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Co-exposure to n-TiO_2 and Cd^{2+} results in interactive effects on biomarker responses but not in increased toxicity in the marine bivalve *M. galloprovincialis*

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HIGHLIGHTS

- The effects of co-exposure to nTiO_2 and Cd^{2+} were evaluated in *Mytilus*.
- Interactions were found on immune/digestive gland biomarkers and embryo development.
- n-TiO_2 did not affect Cd^{2+} bioavailability and accumulation.
- n-TiO_2 and Cd^{2+} did not result in increased adverse effects in marine mussels.

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ABSTRACT

The increasing production of nanoparticles (NPs) will lead to their release into the aquatic environment, where they could modify the bioavailability/bioconcentration and consequent biological impact of other contaminants. Interactive effects of n-TiO_2, one of the most widespread NP type, and Cd^{2+}, a common heavy metal pollutant, have been described in freshwater species, whereas no information is available in marine organisms. In this work, the effects of co-exposure to n-TiO_2 and Cd^{2+} were investigated in the marine bivalve *Mytilus galloprovincialis*. Experimental conditions (100 μg/L, 96 h), were chosen in order to induce early but measurable stress responses (biomarkers) without toxicity. Several biomarkers, from molecular to tissue level, were measured in hemolymph and digestive gland; the effects on embryo development were also evaluated. In hemolymph, Cd^{2+} abolished the increase in immune parameters induced by n-TiO_2 (NO production and lysozyme activity). In the digestive gland, distinct interactive effects of n-TiO_2 and Cd^{2+} were observed on different lysosomal biomarkers (lysosomal membrane stability, lipid accumulation and lysosome/cytoplasm volume ratio) and transcription of the immune genes lysozyme and toll-like receptor (TLR). However, n-TiO_2 did not affect specific metal-induced responses (metallothionein induction) and tissue metal accumulation. Cd^{2+} alone, but not in combination with n-TiO_2, affected embryo development. The interactive effects observed on different biomarkers were not apparently due to differences in bioavailability/bioaccumulation of Cd^{2+} in the presence of n-TiO_2 agglomerates; these effects may result from interactions of either contaminant with both common and distinct targets/mechanisms of action at different levels of biological organization. Overall, the results indicate that co-exposure to n-TiO_2 and Cd^{2+} did not result in increased adverse effects in *M. galloprovincialis*. These data underline the need for further knowledge on the potential interactions of NPs with existing contaminants in marine organisms.

1. Introduction

The increasing production and use of manufactured nanoparticles—NPs (e.g. in industrial applications and consumer products) will lead to their release into the aquatic environment, posing a potential threat to the health of aquatic organisms (Moore, 2006; Scown et al., 2010;

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Delay and Frimmel, 2012; Matranga and Corsi, 2012). Both in the water phase and in the sediments NPs could mix and interact with other environmental pollutants, such as organic xenobiotics and heavy metals; these interactions may lead to changes in bioavailability/bioconcentration/toxicity of these contaminants in aquatic biota (Hartmann and Baun, 2010; Hartmann et al., 2012; Matranga and Corsi, 2012). Filter-feeders in particular are important test organisms in ecotoxicological tests with respect to NPs (Baun et al., 2008), as well as in studies aimed at evaluating the possible interactive effects of NPs with other pollutants (Hartmann et al., 2012).

Nanosized titanium dioxide (n-TiO_2) is one of the most widespread NPs in use (Robichaud et al., 2009; Menard et al., 2011). Increasing evidence suggests that in different biological systems interactions of n-TiO_2 with other chemico/physical factors may result in an increase in toxicity or adverse effects (Liu et al., 2013). The most studied example so far in aquatic organisms is that of co-exposure of freshwater species to n-TiO_2 and Cd^{2+}: these studies showed that Cd^{2+} bioavailability, bioaccumulation and toxicity can be affected by the presence of n-TiO_2, at mg/L levels, with contrasting results obtained in algae, daphnids and fish (Zhang et al., 2007; Hu et al., 2011; Hartmann and Baun, 2010; Hartmann et al., 2012; Yang et al., 2012).

With regard to marine organisms, interactive effects of different types of NPs and organic chemicals have been reported in the model bivalve, the mussel *Mytilus* (Tedesco et al., 2010; Al-Subiai et al., 2012; Canesi et al., 2014). In *Mytilus galloprovincialis*, exposure to n-TiO_2, in a wide concentration range, has been shown to induce significant changes in different biomarkers related to immune and digestive gland function (reviewed in Canesi et al., 2012; Canesi and Prochàzovà, 2013). Moreover, recent data in mussels showed that n-TiO_2 and the organic xenobiotic TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) exerted synergistic or antagonistic effects, depending on experimental condition, cell/tissue and type of measured response; some of these interactions could be ascribed to increased TCDD bioaccumulation in the presence of n-TiO_2, indicating a *Trojan horse* effect (Canesi et al., 2014). However, no information is so far available on the possible interactive effects of n-TiO_2 and heavy metals in marine organisms.

In this work, the effects of n-TiO_2 and Cd^{2+}, alone and in combination, were investigated in *M. galloprovincialis*, in order to evaluate whether co-exposure to the two contaminants may result in increased adverse effects in a marine species. Exposure conditions (100 μg/L, 96 h) were chosen in order to induce significant biomarker responses in mussels without toxicity, on the basis of previous data obtained in mussels with n-TiO_2 (Canesi et al., 2010; Barmo et al., 2013), and Cd^{2+} (Dondero et al., 2006; Kaloyianni et al., 2009). A wide range of biomarkers from molecular to tissue level were measured in hemolymph and digestive gland. Functional immune parameters were evaluated in hemolymph (hemocyte lysosomal membrane stability—LMS, phagocytic activity, nitric oxide production, serum lysozyme activity). In the digestive gland, lysosomal parameters (LMS, neutral lipid and lipofuscin accumulation, lysosome/cytoplasm volume ratio) were determined as general stress biomarkers at the cellular/tissue level. Gene expression was also evaluated both in hemocytes and digestive gland: transcription of the metallothionein (MT) isoforms MT20 and MT10, as a specific biomarker of exposure to heavy metals, and of immune related genes (lysozyme and toll-like receptor isoform i). Concentrations of Ti and Cd in exposure media, as well as in the digestive gland, were measured. The effects on mussel embryo development were also evaluated, as an indication of possible interactions at higher levels of biological organization.

2. Methods

2.1. Preparation and characterization of NPs

Nanosized titanium dioxide (n-TiO_2), namely Aeroxide© P25 (declared purity: 99.9%), kindly provided by Eigenmann & Veronelli (Milan, Italy), was characterized by a combination of analytical techniques (HR-TEM, TEM-EDX, XRD, HR-TEM-SAED, BET, ICP-MS, etc.), as described in Brunelli et al. (2013). Suspensions of n-TiO_2 were prepared in filtered artificial sea water (ASW) (ASTM, 2004), at the concentrations tested for exposure (100 μg/L), and sonicated with a UP200S Hielscher Ultrasonic Technology (Teltow, Germany) for 15 min at 100 W, 50% on/off cycle, cooling the dispersion in an ice bath. Size distribution of n-TiO_2 suspensions was calculated by Dynamic Light Scattering (DLS), performed with a Submicron Particle Sizer Nicomp 370 (Santa Monica, Ca, USA), equipped with a 35 mW He–Ne laser, 632.8 nm laser diode and photodiode detector set at 90 °C (Brunelli et al., 2013).

2.2. Adsorption of Cd^{2+} to n-TiO_2 agglomerates in standard ASW suspensions

Powdered $CdCl_2$ was added to the sonicated n-TiO_2 suspension (100 μg/L), to reach the desired Cd^{2+} final concentration utilized for exposure experiments (100 μg/L). At different time intervals from $CdCl_2$ addition (5 min, 1 h and 24 h), 300 mL of each prepared dispersion was sequentially filtered through 0.45 μm and then 0.22 μm cellulose nitrate filters (Whatman, Maidstone, UK). Each filter was then washed with 30 mL of ASW in order to remove any residual dissolved $CdCl_2$ not sticking to n-TiO_2 particles, and subsequently analyzed for Cd and Ti content by ICS-MS. Each experiment was performed in triplicate.

2.3. Animals and treatments

Mussels (*M. galloprovincialis* Lam.) 4–5 cm long, were purchased in June 2013 from an aquaculture farm in the Thyrrenian Sea (Arborea-OR, Italy) and kept for 3 days in static tanks containing 1 L ASW/mussel at 16 °C and daily fed with 30 mg/mussel/L Marine Liquifry (Interpet, England). Sea water was changed daily.

Stock suspensions of n-TiO_2 in ASW were prepared by sonication as for DLS analysis and immediately spiked in the tanks in order to reach the desired concentration. Cd^{2+} from a standard $CdCl_2$ stock solution (Merck) was suitably diluted in ASW and added to the tanks to reach the desired concentration. Mussels (at least 15 mussels in quadruplicate for each condition) were exposed for 96 h to either n-TiO_2 (100 μg/L nominal concentration levels, corresponding to 60 μg/L Ti on a mass basis), or Cd^{2+} (100 μg/L) and to both n-TiO_2 and Cd^{2+} at the same concentrations. A parallel group of control (untreated) mussels were kept in clean ASW. Sea water was changed each day before addition of the contaminants. Animals were not fed during the experiments. No mortality was observed in different experimental conditions.

After exposure, hemolymph was extracted from the posterior adductor muscle of 15 × 4 mussels from each experimental group, filtered through a sterile gauze and pooled in Falcon tubes at 4 °C.

Aliquots of whole hemolymph were utilized for determination of hemocyte immune parameters (lysosomal membrane stability, phagocytic activity and NO production). The remaining hemolymph was centrifuged at 100 ×*g* for 10 min at 4 °C. The resulting supernatant was utilized for determination of serum lysozyme activity. The hemocyte pellet was resuspended in TRIzol Reagent (Sigma, Milan, Italy) and stored at −80 °C for gene expression analysis.

For histological analyses, small pieces of digestive glands were placed on aluminium chucks, immersed in hexane pre-cooled to −70 °C in liquid nitrogen and maintained at −80 °C (Moore, 1988). For each exposure group, aliquots of digestive glands from 10 mussels were pooled and stored at −20 °C for analysis of total cadmium and titanium content. The remaining digestive glands collected from 15 × 4 mussels from each experimental group were divided into 0.4 g aliquots and stored in liquid nitrogen at −80 °C for MT protein determination and into 0.1 g aliquots stored in TRIzol Reagent (Sigma, Milan, Italy) at −80 °C for RNA extraction.