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Acute exposure to TiO_2 nanoparticles produces minimal apparent effects on oyster, *Crassostrea virginica* (Gmelin), hemocytes

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ABSTRACT

The response of oyster (*Crassostrea virginica*) hemocytes was studied following exposure to anatase nanoparticles (ca. 7.4 nm), surface-coated rutile nanocomposites (UV–Titan M212, ca. 86 nm) and bulk titanium dioxide (TiO_2) particles (anatase and rutile crystalline forms; ca. 2–5 μm). Hemocytes were collected from oysters and exposed to one of the four particle types at concentrations of 0.1, 0.5, and 1.0 mg/L under dark and environmentally-relevant light conditions for periods of two and four hours. Hemocyte mortality, phagocytosis, and reactive oxygen species (ROS) production were then evaluated using flow-cytometric assays. Bulk and nanoparticulate TiO_2 had little effect on viability of oyster hemocytes or on production of ROS. Significant changes in phagocytosis occurred after exposure to anatase nanoparticles for 4 h under dark conditions, and UV–Titan for 2 h under light conditions. Results demonstrate that TiO_2 particles (bulk or nanoscale) produce minimal effects on hemocyte biomarkers examined following acute, *in vitro* exposures.

1. Introduction

Nanoparticles (NP) have applications in a diverse range of consumer products with an economic worth projected to reach $173.95 billion dollars by 2025 (Business Wire, 2016). In particular, titanium dioxide nanoparticles (n-TiO_2) possess novel physicochemical properties that are highly sought after in the manufacture of paint, surfaces, solar-powered devices, cosmetics, and sunscreens. For example, n-TiO_2 are used in paint as opaquers (Carp et al., 2004) and to absorb air pollutants (Bueza et al., 2007), on surfaces to expedite chemical and microbial decomposition (Albrecht et al., 2006; Bueza et al., 2007; Sharma, 2009), in photovoltaic devices to capture ultra-violet radiation (UV; Matsui, 2005), and in cosmetics and sunscreens to absorb UV (Jaroenworaluck et al., 2006; Siddiquey et al., 2007; Labille et al., 2010). As a result, the production rates of n-TiO_2 are projected to increase to 200,000 tons per year to meet demand (Robichaud et al., 2009; Future Markets, 2011). With an increasing demand for production and inclusion in retail products, it is nearly certain that NP are reaching aquatic environments (Moore, 2006; Baun et al., 2008; Mueller and Nowack, 2008). Currently, environmental concentrations of n-TiO_2 in aquatic systems are estimated to be 16 μg/L (Mueller and Nowack, 2008), with environmental loads expected to be in the range of 2,000,000 to 6,000,000 tons over the next decade (Robichaud et al., 2009). Upon entering marine and freshwater ecosystems, however, anthropogenic nanomaterials will be diluted, thereby exposing organisms to likely sublethal concentrations of the particles (Boxall et al., 2007; Handy et al., 2008; Klaper et al., 2008). As a result, there is a growing need to evaluate possible sublethal effects of NP at environmentally relevant concentrations (De Coen and Janssen, 1997; De Coen et al., 2001; Klaper et al., 2008; Canesi et al., 2012).

The toxicity of different crystalline forms of n-TiO_2 has been demonstrated with several different species including *Oncorhynchus mykiss* (rainbow trout; Vevers and Jha, 2008), *Oryzias latipes* (Japanese medaka; Ma et al., 2012), *Arenicola marina* (lugworm; Galloway et al., 2010), and *Daphnia magna* (water flea; Adams et al., 2006; Hund-Rinke and Simon, 2006; Lovern and Klaper, 2006; Warheit et al., 2007; Ma et al., 2012). The adverse effects on bivalves also have been reported (see Canesi et al., 2012; Doyle et al., 2016 for reviews). For example, Libralato et al. (2013) reported that exposure of the larvae of the mussel *Mytilus galloprovincialis* produced malformations and adverse effects during development. Exposure of adult *M. galloprovincialis* to n-TiO_2 produced several effects upon digestive gland cells, including oxidative stress and decreased transcription of antioxidant and immune-related genes (Barmo et al., 2013). Deleterious effects upon bivalve hemocytes also have been reported. Exposure of *M. galloprovincialis* to n-TiO_2 *in vitro* resulted in sublethal effects such as changes in lysozyme activity,

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phagocytosis, production of reactive oxygen species (ROS) and nitric oxide, and upregulation of stress genes (Canesi et al., 2010a; Ciacci et al., 2012). Further, Barmo et al. (2013) reported that exposure of *M. galloprovincialis* to n-TiO_2 decreased lysosomal membrane stability, inhibited phagocytosis, and produced other effects upon hemocytes. In one of the few *in vitro* studies with the eastern oyster, *Crassostrea virginica*, Abbott-Chalew et al. (2012) noted reduced phagocytic activity of hemocytes when exposed to n-TiO_2. These reports suggest that contact with n-TiO_2 may present an unfamiliar challenge to the innate immune systems of bivalves as synthetic NP did not exist throughout the evolutionary process (Moore, 2006).

The effects of n-TiO_2 upon aquatic organisms can be attributed to two properties: 1) effects resulting from high surface area (nanoparticulate reactivity), and 2) effects resulting from photocatalytic activity and production of ROS. Most previous studies, however, do not distinguish between these two effects because researchers seldom reported the amount of UV to which the animals were exposed; although under laboratory conditions and typical indoor lighting, the amount of UV is likely low. The photocatalytic properties of TiO_2 in the presence of UV (290–400 nm), first described by Fujishima and Honda (1972), are well known (Wold, 1993; Konaka et al., 2001; Serpone et al., 2007; Markowska-Szczupaka et al., 2011). Upon exposure to UV, atoms at the surface of TiO_2 absorb the energy of photons and produce electron-hole pairs. In the presence of water and oxygen, the free electron and the electron-hole begin producing ROS (Ma et al., 2012). Typically, exposure of TiO_2 to UV results in the production of oxygen radicals such as singlet oxygen and superoxide anion ($^1O_2$, $^{\bullet}O_2^-$; Konaka et al., 2001), hydroxyl radicals ($^{\bullet}OH$; Uchino et al., 2002), and hydrogen peroxide (H_2O_2; Rao et al., 1980). Taking advantage of these properties, manufacturers have used TiO_2 in a wide variety of commercial and industrial applications, including self-cleaning building materials, air and water purification, (Fujishima et al., 2000; Bueza et al., 2007), antimicrobials (Kim et al., 2003; Adams et al., 2006; Li et al., 2006; Foster et al., 2011; Markowska-Szczupaka et al., 2011); degradation of organic pollutants (Chatterjee and Mahata, 2002); and as anti-cancer agents (Zhang and Sun, 2004; Rozhkova et al., 2009). In addition, TiO_2, particularly in the nanoparticulate form, has been used extensively in sunscreens as a barrier that acts to both absorb and reflect UVA and UVB radiation (Wolf et al., 2001; Serpone et al., 2007; Lin and Lin, 2011). Concerns have arisen, however, over the formation of ROS that potentially can harm adjacent cells (Dunford et al., 1997; Wolf et al., 2001). Consequently, several strategies have been used in the design of sunscreens, such as surface coatings, to remediate the effects of ROS in metal-oxide-based sunscreens (Ukmar et al., 2009; Jacobs et al., 2010).

Oxidative stress, stimulated by ROS, is known to produce a range of harmful effects upon cells. ROS can interact with cell membranes, proteins, and nucleic acids resulting in peroxidation of lipids, distortions of the conformation of proteins, disruption of DNA, interference with signal-transduction pathways, and modulation of gene transcription (Bueza et al., 2007). More specific photocatalytic effects of n-TiO_2 upon aquatic animals also have been reported (Haynes et al., 2017). When activated by UVA radiation, the deleterious effects of n-TiO_2 increases, causing low cell viability in goldfish skin cells (Reeves et al., 2008), genotoxicity in cells of rainbow trout (Vevers and Jha, 2008), and acute toxicity to the crustacean *Daphnia magna* (Amiano et al., 2012). Mortality also was reported for *D. magna*, larvae of the Japanese killifish, and several freshwater benthic invertebrates when exposed to both n-TiO_2 and UV light (Ma et al., 2012; Li et al., 2014a, 2014b).

The purpose of this study was to determine the effects of two different types of TiO_2 particles upon the hemocytes of the eastern oyster, *Crassostrea virginica*. As primary cells involved in the immune response of bivalves, hemocytes are motile cells responsible for the recognition, transport, and disposal of foreign materials through the process of phagocytosis, and the production of proteolytic enzymes and ROS (Fisher, 1986; Chu, 1988; Feng, 1988; Anderson, 1994; Winston et al., 1996). In this research, hemocytes were exposed, *in vitro*, to bulk TiO_2 particles (rutile and anatase), uncoated n-TiO_2 (anatase), and a surface-treated TiO_2 (rutile) nanocomposite at three different concentrations (0.1, 0.5, 1.0 mg/L) for 2 and 4 h. Each treatment combination was duplicated with one being held under dark conditions and the other held under environmentally-relevant light conditions. The particles included in this study are used as whiteners in food or as sunblock in personal-care products (e.g., Weir et al., 2012). To our knowledge, this study is the first to examine the effects of two widely-used forms of nano-titania upon the hemocytes of oysters, and to probe differences between nanoparticulate (dark) and photocatalytic (light) effects.

2. Materials and methods

2.1. Characterization and preparation of TiO_2 particles

Anatase NP were obtained from Meliorum Technologies ($\bar{x}$ particle size = 7.4 nm ± 2.5 SD; 99.9% pure; hereafter referred to as n-anatase). Surface-coated rutile NP, which are used in sunscreens (UV–Titan M212; https://www.in-cosmetics.com/_novadocuments/2843), were obtained from Sachtleben Pigments Oy ($\bar{x}$ particle size = 86 nm ± 32 SD; 93% rutile TiO_2, 6% Al_2O_3, and 1% glycerin; hereafter referred to as n-Titan). Both NP have been characterized previously with respect to size, surface area, composition, and zeta potential (Doyle et al., 2014). Bulk TiO_2 particles, both rutile ($\bar{x}$ particle size = 485 nm ± 89 SD; 99.9% pure) and anatase ($\bar{x}$ particle size = 454 nm ± 67 SD; 99.8% pure) crystalline forms, were obtained from Sigma Aldrich); hereafter referred to as bulk rutile and bulk anatase. Bulk TiO_2 particles were used to separate crystalline-form effects from particle-size effects. Bulk rutile particles are the best approximation of a control for n-Titan because a bulk rutile particle with an alumina oxide and glycerin coating does not exist. Hydrodynamic diameter of both bulk particles was determined using dynamic light scattering (Zetasizer Nano ZS, Malvern) following procedures modified from Doyle et al. (2014).

Stock suspensions of each particle type were prepared in MQ-water at a concentration of 250 mg/L. To prepare working suspensions, stocks were diluted to 10 mg/L with MQ-water, placed on a stir plate, and subjected to ultrasonication (Fisher Scientific FB-505) at 20% power (13.8 W) for 30 min (modified from Wang et al., 2009).

2.2. Maintenance and preparation of the animals

Oysters (ca. 4–6 cm in shell height) were maintained in lantern nets suspended from floating docks in Avery Point Harbor (Groton, CT USA; 18–20 °C, 28.5 ppt). Animals were allowed to acclimate for a minimum of one week prior to experimentation. All fouling organisms and sediment were cleaned from the shells, and a notch cut in the dorsal-posterior edge of the shell. A 22-gauge needle attached to a 5-mL syringe was inserted through the notch and into the adductor muscle, and 1.75 mL of hemolymph was withdrawn (adapted from Hégaret et al., 2003a). A few microliters were spotted onto a microscope slide, and the hemolymph was examined under a compound light microscope for the presence of hemocytes and absence of gut contents and debris. Shell fragments and similar debris, if present, were removed by passing the hemolymph through a 53-μm mesh. The hemolymph was then transferred to a 2.0-mL Eppendorf tube and stored on crushed ice to reduce clumping until it was used, always within one hour, in the assays described below. Four 350-μL aliquots were separated from the hemolymph collected from each animal, one aliquot for each particle concentration to which the cells were exposed (0 ppm, 0.1 ppm, 0.5 ppm, and 1.0 ppm). Extra hemolymph was removed from each animal to compensate for spills or errors in pipetting, and cells from multiple oysters were never mixed. Experiments were conducted on 252 oysters for the dark exposures and 240 oysters for the light exposures. The experimental matrix was as follows: 10 to 12 replicate oysters × 4 particle concentrations × 4 particle types × 3 cellular effects × 2 time intervals for a total of 1008 samples in the dark and 960 samples in the