular content [42], thus influence important mechanisms of proteins synthesis sustaining organisms’ reproduction and growth [43]. In addition, impairment of RNA allocation and cellular P content [41, 44] will impact more broadly the biogenesis of ribosomes, significant repositories of P in ecosystems and intracellular elements proportions, particularly associated to P-rich biomolecules. In the long-term, this will have the potential to affect organisms’ growth rate and fitness [20], thus also biological productivity.

### 6.4.6 Changes in protein structure during nTiO\(_2\) exposure

FT-IR allowed a finer-resolution examination of the potential protein pool structure and compositional changes. Figure 6.7 shows the temporal changes in Amide II/Amide I ratio in all treatments, during both short and long term exposure. The highest nTiO\(_2\) concentrations applied (1000 \(\mu\)gTiO\(_2\)/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 average of 10.6% increase during the other exposure times
analyzed). Although the changes generated by the lower nTiO₂ concentrations were not as significant ($p>0.51$), instances of 17% and 14% increase in the amide II to amide I ratio were observed in treatments at 1 and 10 µgTiO₂/L at day 21, suggesting that with extended exposure time, even very low nTiO₂ concentrations may lead to observable sub-cellular changes.

**Figure 6.7.** Dose-dependent changes in Amide II/Amide I ratio determined from 1540 and 1650 cm⁻¹ FT-IR vibrations intensities for *A. variabilis* cells after exposure to different nTiO₂ concentrations ranging from 0 to 1000 µg/L, during short term (96 hours) and long term (21 days) exposure.

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 proteins denaturation and in membrane proteins secondary structure, are likely induced by nTiO₂ treatment during the initial exposure [45]. Modifications of protein backbones conformation, such as secondary structures profiles and $\alpha$- helix to $\beta$-sheet ratios may have important
implications in cyanobacteria metabolism and response to stress, as well as protein internal utilization and availability [46].

**6.4.7 Changes in lipids during exposure to nTiO₂**

Similar to the observation for carbohydrates over long-term exposure, although a temporal variations in the lipids/amide I ratio was observed for the first 96 hours, a more conservative average value of the lipid/amide I ratio was obtained after 10 days for both treated and untreated cultures (data not plotted). The final average ratio after 10 days seemed to decrease with increasing nTiO₂ doses, from 0.63±0.01 in the control, 0.56±0.04 and 0.42±0.04 in the 1 and 10 µg/L exposure (p< 0.04), to 0.41±0.02 and 0.44±0.05 (p< 0.003) for the treatments of 100 and 1000 µgTiO₂/L exposure, 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. [26]. These results suggest that nTiO₂ promotes changes in carbon allocation, decreasing both carbohydrates and lipids ratios. Alterations in the relative abundance of lipids caused by nTiO₂ exposure might also have contributed to *A. variabilis* structural changes [22]. Changes in the asymmetrical –CH₂ vibrational frequencies caused by NMs 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 [47].
6.4.8 Nano-TiO₂ impact on intracellular element stoichiometry

To further confirm the nTiO₂-induced cellular changes in composition and macromolecule pools, we also evaluated the intracellular nutrient elements balances and stoichiometry of *A. variabilis* upon nTiO₂ exposure at 96 hours and 21 days. Elemental quantitative analysis (Table 6.1) shows differences in intracellular nutrient stoichiometry (C:N, C:P, N:P) between treated and untreated cells, and also with different culture age.

In the control, the 5.6% decrease in the C:N ratio was recorded at 96 hours, while the same ratio increased by 4.5% at 21 days, in respect to the stoichiometry characterizing the beginning of the experiment. However, C:P and N:P ratios at 96 hours and 21 days were higher than those at the initial time. This biochemical variability at various phases of growth has been previously reported on various aquatic species, including green algae [34, 48], and likely linked to changes in the extent of carbon and nitrogen fixation of cells when approaching late exponential growth conditions [49].

Differences in intracellular C:N:P stoichiometry were observed in nTiO₂ treated cells from those in the control. Trends of elemental ratios are often found to be dose-specific, indicating that different doses can trigger different biochemical responses and cells behaviors at various exposure time lengths. Potential interferences of nTiO₂ on elemental analysis were excluded, since at the beginning of the test C:N, C:P and N:P ratios of the control were comparable to those at 10 mgTiO₂/L.

In the short-term exposure (96 hours), nTiO₂ impaired cellular macronutrients stoichiometry significantly at concentrations higher than 100 µgTiO₂/L, with increasing C:P and N:P ratios, and varying C:N differently depending on the dose applied. At 21 days, exposed cells mostly experienced a general decrease in C:N and concurrent increase in C:P and N:P ratios, with significant (p<0.1) differences for treatments at 1, 10 µg/L and 10 mg/L. The classification
with PCA confirms that the same cultures (1 and 10 µg/L) have major separation (along negative PC2) from the control at 21 days exposure, caused by changes in lipids, carbohydrates and proteins pools. Thus, the decreased C/N ratio and the results of the data transformation into principal components after long-term exposure to nTiO2 may suggest the potential reallocation of carbon among macromolecules and, particularly, from storage C-rich products (lipids and carbohydrates) into proteins [50]. Increase in proteins has been considered as one of the mechanisms implemented by cells under conditions of stress [51, 52], and, in conditions of lower C/N, this may also promote the storage of intracellular N-rich polymers to prepare the cell survival in stress conditions.

Given the decrease in the symmetric v P=O of nucleic acid, this study also demonstrates how increased nTiO2 dose likely induces alterations in cellular P-rich RNA, thus causing changes in the intracellular phosphorus quota, and the related C:P and N:P ratios observed. The implications of C:P and N:P variations are well-documented, although not fully elucidated, and certainly known to impair biologically mediated flows of energy and phosphorus in ecosystems and modify organisms’ sensitivity to external nutrients, thus ecological nutrients cycling [53]. On the basis of the growth rate hypothesis (GRH), ribosomal RNA not only governs the P content but also the growth rate of aquatic organisms [54], thus have impact on trophic dynamics and nutrient cycling [44].
Table 6.1. 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 hours and 21 days. Average and standard deviations are of 3 independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>C:N</th>
<th>C:P</th>
<th>N:P</th>
</tr>
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<tbody>
<tr>
<td><strong>Time - 0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.4 ± 1.4</td>
<td>143.3 ± 1.61</td>
<td>19.85 ± 4.0</td>
</tr>
<tr>
<td>10 mg/L</td>
<td>7.5 ± 0.1</td>
<td>155.3 ± 19.2</td>
<td>20.7 ± 2.6</td>
</tr>
<tr>
<td><strong>Time - 96 hrs</strong></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>
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</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>327.9 ± 10.9 (*)</td>
<td>43.5 ± 0.1</td>
</tr>
<tr>
<td><strong>Time - 21 days</strong></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>309.3 ± 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>

(*), (**) Statistical significant values based on Student t-test at p<0.1 and p<0.05

6.5 Conclusions

In summary, a combination of FT-IR and chemical analysis, as well as principal components analysis of the obtained results of macromolecular ratios clearly indicated that an array of impact of nTiO₂ at environmentally-relevant low doses (dose below those causing observable growth inhibition) on *A. variabilis*. The results revealed 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). Additionally, changes in intracellular stoichiometry of macronutrients
and the decreased C/N suggest major reallocations of intracellular carbon for protein production in response to nTiO$_2$-induced stress.

This is an important warning that there might be long-term changes in the intracellular composition of ecologically relevant organisms at very low and environmentally relevant nTiO$_2$ concentrations (i.e., 1-100 µgTiO$_2$/L). These modifications observed may have important ecological implications, since changes in the rates of proteins uptake, synthesis, degradation and the internal quota of both limiting and non-limiting nutrients in phytoplankton were found to strongly influence the dynamics of nutrients uptake from the environment [55]. It is believed that phytoplanktonic species do not modify their internal ratios according to the ecosystem stoichiometry, but they adjust the nutrients ratios in the environment, for example through the extent of nitrogen fixation, in order to meet their metabolic requirements [56]. 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.

6.5 References


Chapter 7

Conclusions and Recommendations

7.1 Conclusions

This study presented a comprehensive investigation of the nanotoxicity and its mechanisms, as well as the ecological implications of nano titanium dioxide (nTiO₂) exposure on the representative aquatic primary producer-cyanobacteria *Anabaena sp.*. In complementary to conventional toxicological approaches, an array of modern molecular biology tools and advanced analytical techniques (i.e., RT-PCR, TEM, AFM, Raman, FT-IR, elemental analysis) were explored and employed to reveal sub-cellular and adaptive changes in response to long-term nTiO₂ exposure at sub-cytotoxic and environmentally-relevant dose levels. The techniques and the research approach presented in this study can be widely applied for the ecotoxicity assessment of other NMs, as well as for the evaluation of the adaptability of primary producers to other environmental stimuli, such as other emerging anthropogenic pollutants of concern.

The main findings and conclusions are summarized as following:

- Cytotoxic effects of nTiO₂ on cell growth rate and nitrogen fixation ability were systematically evaluated at various nTiO₂ concentrations and exposure time length. The results indicated that both growth rate (EC₅₀-96h of 0.62 mgTiO₂/L) and nitrogen fixation activity (EC₅₀-96h of 0.4 mgTiO₂/L) were inhibited by nTiO₂ exposure. The Hom’s law (CⁿTᵐ) 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 nTiO₂ concentration in toxicity. The CT-dependent inhibition effect implies that extended exposure time can lead to
severe impacts even at very low NMs concentrations, comparable to those obtained at higher doses with acute exposure.

- The formation of reactive oxygen species (ROS) and oxidative stress have been proposed as the primary mechanisms inducing toxicity in cells exposed to nTiO$_2$. The oxidative status of *Anabaena sp.* cells induced by different concentrations of nTiO$_2$ at various exposure times was monitored through intracellular (endogenous) and extracellular (exogenous) reactive oxygen species (ROS) production analysis. The production of total ROS in *Anabaena sp.* cells increased with increasing nTiO$_2$ doses and exposure time, and the intracellular ROS contributed to only a small fraction (<10%) of the total ROS measured. The dose-dependent ROS production confirms that nTiO$_2$ causes oxidative stress to *Anabaena sp.* and, indicates oxidative damages as a main toxic action directly or indirectly leading to cell surface and membranes as well as other cellular damages.

- The physiological, structural and nanomechanical alterations induced in *Anabaena sp.* cells by nTiO$_2$ exposure were investigated using high resolution TEM imaging and probed at the nano-scale level using AFM. Nano-TiO$_2$ exposure led to observable alterations in various intracellular- and membrane-level structures, which include the opening of intrathylakoidal spaces that may reflect the loss of cell photosynthetic capability, appearance of membrane crystalline inclusions, membrane mucilage layer formation, opening of intrathylakoidal spaces, and internal plasma membrane disruption. Particularly, nTiO$_2$ induced a concentration- and time-dependent increase in both the occurrence and intracellular levels of a functionally-relevant nitrogen-rich biomolecule, named cyanophycin grana protein (CGP), which is involved in cell N storage and promptly responsive to the toxicity exerted by conditions of environmental stresses. Algal cells surface morphology and mechanical
properties were modified, as indicated by the increase in cell surface roughness and shifts in cell spring constant (i.e., cytoplasmic turgor pressure) upon nTiO$_2$ exposure. These structural impairments and cytotoxic effects observed are likely mediated by the excessive production of reactive oxygen species and, particularly the membrane damages might facilitate NMs internalization by non-specific modes of entry.

- TEM and Raman imaging showed the internalization of nTiO$_2$ particles through multilayered membranes in algal cells, and also revealed the disaggregation of nTiO$_2$ aggregates when in close contact with microbial cells, potentially as a result of biomolecules (i.e., DNA) excreted by organisms that may serve as biodispersant. This process of NMs internalization still remains unclear, since cyanobacteria are believed not to have specific internalization mechanisms for nanoparticles or colloids. These evidences seem to suggest mechanisms for NMs transport into bacterial cells via diffusion processes after loss of membrane integrity. Additionally, it is still under debate whether intracellular uptake is a condition that truly causes toxicity. Nevertheless, even at these conditions, their translocation to higher trophic members is possible and, in the long-term, may possibly lead to their biomagnification and transport along the food web chain.

- The observed increase in intracellular nitrogen storage polymer (CGPs) and the inhibitory effects on nitrogen fixation rates suggested that nitrogen metabolism is sensitive and likely impacted by nTiO$_2$ exposure. The examination of the transcriptional level changes of key biomarker genes involved in the global regulation of the cell N status, in N assimilation- and N storage-specific pathways indicated that cellular C/N balance, aminoacids and nitrogen metabolism were impaired by nTiO$_2$ exposure, even at environmentally-relevant low dose levels. A more distinctive dose-dependent toxic impact was observed in light cycle, not at
dark, suggesting that complicated sub-cellular responses of cyanobacteria in exposure to nTiO₂ were associated with not only the external stressor but also light, internal circadian metabolism regulation and energy (ATP) levels. A significant increase in cell proteins was observed immediately upon nTiO₂ exposure, likely associated with stress response and homeostatic activities that require higher level of enzymes. Dose-dependent increasing levels of N-rich aminoacids (aspartic acid and arginine) and of the N-storage polymer cyanophycin grana protein (CGP) in exposed cells were measurable cellular responses to nTiO₂-induced stress and intracellular carbon and nitrogen imbalance. The occurrence and intracellular levels of the nitrogen-rich CGPs were both NMs concentration- and exposure time-dependent, suggesting that CGPs may play an important role in the stress and detoxifying response mechanisms of nTiO₂ exposure.

The alteration of N metabolism and stimulated intracellular N storage polymer formation implies potential cellular biochemical pools and nutrients stoichiometry changes. These specific subcellular responses are associated with cellular stress response and efforts to regain homeostatic states and equilibriums.

- Cellular composition changes assessment using FT-IR fingerprints, as well as chemometric analysis, revealed high-resolution changes in the allocation of major biochemical pools and chemical markers upon short- (96 hours) or long-term (21 days) exposure to nTiO₂ at environmentally-relevant sub-cytotoxic low doses (dose below those causing observable growth inhibition). Principal component analysis on over 6000 FT-IR spectra under various treatment conditions showed clusters of data associated with different dosed nTiO₂ concentrations and exposure time and the main biochemical contributors to the data variation. The results revealed subtle intracellular modifications in the 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). The results obtained suggest the potential reallocation of carbon among macromolecules and, particularly, from storage C-rich products (lipids and carbohydrates) into proteins. Increase in the total protein levels has been also demonstrated, and explained as a response mechanism of cells in response to the nTiO$_2$-induced stress. The results of the intracellular composition changes induced by long-term exposure to nTiO$_2$ may have important ecological implications in terms of the influence on nutrients uptake and storage, and specie stoichiometry, with potential effects on the ecological nutrient distributions, trophic interactions and communities’ structures.

- The sub-cellular biochemical and metabolic changes observed implied the potential of nTiO$_2$ to induce changes in intracellular macronutrients stoichiometry (C:N:P). A quantitative elemental analysis confirmed that during short-term exposure (<96 hours), statistically significant changes in C/N, C/P ratios were detected, although the dose-dependence trends were not consistent. However, after long-term exposure (21 days), more consistent dose-dependent patterns were observed that exhibited a general decrease in C:N and concurrent increase in C:P and N:P ratios as nTiO$_2$ dose increases. These compositional changes in nutrients ratios of food web base organisms, will potentially translate into the alteration of the food quality for higher trophic organisms, thus reflect on interspecies dynamics and communities’ structures. Additionally, since P-ribosomal RNA and the internal distribution of nutrients strictly governs the growth rate of aquatic organisms, more chronic population-level impacts are therefore warranted not only on these primary producers, but also on other aquatic species of the ecosystems that they support.
In summary, this study provides a more in depth understanding on the ecotoxicity of NMs on aquatic members showing a series of molecular and biochemical responses associated to the organism’s structure and composition under nTiO$_2$ exposure. Particular emphasis was given to those cellular events that are known to have broad ecological implications, such as the alteration of the organisms’ metabolic function linked to cell N status of the cell, the reallocation of intracellular biochemical pools and nutrients stoichiometry changes.

7.2 Recommendations for Further Studies

Nanoecotoxicology is still at its infancy with many aspects hardly explored. Fundamental research on toxicity and ecological implications of NMs to various ecologically relevant organisms, such as prokaryotic and eukaryotic primary producers, is rather scarce. This study provided insights and evidence of potential permanent adaptive cellular changes in primary producer as a result of long-term exposure to NMs at environmentally-relevant low doses, which have profound impact on not only their ecological functions, but also rebound effects on high trophic levels communities’ structure and integrity, with the translocation of macronutrients. The results pointed out the importance in applying more comprehensive and more holistic toxicity assessment approaches for assessing nanoeffecticity in order to reveal the subtle and subcellular effects and potential “true” ecological implications beyond just phenotypic toxic endpoints on one populations or species. It warrants further and more extensive investigation on the nanotecotoxicity of various NMs to other key functionally-relevant ecological organisms, as well as long-term studies for ecological system with multiple memories from different trophic levels. In addition to cyanobacteria, other important eco-relevant species perform primary productivity and deserve in depth study, such as algae and higher plants. Their typical eukaryotic
structure and metabolic capabilities, which differ from those of prokaryotic cyanobacteria, may either add newer and more specific toxic responses or show lower vulnerability to the previously recognize NMs mode of action. Furthermore, ecosystem-like set-up including organisms of various trophic levels and different feeding strategies, is needed to provide important insights on nutrients dynamics and distribution in nTiO$_2$-contaminated ecosystems, on the potential NMs bio-transfer among organisms of different trophic levels, and will finally elucidate more complex species interactions (i.e., symbiosis, species competitions, etc.).

The use of techniques that provide more sub-cellular and molecular level information is needed to better understand the organisms’ response to NMs and the modes of action involved during exposure.

It is yet to be further evaluated if the effects that are observed in laboratory settings and conditions, during short-term exposure to NMs, truly reflect actual organisms’ responses in the real environment and if it can be extrapolated to long-term effects. Therefore, more long-term exposure studies with a mixture of various trophic-level species with different media matrixes will be required to determine if and how they would be affected by the matrix composition.

The majority of nanoecotoxicological research has focused on those NMs, such as nTiO$_2$ or nAg, that have more likelihood to be released in the environment due to their large application in industrial processes and consumers goods. However, other nanomaterials (i.e., carbon-based NMs, quantum dots) have been shown to be highly toxic to living cells, although their environmental presence is not expected to be as widespread, due to their lower incorporation into products, as those of nTiO$_2$ or nAg. Thus, the investigation of the toxicity mechanisms associated to other NMs (i.e., metal and carbon-based nanomaterials, quantum dots, dendrimers)
is required, since their intrinsic properties and specific environmental behaviors are expected to influence their interactions with living organisms and have strong impacts.

Currently, limited information is available on the environmental concentrations of NMs and their fate and transport, mainly due to the lack of available methods able to detect and quantify trace concentrations of nanoparticles and distinguish them from the correspondent coarse counterparts in complex environmental matrixes. Also, the current difficulties and lack of feasible techniques for separating and quantifying NMs from cells and test matrix still limits the observation of nTiO$_2$ toxic effects carried-over after the exposure. Thus, the nanoeffectivity field that investigates the interaction of ecological species with released NMs in real environments is still at its early stage and is expected to progress with advancements in NMs analysis tools.

In conclusion, the approach and the methodology developed or refined in this study can be further applied for the ecotoxicity assessment of other NMs or other emerging and anthropogenic pollutants of concern. These mechanism- and biochemical-based assessment tools are, in fact, ideal for the evaluation of the adaptability of primary producers to changing environmental conditions and emerging environmental stimuli.
APPENDIX
Appendix A

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

**Figure A1.** Correlation of growth inhibition and N fixation inhibition values (as percentage to control) at any given CT value.
Figure A2. EC\textsubscript{50} values obtained based on nitrogen fixation inhibition of *Anabaena variabilis* cells exposed to concentrations of nTiO\textsubscript{2} ranging from 0-500 mg/L and with exposure time length from 3 hours to 6 days. (Inset, figure shown at a different scale).
Appendix B

Impact of Nano Titanium Dioxide Exposure on Cellular Structure of *Anabaena variabilis* and Evidence of Internalization

**Figure B1.** Intracellular ROS production rate in *Anabaena variabilis* cells exposed to various concentration of nTiO$_2$ ranging from 0 to 200 mg/L.
Figure B2. Representative electronmicrographs showing the effects of nTiO$_2$ exposure on *Anabaena* v. membranes in vegetative cell (a) and heterocyst (b). Intracellular material freely released upon membrane disruption (c). nTiO$_2$ punctures onto cells membrane (d, e). Mucilage formation surrounding *Anabaena variabilis* cell (f).
Figure B3. TEM images showing nTiO$_2$ aggregates and possible disaggregation in immediate adjacent area next to three distinct *Anabaena variabilis* cells exposed to 1 mg nTiO$_2$/L (a) and 50 mg nTiO$_2$/L (b, c) for 96 hours. Samples were visualized before treatment with uranyl acetate and lead citrate to avoid formation of staining precipitate. Scale bars: 100 nm.
Appendix C

Nano-Titanium Dioxide Exposure Impact Nitrogen Storage and Metabolism in Cyanobacteria

Figure C1. Melting curve of cphA reaction products of samples run in this study
Figure C2. Percentage of total proteins increase in respect of the control for samples exposed to nTiO₂ concentrations ranged from 0.01 to 10 mg/L at different times of light/dark regimens. Standard deviation is of three replicate analysis.
**Table 1.** Genes and respective RT-PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme</th>
<th>Function/Reaction</th>
<th>Tm (°C)</th>
<th>F* / R**</th>
<th>Sequence (5' - 3')</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>cphB</td>
<td>Cyanophycinase</td>
<td>[L-Asp(4-L-Arg)]ₙ + H₂O = [L-Asp(4-L-Arg)]ₙ₋₁ + L-Asp(4-L-Arg)</td>
<td>61</td>
<td>F</td>
<td>5' - TTTGGGACACGGGACATACA - 3'</td>
<td>Abd-El-Karem et al. (2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - CCAAATCGAGGCGCATGAC - 3'</td>
<td></td>
</tr>
<tr>
<td>cphA</td>
<td>Cyanophycin synthetase</td>
<td>ATP + [L-Asp(4-L-Arg)]ₙ + L-Asp → ADP + phosphate + [L-Asp(4-L-Arg)]ₙ₋₁ + L-Asp</td>
<td>61</td>
<td>F</td>
<td>5' - ATGGGGCATCACATGATTGCTGGCG - 3'</td>
<td>Abd-El-Karem et al. (2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - GGAGATGGGAATCACCACATCTCTAC - 3'</td>
<td></td>
</tr>
<tr>
<td>ntcA</td>
<td>Global nitrogen</td>
<td>Encodes NtcA protein, global nitrogen regulator</td>
<td>57</td>
<td>F</td>
<td>5' - AGCGCCAAATTGAACAGGTAG - 3'</td>
<td>Dines et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>regulator</td>
<td></td>
<td></td>
<td>R</td>
<td>5' - CTCACCAATCTGGAAACC CAT - 3'</td>
<td></td>
</tr>
<tr>
<td>glnB</td>
<td>PII protein</td>
<td>Encodes the PII protein, central signal transmitter in nitrogen control</td>
<td>58</td>
<td>F</td>
<td>5' - AAATCGCTTTAGTCAAGCGC - 3'</td>
<td>Vinila et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - CAACTACAATCTCCACCT CTA - 3'</td>
<td></td>
</tr>
<tr>
<td>glnA</td>
<td>Glutamine synthetase</td>
<td>Glutamate + ATP + NH₃ → Glutamine + ADP + phosphate</td>
<td>61</td>
<td>F</td>
<td>5' - GCCGTACCACATCGAAAAAACATCA - 3'</td>
<td>Paz-Yepes et al. (2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - AAATCGCCGGTTTGGGAT AAGAT C - 3'</td>
<td></td>
</tr>
<tr>
<td>all2934</td>
<td>Glutaminase</td>
<td>Glutamate + H₂O → Glutamate + NH₃</td>
<td>59</td>
<td>F</td>
<td>5' - CTCAATCCGTTGACTGAAATGTT - 3'</td>
<td>Zhou et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - CCCATTCCTCCATTTCCACTCAT A - 3'</td>
<td></td>
</tr>
<tr>
<td>nifH</td>
<td>Dinitrogenase</td>
<td>Encodes the Fe protein of dinitrogenase reductase</td>
<td>57</td>
<td>F</td>
<td>5' - GTTGCGACCTAAAGCTGAC - 3'</td>
<td>Suzuki et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - GGGTGGAATGATACCACGG - 3'</td>
<td></td>
</tr>
<tr>
<td>nifK</td>
<td>Dinitrogenase reductase</td>
<td>Encodes the β subunit of dinitrogenase</td>
<td>57</td>
<td>F</td>
<td>5' - TCAACACGCGAATACAC ACC - 3'</td>
<td>Pratte et al. (2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - AACGCACCTGGGAACCC TT - 3'</td>
<td></td>
</tr>
<tr>
<td>rnpB</td>
<td>Housekeeping gene</td>
<td>Encodes for the RNase P (removes 5' leader sequences from tRNA precursors during tRNA biosynthesis)</td>
<td>57</td>
<td>F</td>
<td>5' - AGGGAGAGTAGCGGTTG3-3'</td>
<td>Gonzales et al. (2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - AAAAGAGGAGAGTAGGTTGG - 3'</td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>Genomic DNA</td>
<td>-</td>
<td>65</td>
<td>F</td>
<td>5' - CCTACGGGAGGCGACAG - 3'</td>
<td>Qing et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>5' - ATTACCAGCCGCTGCTGG - 3'</td>
<td></td>
</tr>
</tbody>
</table>

*Forward; **Reverse
Appendix D

Nano-Titanium Dioxide Exposure Induced Intracellular Biochemical Composition Changes in Cyanobacteria

Table D1. Physical and chemical characterization of nTiO$_2$ tested in this study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polydisperisty index (PdI) after dispersion in culture media</td>
<td>0.479</td>
</tr>
<tr>
<td>Specific surface area (SSA)</td>
<td>274.2 m$^2$g$^{-1}$</td>
</tr>
<tr>
<td>Average size of NM aggregates in culture media*</td>
<td>192±0.8 nm</td>
</tr>
<tr>
<td>Average size of NM aggregates at 96 hours**</td>
<td>435.0±275.5 nm</td>
</tr>
</tbody>
</table>

* Dynamic Light Scattering measurement
** Transmission Electron microscopy measurement

Note: Transition metals of the bulk material and other physical-chemical parameters (organic and elemental carbon, surface charge) determined for nTiO$_2$ suspension in phosphate buffer saline were also reported in Bello et al. (Reference [18]).

Table D2. Correlation parameter (R) of chlorophyll $a$ and protein measurement with cell count of $A$.variabilis exposed to different nTiO$_2$ concentration for 13 days exposure.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10 µgTiO$_2$/L</th>
<th>100 µgTiO$_2$/L</th>
<th>1000 µgTiO$_2$/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell count - Protein</td>
<td>0.69</td>
<td>0.82</td>
<td>0.73</td>
<td>0.48</td>
</tr>
<tr>
<td>Cell count - Chlorophyll $a$</td>
<td>0.85</td>
<td>0.93</td>
<td>0.87</td>
<td>0.56</td>
</tr>
</tbody>
</table>
### Table D3. FTIR spectrum and frequencies bands assignments for *A. variabilis*

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignments</th>
<th>Functional groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1032 v C-O-C</td>
<td></td>
<td>Polysaccharides</td>
</tr>
<tr>
<td>1078 v P=O</td>
<td></td>
<td>Nucleic acid</td>
</tr>
<tr>
<td>1150 v C-O-C, v C-O</td>
<td></td>
<td>Polysaccharides, Nucleic acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphodiester backbone of nucleic acid (DNA, RNA); may also be due to the presence of phosphorylated proteins and polyphosphate storage products</td>
</tr>
<tr>
<td>1240 v(_{as}) P=O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1320 δ N-H, v C-H</td>
<td></td>
<td>Proteins (Amide III band)</td>
</tr>
<tr>
<td>1398 δ CH(<em>{2}), δ CH(</em>{3}), δ C-O</td>
<td></td>
<td>Proteins, carboxylic groups</td>
</tr>
<tr>
<td>1455 δ(<em>{as}) CH(</em>{2}), δ(<em>{as}) CH(</em>{3})</td>
<td></td>
<td>Methyl and methylene groups of lipids and proteins</td>
</tr>
<tr>
<td>1540 δ N-H, v C-N</td>
<td></td>
<td>Amide associated with proteins (Amide II)</td>
</tr>
<tr>
<td>1650 v C=O</td>
<td></td>
<td>Amide associated with proteins (Amide I); may also contain contributions from C=C stretches of olefinic and aromatic compounds</td>
</tr>
<tr>
<td>1740 v C=O</td>
<td></td>
<td>Ester functional groups primarily from lipids and fatty acids, membrane lipids</td>
</tr>
<tr>
<td>2850 v CH(<em>{3}) and v CH(</em>{2})</td>
<td></td>
<td>Methyl and methylene groups in fatty acids and methyl groups of lipids</td>
</tr>
<tr>
<td>2876 v CH(<em>{3}) and v CH(</em>{2})</td>
<td></td>
<td>Methyl and methylene groups in fatty acids and methyl groups of lipids</td>
</tr>
<tr>
<td>2920 v(<em>{as}) CH(</em>{2})</td>
<td></td>
<td>Methylene groups of lipids</td>
</tr>
<tr>
<td>2956 v(<em>{as}) CH(</em>{3})</td>
<td></td>
<td>Methyl groups of lipids</td>
</tr>
</tbody>
</table>
**Table D4.** $p$-value (t-test) of statistical significance for the protein/chlorophyll $a$ ratio of exposed samples compared to control for different exposure times.

<table>
<thead>
<tr>
<th>p-value (t-test)</th>
<th>1 µg/L</th>
<th>10 µg/L</th>
<th>100 µg/L</th>
<th>1000 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Over 21 days exposure</td>
<td>0.14</td>
<td>0.00</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Over 96 hours exposure</td>
<td>0.23</td>
<td>0.02</td>
<td>0.04</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Figure D1.** Growth inhibition of *A. variabilis* (based on chlorophyll $a$) as a function of nTiO$_2$ dose concentrations for 21 days exposure, expressed as a percentage of the growth in the control treatment.
Figure D2. Dose-dependent changes in the protein/chlorophyll a ratio. Protein/chlorophyll a ratio over time for different nTiO₂ concentrations.