Journal of the Marine Biological Association of the United Kingdom, 2016, 96(1), 137–149.
doi:10.1017/S0025315415001174

Exposure of bivalve shellfish to titania nanoparticles under an environmental-spill scenario: Encounter, ingestion and egestion

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*Nanoparticles have applications in a diverse range of products including medications, detergents, cosmetics, paint, sunscreen and electronics, with an economic worth projected to reach $2.5 trillion dollars in 2015. Research into the effects of manufactured nanomaterials on the environment, however, has failed to keep pace with the high volume of commercial production. Whereas a number of studies have examined the effects of nanoparticles on aquatic species, little work has focused on the way in which benthic marine species encounter, ingest and depurate these materials. The purpose of this study was to examine the ingestion and depuration of titania nanoparticles (anatase) by the blue mussel (*Mytilus edulis*) and the eastern oyster (*Crassostrea virginica*) during a spill scenario (an acute exposure to elevated concentrations). Bivalves were exposed to nanoparticles either incorporated into marine snow, an environmentally relevant medium for pollutants, or added directly to seawater at a concentration of 4.5 mg L^{-1} for 2 h. After feeding, the animals were transferred to filtered seawater and allowed to depurate. Faeces and tissues were collected at 0, 6, 24, 72 and 120 h, post-exposure, and analysed for concentrations of titanium by inductively coupled plasma-mass spectrometry. Results indicated that the capture and ingestion of titania nanoparticles by both species was not dependent on the method of delivery (incorporated into marine snow or freely suspended). Additionally, greater than 90% of the titania nanoparticles, on average, were eliminated from the tissues after 6 h, and only trace amounts remained after 72 h. These data demonstrate that mussels and oysters readily ingest titania nanoparticles, but rapidly depurate the material within hours of an acute exposure suggesting that little would be transferred to secondary consumers including humans. Further research is required to determine if other species of suspension-feeders handle titania nanoparticles in a manner similar to bivalves.*

Keywords: nanoparticles, environmental spill, bivalves, ingestion, bioaccumulation, depuration

Submitted 10 April 2015; accepted 27 June 2015; first published online 11 August 2015

INTRODUCTION

Emerging contaminants such as manufactured nanomaterials are of growing concern in the marine environment, not only because they can directly impact resident organisms, but also because they can potentially be transferred to humans via the food chain. One group of nanomaterials that has received scrutiny is the particles composed of titania. At the nanoscale, titania possesses unique physicochemical and photocatalytic properties that have been implemented in a range of industrial and commercial applications. For example, titania nanoparticles (TiO_2 NPs) are used in paint as pigments and opaquers (Carp *et al.*, 2004), in cosmetics and sunscreens to absorb UV radiation (Jaroenworaluck *et al.*, 2006; Siddiquey *et al.*, 2007; Labille *et al.*, 2010), as antimicrobials (Amézaga-Madrid *et al.*, 2003; Kim *et al.*, 2003; Kühn *et al.*, 2003; Robertson *et al.*, 2005; Adams *et al.*, 2006; Li *et al.*, 2006; Foster *et al.*, 2011) and in the degradation of organic pollutants (Chatterjee & Mahata, 2002). TiO_2 NPs can exist in either the rutile, anatase, or brookite crystalline phases (Markowska-Szczupak *et al.*, 2011), of which rutile and anatase are the most common (EPA, 2010). Both the rutile and anatase crystalline phases demonstrate a tetragonal structure, however, the anatase form tends to be less dense and more photocatalytically active than the rutile structure (Serpone *et al.*, 2007; Dankovic & Kuempel, 2011).

Although the precise production rates of manufactured nanomaterials are not typically released, estimates of TiO_2 NPs in the USA alone are projected to reach 2.5 million tonnes annually by the year 2025 (Robichaud *et al.*, 2009). Mueller & Nowack (2008) estimated that the concentration of TiO_2 NPs in aquatic environments were between 0.7–16 μg L^{-1} for realistic and high exposure scenarios, respectively, however, the estimates were made in 2008, and represent end-use concentrations of TiO_2 NPs in Switzerland only (Robichaud *et al.*, 2009). Naturally derived titanium weathered from the earth's crust (Schroeder *et al.*, 1963; Fishbein *et al.*, 1982; Orians *et al.*, 1990) is estimated to be 4–8 picomolar (~0.3–0.6 ng L^{-1}; Orians *et al.*, 1990) at the ocean's surface, with lower nanomolar concentrations present in estuarine environments (Yokoi & van den Berg, 1991; Skrabal *et al.*, 1992; Skrabal, 1995). Thus, conservative estimates show that anthropogenic loads of TiO_2 NPs in aquatic environments could be three orders of magnitude higher than the

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concentration of naturally derived titanium. As populations in coastal and estuarine regions continue to rise (National Research Council, 2007), the prevalence of novel contaminants such as NPs entering aquatic systems in sewage effluents, industrial waste, and surface runoff have also increased (Kolpin *et al.*, 2002; Farré *et al.*, 2008).

Nanomaterials entering the marine environment are exposed to dissolved, colloidal and particulate organic matter that will increase their potential for homo- and hetero-aggregation (Brant *et al.*, 2005; Xie *et al.*, 2008; Sharma, 2009; Doyle *et al.*, 2014). Additionally, physical and biological processes aggregate particulate matter suspended in the water (including nanoparticles) into larger masses known as marine snow (Alldredge & Silver, 1988; Jackson, 1990; Doyle *et al.*, 2014). In fact at certain times of the year, large proportions (>70%) of natural particulates are incorporated in marine snow (Alldredge *et al.*, 1993; Crocker & Passow, 1995). Marine snow has a complex three-dimensional structure that is physically and chemically distinct from the surrounding water (Silver *et al.*, 1978; Alldredge, 2000; Ploug, 2001), is important for the vertical transport of material to the benthos (Kiørboe *et al.*, 1990; Passow & Wassmann, 1994; Crocker & Passow, 1995; Waite *et al.*, 2000), and can facilitate the trophic transfer of dissolved and particulate matter to benthic suspension-feeders (Alber & Valiela, 1994, 1996; Kach & Ward, 2008). Certain types of metal-oxide NPs have a high agglomeration potential in seawater (Sillanpää *et al.*, 2011; Shih *et al.*, 2012; Doyle *et al.*, 2014). Formation of larger-diameter NP agglomerates can cause an increased collision rate with other particles suspended in the water column (Jackson, 1990), and a higher incorporation rate in marine snow. Nanoparticles that are incorporated in marine snow will sink faster than the same particles that are freely suspended (Stokes Law; Hill, 1998; Waite *et al.*, 2000). Higher sinking rates would increase deposition rates to the bottom, exposing benthic organisms to a higher concentration of NPs over a shorter time period. Additionally, tides and storm events can resuspend settled marine snow (Newell *et al.*, 2005; Lyons, 2008), potentially re-exposing benthic organisms to previously deposited, recalcitrant nanomaterials (Scientific Committee on Emerging and Newly Identified Health Risks, 2006). Given the importance of marine snow to nutrient cycling in near-shore and open-ocean ecosystems (Fowler & Knauer, 1986), understanding how NPs affect the organisms that consume marine snow is important for a full assessment of the environmental impacts of engineered nanomaterials.

Previous studies demonstrate that some aquatic organisms can be negatively impacted when exposed to certain types of manufactured nanomaterials. For example, exposure to TiO_2 NPs has resulted in deleterious effects in vertebrates such as *Oncorhynchus mykiss* (rainbow trout; Federici *et al.*, 2007; Vevers & Jha, 2008), *Oryzias latipes* (Japanese medaka; Ma *et al.*, 2012), and *Danio rerio* (zebrafish; Bar-Ilan *et al.*, 2013). In addition, the toxicity of TiO_2 NPs has also been demonstrated in aquatic invertebrates such as *Daphnia magna* (water flea; Adams *et al.*, 2006; Hund-Rinke & Simon, 2006; Lovern & Klaper, 2006; Warheit *et al.*, 2007; Ma *et al.*, 2012), and *Arenicola marina* (lugworm; Galloway *et al.*, 2010). With production of TiO_2 NPs projected to escalate over the next decade (Robichaud *et al.*, 2009), organisms in coastal estuarine and marine environments will likely be exposed to increased concentrations of TiO_2 NPs. A more detailed review of the effects of nanoparticles on species other than bivalves can be found in the publications of Baun *et al.* (2008), Handy *et al.* (2008) and Sharma (2009).

A group of aquatic organisms that may be particularly vulnerable to NP toxicity is the suspension-feeding bivalve molluscs (Canesi *et al.*, 2012). Bivalves often dominate the macrobenthos, playing significant roles in ecosystem processes, and filtering large volumes of water per unit time (e.g. 3–7 L water $hour^{-1}$ per g dry weight; Newell, 1988; Riisgård, 1988; Newell *et al.*, 2005; Cranford *et al.*, 2011). Dense populations interact strongly with near-shore water columns, removing phytoplankton, depositing faeces and pseudofaeces (material rejected prior to ingestion), cycling dissolved nutrients (Dame, 1993, 1996; Prins *et al.*, 1998; Newell, 2004), and contributing to the concentration of transparent exopolymer particles (TEP; McKee *et al.*, 2005; Heinonen *et al.*, 2007; Li *et al.*, 2008). Additionally, bivalves process large amounts of organic matter, converting some of it into body tissues that can be used by higher trophic levels including humans. Many bivalve species are commercially important, providing a source of jobs and food to people worldwide. For example, the global catch of bivalves as of 2010 was approximately 1.7 million tonnes, whereas worldwide aquaculture production of bivalves was about 13 million tonnes (FAO, 2012). These characteristics make suspension-feeding bivalves an important group of organisms to study. Defining how bivalves interact with, and are affected by, manufactured nanomaterials is critical to an understanding of the potential broad-scale impacts of these materials on water quality and productivity of coastal ecosystems. Such data are also important to define which types of nanomaterials are bioaccumulated and could be transferred up the food chain to higher-level consumers including humans.

Several studies have demonstrated the cytotoxic and genotoxic effects of TiO_2 NPs on suspension-feeding bivalves (see Table 1 for a more complete review). For example, the haemocytes of *Crassostrea virginica* (eastern oyster) were found to have reduced phagocytosis after exposure to TiO_2 NPs (Abbott-Chalew *et al.*, 2012). *Mytilus galloprovincialis* (Mediterranean mussel) demonstrated oxidative stress, reduced transcription of immune-function genes, decreased lysosomal membrane stability, reduced haemocyte phagocytosis, activation of MAPK stress genes, larval malformations, and decreased numbers of mitochondria following exposure to TiO_2 NPs (Canesi *et al.*, 2010a, b, 2014; Ciacci *et al.*, 2012; Barmo *et al.*, 2013; Libralato *et al.*, 2013). A reduction in haemocyte viability and phagocytosis as well as the production of reactive oxygen species (ROS) were observed in *Perna viridis* (Asian green mussel; Wang *et al.*, 2014). *Dreissena polymorpha* (zebra mussel) showed reduced haemocyte phagocytosis and activation of the MAPK stress genes after exposure to TiO_2 NPs (Couleau *et al.*, 2012). Despite the observed cellular and genotoxic effects, no studies have addressed the ways in which suspension-feeding bivalves encounter TiO_2 NPs in the environment. Furthermore, no data exist concerning the ingestion and depuration rates of TiO_2 NPs in exposed bivalves.

The goal of the research presented here was to understand the ingestion and depuration rates of *Mytilus edulis* (blue mussel) and *Crassostrea virginica* (eastern oyster) exposed to a high concentration of TiO_2 NPs over an acute time interval. Dosing the animals at a high concentration for a short period of time was used as a proxy for an environmental spill

https://doi.org/10.1017/S0025315415001174 Published online by Cambridge University Press

Table 1. A summary of the cytotoxic and genotoxic effects of nanoparticles on various marine species of bivalves.

Species	Nanoparticle	Concentration	Exposure	Effect	Reference
Oyster					
Crassostrea virginica	Ag, TiO_2	1–400 μg L^{-1}	15–120 min	Reduced phagocytosis	Abbott-Chalew *et al.* (2012)
	Ag	0.02–20 μg L^{-1}	48 h	Decreased LMS, ROS production, reduction in total proteins	McCarthy *et al.* (2013)
	C_{60} Fullerene	1–500 μg L^{-1}	4 days	Decreased LMS, accumulation in lysosomes, impaired development	Ringwood *et al.* (2009)
	Ag	1.6 ng L^{-1}–16 μg L^{-1}	48 h	Impaired development, Decreased LMS, increased metallothionein gene expression	Ringwood *et al.* (2010)
Mussel					
Mytilus edulis	Fe	1 mg L^{-1}	1–12 h	Bioaccumulation, lipid peroxidation, decreased LMS	Kádár *et al.* (2010)
	SiO_2	Not reported	12 h–16 days	Decreased LMS, oxidative stress, increased phagocytosis, apoptosis	Koehler *et al.* (2008)
	Au	750 μg L^{-1}	24 h	Oxidative stress	Tedesco *et al.* (2008)
	Au	750 μg L^{-1}	24 h	Bioaccumulation, decreased LMS, oxidative stress	Tedesco *et al.* (2010)
	Polystyrene	0.1–0.3 g L^{-1}	8 h	Increased pseudofaeces, reduced filtration	Wegner *et al.* (2012)
	Ag	0.7 μg L^{-1}	3.5 h	Bioaccumulation, presence in extrapallial fluid	Zuykov *et al.* (2011b)
	Ag	0.7 μg L^{-1}	3.5 h	Alterations of shell nacre	Zuykov *et al.* (2011a)
Mussel					
Mytilus galloprovincialis	C_{60} Fullerene	100 μg L^{-1}–1 mg L^{-1}	3 days	DNA damage, histological abnormalities, bioaccumulation, decreased clearance rates	Al-Subiai *et al.* (2012)
	TiO_2	1–100 μg L^{-1}	96 h	Oxidative stress, reduced transcription of immune-function genes, decreased LMS, reduced phagocytosis, pre-apoptotic effects	Barmo *et al.* (2013)
	NCB	1–10 mg L^{-1}	60 min	Increased ROS production, decreased mitochondria, activation of MAPK stress genes	Canesi *et al.* (2008)
	C_{60} Fullerene, TiO_2, SiO_2	1–10 mg L^{-1}	30 min–4 h	Increased oxidative stress, activation of MAPK stress genes	Canesi *et al.* (2010a)
	NCB, C_{60} Fullerene, TiO_2, SiO_2	50 μg L^{-1}–5 mg L^{-1}	24 h	Decreased LMS, oxidative stress	Canesi *et al.* (2010b)
	TiO_2	10 mg L^{-1}; 100 μg L^{-1}	30–60 min; 96 h	Decreased LMS, reduced phagocytosis, genotoxicity, bioaccumulation	Canesi *et al.* (2014)
	TiO_2, SiO_2, ZnO, CeO_2	1–10 mg L^{-1}	30 min – 4 h	Decreased LMS, hormetic effects on phagocytosis, increased ROS production, decreased mitochondria	Ciacci *et al.* (2012)
	CuO	10 μg L^{-1}	15 days	Bioaccumulation, oxidative stress, neurotoxicity	Gomes *et al.* (2011)
	CuO, Ag	10 μg L^{-1}	15 days	DNA damage	Gomes *et al.* (2013a)
Mussel					
Mytilus galloprovincialis	Ag	10 μg L^{-1}	15 days	Bioaccumulation, changes in protein expression	Gomes *et al.* (2013b)
	ZnO	0.1–2 mg L^{-1}	84 days	Bioaccumulation, reduced growth, mortality	Hanna *et al.* (2013)
	CdSe Quantum Dots, Fe	92 ng L^{-1}–2 mg L^{-1}	18 & 24 h	Bioaccumulation	Hull *et al.* (2013)
	TiO_2	0.5–64 mg L^{-1}	48 h	Larval malformations	Libralato *et al.* (2013)
	CeO_2, ZnO	1–10 mg L^{-1}	4 days	Bioaccumulation	Montes *et al.* (2012)
	C_{60} Fullerene	1 & 10 mg L^{-1}	60 min	Decreased LMS	Moore *et al.* (2009)
	SWCNH	1–10 mg L^{-1}	24 & 48 h	Reduced ROS production, decreased LMS	Moschino *et al.* (2014)
Perna viridis	TiO_2	2.5 & 10 mg L^{-1}	24–216 h	Decreased hemocyte viability, decreased phagocytosis, reduced ROS production	Wang *et al.* (2014)
Clam					
Macoma balthica	Ag, CuO	200 μg g^{-1}	35 days	Bioaccumulation	Dai *et al.* (2013)

Continued

Table 1. Continued

Species	Nanoparticle	Concentration	Exposure	Effect	Reference
Mercenaria mercenaria	Au	5.6 μg/kg^{-1}	11 days	Bioaccumulation	Burns *et al.* (2013)
Ruditapes philippinarum	Au	6 & 30 μg L^{-1}	28 days	Bioaccumulation, oxidative stress, accumulation in lysosomes	García-Negrete *et al.* (2013)
Scrobicularia plana	CuO	10 μg L^{-1}	16 days	Oxidative stress, changes in burrowing, decreased feeding	Buffet *et al.* (2011)
Clam *Scrobicularia plana*	CuO	10 μg L^{-1}	21 days	Bioaccumulation, increased ROS, apoptosis, DNA damage, decreased feeding, changes in burrowing	Buffet *et al.* (2013)
	Au	100 μg L^{-1}	16 days	Bioaccumulation, oxidative stress, changes in burrowing	Pan *et al.* (2012)
Scallop *Chlamys islandica*	Ag	110 & 151 ng L^{-1}	12 h	Bioaccumulation followed by depuration	Al-Sid-Cheikh *et al.* (2013)

scenario. Under such conditions, animals would be subjected to a large amount of material, but natural processes such as tidal flushing, currents, and dilution would render the exposure time relatively short. This study is unique in that it also tests how several ecologically relevant modes of delivery (i.e. marine snow, aged suspended, un-aged suspended) affect encounter and ingestion of nano-titania by bivalves.

MATERIALS AND METHODS

Production of marine snow

A stock solution was prepared by suspending TiO_2 NPs (Meliorum Technologies, 99.9% pure anatase) in MQ-water at a concentration of 250 mg L^{-1}. X-ray diffraction (XRD) analysis of the TiO_2 NPs showed the characteristic anatase crystalline phase and a mean particle size of 7.4 nm ± 2.53 (Doyle *et al.*, 2014). The stock suspension was placed on a stir plate and subjected to ultrasonication (Fisher Scientific FB-505; calibrated according to Taurozzi *et al.*, 2012) at 13.8 Watts for 30 min (modified from Wang *et al.*, 2009). Following ultrasonication, TiO_2 NPs from the stock suspension were added to filtered-seawater (210-μm mesh) to achieve a final concentration of ~4.5 mg L^{-1} (4.4–4.7 mg L^{-1}). The working solution was mixed on a stir plate and then poured into 1-L Nalgene rolling bottles in quarter-litre aliquots. The solution was stirred and agitated after dispensing each aliquot to ensure that the NPs remained well mixed. This process was repeated until all the rolling bottles were full. Bottles designated as rolled samples (hereafter referred to as marine snow samples) were placed on a roller table for 72 h at 15 rpm (Shanks & Edmondson, 1989). Unrolled bottles consisted of the same solutions as described above, but instead of rolling, the bottles were placed next to the roller table for 72 h. A second treatment was prepared as described above to calculate the per cent incorporation, which was determined by the concentration of TiO_2 NPs in the marine snow when compared with the initial concentration of TiO_2 NPs (4.5 mg L^{-1}) added to the water. The TiO_2 NPs and marine snow were characterized using a suite of analytical techniques (dynamic light scattering, zeta potential, field-emission scanning electron microscopy and inductively coupled plasma-mass spectrometry) as previously described (Doyle *et al.*, 2014).

Feeding experiments

Mussels (5.0–6.5 cm in shell length) were collected from a local population at Avery Point (Groton, CT, USA), and oysters (5.0–6.5 cm in shell height) were obtained from the Noank Shellfish Cooperative (Noank, CT, USA). Prior to the experiments, bivalves were cleaned of all fouling organisms. A Velcro® strip was attached to one of the animals' shells using a two-part marine epoxy (Ward & Kach, 2009). Animals were held in an environmental chamber and fed *Tetraselmis* sp. for several days in order to acclimate to a temperature between 18° to 20°C. Before the commencement of the feeding experiments, the bivalves were secured to craft sticks with Velcro® and transferred to a large holding tray filled with aerated seawater, fed *Tetraselmis* sp., and allowed to acclimate at least 1 h prior to the beginning of the experiments.

https://doi.org/10.1017/S0025315415001174 Published online by Cambridge University Press

Each animal was exposed to one of four treatments. Two of the four treatments (marine snow and unrolled) were described above. The third treatment consisted of TiO_2 NPs spiked directly into 1-L Nalgene bottles containing filtered seawater (210-μm mesh) just prior to the start of the feeding assay (hereafter referred to as freely suspended). The last treatment, which served as blanks, consisted of 1-L Nalgene bottles containing filtered-seawater devoid of NPs to account for background concentrations of titanium. Bottles containing these four treatments were arranged on multi-position stir plates. Each bottle was supplied with gentle aeration using glass Pasteur pipettes and a stir bar. The stir plates were programmed to agitate the water for 10 s every 15 min to prevent the marine snow from settling on the bottom for too long. The water was then spiked with 10-μm polystyrene beads at a concentration of 2000 beads L^{-1}. The 10-μm polystyrene beads have a diameter large enough to ensure a capture efficiency of approximately 100% in both species, and were used as a means of determining feeding activity (Ward & Kach, 2009).

Animals with their shells open and mantles extended were transferred from the holding tray into the bottles. One bivalve was placed into each bottle and its craft stick secured to the rim by means of a wooden clip so that the animal was in the centre of the bottle (Ward & Kach, 2009). Animals were allowed to feed for 2 h with time commencing after they showed signs of suspension feeding (i.e. shells open, mantles extended). After 2 h, the animals were transferred from the bottles to clean 1-L beakers containing filtered seawater (0.22-μm membrane) at 18°–20°C. Faeces were collected immediately from the 1-L Nalgene bottles and labelled as the 0-h sample (time, post-exposure). Animals were fed a diet of *Tetraselmis* sp. at a concentration of 10 000 cells L^{-1}, and faeces were collected at 6, 24, 72 and 120 h post-exposure to examine the depuration rates of the TiO_2 NPs. In total, 102 animals of each species were used in the experiment described above; six animals at each time interval for each treatment. The experiment was repeated three times to ensure a large enough sample size for statistical analysis (total of 306 animals of each species).

Sample analysis

Animals were euthanized after feeding at 0, 6, 24, 72 and 120 h, post-exposure. The visceral mass, mantle and gills were removed by dissection and placed in 20-mL scintillation vials. Tissues were stored at −20°C overnight and then lyophilized for 48 h to remove any remaining moisture. A dry mass was obtained, and the organs were digested in 2 mL of 18 M H_2SO_4 and 16 M HNO_3 in a 3:7 ratio (volume/volume) for 24 h (Lawrence *et al.*, 1999). Following digestion, the samples were agitated on a vortex, and the acid digest was diluted to a 1% solution using MQ-water. Faeces produced by each animal were also collected at each time interval and placed into individually labelled 15-mL Falcon tubes. The tubes were centrifuged at 3220 g for 5 min, and the supernatant was removed. The faeces were washed once with 5 mL of MQ-water, and centrifuged a second time at 3220 g for 5 min. The supernatant was again removed, and the faeces were lyophilized for 48 h to remove any remaining moisture. A dry mass was obtained, and the faeces were digested in 2 mL of 18 M H_2SO_4 and 16 M HNO_3 in a 3:7 v/v ratio for 24 h. The acid digest was then diluted to a 1% solution using MQ-water. A subsample of the dilution was collected and the average number of 10-μm polystyrene beads in the faeces was determined using a haemocytometer. Animals that had an average of less than one bead in their faeces (equivalent to the ingestion of <1% of available beads), or no TiO_2 present in the visceral mass or faeces at $T = 0$ (immediately following exposure to NPs) were considered not to have fed during the experimental period and were removed from the analyses (one mussel and eight oysters). Background concentrations of TiO_2 detected in the faeces and tissues of blank animals (not exposed to NPs) were averaged and subtracted from the concentrations of TiO_2 measured in exposed animals. This step was taken to ensure that only the titanium from the NPs was being measured in the exposed animals. Concentrations of TiO_2 were then standardized to the dry mass of the tissue and faecal material to account for differences in the size of experimental animals (see Supplementary Table S1).

Tissue and faeces samples were analysed for titanium using an ELAN DRC II inductively coupled plasma-mass spectrometer (ICP-MS; Perkin Elmer) to examine the concentration of TiO_2 present. The ICP-MS was tuned to detect the titanium-47 isotope in the tissue and faeces samples to avoid interference from the high levels of the titanium-48 isotope found in natural seawater. The analytical error of the ICP-MS was calculated as 0.91 ± 0.06 mg L^{-1} (mean ± standard deviation of six solutions containing TiO_2 at a concentration of 1.0 mg L^{-1}). The limits of detection of the ICP-MS were calculated as 3.75×10^{-2} μg g^{-1} (three times the mean of the standard deviation of three replicate solutions containing TiO_2 at a concentration of 1.0 mg L^{-1}, and converted to μg g^{-1} assuming the density of MQ-water is 1000 g L^{-1}).

Statistical analysis

Two-way analysis of variance (ANOVA) tests were used to compare the effects of treatment and time on the concentration of TiO_2 NPs measured in the gills, mantles, visceral masses and faeces of the mussels and oysters. Effects of the two independent variables (time, treatment) within a given tissue/faecal sample and bivalve species were of primary interest, so two-way procedures were applied. If no differences were found between treatments at each time period, data were pooled and reanalysed to examine effects of time and species on TiO_2 concentrations within tissue/faecal samples. Following ANOVA analyses, a Tukey's HSD *post hoc* test was applied to examine differences between levels of the independent variables. 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 square-root or natural-log transformation. In all tests, an alpha level of 0.05 was used.

RESULTS

Incorporation into marine snow

The marine snow produced in the laboratory ranged in size from approximately 1–10 mm. Incorporation efficiency of TiO_2 NPs in laboratory-made marine snow was ~52% ± 5.7% (standard error; N = 9) after 72 h (~2.3 mg of the

https://doi.org/10.1017/S0025315415001174 Published online by Cambridge University Press

4.5 mg in each 1-L bottle). This efficiency is similar to that obtained in previous studies examining the incorporation of nano-titania (anatase form) into marine snow (Doyle *et al.*, 2014). Analysis using field emission scanning electron microscopy with energy dispersive X-ray spectroscopy (FESEM-EDX) revealed that agglomerates of TiO_2 NPs were distributed throughout the organic matrix of the marine snow (Doyle *et al.*, 2014).

Encounter, ingestion and egestion

No significant difference was found in the number of 10-μm polystyrene beads removed from suspension by animals delivered TiO_2 in the marine snow, unrolled or freely suspended treatments (ANOVA, data not presented). This finding indicates that the animals all fed at the same rate regardless of the treatment to which they were exposed. The mean concentration of background TiO_2 in the tissues and faeces of the blank animals (not exposed to NPs) was 1.6% ± 0.7 (mean ± SE) of the mean concentration of TiO_2 measured in the same samples from animals exposed to NPs. Overall, data analyses of the concentration of TiO_2 NPs on the gills, in the visceral mass, and in the faeces of both mussels and oysters yielded similar results. These included significant time effects, no significant treatment effects (marine snow *vs* unrolled *vs* free), and no significant interaction effects between the two independent variables (two-way ANOVA; Table 2; Figure 1). The only exceptions to this general trend were for the gills of oysters, which demonstrated no significant effect of time on the concentration of TiO_2, and the faeces of mussels, which demonstrated a significant interaction effect between time and treatment (Table 2). In general, pairwise comparisons indicated that the concentration of TiO_2 in tissues and faeces immediately after the 2-h feeding exposure (0-h) was significantly greater than that after 6, 24, 72, and 120 h of exposure (Tukey's, $P < 0.05$; Figure 1, Supplementary Figures S1, and S2). Concentrations were typically lowest or not detectable after 72 h. As mentioned above, treatment had no significant effect on the concentration of TiO_2 measured in the tissues or faeces. The only exception being a slight but significant difference in the egested concentrations of TiO_2 in the marine snow and freely suspended treatments of mussels at 24 h (Supplementary Figure S2A). Therefore treatment data for each sampling period were pooled in order to more easily compare elimination of NPs over time between the two species (Figure 2, Supplementary Table S1).

Analyses of the pooled TiO_2-concentration data for the gills, the visceral mass, and the faeces also demonstrated common trends. In all cases, significant time effects were found (two-way ANOVA; Table 3, Figure 2), with significantly higher concentrations immediately after the 2-h feeding exposure (0-h; Tukey's, $P < 0.05$). A significant species effect was also found for the faecal samples, and significant interaction effects found for both visceral mass and faecal samples (Table 3). The concentration of TiO_2 on the gills was more than an order of magnitude lower than in the visceral mass (Figure 2A). At 0-h post exposure, a significant difference in the concentration of TiO_2 in the visceral mass was found between mussels and oysters (Tukey's, $P < 0.01$; Figure 2B). A similar difference was found in the faeces at

Table 2. Results of two-way analysis of variance tests for (A) mussel and (B) oyster data. The concentration of TiO_2 nanoparticles in each tissue type (gill, visceral mass) and faeces was compared over five time periods (0, 6, 24, 72, 120 h) and between three treatments (marine snow, unrolled and freely suspended)

Source	df	MS	*F*	*P*
(A) Mussel				
Gill				
Time	4	0.083	4.115	0.005
Treatment	2	0.002	0.115	0.891
Time × treatment	8	0.003	0.125	0.998
Error visceral mass	75	0.020		
Time	4	3.586	24.63	0.000
Treatment	2	0.037	0.257	0.774
Time × treatment	8	0.168	1.157	0.337
Error faeces	75	0.146		
Time	4	331.3	199.6	0.000
Treatment	2	0.324	0.195	0.823
Time × treatment	8	4.733	2.851	0.005
Error	252	1.660		
(B) Oyster				
Gill				
Time	4	0.009	0.686	0.604
Treatment	2	0.000	0.011	0.989
Time × treatment	8	0.009	0.644	0.738
Error visceral mass	67	0.013		
Time	4	0.907	7.635	0.000
Treatment	2	0.016	0.132	0.876
Time × treatment	8	0.041	0.344	0.946
Error faeces	67	0.119		
Time	4	70.06	75.54	0.000
Treatment	2	1.260	1.358	0.259
Time × treatment	8	0.618	0.666	0.721
Error	247	0.927		

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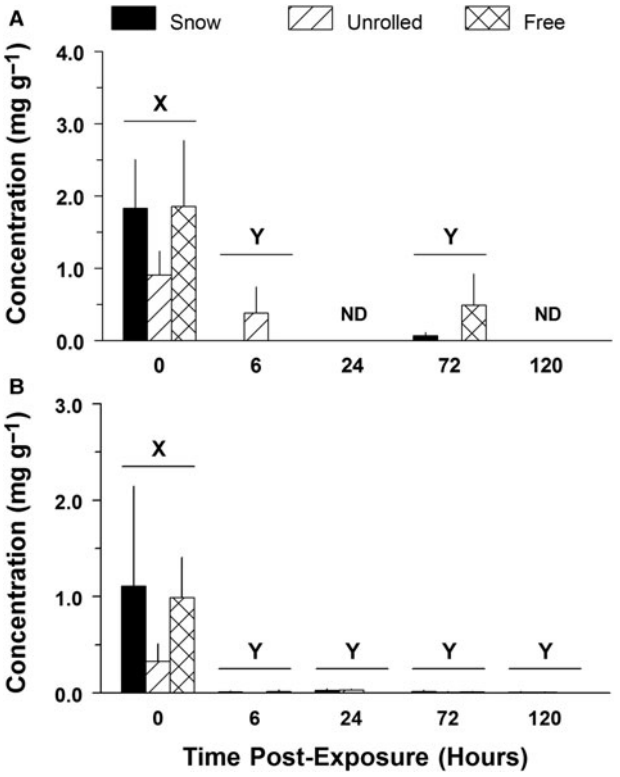

Fig. 1. Concentration of TiO_2 NPs in the visceral masses of mussels (A) and oysters (B) in three different treatments over time (note difference in scale). Bars designated by different letters are significantly different at $P < 0.05$. ND indicates no titanium was detected. Data are means ± standard error (N = 4–6). Snow = NPs incorporated into marine snow; Unrolled = NPs aged in seawater for the same length of time as those in the marine snow treatment but not incorporated into marine aggregates; Free = NPs suspended in seawater just prior to the start of the experiment.

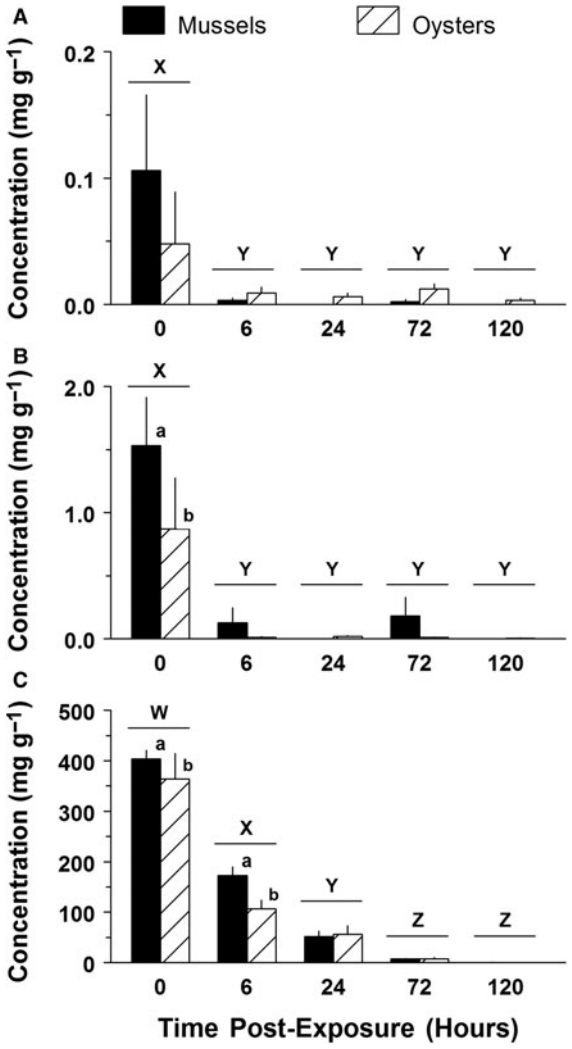

Fig. 2. Concentration of TiO_2 NPs in the gills (A), the visceral mass (B), and the faeces (C) of mussels and oysters over time (treatments pooled). Bars designated by different letters are significantly different at $P < 0.05$. Capital letters denote differences in time, whereas lower case letters show differences between species within a given time. Data are grand means ± standard error. For number of replicates see Figure 1, Supplementary Figures S1 and S2, and Supplementary Table S1.

both 0- and 6-h post exposure (Tukey's, $P < 0.01$; Figure 2C). These data suggest that mussels ingested more NPs than oysters over the 2-h exposure period. The concentration of TiO_2 in the faeces of both mussels and oysters diminished more gradually over time compared with that on the gills and in the visceral mass (Figure 2). Visual observation showed a colour transition in the faeces, likely due to the presence of TiO_2 NPs, over the course of the 120-h depuration period. For example, the faeces produced at both the 0- and 6-h time intervals were white in colour, while a mix of both white and greenish-brown faeces was observed at 24 h. These observations correspond to the significantly higher concentrations of TiO_2 found at 6 h, post exposure, compared with >6 h, and at 24 h, post exposure, compared with >24 h. The faeces produced during the 72- and 120-h time intervals were the typical greenish-brown hue.

Subsamples of mantle from the mussels and oysters (16 of each species) were examined at 0, 6, 24, 72 and 120 h, and measurable concentrations of TiO_2 NPs were detected only in the 0-h samples. Data analyses of the concentration of TiO_2 in the mantle of mussels and oysters at 0 h revealed no significant treatment effects ($P > 0.1$; one-way ANOVA).

DISCUSSION

The results of this study demonstrate that mussels and oysters are able to capture and ingest TiO_2 NPs regardless of how they encounter the material (incorporated in marine snow or freely suspended). Mussels are able to ingest significantly more NPs than oysters over a 2-h exposure period. Once the TiO_2 NPs are ingested, both species of bivalves are able to eliminate the majority of NPs within the first 6 h from their gills, mantles and visceral masses. Additionally, the majority of TiO_2 NPs are depurated in the faeces over the course of 72 h, with only trace amounts remaining after this time. Data demonstrate that after an acute exposure to a high concentration of TiO_2 NPs, accumulation in the tissues of mussels and oysters does not occur.

Counter to our main alternative hypothesis, we found no increase in ingestion when NPs were incorporated in marine snow. This finding was likely a result of the agglomeration potential of TiO_2 NPs in seawater (Christian *et al.*, 2008; Handy *et al.*, 2008; Tiede *et al.*, 2009; Sillanpää *et al.*, 2011). When TiO_2 NPs are immersed in seawater, dissolved organic matter (DOM) begins to coat the particles, creating a uniform negative charge at the surface of the particle

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Table 3. Results of two-way analysis of variance tests for mussel and oyster data (treatments pooled). In each tissue type (gill, visceral mass) and faeces, the concentration of TiO_2 NPs was compared between the two species (mussels, oysters) over five time intervals (0, 6, 24, 72, 120 h).

Source	df	MS	*F*	*P*
Gill				
Time	4	0.071	4.615	0.002
Species	1	0.013	0.829	0.364
Time × species	4	0.019	1.256	0.290
Error visceral mass	162	0.015		
Time	4	4.070	31.94	0.000
Species	1	0.367	2.881	0.092
Time × species	4	0.371	2.910	0.023
Error Faeces	162	0.127		
Time	4	180.6	280.6	0.000
Species	1	4.304	6.687	0.010
Time × species	4	4.564	7.092	0.000
Error	502	0.644		

(Handy *et al.*, 2008). The negatively charged surface then begins to attract cations dissolved in solution promoting Columbic attraction and enhanced agglomeration (Handy *et al.*, 2008; Lead & Smith, 2009). As agglomeration of the NPs increases so does the particle diameter making it more likely that the TiO_2 NPs will be encountered by the gills of the bivalve and ingested. Our data examining the physiochemical behaviour of TiO_2 NPs immersed in natural seawater show the formation of agglomerates ranging in size from ~0.5–3 μm (Doyle *et al.*, 2014). Particles >1.5 μm can be captured by both mussels and oysters at an efficiency of between 50 and 75% (see Ward & Shumway, 2004 for review). Thus, the agglomeration of TiO_2 NPs in natural seawater is as effective as marine snow at increasing the particle diameter, and enabling capture on the gills of mussels and oysters. Over the course of 2 h, mussels can filter a litre of water approximately 3 times, whereas oysters can filter a litre of water approximately 10 times (assuming a dry tissue mass of ~0.5 g for mussels and 1.0 g for oysters; see Newell, 1988; Newell *et al.*, 2005). Considering these clearance rates and the intermittent stirring of water to counter the effects of particle settling, we conclude that bivalves were exposed to all of the NPs added to each bottle (~4.5 mg). They did not, however, ingest all of the material, likely because of the production of pseudofaeces and the lower capture efficiency of agglomerates <1.0 μm in size. Our results support the findings of other research which report that bivalves can effectively capture a variety of NP types including Au, ZnO, CeO_2, TiO_2, SiO_2, carbon black and C60 fullerene (Koehler *et al.*, 2008; Canesi *et al.*, 2010b; Tedesco *et al.*, 2010; Montes *et al.*, 2012). It is likely that these particles were captured and ingested by the bivalves because they were in an agglomerated form. In contrast, NPs that remain more monodispersed in seawater would be captured at very low efficiencies unless they were incorporated in marine snow (Ward & Kach, 2009).

The bulk of the TiO_2 NPs were removed from the gills and visceral masses of both mussels and oysters between 0 and 6 h, post-exposure. On the gills, the average residence time of particles is on the order of minutes, as material is rapidly transported to the labial palps or mantle (Milke & Ward, 2003). In the gut, food particles with high nutritive value are retained for extracellular digestion in the stomach, followed by intracellular digestion in the cells of the digestive gland. Conversely, particles with little or no nutritive value are subjected to minimal extracellular digestion in the stomach and transported to the intestine for egestion (Bricelj *et al.*, 1984; Brillant & MacDonald, 2002, 2003; Ward & Shumway, 2004). Furthermore, bivalves retain larger, less dense particles longer than smaller, denser particles because organic matter tends to be larger and lighter than inorganic particles that contain little nutritive value. Thus, larger, less dense material remains suspended in the stomach for more thorough processing, whereas smaller, denser particles settle into ciliary selection tracts where they are transported rapidly to the intestine for egestion (Reid, 1965; Brillant & MacDonald, 2000). The separation of particles in the gut of bivalves based on size and density increases digestive efficiency and reduces digestive investment in material with little to no nutritive value (Brillant & MacDonald, 2000). Previous studies regarding gut-retention time (GRT) report that *M. edulis* retain natural food particles for a period of approximately 2.5 h (Bayne *et al.*, 1989), whereas *C. virginica* was found to retain natural food particles for approximately 9 h (Owen, 1966, 1974; Morton, 1977) depending on feeding rate and tidal cycle. Therefore, in the current study, bivalves handled the bulk of TiO_2 NPs as small, dense particles with little nutritive value, moving the material quickly to the intestine for egestion. Mass-balance analysis demonstrates the rapid depuration process that occurred with both mussels and oysters (Figure 3). Immediately following exposure (0 h), >70% of the ingested TiO_2 NPs had been eliminated in the faeces, and at 6 h, >90% of the ingested TiO_2 NPs had been egested. Only trace amounts of TiO_2 NPs were associated with the gills, visceral masses, and faeces 24 h after exposure.

The concentration of NPs to which bivalves were exposed in this study was greater than those deemed environmentally relevant (low $\mu g\ L^{-1}$; see Mueller & Nowack, 2008). Such conditions are possible, however, in a scenario where NPs are released into the near-shore environment during a spill (e.g. nanoparticle manufacturers located close to rivers or estuaries experiencing a failure). The effluent would be dispersed through the action of currents and tides, exposing coastal organisms to a high concentration of NPs over a short time interval. The impacts of spill-scenario concentrations of NPs on aquatic and terrestrial organisms have been examined in previous studies with effects ranging from the production of reactive oxygen species and activation of stress genes to delays in moulting and reduced fecundity (Oberdörster

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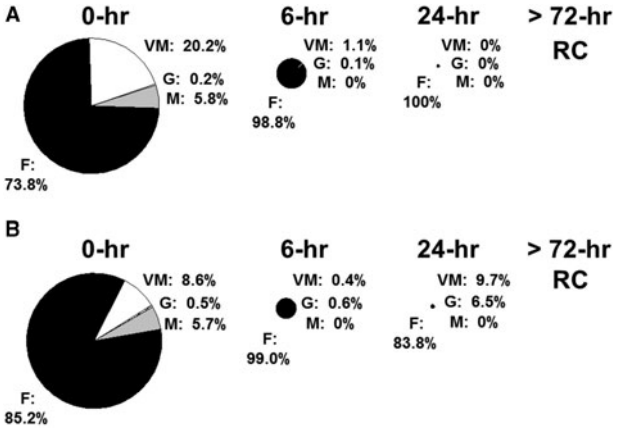

Fig. 3. Percentage of TiO_2 NPs measured in the tissues and faeces of mussels (A) and oysters (B) at each time interval (treatments pooled). The relative amount of TiO_2 at each time interval, as a proportion of the total amount of TiO_2 ingested by each group of bivalves over the experimental period, is represented by the size of each circle. VM, visceral mass; G, gill; M, mantle; F, faeces; RC, residual concentrations detected.

et al., 2006; Warheit *et al.*, 2007; Canesi *et al.*, 2008; Klaper *et al.*, 2009).

Although mussels and oysters do not bioaccumulate the anatase form of TiO_2 NPs when subjected to a spill scenario, the results of this study cannot be extrapolated to exposure conditions predicted for most marine environments (i.e. continuous exposure to concentrations <1.0 mg L^{-1}). Therefore, the experiments outlined in the present study should be repeated at lower concentrations for a longer period of time (>2 h to days) to determine if any measurable bioaccumulation occurs. Additionally, the current research examined only one form of one type of NP, representing a small fraction of the NPs that are presently being developed, produced and included in consumer products. For example, nano-Ag in textiles (Benn & Westerhoff, 2008; Geranio *et al.*, 2009), ZnO in sunscreens and cosmetics (Serpone *et al.*, 2007), and the degradation of plastics into micro- and nanosized particles (Moore, 2008; Wegner *et al.*, 2012) are potentially entering coastal marine systems. Currently, there are too few data regarding how marine organisms encounter, ingest, egest and accumulate these other types of nanomaterials.

There is a clear need for more research on the interactions between marine organisms and manufactured NPs, especially at environmentally relevant concentrations (Gottschalk *et al.*, 2009; Canesi *et al.*, 2012; Handy *et al.*, 2012). Future studies on bivalves and other suspension-feeders should take into account the agglomeration potential of the NP to which the animals are being exposed in order to better understand encounter and capture efficiency. Large agglomerates of nanoparticles are deposited to the benthos more rapidly and are captured by suspension-feeders more efficiently than the primary particles. This is a direct divergence from the more traditional position which suggests that aggregation increases particle diameter, and subsequently decreases bioavailability (Brant *et al.*, 2005; Navarro *et al.*, 2008; Nel *et al.*, 2009). TiO_2 NPs are believed to be recalcitrant in natural systems (Scientific Committee on Consumer Safety, 2013). Therefore, resuspension events, such as storms, could serve to re-expose benthic suspension- and deposit-feeding animals to previously sedimented nanomaterials. The potential for re-exposure suggests the need for more long-term studies that consider: (1) the routes of entry and fate of nanomaterials in the marine environment, and (2) the way in which marine animals encounter, handle, ingest and egest the nanomaterials. Such research would provide valuable information regarding bioaccumulation and potential for food-chain transfer, and would be essential for commercially important marine species that are consumed by humans.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0025315415001174

ACKNOWLEDGEMENTS

We would like to thank Bridget Holohan, Prentiss Balcom, Meghan Danley, Anusha Perumalla, James Markow (Noank Shellfish Cooperative), and Gregg Rivara (Suffolk County Marine Environmental Learning Center) for their assistance with various aspects of this study.

FINANCIAL SUPPORT

This research was supported by grants from Connecticut Sea Grant (R/P-1, NA10OAR4170095) and the National Science Foundation's Environmental Nanotechnology program (CBET-1336358) to J. Evan Ward and Robert Mason; and a grant from NOAA's Oceans and Human Health program for the Interdisciplinary Research and Training Initiative on Coastal Ecosystems and Human Heath (I-RICH) to J. Evan Ward. We appreciate this support.

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https://doi.org/10.1017/S0025315415001174 Published online by Cambridge University Press