Assessment of nanomaterial uptake and depuration kinetics in protozoa by image analysis

Introduction

Engineered nanomaterials (ENMs) are manufactured metallic and non-metallic substances that range from 1-100 nm in size on at least one physical dimension. Their small size and larger surface area can foster greater chemical reactivity and enhanced biological membrane permeability which increases cytotoxicity in organisms (Hurt et al. 2006). ENMs’ fast-paced development and emerging applications in industry and research make their risk assessment in the environment increasingly relevant. The ENMs studied in this research project were graphene (GNM), multiwall carbon nanotubes (MWCNTs), hexagonal boron nitride (hBN) and boron nitride nanotubes (BNNTs). Carbon black (CB), a longstanding manufactured nanomaterial used for many industrial applications, was used as a toxicological control since it has been deemed non-toxic and non-bioaccumulative.

CB nanoparticles are inert colloids formed by incomplete hydrocarbon combustion, and are used extensively in industry for pigmentation purposes. Despite also being carbon-based, GNM and MWCNTs are expected to exhibit different environmental outcomes due to their structures. Graphene is structurally an atom thick, with sp²-hybridized carbon atoms configured hexagonally by covalent bonds. In plant-soil interactions, GNM were accumulated in the roots and nodules of wheat and soybean plants, respectively (Chen et al. 2017; Wang et al. 2017). This resulted in altered root and cellular structures, heightened oxidative stress (Chen et al. 2017), earlier flowering time and altered nitrogen fixation potential (Wang et al. 2017). Also, GNM have displayed cytotoxic effects on aquatic organisms due to membrane damage (Zhao et al. 2014).

Structurally, MWCNTs are comprised of numerous graphene layers rolled into tubes, equidistant from each other. MWCNT-amended soils resulted in shorter soybean plants, with smaller leaves, and decreased nitrogen fixation potential (Wang et al. 2017). Their bioaccumulation potential was established in the protozoan, Tetrahymena thermophila, through direct uptake and trophic transfer of MWCNTs during grazing on the ENM-exposed bacteria (Mortimer et al. 2016a). MWCNTs alter bioenergetics of bivalve organisms, manifesting as oxidative damage evidenced by increased lipid peroxidation; neurotoxin generation occurred due to inhibited cholinesterase activity (De Marchi et al. 2017).

The effects of boron nitride-based nanomaterials in the environment are not well-studied, calling for better understanding as they are often looked to as alternatives to carbon-based nanomaterials. hBN are structurally sp²-hybridized boron and nitrogen atoms that are configured in a honeycomb-like structure held together by ionic and covalent bonds. No current research identifies possible ecotoxicological effects of hBN. BNNTs are structurally similar to MWCNTs, i.e. concentric tubes, except instead of solely carbon atoms, they are alternating boron and nitrogen atoms. In vitro
effects of BNNTs on human cell lines, particularly human embryonic kidney cells and lung alveoli, showed that cytotoxicity occurred (Horvath et al. 2011). The BNNT exposure triggered multinucleated giant cell formation in macrophages and overproduction of eosinophilia in fibroblasts (Horvath et al. 2011). No studies have been found that regard environmental effects of BNNTs.

As iterated above, ENMs have been shown (or lack thereof due to lack of research) to have different effects in the environment which are largely dependent on the ENM type and the environmental matrices they enter (Mortimer et al. 2016a; Wang et al. 2017). An approach integral to ENM risk evaluation, particularly in the scope of their fate and impact in natural ecosystems, is the determination of their uptake and depuration kinetics. These processes provide insight on ENM bioavailability, exposure, susceptibility for trophic transfer and other biotic effects. However, acquiring this information for current non-metallic ENMs is challenging and requires use of radioactive labeling coupled with image analysis (Mortimer et al. 2016a) and complex instrumentation. Therefore, the development of alternative methods that determine the internalization, retention and depuration kinetics of carbon-based and boron nitride ENMs are important.

The primary goal of this research project is to compare, for carbon-based and boron nitride ENMs, compartmentalization and elimination of these nanomaterials using an image analysis approach previously confirmed for $^{14}$C-labeled MWCNTs (Mortimer et al. 2016a). In the prior work, there was a strong correlation between the analytical quantification of MWCNTs internalized by the protozoa, *Tetrahymena thermophila*, using accelerator mass spectrometry and their simultaneous polarized microscopic image analyses (Mortimer et al. 2016a). Similar to the previous study, this research project used the fresh-water protozoan *T. thermophila* as a test organism. These phagocytic, unicellular eukaryotes have a translucent anatomy that allow visualization and quantification of opaque ENMs such that the time course of uptake and elimination can be studied using phase-contrast microscopy. Using imaging and image analysis, we can determine the bioaccumulative nature of the studied ENMs. Uptake into the protist *T. thermophila* would suggest that the subject ENMs could be bioavailable to organisms in higher trophic levels.

**Research Questions**

The following are the key research questions that were outlined for the summer research:

1. What are the uptake and elimination rates across the studied ENMs, as quantified using microscopic image analyses?
2. How do the ENM chemistries affect uptake and elimination rates of the studied ENMs in *T. thermophila*?
3. How does ENM morphology affect uptake and elimination rates of the studied ENMs in *T. thermophila*?

**Methods & Results**

1. What are the uptake and elimination rates of ENMs ingested by *T. thermophila*, as quantified using microscopic image analyses?
**Approach:** ENMs (at 200 mg/L) were dispersed in nanopure water with 400 mg/L of alginic acid by probe sonication. *T. thermophila* SB210E cells were inoculated into protease peptone-based growth media (SSP) in sterile, polystyrene petri plates and grown in a stationary humidity chamber for 17 h at 30°C. The culture, at its late exponential phase, was then centrifuged at 1000 rpm for 8 min. The centrate was decanted, and the cell pellet was dispersed into Dryl’s starvation medium. A 10 mL aliquot of the cell suspension was added to sterile, polystyrene petri plates and starved in a humidity chamber at 30°C for 17 h. The starved cell suspension was then centrifuged at 280 g for 8 min at 5-10°C. The supernatant was discarded and the pellet suspended in fresh Dryl’s starvation medium. The cell density of the protozoan culture was adjusted to 10⁶ cells/mL. An aliquot of 100 µL of the *T. thermophila* suspension was added to each well of a sterile, polystyrene 12-well plate. For each nanomaterial, an aliquot of 100 µL of 100 mg/L of nanomaterial + Dryl’s suspension was pipetted into 3 wells and placed in the stationary humidity chamber at 30°C. Uptake sampling occurred at 30, 45 and 60 min. The protozoan culture was purified of unassociated nanomaterials and fecal pellets by centrifugation in OptiPrep™ density gradient medium as detailed in Mortimer et al. (2016b). After density-gradient centrifugation, the protozoa were transferred to fresh Dryl’s medium and the clearance of nanomaterials was studied for samples acquired after 15, 30, and 60 minutes. The cells were fixed with glutaraldehyde and prepared for microscopy. Using phase-contrast microscopy, 20 to 30 cells were imaged per sample with a 100X oil immersion objective.

**Results:** The outlined method allowed for ENM uptake and depuration to be visualized, affirming the observations in Mortimer et al. (2016a) (Fig. 1). The food vacuoles were clearly seen under the microscope providing insight on uptake and depuration kinetics of ENMs in *T. thermophila*. More detailed results will be disseminated in a publication that is currently in preparation.

2. How do the ENM chemistries affect uptake and elimination rates of the studied ENMs in *T. thermophila*?
**Approach:** The overlaid microscopic images (Fig. 2A) of *T. thermophila* exposed to ENMs at the different uptake and depuration time points were analyzed using ImageJ. The parameters of interest were the total cell area and the area of filled vacuoles in the cell. A hand-drawn outline of the cell was done (Fig. 2B), isolating the food vacuoles from the background, then converted into a binary image (Fig. 2C). Through particle analysis of the binary image, the area of the filled food vacuoles was determined (Fig. 2D).

![Fig. 2: Determination of filled food vacuole area using ImageJ.](image)

**Results:** There were few differences between the uptake rates of the different ENMs. There were, however, notable differences in depuration rates between the different ENMs. A detailed comparative analysis will be disseminated in a publication that is currently in preparation.

3. **How does ENM morphology affect uptake and elimination rates of the studied ENMs in *T. thermophila***?

**Approach:** Due to notable differences in elimination rates between the different ENMs in this study, and based on observations of food vacuole shapes that appeared to morphologically resemble the ENM morphologies (i.e. platelet graphene ENMs, versus bundled MWCNTs), the fractal dimensions of the food vacuoles of *T. thermophila* were measured. Fractal analysis was tested as an approach that could reveal overall morphological differences in food vacuoles based on the specific internal ENMs. Using the microscopic images for samples acquired after 45 minutes during the elimination process, and using the ImageJ plugin, FracLac, the fractal dimension of filled food vacuoles was calculated assuming the binary outlines of hand-selected aggregates used in the original image analysis. The fractal analysis was performed substantially by an undergraduate assistant, Anne Trinh.

**Results:** There were marked differences in fractal dimension values between CB, MWCNTs and GNM*s, which correlate with their differences in depuration rates. A detailed comparative analysis will be disseminated in a publication that is currently in preparation.
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References