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

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Highlights:

- The effects of titania nanoparticles on oyster (*Crassostrea virginica*) hemocytes was investigated
- Cells were exposed to anatase and UV-Titan M212 nanoparticles at 0.1, 0.5, and 1.0 mg/L
- Exposures included dark and light conditions for periods of two and four hours
- Few effects on mortality, phagocytosis, and reactive-oxygen-species production were detected
- Titania nanoparticles produced minimal effects on biomarkers examined following acute, *in vitro* exposures

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 hours under dark conditions, and UV-Titan for 2 hours under light conditions. Results demonstrate that TiO_2 particles (bulk or nanoscale) produce minimal effects on hemocyte biomarkers examined following acute*, in vitro* exposures.

Keywords: oyster, titania, nanoparticles, hemocytes, photoactivation, ROS

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 UV radiation (Matsui, 2005), and in cosmetics and sunscreens to absorb UV radiation (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, phagocytosis, production of 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 reactive oxygen species (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 ultra-violet radiation (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-Szczupak 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 (•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-Szczupak 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 (n-TiO_2), 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 (medaka), and several freshwater benthic invertebrates when exposed to both n-TiO2 and UV light (Ma et al., 2012; Li et al., 2014a,b).

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 hr. 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 scatter (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 Watts) for 30 minutes (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 x 4 particle concentrations x 4 particle types x 3 cellular effects x 2 time intervals for a total of 1008 samples in the dark and 960 samples in the light exposures.

2.3 Hemolymph Exposure to TiO_2 particles

Hemolymph aliquots of 350 µL were placed into either 5-mL flow cytometry tubes, for exposures in the dark, or into 48-well cell culture plates, for exposures in the light (Falcon, BD BioSciences). One of the aforementioned particle types (bulk anatase, bulk rutile, n-anatase, or n-Titan) was then added at a nominal concentration of 0.1, 0.5, or 1.0 mg/L. The actual amount of hemolymph added to each tube or well was slightly different for each concentration so that the total volume of liquid in each was the same. No filtered seawater or phosphate buffer was used to dilute the hemolymph (Hégaret et al., 2003a). Given the small volume of working suspension added to each well, ionic strength of the hemolymph was only minimally effected (e.g., at 1.0 mg/L hemolymph salinity was 35.7 ppt). Cells from each oyster were exposed to all concentrations of one particle type, making these experiments a repeated measures design. The culture tubes and plates were divided into two sets, with one set being exposed for 2 hr and the second set being exposed for 4 hr at a temperature of ca. 20° C. Controls consisted of only hemocytes (no TiO_2 particles) incubated in the dark or light for 2- and 4-hr durations.

2.4 Exposure to Simulated Solar Radiation

Solar-simulation lamps (150W Solarlux®, Class B metal halide full spectrum lamps, EYE Applied Optics) were used to simulate the spectral output of natural sunlight found below the surface of the water (ca. 350-800 nm). A Jaz Spectrometer (Ocean Optics, Inc, ca. 320-1000 nm) was used to examine the spectral power curve of the solar lamps, and the distance between the

48-well plates and lamps was adjusted to 55 cm to match the modeled irradiance (Hydrolight, Sequioa Scientific) found at mid-latitude marine waters during summer months at a depth of ca. 0.6 m below the surface (Haynes et al., 2017). Total irradiance within the photoactivation range of TiO_2 was ca. 3 mW/cm^2 (350-420 nm). Each circular well was 1.1 cm in diameter giving an irradiance level of 2.85 mW per well. An LS-1-Cal lamp (NIST traceable) was used to calibrate the spectrometer. The 48-well plates (without lids) were floated in a water bath and held in position under the lamps by small racks. Ice was added to the water bath at intervals of 10 to 15 minutes throughout the 2- and 4-hour exposures, and temperature was monitored continuously to ensure it was maintained at ca. 20° C.

2.5 Flow Cytometry Assays

The effects of nanoparticle exposure on oyster hemocytes were assessed using the cellular viability, phagocytosis, and ROS assays. All samples were analyzed using a FACScan flow cytometer (Becton Dickinson Biosciences; San Jose, CA), and analyzed with the WinMDI 2.8 software package.

2.5.1 Cellular Viability Assay

The cellular viability assay measured the number of living and dead hemocytes present following exposure to the TiO_2 particles. Briefly, 300 µL of 0.2-µm filtered seawater was combined with 4 µL of SYBR green, 4 µL of propidium iodide, and 100 µL of the hemocyte-TiO_2 suspension. The samples were then incubated for one hour at room temperature (ca. 20° C) in the dark. Living hemocytes were identified by characteristic SYBR green (FL1) fluorescence, and dead hemocytes were identified from propidium-iodide (FL3) fluorescence (modified from Hégaret et al., 2003a). Data are presented as mortality which is the fraction of cells identified as dead, and calculated in the following manner:

% Dead Hemocytes = (# propidium-iodide-fluorescent cells / total hemocyte count) x 100

2.5.2 Phagocytosis Assay

The phagocytosis assay measured the percentage of highly-phagocytic cells present in the hemolymph following exposure to TiO_2 particles. Briefly, 150 µL of 0.2-µm filtered seawater

was combined with a 30-µL suspension of fluorescent microspheres (1 x 10^7 beads/mL; Polysciences Fluoresbrite YG Microspheres, 2 µm) and 150 µL of the hemocyte-TiO_2 suspension. The samples were incubated for two hours at room temperature (ca. 20° C) in the dark. To eliminate the possibility of false positives resulting from the adherence of fluorescent beads to the cell membrane, the presence of three or more fluorescent beads in each hemocyte was used as the criterion to categorize a hemocyte as "highly-phagocytic" (modified from Hégaret et al., 2003b).

2.5.3 Reactive Oxygen Species (ROS) Assay

A fluorescent assay was used to measure the amount of ROS production in the hemocytes following exposure to TiO_2 particles. Briefly, 300 µL of 0.2-µm filtered seawater was combined with 4 µL of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and 100 µL of the hemocyte-TiO_2 suspension. The samples were incubated for two hours at room temperature (ca. 20° C) in the dark. The production of ROS in the hemocytes was measured by detecting the intensity of fluorescence of 2′,7′-dichlorofluorescein (DCF; modified from Hégaret et al., 2003b).

2.6 Statistics

Given the number of assays conducted in this study, it was not possible to collect and assay all oysters at one time (total of 492 animals). Therefore, data obtained from flow-cytometry assays were standardized to their respective mean control values, i.e. oysters sampled at the same time but hemocytes not exposed to TiO_2 particles, to account for temporal differences in biomarkers as a result of natural variations in water temperature, food supply, and other environmental factors. Standardized values were used for statistical analyses described below.

A mixed-model, two-way repeated measures analysis of variance (ANOVA) was used to compare the within-subject random effects (particle concentrations) and between-subject fixed effects (particle type; i.e., bulk vs NP) upon the mortality, phagocytic activity, and ROS production of oyster hemocytes. For each dependent variable, separate analyses were completed for data within each exposure time and particle type (e.g., bulk anatase vs n-anatase). Following ANOVA, a Tukey's HSD post-hoc test was applied to examine differences between levels of the independent variables and interaction effects. A Dunnett's post-hoc test was used to compare the viability, phagocytic activity, and ROS production of oyster hemocytes exposed to TiO_2 particles

to those of control (unexposed) hemocytes. To further probe the effects of particle type, concentration, and exposure time upon the three response variables, a multivariate analysis of variance (MANOVA) was performed. Prior to statistical analyses, data were assessed for homoscedasticity and normality using an Equality-of-Variance test and Kurtosis test, respectively. Data sets that did not meet the underlying assumptions were transformed by means of a natural-log transformation. In all tests, an alpha level of 0.05 was used.

3. Results

3.1 Mortality

Hemocyte mortality at the end of all experiments was low. For the controls (no TiO_2 particles), the percentage of dead hemocytes observed under dark and light exposures ranged from 0.2 to 0.6% and 0.7 to 1.6%, respectively. For treatments with TiO_2 particles, the percentage of dead hemocytes found under dark and light exposures ranged from 0.2 to 0.9% and 0.6% to 2.1%, respectively. With regard to standardize data under dark conditions, there were no significant effects of particle concentration or type (bulk, NP) upon mortality of oyster hemocytes after either 2 or 4 hr of exposure (ANOVA, $p>0.05$, Table 1A). No significant interaction effects were found between these two variables. Under light conditions, hemocytes exposed to anatase particles for 2 hr and rutile particles for 4 hr demonstrated a small but significant increase in mortality with increasing concentration (Tukey's HSD, $p<0.05$, Table 1B). No difference in mortality was found between the bulk particles and NP, and the increase in mortality of hemocytes was driven largely by effects of the bulk particles. No significant interaction effects were found between particle type and concentration ANOVA, $p>0.05$).

3.2 Phagocytic Activity

When hemocytes were exposed to anatase particles for 2 hr in the dark, there was a significant effect of concentration (ANOVA, $p<0.05$, Table 2A, Fig. 1A), but no effect of particle type (bulk, NP) upon phagocytic activity. There were also no significant interaction effects between these two variables. Pairwise comparisons showed that the phagocytic activity of hemocytes exposed to anatase particles at 1.0 mg/L was significantly higher than that of hemocytes exposed to anatase particles at 0.1 mg/L (Tukey's HSD, $p<0.05$, Fig. 1A). No other significant effects were found. After 4 hr of exposure to anatase particles, there were significant

effects of concentration and particle type on phagocytic activity, and a significant interaction effect between these two variables (ANOVA, $p<0.05$, Table 2A, Fig. 1B). Pairwise comparisons revealed significant differences in phagocytosis between hemocytes exposed to bulk anatase and those exposed to n-anatase at all three concentrations (Tukey's HSD, $p<0.05$, Table 2A, Fig. 1B). Phagocytic activity of hemocytes exposed to n-anatase increased in a dose-dependent manner, and at 0.5 and 1.0 mg/L was significantly higher than the phagocytic activity of control hemocytes (Dunnett's, $p < 0.05$; Fig. 1B).

When hemocytes were exposed to rutile particles for 2 hr in the dark, phagocytic activity was significantly affected by concentration (ANOVA, $p<0.05$, Table 2A, Fig. 1C), with a slightly but significantly higher activity at 1.0 mg/L compared to the two lower concentrations (Tukey's HSD, $p<0.05$, Table 2A, Fig. 1C). There were no significant effects of particle type (bulk, NP) or significant interaction effects upon phagocytosis. After 4 hr of exposure to rutile particles, no significant concentration, particle type, or interactive effects were found (ANOVA, $p>0.05$, Table 2A, Fig. 1D).

The significant effects of anatase and rutile particles upon hemocyte phagocytosis under dark conditions largely diminished under light conditions. When hemocytes were exposed to anatase particles for 2 or 4 hr, no significant concentration, particle type, or interactive effects were found (ANOVA, $p>0.05$, Table 2B, Fig. 2A, B). After 2 hr of exposure to rutile particles, only particle type (bulk, NP) significantly affected phagocytic activity of hemocytes (ANOVA, $p<0.05$, Table 2B, Fig. 2C). Pairwise comparisons demonstrated that hemocytes exposed to n-Titan had significantly lower phagocytic activity than hemocytes exposed to bulk rutile particles at concentrations of 0.1 and 1.0 mg/L (Tukey's HSD, $p<0.05$, Table 2B, Fig. 2C). After 4 hr of exposure to rutile particles, however, no significant concentration, particle type, or interactive effects were found (ANOVA, $p>0.05$, Table 2B, Fig. 2D).

3.3 Production of ROS

When hemocytes were exposed to anatase particles for 2 or 4 hr in the dark, there were no significant concentration, particle type, or interactive effects upon ROS production (ANOVA, $p>0.05$, Table 3A, Fig. 3A, B). After 2 hr of exposure to rutile particles, there was a significant effect of concentration (ANOVA, $p<0.05$, Table 3A, Fig. 3C), but no effect of particle type (bulk, NP) on ROS production by hemocytes. No significant interaction effects were found

between these two variables. Pairwise comparisons found that ROS production by hemocytes exposed to rutile particles (bulk & n-Titan) at 1.0 mg/L was significantly different than that of hemocytes exposed to the same particles at 0.1 and 0.5 mg/L (Tukey's HSD, $p<0.05$, Table 3A, Fig. 3C). After 4 hr of exposure to rutile particles, however, no significant concentration, particle type, or interactive effects were found (ANOVA, $p>0.05$, Table 3A, Fig. 3D).

Under light conditions, there was a significant effect of particle type upon ROS production by hemocytes (ANOVA, $p<0.05$, Table 3B, Fig. 4A), but no effects of concentration or interactions between the two variables. Pairwise comparisons showed that hemocytes exposed to bulk anatase had significantly lower ROS production compared to hemocytes exposed to n-anatase at a concentration of 0.5 and 1.0 mg/L (Tukey's HSD, $p<0.05$, Table 3B, Fig. 4A). After 4 hr of exposure to anatase particles, however, no significant concentration, particle type, or interactive effects were found (ANOVA, $p>0.05$, Table 3A, Fig. 4B). Similarly, when hemocytes were exposed to rutile particles for 2 or 4 hr, there were no significant concentration, particle-type, or interactive effects on ROS production (ANOVA, $p>0.05$, Table 3B, Fig. 4C, D).

3.4 Multivariate Analysis of Mortality, Phagocytic Activity, and ROS Production

Multivariate analysis largely confirmed the results of the univariate, mixed-model ANOVA. Significant differences between bulk and NP treatments occurred only for phagocytic activity of hemocytes, and only when exposed to anatase particles at 1.0 mg/L in the dark, and rutile particles at 0.1 and 0.5 mg/L in the light (Table 4). At 0.5 mg/L there was also a significant effect of exposure time (2 vs 4 hr) upon phagocytosis. One other significant effect was detected; for ROS production, there was a significant interaction effect between particle concentration and exposure time when hemoctyes were exposed to anatase particles at 0.5 mg/L in the light (Table 4).

4. Discussion

Results of this *in vitro* study indicate that exposure of oyster hemocytes to bulk and n-TiO_2 for up to 4 hr under dark and environmentally-relevant light conditions produce minimal apparent effects upon cell mortality, phagocytosis, and ROS production. Even at 1.0 mg/L, a concentration at the upper end of environmental relevancy, few effects were observed after 4 hr of exposure. Surprisingly, photoactivation of the TiO_2 did not increase mortality or sub-lethal

effects upon the hemocytes. Although there were few significant effects overall, notable exceptions were apparent.

Mortality of oyster hemocytes was least affected by experimental treatments, with only a slight but significant increase when cells were exposed to anatase (2 hr) and rutile (4 hr) particles under light conditions at 1.0 mg/L compared to lower concentrations. In both cases, no significant differences were detected between the effects produced by bulk particles and those produced by NP, demonstrating that no nano-effect occurred. Additionally, actual mean mortality of hemocytes (not standardized) in these treatments increased to a maximum of only 2.1% (± 1.9%, SD), which was the highest mortality observed in any treatment. The mortality of hemocytes observed in our experiments was consistent with, or lower than that reported previously (Anderson, 1994; Anderson et al., 1994; Hégaret et al., 2011; Croxton et al., 2012).

Production of ROS by oyster hemocytes was also little affected by TiO_2 particles or by environmentally-relevant light exposure. Although under dark conditions, there was a slight but significant increase in ROS when cells were exposed for 2 hr to rutile particles at 1.0 mg/L compared to lower concentrations, no significant differences were detected between the effects produced by bulk particles and those produced by NP. Again, these data demonstrate that no nano-effect occurred. Under light conditions, there was a strong and significant decrease in ROS production by hemocytes exposed for 2 hr to bulk anatase particles compared to n-anatase at both 0.5 and 1.0 mg/L. Surprisingly, this reduction in ROS production was not observed when hemocytes were exposed to the same treatments for 4 hr, which led to the significant interaction effect detected by the MANOVA between particle type and time of exposure in the 0.5 mg/L treatment (Table 4). These findings indicate that although TiO_2 particles produce ROS when photoactivated by UV light (Ma et al., 2012), they did not stimulate ROS production by oyster hemocytes. Several studies have previously reported either no change in the level of ROS production in bivalve hemocytes exposed to xenobiotics (Coles et al., 1995; Croxton et al., 2012), or a significant increase in ROS production following hemocyte exposure to toxicants (Canesi et al., 2008; Canesi et al., 2010a; Ciacci et al., 2012). Taken as a whole, these findings suggest the production of ROS by bivalve hemocytes exposed to xenobiotics may be dependent upon several interrelated factors such as bivalve species, time of exposure, the specific pollutant encountered, and the concentration of the pollutant.

Phagocytic activity was the cellular function most affected by TiO_2 particles. Under dark conditions, a significant increase in phagocytosis was observed in response to an increase in concentration of anatase particles (2 hr exposure) and rutile particles (2 hr exposure). In both cases, however, no significant differences were detected between the effects produced by bulk particles and those produced by NP. A nano-effect was observed when hemocytes were exposed to n-anatase for 4 hr. At all three concentrations, phagocytic activity was significantly higher when hemocytes were exposed to NP compared to the bulk anatase particles. Additionally, when exposed to n-anatase at concentrations of 0.5 and 1.0 mg/L, hemocytes demonstrated significantly higher phagocytic activity compared to the control hemocytes. The dose-dependent nature of the response could be attributed to a greater probability of encounter between the hemocytes and the TiO_2 particles as a result of the higher concentrations. The strong phagocytic response of hemocytes to n-anatase, however, was not observed with n-Titan (rutile). This finding may be a result of the different sizes and surface areas of the two NP. The n-anatase used in this study had a mean size of 7.4 nm (± 2.5 nm, SD) and a BET surface area of 121. 2 m^2/g (± 0.6 m^2/g, SD); whereas, the n-Titan had a mean size of 86.0 nm (± 32.0 nm, SD) and a BET surface area of 62.5 m^2/g (± 1.2 m^2/g, SD; Doyle et al., 2014). Given the much higher surface area, n-anatase would be more reactive than n-Titan under dark conditions. As such, the increase in phagocytosis in response to n-anatase could indicate induction of a protective immune response to foreign particles. Surprisingly, the response to n-anatase was not observed under the light conditions. Additionally, when exposed to light and n-Titan for 2 hr, hemocytes demonstrated a significant decrease in phagocytosis compared to the bulk rutile treatment at both 0.1 and 1.0 mg/L. This decrease, however, was not significantly different than the controls and was likely caused by the slight increase in phagocytosis produced by the bulk rutile particles. Hemocytes seem to recover from this effect after 4 hr of exposure.

In vitro assays utilizing cells of aquatic animals have been used in a range of studies as a way to rapidly assess the sub-lethal effects of natural and xenobiotic materials (Larson et al., 1989; Fisher et al., 1990; Anderson et al., 1994; Winston et al., 1996; Sauvé et al., 2002; Hégaret and Wikfors, 2005; Hégaret et al., 2007; Reeves et al., 2008; Vevers and Jha, 2008; Hégaret et al., 2011). With respect to n-TiO_2, various deleterious effects have been reported under both light and dark conditions, including effects on goldfish skin cells (*Carassius auratus*; Reeves et al., 2008), rainbow trout gonadal tissue (*Oncorhynchus mykiss*; Vevers and Jha, 2008), mussel

hemocytes (*M. galloprovincialis*; Canesi et al., 2010a; Ciacci et al., 2012), and oyster hemocytes (*Crassostrea virginica*; Abbott-Chalew et al., 2012) and digestive-gland tissues (Johnson et al., 2015). In most cases, deleterious effects were observed at n-TiO_2 concentrations above 1.0 mg/L (Vevers and Jha, 2008; Canesi et al., 2010a; Ciacci et al., 2012). Other studies, however, have reported no effect on cells at high n-TiO_2 concentrations. For example, Reeves et al. (2008) observed no toxic effects on goldfish cells even at concentrations exceeding 100 mg/L, unless the exposure was in combination with UVA light. Wang et al. (2014) exposed hemocytes of the mussel, *Perna viridis*, to n-TiO_2 (P25) at concentrations of 2.5 and 10 mg/L and found no significant effects on hemocyte mortality, phagocytosis, ROS production or other immune variables after 2 hr. One factor that might influence effects measured *in vitro* at high NP concentrations, is particle agglomeration. As concentration increases, more agglomerates form, which could effectively reduce particle interactions with cells resulting in greater effects at lower concentrations. Additionally, there may be species-specific sensitivity to various forms of n-TiO_2. Such factors could explain the mixed results obtained in previous *in vitro* studies outlined above.

Comparing results of *in vitro* studies within a species at environmentally relevant concentrations of n-TiO_2 ($\leq$ 1.0 mg/L) also demonstrate inconsistencies. In a pilot study, Abbott-Chalew et al. (2012) exposed hemocytes of the oyster, *C. virginica*, to n-TiO_2 (form not indicated) at concentrations of 1 to 400 µg/L for periods of 15 to 120 min. They found significant reduction in the phagocytosis of *Cryptosporidium parvum* oocysts at all concentrations except 10 µg/L. Johnson et al. (2015) reported significant lysosomal destabilization of digestive-gland tissue isolated from the oyster, *C. virginica*, when exposed to n-TiO_2 (form not indicated) at 50, 500 and 5000 µg/L for 24 hr under dark conditions, but no effect at 5 µg/L. In the current research, minimal effects were observed when oyster hemocytes where exposed to n-anatase and n-Titan at concentrations of 100, 500, and 1000 µg/L for 2 and 4 hr. In order to reduce between-study variation, future *in vitro* work should strive to standardize particle preparation, exposure time, and the crystalline form of n-TiO_2 to which cells are exposed.

One shortcoming of the studies cited above, is that few exposed cells to bulk TiO_2 (i.e., > 100 nm). Therefore, the reported effects could be a result of the titania itself (material effect) and not a result of size (NP effect). In the current research, bulk titania was used to examine a materials

effect, and in one treatment (2 hr, anatase, light exposure), bulk anatase significantly decreased ROS production by hemocytes at 0.5 and 1.0 mg/L compared to n-anatase. Additionally, TiO_2 concentrations and light were held to environmentally-relevant levels, and exposure time was held to a maximum of 4 hr so as to reduce stress on hemocytes which show increasing mortality after ca. 6 hr *ex vivo* (Wikfors, unpublished hemocyte incubation time trials).

In vivo experiments have been used as a way to increase cellular exposure times, and these studies have demonstrated more consistent deleterious effects of n-TiO_2. Barmo et al., (2013) exposed the mussel, *M. galloprovincialis*, to n-TiO_2 (P25) at concentrations as low as 1.0 µg/L for 96 hr and reported significant negative effects on digestive gland cells and hemocytes. Effects of higher concentrations of n-TiO_2 on cells of whole animals exposed *in vivo* have also been reported (Canesi et al., 2010b; Canesi et al., 2012; Couleau et al., 2012; D'Agata et al., 2014; Wang et al., 2014; Johnson et al., 2015). Similar to work conducted *in vitro*, few of the studies cited above exposed cells to bulk TiO_2 (i.e., > 100 nm). Therefore, an actual nano effect is ambiguous. One exception is the study by D'Agata et al. (2014), who found that bulk TiO_2 (ca. 151 nm) at 10 mg/L elicited the same effects upon mussel hemocytes and stronger effects upon transcriptional expression of mitochondrial genes than n-TiO_2 (P25). Given these results, future studies should test for the effects of bulk TiO_2 to separate a materials effect from a NP effect.

5. Conclusions

This study examined the effects of acute exposure to bulk and nanoparticulate TiO_2 upon mortality, phagocytosis, and ROS production of oyster hemocytes. Overall the results indicate minimal apparent effects upon these three immune-function biomarkers. In particular, hemocyte mortality and ROS production remained stable following exposure to bulk and nanoparticulate TiO_2. Hemocyte phagocytosis was significantly changed under some conditions, but these effects were not consistent with regards to exposure time (2 vs 4 hr) or light conditions (dark vs light exposure). Result of the current study with the oyster, *C. virginica*, support the conclusions of Wang et al. (2014) for the mussel, *P. viridis*. Generally, the response of hemocytes to n-TiO_2 *in vitro* do not necessarily represent the possible *in vivo* effects observed with longer exposure time upon the whole organism. Even when photoactivated, the effects of short-term exposure to n-TiO_2 upon oyster hemocytes are minimal. Future studies with nanomaterials should continue

focusing on chronic, *in vivo* exposures and consider other factors that cause stress in bivalves, activate the immune system, and work synergistically with NP such as changes in temperature, pH, food availability, parasitic infection, and other xenobiotics (e.g., Canesi et al., 2014, Hu et al., 2017). Bulk material should always be tested along with the chosen nanomaterial so as to differentiate a material effect from a size (nano) effect. Experiments that adhere to environmentally-relevant concentrations and conditions will provide a more accurate assessment of the actual effects of NP upon physiological processes of bivalves.

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Table 1. Mortality of hemocytes exposed to three concentrations (within-subject effects) of two different particle sizes (bulk vs NP, between-subject effects), for two different exposure periods under both dark (A) and light (B) regimes. Statistical comparisons of data within each exposure time were conducted using a mixed-model analysis of variance. Means designated with different capital letters indicate significant within-subject differences ($p<0.05$, within an exposure time), whereas means designated with different lowercase letters indicate significant between-subject differences ($p<0.05$, within an exposure time and within a concentration). Means without letter designations indicate that there were no significant within- or between-subject effects ($p>0.05$). Data were standardized to their respective mean control value and are presented as mean ratios (index values) ± standard error (SE). Ten to twelve replicates were used for each concentration within each exposure time and within each particle comparison.

A. Dark Exposure

1. Bulk Anatase vs n-Anatase

Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.15 (0.18)	Bulk Anatase n-Anatase	0.91 (0.19) 1.44 (0.30)
	0.5	1.16 (0.14)	Bulk Anatase n-Anatase	1.04 (0.21) 1.30 (0.16)
	1.0	1.32 (0.16)	Bulk Anatase n-Anatase	1.33 (0.21) 1.30 (0.26)
4	0.1	1.46 (0.24)	Bulk Anatase n-Anatase	1.52 (0.40) 1.39 (0.26)
	0.5	1.25 (0.18)	Bulk Anatase n-Anatase	1.21 (0.30) 1.30 (0.20)
	1.0	1.54 (0.29)	Bulk Anatase n-Anatase	1.33 (0.39) 1.79 (0.43)

2. Bulk Rutile vs n-Titan

Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.12 (0.17)	Bulk Rutile n-Titan	0.95 (0.19) 1.29 (0.27)
	0.5	1.17 (0.15)	Bulk Rutile n-Titan	1.03 (0.12) 1.31 (0.28)
	1.0	1.28 (0.17)	Bulk Rutile n-Titan	1.17 (0.19) 1.38 (0.28)
4	0.1	1.07 (0.17)	Bulk Rutile n-Titan	1.02 (0.14) 1.11 (0.32)
	0.5	0.97 (0.14)	Bulk Rutile n-Titan	1.01 (0.20) 0.92 (0.22)
	1.0	0.90 (0.15)	Bulk Rutile n-Titan	1.03 (0.27) 0.76 (0.12)

B. Light Exposure				
1. Bulk Anatase vs n-Anatase				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	0.88 (0.10)^{A}	Bulk Anatase n-Anatase	0.81 (0.12) 0.95 (0.17)
	0.5	0.99 (0.11)^{AB}	Bulk Anatase n-Anatase	1.11 (0.16) 0.88 (0.15)
	1.0	1.22 (0.16)^{B}	Bulk Anatase n-Anatase	1.38 (0.30) 1.07 (0.11)
4	0.1	1.03 (0.21)	Bulk Anatase n-Anatase	1.10 (0.31) 0.96 (0.30)
	0.5	1.05 (0.18)	Bulk Anatase n-Anatase	0.87 (0.19) 1.24 (0.31)
	1.0	1.32 (0.24)	Bulk Anatase n-Anatase	1.21 (0.27) 1.44 (0.41)
2. Bulk Rutile vs n-Titan				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.13 (0.17)	Bulk Rutile n-Titan	1.26 (0.19) 1.00 (0.28)
	0.5	1.13 (0.17)	Bulk Rutile n-Titan	1.31 (0.14) 0.95 (0.30)
	1.0	1.16 (0.20)	Bulk Rutile n-Titan	1.04 (0.14) 1.27 (0.38)
4	0.1	0.97 (0.14)^{A}	Bulk Rutile n-Titan	0.86 (0.21) 1.08 (0.20)
	0.5	1.19 (0.21)^{AB}	Bulk Rutile n-Titan	1.35 (0.39) 1.03 (0.17)
	1.0	1.23 (0.15)^{B}	Bulk Rutile n-Titan	1.21 (0.21) 1.24 (0.23)

Table 2. Phagocytosis activity of hemocytes exposed to three concentrations (within-subject effects) of two different particle sizes (bulk vs NP, between-subject effects), for two different exposure periods under both dark (A) and light (B) regimes. See Table 1 for explanation of statistical comparisons. Data were standardized to their respective mean control value and are presented as mean ratios (index values) ± standard error (SE). Ten to twelve replicates were used for each particle-type mean within each level of concentration.

A. Dark Exposure				
1. Bulk Anatase vs n-Anatase				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.07 (0.11)^{A}	Bulk Anatase n-Anatase	1.01 (0.13) 1.14 (0.18)
	0.5	1.14 (0.12)^{AB}	Bulk Anatase n-Anatase	1.09 (0.14) 1.21 (0.20)
	1.0	1.21 (0.13)^{B}	Bulk Anatase n-Anatase	1.14 (0.15) 1.30 (0.24)
4	0.1	1.18 (0.18)^{A}	Bulk Anatase n-Anatase	0.91 (0.20)^{a} 1.52 (0.28)^{b}
	0.5	1.31 (0.21)^{B}	Bulk Anatase n-Anatase	0.85 (0.16)^{a} 1.86 (0.36)^{b}
	1.0	1.57 (0.20)^{C}	Bulk Anatase n-Anatase	1.04 (0.21)^{a} 2.20 (0.27)^{b}
2. Bulk Rutile vs n-Titan				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.05 (0.15)^{A}	Bulk Rutile n-Titan	1.00 (0.21) 1.11 (0.23)
	0.5	0.94 (0.12)^{A}	Bulk Rutile n-Titan	0.85 (0.17) 1.04 (0.18)
	1.0	1.27 (0.15)^{B}	Bulk Rutile n-Titan	1.35 (0.21) 1.19 (0.22)
4	0.1	0.96 (0.13)	Bulk Rutile n-Titan	1.04 (0.24) 0.87 (0.09)
	0.5	1.11 (0.14)	Bulk Rutile n-Titan	1.08 (0.27) 1.14 (0.12)
	1.0	1.12 (0.13)	Bulk Rutile n-Titan	1.11 (0.21) 1.13 (0.16)

B. Light Exposure				
1. Bulk Anatase vs n-Anatase				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.11 (0.08)	Bulk Anatase n-Anatase	1.15 (0.12) 1.08 (0.12)
	0.5	1.11 (0.09)	Bulk Anatase n-Anatase	1.11 (0.14) 1.11 (0.12)
	1.0	0.97 (0.08)	Bulk Anatase n-Anatase	1.07 (0.13) 0.87 (0.09)
4	0.1	1.07 (0.10)	Bulk Anatase n-Anatase	1.24 (0.17) 0.89 (0.10)
	0.5	1.19 (0.12)	Bulk Anatase n-Anatase	1.35 (0.19) 1.02 (0.13)
	1.0	1.23 (0.16)	Bulk Anatase n-Anatase	1.34 (0.22) 1.12 (0.24)
2. Bulk Rutile vs n-Titan				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	0.99 (0.16)	Bulk Rutile n-Titan	1.29 (0.23)[a] 0.69 (0.18)[b]
	0.5	0.80 (0.12)	Bulk Rutile n-Titan	1.03 (0.18) 0.58 (0.11)
	1.0	0.99 (0.14)	Bulk Rutile n-Titan	1.25 (0.18)[a] 0.73 (0.19)[b]
4	0.1	1.09 (0.12)	Bulk Rutile n-Titan	1.22 (0.15) 0.95 (0.18)
	0.5	1.15 (0.10)	Bulk Rutile n-Titan	1.28 (0.15) 1.03 (0.14)
	1.0	1.12 (0.11)	Bulk Rutile n-Titan	1.18 (0.12) 1.06 (0.18)

Table 3. Reactive oxygen species production by hemocytes exposed to three concentrations (within-subject effects) of two different particle sizes (bulk vs NP, between-subject effects), for two different exposure periods under both dark (A) and light (B) regimes. See Table 1 for explanation of statistical comparisons. Data were standardized to their respective mean control value and are presented as mean ratios (index values) ± standard error (SE). Ten to twelve replicates were used for each particle-type mean within each level of concentration.

A. Dark Exposure				
1. Bulk Anatase vs n-Anatase				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	0.96 (0.08)	Bulk Anatase n-Anatase	0.90 (0.14) 1.02 (0.09)
	0.5	1.02 (0.10)	Bulk Anatase n-Anatase	1.03 (0.17) 1.00 (0.09)
	1.0	0.97 (0.08)	Bulk Anatase n-Anatase	0.95 (0.13) 1.00 (0.08)
4	0.1	0.94 (0.07)	Bulk Anatase n-Anatase	0.87 (0.11) 1.01 (0.06)
	0.5	0.98 (0.08)	Bulk Anatase n-Anatase	0.95 (0.15) 1.02 (0.06)
	1.0	0.97 (0.07)	Bulk Anatase n-Anatase	0.90 (0.12) 1.06 (0.07)
2. Bulk Rutile vs n-Titan				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.00 (0.09)^{A}	Bulk Rutile n-Titan	1.06 (0.15) 0.93 (0.10)
	0.5	1.10 (0.08)^{A}	Bulk Rutile n-Titan	1.15 (0.11) 1.05 (0.11)
	1.0	1.24 (0.09)^{B}	Bulk Rutile n-Titan	1.22 (0.10) 1.25 (0.15)
4	0.1	1.11 (0.12)	Bulk Rutile n-Titan	1.07 (0.12) 1.16 (0.22)
	0.5	1.09 (0.13)	Bulk Rutile n-Titan	0.99 (0.11) 1.19 (0.23)
	1.0	1.14 (0.12)	Bulk Rutile n-Titan	1.04 (0.12) 1.24 (0.22)

B. Light Exposure				
1. Bulk Anatase vs n-Anatase				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	1.07 (0.17)	Bulk Anatase n-Anatase	0.82 (0.21) 1.32 (0.25)
	0.5	0.83 (0.18)	Bulk Anatase n-Anatase	0.34 (0.13)[a] 1.31 (0.25)[b]
	1.0	0.80 (0.13)	Bulk Anatase n-Anatase	0.54 (0.13)[a] 1.10 (0.19)[b]
4	0.1	1.04 (0.09)	Bulk Anatase n-Anatase	1.09 (0.14) 1.00 (0.11)
	0.5	0.94 (0.09)	Bulk Anatase n-Anatase	1.04 (0.13) 0.83 (0.13)
	1.0	0.95 (0.09)	Bulk Anatase n-Anatase	0.99 (0.14) 0.91 (0.13)
2. Bulk Rutile vs n-Titan				
Exposure Time (hours)	Within-subject Effects		Between-subject Effects	
	Concentration (mg/L)	Index Value [Mean (± SE)]	Particle Type	Index Value [Mean (± SE)]
2	0.1	0.96 (0.13)	Bulk Rutile n-Titan	0.90 (0.19) 1.03 (0.18)
	0.5	1.00 (0.13)	Bulk Rutile n-Titan	0.97 (0.18) 1.03 (0.20)
	1.0	1.03 (0.12)	Bulk Rutile n-Titan	0.99 (0.21) 1.07 (0.15)
4	0.1	0.95 (0.11)	Bulk Rutile n-Titan	0.77 (0.12) 1.13 (0.18)
	0.5	0.89 (0.10)	Bulk Rutile n-Titan	0.75 (0.14) 1.03 (0.14)
	1.0	0.80 (0.12)	Bulk Rutile n-Titan	0.73 (0.21) 0.86 (0.13)

Table 4. Summary of two-way MANOVA results showing effects of two independent variables (particle type, time) on the three dependent variables (cell mortality, phagocytosis, ROS production) for each level of particle concentration (mg/L). Data were standardized to their respective mean control value before analysis. In cases where significant model effects were detected, the most effected dependent variable is listed (n.s. = not significant). A) Anatase particles (bulk vs NP); B) Bulk rutile vs n-Titan.

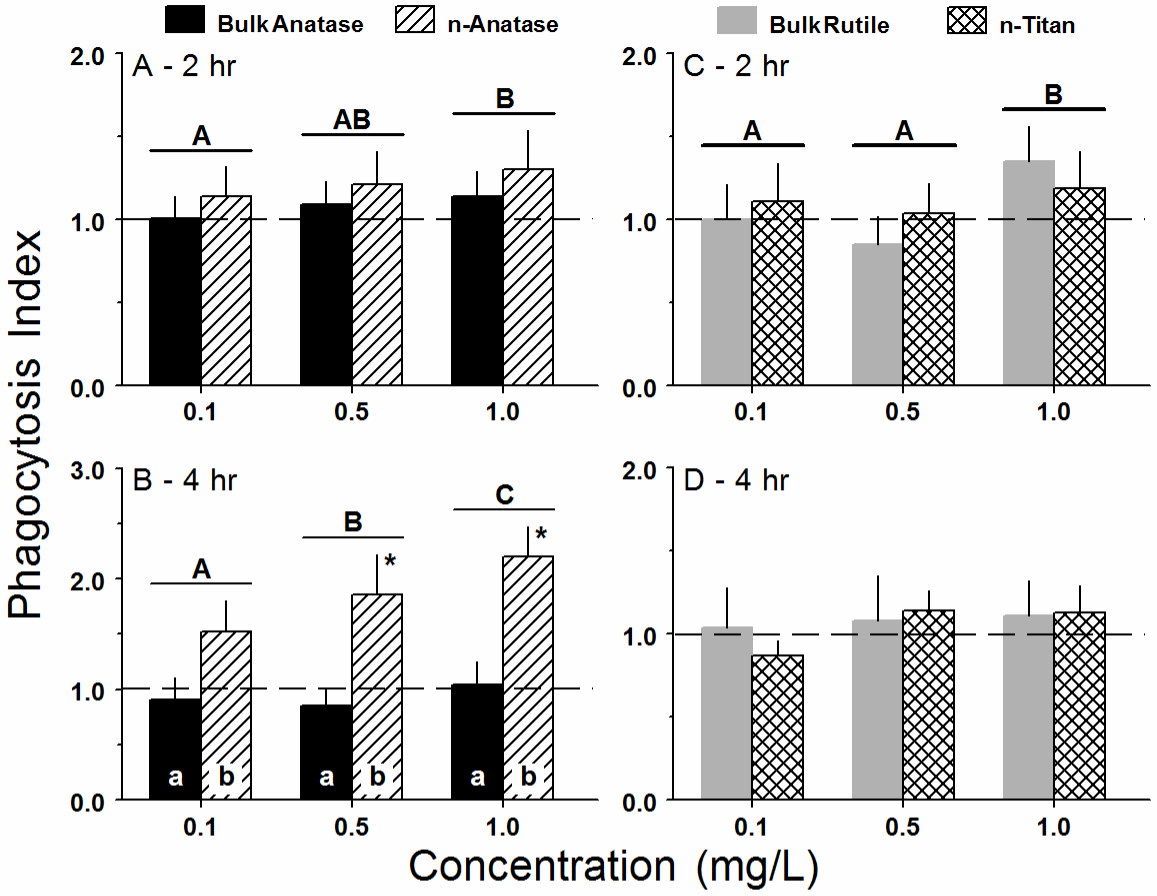
A) Anatase	Independent variables			Most effected dependent variable
Regime Concentration (mg/L)	Particle (bulk vs NP)	Time (2 vs 4 hr)	Interaction	
Dark				
0.1	n.s.	n.s.	n.s.	--
0.5	n.s.	n.s.	n.s.	--
1.0	$P < 0.05$	n.s.	n.s.	Phagocytosis
Light				
0.1	n.s.	n.s.	n.s.	--
0.5	n.s.	n.s.	$P < 0.01$	ROS
1.0	n.s.	n.s.	n.s.	--
B) Rutile/Titan	Independent variables			Most effected dependent variable
Regime Concentration (mg/L)	Particle (bulk vs NP)	Time (2 vs 4 hr)	Interaction	
Dark				
0.1	n.s.	n.s.	n.s.	--
0.5	n.s.	n.s.	n.s.	--
1.0	n.s.	n.s.	n.s.	--
Light				
0.1	$P < 0.05$	n.s.	n.s.	Phagocytosis
0.5	$P < 0.01$	$P < 0.05$	n.s.	Phagocytosis
1.0	n.s.	n.s.	n.s.	--

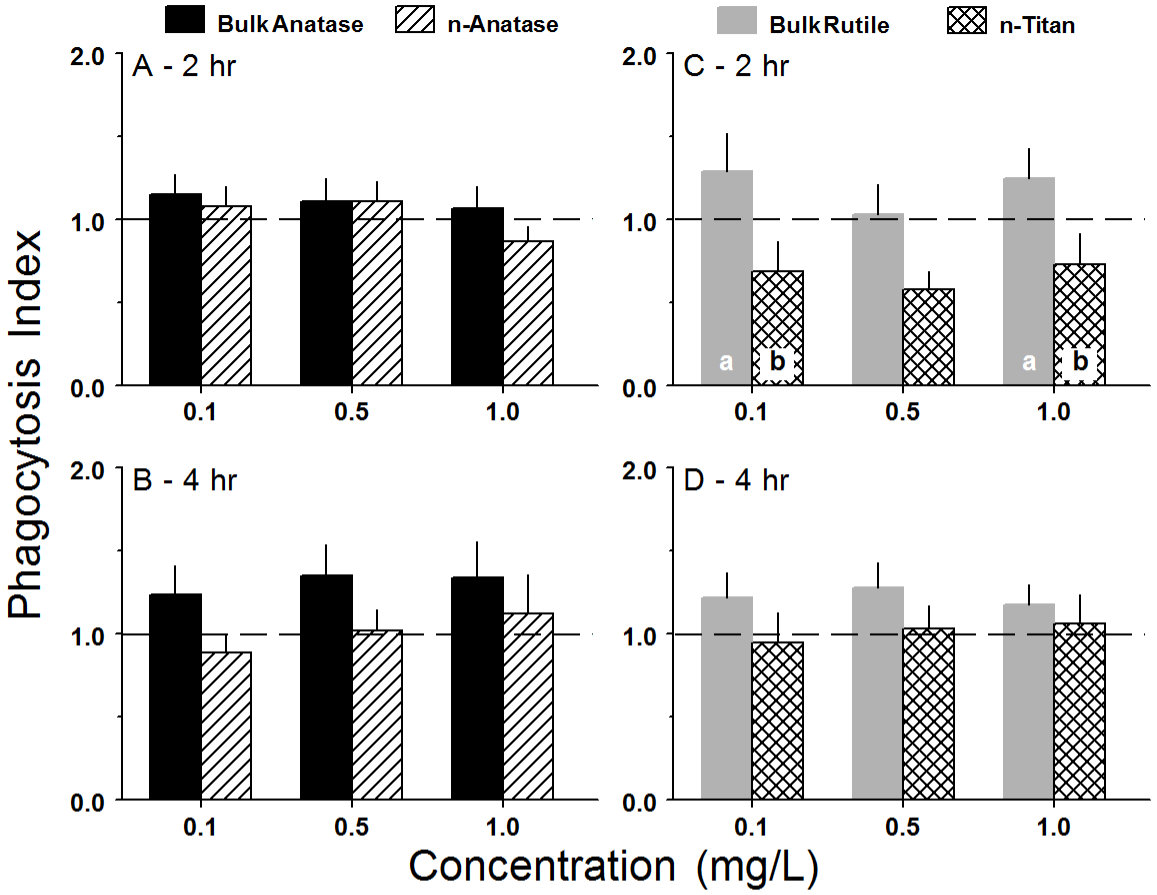
Figure 1: Phagocytic activity of oyster hemocytes under dark conditions following exposure to bulk anatase particles and n-anatase for (A) two-hour and (B) four-hour periods, and exposure to bulk rutile particles and n-Titan for (C) two-hour and (D) four-hour periods. Means designated with different capital letters indicate significant within-subject differences ($p<0.05$, within an exposure time), whereas means designated with different lowercase letters indicate significant between-subject differences ($p<0.05$, within an exposure time and within a concentration). Means without letter designations indicate that there were no significant within- or between-subject effects ($p>0.05$). Asterisks indicate significant changes in phagocytosis compared to the respective control values (dashed line). Data were standardized to their respective mean control value and are presented as mean ratios (index values) ± standard error (n = 10-12).

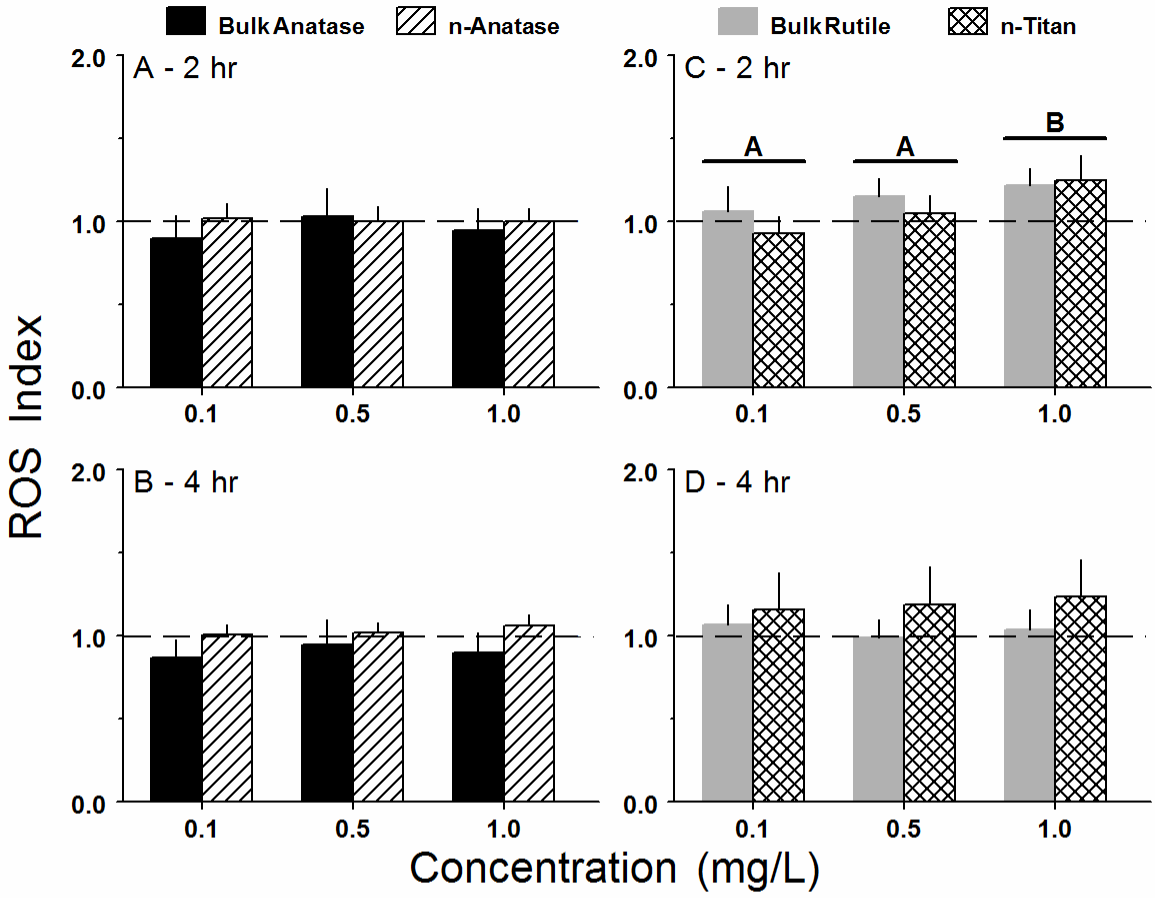
Figure 2: Phagocytosis activity of oyster hemocytes under light conditions following exposure to bulk anatase particles and n-anatase for (A) two-hour and (B) four-hour periods, and exposure to bulk rutile particles and n-Titan for (C) two-hour and (D) four-hour periods. See Figure 1 for explanation of statistical comparisons. Data were standardized to their respective mean control value and are presented as mean ratios (index values) ± standard error (n = 10-12).

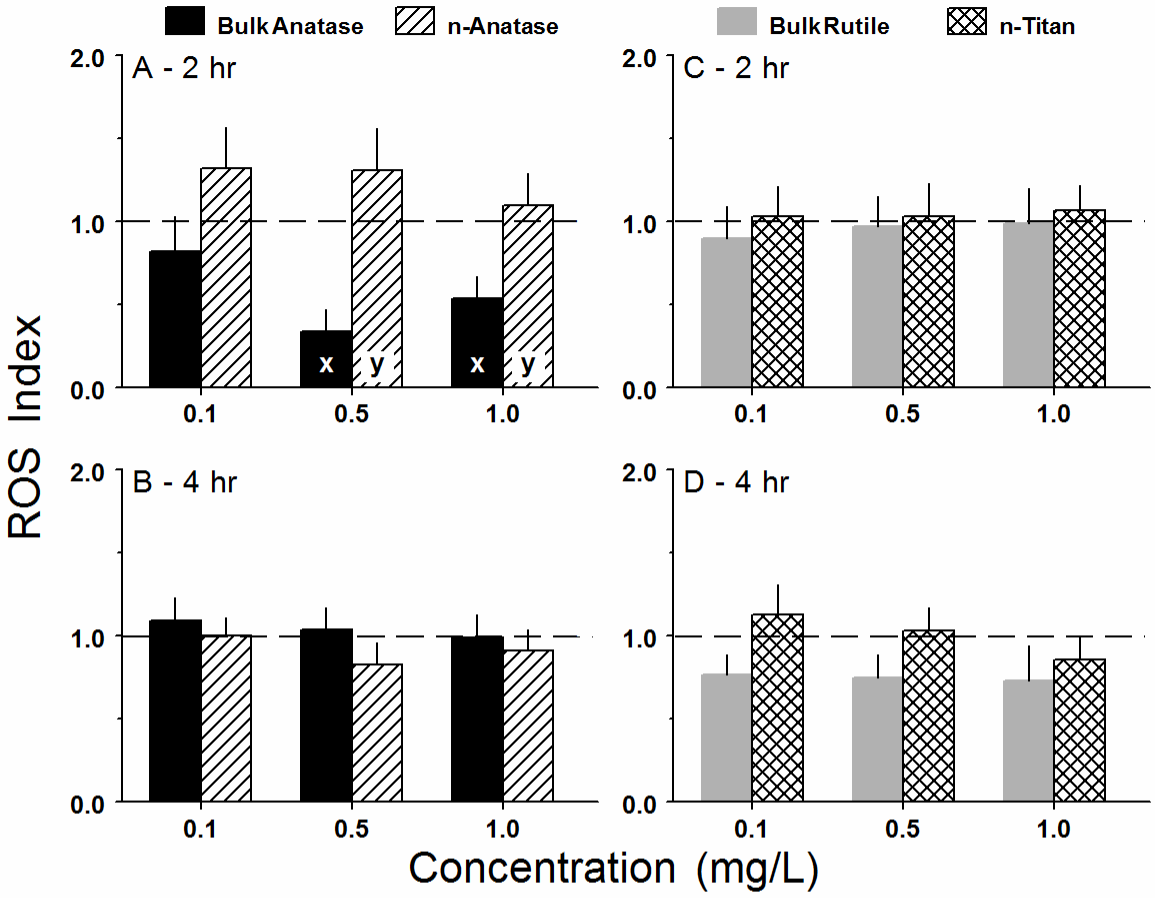
Figure 3: Reactive oxygen species (ROS) production by oyster hemocytes under dark conditions following exposure to bulk anatase particles and n-anatase for (A) two-hour and (B) four-hour periods, and exposure to bulk rutile particles and n-Titan for (C) two-hour and (D) four-hour periods. See Figure 1 for explanation of statistical comparisons. Data were standardized to their respective mean control value and are presented as mean ratios (index values) ± standard error (n = 10-12).

Figure 4: Reactive oxygen species (ROS) production by oyster hemocytes under light conditions following exposure to bulk anatase particles and n-anatase for (A) two-hour and (B) four-hour periods, and exposure to bulk rutile particles and n-Titan for (C) two-hour and (D) four-hour periods. See Figure 1 for explanation of statistical comparisons. Data were standardized to their respective mean control value and are presented as mean ratios (index values) ± standard error (n = 10-12).


Bulk Anatase
n-Anatase
Bulk Rutile
n-Titan
A - 2 hr
B - 4 hr
C - 2 hr
D - 4 hr
Phagocytosis Index
Concentration (mg/L)
0.1
0.5
1.0
0.0
1.0
2.0
3.0
A
AB
B
C
a
b
*


Bulk Anatase
n-Anatase
Bulk Rutile
n-Titan
A - 2 hr
B - 4 hr
C - 2 hr
D - 4 hr
Phagocytosis Index
Concentration (mg/L)
2.0
1.0
0.0
0.1
0.5
1.0
a
b
a
b


Bulk Anatase
n-Anatase
Bulk Rutile
n-Titan
A - 2 hr
B - 4 hr
C - 2 hr
D - 4 hr
A
A
B
2.0
1.0
0.0
0.1
0.5
1.0
ROS Index
Concentration (mg/L)


Bulk Anatase
n-Anatase
Bulk Rutile
n-Titan
A - 2 hr
B - 4 hr
C - 2 hr
D - 4 hr
ROS Index
Concentration (mg/L)
2.0
1.0
0.0
0.1
0.5
1.0
x
y
x
y