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Research paper

Photosynthetic efficiency predicts toxic effects of metal nanomaterials in phytoplankton

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ABSTRACT

High Throughput Screening (HTS) using in vitro assessments at the subcellular level has great promise for screening new chemicals and emerging contaminants to identify high-risk candidates, but their linkage to ecological impacts has seldom been evaluated. We tested whether a battery of subcellular HTS tests could be used to accurately predict population-level effects of engineered metal nanoparticles (ENPs) on marine phytoplankton, important primary producers that support oceanic food webs. To overcome well-known difficulties of estimating ecologically meaningful toxicity parameters, we used novel Dynamic Energy Budget and Toxicodynamic (DEBtox) modeling techniques to evaluate impacts of ENPs on population growth rates. Our results show that population growth was negatively impacted by all four ENPs tested, but the HTS tests assessing many cell/physiological functions lacked predictive power at the population level. However, declining photosynthetic efficiency, a traditional physiological endpoint for photoautotrophs, was a good predictor of population level effects in phytoplankton. DEBtox techniques provided robust estimates of EC_{10} for population growth rates in exponentially growing batch cultures of phytoplankton, and should be widely useful for ecotoxicological testing. Adoption of HTS approaches for ecotoxicological assessment should carefully evaluate the predictive power of specific assays to minimize the risk that effects at higher levels of biological organization may go undetected.

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1. Introduction

Novel chemicals and materials, including engineered nanomaterials, are being developed at an ever-increasing rate, but few are thoroughly evaluated for their potential to inflict harm to the environment. This is due in large part to traditional methods of ecotoxicological risk assessment that are time consuming and expensive. Accordingly, high-throughput screening (HTS) methods have been a promising approach for rapid risk assessment of new compounds, including nanomaterials (Nel et al., 2013; Knudsen et al., 2015). These approaches attempt to predict and explain responses of whole organisms and ecological systems by measuring responses at lower levels of biological organization, typically using in vitro assays to identify high-risk compounds and prioritize in vivo tests (Judson et al., 2013). The assays can also be used to identify mechanisms of cellular injury that can inform *in silico* models for predictive toxicology (Nel et al., 2013; George et al., 2011). Potential advantages include lower cost, less reliance on animal models, higher replication, and therefore greater confidence in results. HTS has proven highly useful in drug testing and human medical science (Nel et al., 2013). However, in ecotoxicology, most research has thus far focused on the in vitro level and relatively little has been done to evaluate the effectiveness of HTS methods in predicting higher-level ecological impacts.

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HTS techniques measure cellular injury responses to toxicant exposure, e.g. fluorescent-probe-based proxies for mitochondrial membrane potential, intracellular calcium fluxes, and membrane permeability (George et al., 2010) that can be measured in high-capacity plate readers. Whether positive signals in HTS translate into impacts at higher levels of biological organization, where compensatory mechanisms may mitigate impacts, remains uncertain (Forbes and Calow, 2012). The need for HTS studies to be directly linked to organismal and population-level impacts is clear but has rarely been conducted. Phytoplankton offer a eukaryotic cell system where cellular/physiological endpoints can be rapidly assessed using an HTS approach, and organismal function (photosynthetic efficiency) and population-level growth can be readily determined.

Phytoplankton are single-celled planktonic photoautotrophs that are the dominant primary producers in the oceans. As small (0.2–200 μm) single or clustered cells with high surface-to-volume ratios suspended in water, they are vulnerable to pollution, especially in coastal zones where they are abundant (Lucas et al., 2011) and contaminants may be concentrated (Bowen and Depledge, 2006). Impacts to phytoplankton may cascade through marine food webs, since reductions in population growth rate of phytoplankton cells reduce resources available for consumers (Edwards and Richardson, 2004), and accumulation of contaminants in phytoplankton can lead to trophic transfer and consequent toxic effects on consumers (Hanna et al., 2013a; Bielmyer-Fraser et al., 2014). Metals are a common class of contaminants that impact phytoplankton and accumulate in coastal marine food webs, and metallic nanomaterials are an emerging form of metal contamination in coastal marine ecosystems (Gottschalk et al., 2009; Gottschalk et al., 2013). Nanomaterials are widely employed for their enhanced mechanical, optical, and electromagnetic properties relative to larger forms of similar materials (Pitkethly, 2004). Engineered nanoparticles (ENPs) are nanomaterials created with all three dimensions at the nanoscale (1–100 nm). Because of their adoption in wide array of applications, including electronics, chemistry, cosmetics, and biomedicine (West and Halas, 2003; Mauter and Elimelech, 2008; Barreto et al., 2011), ENPs and other nanomaterials are emerging as a new class of contaminants, with potentially negative environmental consequences (Klaine et al., 2008). Metal-based nanomaterials in particular are commonly used because they are synthesized relatively easily and have myriad industrial and consumer applications (Rao et al., 2004). As the volume of nanomaterials production and subsequent discharge into the marine environment increases, so does the potential for wide-ranging ecological impacts (Knudsen et al., 2015). Exposure of aquatic organisms to nanomaterials is difficult to quantify, but modeling efforts have estimated concentrations of metal oxide ENPs in aquatic systems at levels of significant concern (Gottschalk et al., 2009; Gottschalk et al., 2013).

Here we investigate the potential of four fluorescence-based HTS assays of cellular health to predict population-level impacts of ZnO, Ag, CeO and CuO ENPs, specifically the population growth rate of a geographically widespread prymnesiophyte phytoplankton species, *Isochrysis galbana*. The HTS assays are designed to measure different aspects of cellular functioning and integrity, including mitochondrial membrane potential, occurrence of reactive oxygen species (ROS), cellular efflux pump activity and cell membrane permeability. Since photosynthetic processes are well-known targets of metal toxicity (Clijsters and Vanassche, 1985), we also used a non-HTS, physiological assay to measure impacts on photosynthetic efficiency. Cell densities measured during the exponential growth phase in batch cultures were analyzed within a Dynamic Energy Budget and Toxicodynamic (DEBtox) modeling framework to determine impacts of ENPs on population growth rates. Building on models we used previously (Muller et al., 2010; Miller et al., 2010), we developed new formalism to obtain robust estimates of EC_{10} for population growth rates exposed to the suite of commonly used metallic ENPs.

2. Materials and methods

All the ENPs used were characterized for the central materials library maintained by University of California's Center for Environmental Implications of Nanotechnology (UC CEIN)(Godwin et al., 2009). ZnO and CeO_2 ENPs were obtained from Meliorum Technologies (Rochester, NY, USA) and characterized for size, morphology, and chemical composition (Montes et al., 2012). Briefly, the primary ZnO ENPs were spheroid with a mean dry diameter of 24 ± 3 nm and a surface area of 42.1 $m^2\ g^{-1}$. Their agglomerate hydrodynamic diameter was 205 ± 14 nm in nanopure water (18.2 MΩ cm). The primary CeO_2 ENPs were rods ($67 \pm 8 \times 8 \pm 31$ nm) with a surface area of 93.8 $m^2\ g^{-1}$ and an agglomerate hydrodynamic diameter of 231 ± 16 nm in NanoPure water. CuO ENPs were obtained from Sigma-Aldrich (St. Louis, MO, USA) and described as < 50 nm and 99.8% pure; characterization with ICP-OES and TEM showed that they were 84.8 ± 2.7% pure (impurities included Na, Ca, Si, and Mg) and 20–100 nm in diameter (Hanna et al., 2013b). AgO ENPs were obtained from QuantumSphere Inc. (Santa Ana, CA) and described as 20–30 nm in diameter. TEM characterization showed that the AgO ENPs were 20–70 nm in diameter; no detectable impurities were found (Bielmyer-Fraser et al., 2014). The behavior in seawater of all particles used in this study has been previously characterized and is summarized in Table 1. All aggregate significantly in seawater. ZnO dissolves rapidly, whereas CuO and Ag dissolve very slowly and CeO dissolution was undetectable (Table 1).

2.1. Phytoplankton culture

Axenic cultures of *Isochrysis galbana* were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, USA), and were maintained in standard media (f/2) made with filtered (0.22 μm filtered) natural seawater, which was autoclaved prior to inoculation. For cellular metal accumulation measurements, phytoplankton were cultured in synthetic seawater made by mixing Instant Ocean salt with 18 mΩ Milli-Q water and aerating at least 24 h before use. To provide inoculant for experiments, algae were incubated in polycarbonate flasks under cool white fluorescent lights (14:10 light:dark, 100–120 μmol $m^{-2}\ s^{-1}$) at 20 °C with aeration for 5–7 d, until log phase growth prevailed. Cell densities were measured directly by hemacytometer (Reichert, NY, USA) at 200× magnification or using a fluorometer (Turner Trilogy) calibrated with hemacytometer data.

2.2. ENP preparation and dispersion

ENP dispersions were prepared within 45 min of the start of each experiment. ENP stock suspensions were prepared by adding 10 mg of metal oxide ENPs to 10 mL of ultrapure 18 mΩ Milli-Q water, vortexing for 30 s, sonicating for 45 min, and adding 100 μL of 2 mg L^{-1} alginate (previously made stock in 18 mΩ Milli-Q water), and vigorously vortexing again for 1 min.

2.3. Phytoplankton population growth

All experiments were conducted at 20 °C, 34 ppt salinity, under the same illumination schedule described above. All glassware was acid-washed, rinsed with purified water (Barnstead nanopure, resistivity >18 MΩ cm), and autoclaved before use. Experiments were run in 125 mL polycarbonate flasks, media volume 50 mL, and were mixed at ~150 rotations per min on a rotary shaker (New Brunswick Scientific Co., NJ, USA). ENP concentrations tested were

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