Reviewer 4 Comments:

Your chosen relevant concentration seems to be really high. Kiser et al detected TiO2 NM in waste water streams but they did not differentiate between NP and larger sizes, as I remember correctly. Therefore I would expect a lower relevant concentration in the environment.

Author's Response:

Thank you, but I disagree with this comment. Our relevant concentration (50 µg/L TiO_2NP) represents a nominal Ti concentration of 30 µg/L, which falls within the range of concentrations measured in effluent (10 to 100 µg/L Ti). Additionally, Kiser et al. report that they observed both single nanoparticles and aggregates in effluent ranging in size from 50 to 300 nm. Thank you for pointing out the missing rationale for our chosen relevant concentration. I have modified the manuscript on lines 194 to 197 to include this missing rationale.

From Kiser 2009: "Although the majority of Ti sorbed to biomass, we found that 10-100 μg/L Ti still remained in effluents. This study, therefore, defines environmentally relevant concentrations for studying the toxicity of TiO2 nanoparticles to organisms".

"Single nanoparticles plus spherical aggregates (50 nm to a few hundred nanometer in size) composed of sub-50 nm spheres of Ti and oxygen only (presumably TiO2) were observed in all samples".

Reviewer 4 Comments:

In my opinion, the Discussion section could have been more explored in terms of other intrinsic characteristics of TiO2 NP as mentioned above. In many studies, the aggregation of NPs can lead to several toxic effects to aquatic/terrestrial biota. Not only the metal concentration matters but also the other properties of nanoparticles that can be of the crucial relevance to interact with the environment. I think that the study is very complex and interesting, but a lack of basic experiments makes the discussion poor.

Author's Response:

Thank you for the thoughtful feedback. In the discussion, we have added lines 439-445 to address how some of the intrinsic characteristics of the TiO2NP were affected by the wastewater effluent, and how other environmental variables affected TiO2NP behavior and aggregation in effluent (lines 446-458). On lines 487-502 we discuss the mechanisms of toxicity as they relate to TiO2NP toxicity (TiO2NP aggregate-mediated shading and ROS release that affected the exterior of the algal cells).

With regard to using lab experiments, I agree that they're very important. However, part of the reason we conducted a mesocosm study was to address the toxicity of TiO2NP in a natural setting more representative of a realistic environmental scenario. A number of lab studies were already conducted that have provided data on the concentrations that cause toxicity, and we are building on that foundation to provide an additional layer of necessary complexity to ask different but related questions.

The complexity of ecosystems is impossible to replicate in the laboratory, and any results from these types of studies should be regarded as a basis for the state of the knowledge until they can be confirmed in large-scale field experiments. Field studies are a critical component of environmental research because they incorporate the complexity found in nature and can be carried out at the most environmentally-relevant spatial and temporal scales (Carpenter *et al.* 1996; Joern & Hoagland 1996; Schindler 1998).

We feel that our results represent a novel ecological scenario that has not yet been reported in the literature, and we hope that our explanation for the study design is satisfactory.

Carpenter SR. 1996. Microcosm Experiments Have Limited Relevance for Community and Ecosystem Ecology. Ecology 77(3): 677-680.

Joern A and KD Hoagland. 1996. In defense of whole-community bioassays for risk assessment. *Environmental Toxicology & Chemistry* 15: 407-409.

Schindler D. 1998. Whole-Ecosystem Experiments: Replication Versus Realism: The Need for Ecosystem-Scale Experiments. *Ecosystems* 1(4): 323-334. https://doi.org/10.1007/s100219900026