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Combined effects of n-TiO_2 and 2,3,7,8-TCDD in *Mytilus galloprovincialis* digestive gland: A transcriptomic and immunohistochemical study

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ABSTRACT

Despite the growing concern over the potential biological impact of nanoparticles (NPs) in the aquatic environment, little is known about their interactions with other pollutants. In the marine mussel *Mytilus galloprovincialis*, exposure to nanosized titanium dioxide (n-TiO_2), one of the most widespread type of NPs in use, in combination with and 2,3,7,8-tetrachlorodibenzo-p-dioxins (TCDD), chosen as model organic xenobiotic, was shown to induce significant changes in different biomarkers in hemocytes, gills and digestive gland, with distinct effects depending on cell/tissue and type of response measured. In this work, the interactive effects of n-TiO_2 and TCDD at the tissue level were further investigated in mussel digestive gland using an integrated approach transcriptomics/immunohistochemistry. Mussels were exposed to n-TiO_2 (100 μg L^{-1}) and TCDD (0.25 μg L^{-1}), alone and in combination, for 96 h. Transcriptomic analysis identified 48-, 49- and 62 Differentially Expressed Genes (DEGs) in response to n-TiO_2, TCDD and n-TiO_2/TCDD, respectively. Gene Ontology (GO) term analysis revealed distinct biological processes affected in different experimental conditions. n-TiO_2 mainly up-regulated cytoskeletal genes, while TCDD up-regulated endocrine and signal transduction related processes. Co-exposure induced transcriptional changes common to individual treatments, and identified a newly generated process, response to chemical stimulus. Transcription of selected genes was verified by qPCR. Moreover, expression of tubulin, as an example of target protein of interest identified by gene transcription data, was evaluated in tissue sections by immunolabelling. Tissue TCDD accumulation was evaluated by immunofluorescence with an anti-dioxins antibody.

The results demonstrate both distinct and interactive effects of n-TiO_2 and TCDD in mussel digestive gland at the molecular and tissue level, identify the main molecular targets involved, and underline how exposure to the n-TiO_2/TCDD mixture does not result in increased TCDD accumulation and overall stressful conditions in the tissue. These represent the first data on transcriptional responses of marine invertebrates to exposure not only to n-TiO_2 as a model of NP, but also to a legacy contaminant like TCDD.

1. Introduction

Due to the increasing production of nanoparticles (NPs) and their potential release to the aquatic environment, the evaluation of their biological impact on aquatic organisms represents a major concern. Based on their exceptional physicochemical properties, NPs are likely to interact (i.e. adsorption) with other pre-existing contaminants, thus possibly affecting their bioavailability/uptake and consequent biological effects (Hartmann and Baun, 2010; Matranga and Corsi, 2012). Evidence obtained so far in aquatic organisms indicate complex and often unexpected interactive responses of NPs with other pollutants, mainly depending on type of NP and contaminant and the endpoint measured, as well as differences in bioaccumulation. However, little information is available on the possible combined effects of NPs and other contaminants, including persistent organic xenobiotics, in marine organisms (reviewed in Canesi et al. (2015)).

Suspension feeding bivalve mollusks have been shown to represent a significant target for different types of NPs (Baun et al., 2008; Canesi et al., 2012; Corsi et al., 2014). In the marine bivalve *Mytilus galloprovincialis*, the possible interactive effects of titanium dioxide (n-TiO_2) and 2,3,7,8-tetrachlorodibenzo-p-dioxins (2,3,7,8-

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TCDD), chosen as model NP and organochlorine contaminant, respectively, have been recently investigated (Canesi et al., 2014).

N-TiO_2 is one of the most widely used NPs, with predicted levels in the aquatic environment at low $\mu g\ L^{-1}$ concentrations (Robichaud et al., 2009; Sun et al., 2014), as well as as one of the most extensively studied metal oxide NPs from the perspective of ecotoxicity (Menard et al., 2011; Grimaldi et al., 2013; Fouqueray et al., 2012; Pinsino et al., 2015). Dioxins, considered one of the most hazardous organochlorine compounds, are ubiquitous environmental contaminants, that persist and bioaccumulate through aquatic food chains (Schecter et al., 2006; Domingo and Bocio, 2007; US EPA, 2010), and are generally present in bivalve tissues at pg/g concentrations (Wade et al., 2014; Cano-Sancho et al., 2015). In the light of the environmental relevance of both n-TiO_2 and TCDD as emerging and legacy contaminants, respectively, understanding their effects, alone and in combination, in marine invertebrates deserves further attention.

In our first study on *M. galloprovincialis* co-exposed to n-TiO_2 and TCDD, a wide range of biomarkers (such as immune-, lysosomal-, stress response-, genotoxicity-, biotransformation-, oxidative stress- related biomarkers) were evaluated in hemocytes, gills and digestive gland. Distinct interactive effects were observed, depending on cell/tissue and type of measured response (Canesi et al., 2014). Some of these interactions may have been related to higher TCDD accumulation observed in whole mussel tissues in the presence of n-TiO_2, suggesting a possible *Trojan horse* effect (Canesi et al., 2014). In the digestive gland, n-TiO_2 and TCDD showed combined effects on different aspects of lysosomal physiology; however, no interactions were observed on transcription of stress-response and antioxidant genes, and the possible molecular targets for either contaminant, alone and in combination, were not identified.

In this work, in order to investigate more thoroughly the impact of co-exposure to n-TiO_2 and TCDD in mussel digestive gland, an integrated approach transcriptomics/ immunohistochemistry was utilized. Mussels were exposed to n-TiO_2 ($100\ \mu g\ L^{-1}$) or to 2,3,7,8-TCDD ($0.25\ \mu g\ L^{-1}$), alone and in combination, for 96 h, in the same experimental conditions previously described (Canesi et al., 2014). Transcriptomic analysis was performed using a *M. galloprovincialis* cDNA microarray (Venier et al., 2006) previously utilized to evaluate the effects of contaminant mixtures (Dondero et al., 2010, 2011; Canesi et al., 2011). Transcription of selected genes was also quantified by qRT-PCR. On the basis of the results obtained on gene transcription, expression of target proteins of interests (tubulin) was evaluated by immunolabelling. Accumulation of TCDD was evaluated in digestive gland tissue sections by immunofluorescence analysis using an anti-dioxins antibody.

2. Materials and methods

2.1. Characterization of n-TiO_2 primary particles, agglomeration of n-TiO_2 standard suspensions in ASW and interactions between n-TiO_2 and TCDD in ASW

Nanosized Titanium Dioxide (n-TiO_2), Aeroxide® P25 namely Aeroxide © (declared purity of 99.9%) was kindly provided from Eigenmann & Veronelli (Milan, Italy). The obtained batch was characterized by a combination of analytical techniques (HR-TEM, TEM-EDX, XRD, HR-TEM-SAED, BET, ICPMS) as previously described (Barmo et al., 2013). Stock suspensions of n-TiO2 were freshly prepared in filtered artificial seawater-ASW (ASTM, 2004) at $10\ mg\ L^{-1}$, sonicated for 15 min at 100 W, 50% on/off cycle while cooling the dispersion in an ice bath, with a UP200S Hielscher Ultrasonic Technology (Teltow, Germany). Size distribution of n-TiO2 suspensions were evaluated by Dynamic Light Scattering (DLS) analysis 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 as previously described (Brunelli et al., 2013). The obtained results, previously reported in Canesi et al. (2014) and Della Torre et al., (2015), are summarized in Table S1. Size distribution of n-TiO_2 by TEM analysis ranged approximately from 10 to 65 nm (27 nm average), with shape partly irregular and semi-spherical. The main crystallographic phases were confirmed to be anatase and rutile (4:1 ratio), in accordance with the manufacturer's data. BET analysis indicated a specific surface area of $61 \pm 0.2\ m^2/g$, a pore size of 0.5 ± 0.1 ml/g and a bimodal pore size distribution in the 2–4 and 10–90 nm size range, respectively. According to these results, the selected n-TiO_2 sample could be classified as mesoporous. DLS analysis of a n-TiO_2 suspension ($100\ \mu g\ L^{-1}$ in ASW), indicated the general formation of agglomerates, starting immediately after n-TiO_2 addition (180 ± 21 nm), and whose average size increased after 25 h (207 ± 26 nm) and 50 h (304 ± 38 nm).

Chemical interaction between n-TiO_2 and 2,3,7,8- TCDD in ASW and in the presence of vehicle DMSO (0.001‰) was investigated by UV–vis adsorption spectroscopy and Nuclear Magnetic Resonance (NMR) spectroscopy as described in Della Torre et al. (2015), indicating no interaction between nano-TiO_2 and TCDD in ASW exposure medium.

2.2. Animals and treatments

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

Exposure experiments were performed as previously described (Canesi et al., 2014). 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. TCDD (Wellington Laboratories, Ontario, Canada) purchased in dimethyl sulfoxide (DMSO) ($32.2 \pm 1.6\ mg\ mL^{-1}$) was suitably diluted in ASW and spiked in 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 at $100\ \mu g\ L^{-1}$ nominal concentration levels, or TCDD $0.25\ \mu g\ L^{-1}$ and to both n-TiO_2 and TCDD at the same concentrations. Two parallel groups of control (untreated) and vehicle-treated (0.001% DMSO) 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 treatments, digestive glands were rapidly removed and frozen for subsequent analyses. For transcriptomics, tissues were kept at −20 °C in a RNA preserving solution (RNA Later, Sigma-Aldrich); for histochemistry, tissues were mounted on aluminum chucks, frozen in super-cooled n-hexane and stored at −80 ° C.

2.3. Histological analysis

Frozen digestive gland sections (10 μm) of ten mussels from each exposure condition were cut by cryostat (Leica CM3050) and flash-dried by transferring them onto poly-L-lysine-coated microscope slides at room temperature. After fixation (4% paraformaldehyde-PFA in phosphate buffer saline-PBS, pH 7.2, 20 min), sections were stained with hematoxylin and eosin (Chan, 2014) or Fontana-Masson (for melanin staining), mounted in DPX and then viewed under 400 × magnification by a Axiolab photo-microscope (Zeiss).