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Oxidative stress induced by titanium dioxide nanoparticles increases under seawater acidification in the thick shell mussel *Mytilus coruscus*

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ABSTRACT

Biochemical responses of the mussel *Mytilus coruscus* exposed to different concentrations of titanium dioxide nanoparticles (nano-TiO_2) (0, 2.5, 10 mg L^{-1}) and two pH levels (pH 8.1 and pH 7.3) for 14 days. Mussel responses were also investigated after a 7 days recovery period (pH 8.1 and no nanoparticle). Exposure to nano-TiO_2 led changes in antioxidant indexes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH)), biotransformation enzyme activity (GST) and malondialdehyde level (MDA) in gills and digestive glands. An increase in MDA level and a decrease in SOD and GSH activities were observed in gill of mussels exposed to 10 mg L^{-1} nano-TiO_2. This effect was more severe in mussels kept at pH 7.3 as compared to pH 8.1. A different response was observed in the digestive gland as SOD, CAT and GSH levels increased in mussels exposed to nano-TiO_2. These contrasting results in digestive glands and gills were only evident at high concentration of nano-TiO_2 and low pH. A 7 days recovery period was not sufficient to fully restore SOD, GPx, GST, GSH and MDA levels to levels before exposure to nano-TiO_2 and low pH. Overall, our results confirmed that seawater acidification modulates effects of nanoparticles in mussels, and that gills are more sensitive to these stressors as compared with digestive glands.

1. Introduction

Nanoparticles (NPs) are defined as materials smaller than 100 nm on at least one dimension. They have novel physicochemical properties leading to numerous applications in medical, industrial, agricultural, and manufacturing (Long et al., 2006). As a consequence, NPs enter estuarine, coastal, and marine environments (Hall et al., 2009; Kaegi et al., 2008; Menard et al., 2011) and can have negative impacts on marine species and ecosystems (Mueller and Nowack, 2003). Titanium dioxide nanoparticles (nano-TiO_2) are one of the most extensively used metal oxide NPs in industrial products. Concentrations of nano-TiO_2 in raw sewage waters are ranging from 181 to 1233 µg L^{-1} (Westerhoff et al., 2011). Some recent studies considered the impact of nano-TiO_2 on aquatic organisms (French et al., 2009; Minetto et al., 2016; Peng et al., 2017). However, the understanding of toxicity impacts of nano-TiO_2 is still limited and does not consider interaction with other global and local stressors.

Ocean acidification (OA) is one of the emerging global changes that are threatening marine environments. It is a consequence of an increase in partial pressure of carbon dioxide into the atmosphere and seawater due to human emissions. It leads to a decrease in carbonate ion concentration and pH in seawater (Zeng et al., 2015). Models predict that by 2100, average surface oceanic pH will decrease by 0.4 unit (Pörtner et al., 2014). A large body of evidence is documenting impacts of OA on marine species. These include decreased reproduction success (e.g. Ventura et al., 2016; Xu et al., 2016), reduced calcification rates (e.g. Martin and Gattuso, 2009; Rodolfo-Metalpa et al., 2015), impaired energy metabolism and immune response (e.g. Huang et al., 2016; Wang et al., 2015) and, shifts in community structure (e.g. Hall-Spencer et al., 2008). However, OA has the potential to interact with other local

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and global stressors (Bednaršek et al., 2016; Gunderson et al., 2016; Norris et al., 2013; Zeng et al., 2015). Interactions between OA and other environmental changes, such as warming (e.g. Hu et al., 2015), deoxygenation (e.g. Sui et al., 2017), salinity change (e.g. Velez et al., 2016a) or emerging contaminants such as toxic metal (e.g. Götze et al., 2014; Nardi et al., 2017; Shi et al., 2016; Velez et al., 2016b) and metal oxide (e.g. Hu et al., 2017; Kadar et al., 2010) is currently understudied.

Bivalve molluscs are shell-forming organisms highly sensitive to OA (e.g. Baun et al., 2008; Canesi et al., 2012; Guzman et al., 2006; Sharma, 2009; von der Kammer et al., 2010). Bivalves exposure to nano-TiO_2 can lead to the generation of reactive oxygen species (ROS) and influence of oxidation state (Girardello et al., 2016; Huang et al., 2016; Johnson et al., 2015). It has already be shown that the production of ROS can be enhanced by OA (Sun et al., 2017). An overproduction of ROS results in oxidative stress in tissues and can cause serious damages by depleting antioxidant agents and enzymatic defenses (Qiu et al., 2016; Vale et al., 2016). Nevertheless, there are no detailed studies on the interactive effects toxic effects of nano-TiO_2 and OA on antioxidant responses in mussels.

2. Materials and methods

2.1. Experimental animals

Mussels (shell length 6.98 ± 0.66 cm, wet weight 66.62 ± 2.76 g) were collected from the Shengsi island, Zhejiang Province, China. They were acclimated to the laboratory conditions for 7 days at a salinity 25 ± 1 psμ and a temperature 20 ± 0.5 °C in 500 L tanks equipped with filtering systems. Mussels were fed daily with *Chrysophyta* spp (5.0×10^5 cells ml^{-1}).

2.2. Nano-TiO_2 and pH treatments

Stock aqueous suspensions (10 g L^{-1}) of nano-TiO_2 P25 (purity > 99.5%; Sigma Aldricht) were prepared using artificial seawater and then sonicated (UP200S, Hielscher Ultrasonic Technology, Teltow, Germany) for 15 min at 100 W in ice bath. The 10.0 g L^{-1} sonicated stock solution was used to reach the nominal concentrations (0, 2.5 and 10 mg L^{-1}) in each experimental unit. This concentration range is traditionally used for acute exposure in aquatic animals (e.g. Federici et al., 2007; Gornati et al., 2016; Chen et al., 2011; Jimeno-Romero et al., 2016). X-ray diffraction analysis (XRD) patterns of nano-TiO_2 were obtained by X'Pert PRO X-ray Diffractometer (PANalytical B.V.). Scanning electron microscopy (SEM, Hitachi JSM-7500F) was used to check primary particle size and morphology. Particle size was also analysed by transmission electron microscope (TEM, Low Voltage Tem5, LVEM5) equipped with a CCD camera. The zeta-potential and hydrodynamic diameter of the nano-TiO_2 under different pH conditions were evaluated by dynamic light scattering (DLS) analysis using a Zetasizer Nano ZEN 3600 (Malvern Instruments, UK). Dynamic light scattering technique (DLS, Microtrac S3500, UK) was also used for the evaluation of the particles size distribution at the two tested pH. NP concentrations in water were measured using the standard test method for determination by atomic absorption spectroscopy of titanium dioxide content (Range et al., 2014).

Two pH values treatments were compared for each nano-TiO_2 treatment for a total of 6 treatments (2 pH × 3 TiO_2): pH 8.1, corresponding to present average; and pH 7.3, corresponding to the extreme low pH predicted at the sampling site by the year 2100. Mussels were transferred to the low pH treatment without previous acclimation. The seawater pH was automatically regulated by bubbling pure CO_2 gas using pCO_2/pH system (DAQ-M) equipped with WTW pH 3310 m and SenTix 41 pH electrodes (Loligo Systems Inc.). Seawater salinity was measured using a multiparameter instrument (model 5200 A, YSI, USA). Total alkalinity (A_T) was determined by titration method. Other parameters of the seawater carbonate chemistry (pCO_2, dissolved inorganic carbon (DIC), saturation states of calcite (Ωca) and aragonite (Ωar)) were calculated from A_T and pH_{NBS} using CO_2sys (Pierrot et al., 2006; Wang et al., 2015).

Three replicated 30 L aquariums with 30 mussels were used per treatment. The seawater was changed every day, and new aliquots of nano-TiO_2 solutions were added to maintain the nano-TiO_2 at relatively constant levels over a 14 days exposure. This exposure period was followed by a depuration period of 7 d without nano-TiO_2 and under pH 8.1. Gill and digestive gland tissue samples were collected at days 1, 3, 7, 14, 17 and 21 d) for the analysis of biochemical parameters.

2.3. Tissue sampling and biochemical assays

For each replicated aquarium, gills and digestive glands were removed from three mussels kept on ice and pooled to reduce individual variation. Samples were immediately frozen in liquid nitrogen and stored at −80 °C. Tissues were later defrosted on ice and homogenized in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM Dithiothreitol (DTT, Sigma) and 40 μg/ml Aprotinin (Sigma) using a motor driven Teflon Potter-Elvehjem homogenizer. Homogenized samples were sonicated for 2 min at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles and were then centrifuged at 10,000 g for 20 min at 4 °C. Supernatants were used for biochemical analysis.

Assays were performed using commercial kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and following manufacturer's instructions. Optical density values were measured using a microplate reader (Flexstation® 3, Molecular Devices, California, USA). The protein content of enzyme crude extract was determined using Coomassie Brilliant Blue (G-250) method as reported by (Bradford, 1976) with bovine serum albumin as standard.

The SOD activity was measured using the nitro blue tetrazolium (NBT) method modified from Beauchamp and Fridovich (1971). One unit (U) of SOD activity was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%. The CAT activity was determined by measuring the initial rate of the decrease in absorbance at 405 nm as a spectrophotometric assay of hydrogen peroxide (H_2O_2) over 1 min at 30 °C (Góth, 1991). One U of enzyme activity was defined as the degradation of 1 μmol H_2O_2 per second per mg tissue protein. The activity of GPx was measured by quantifying the rate of H_2O_2-induced oxidation of GSH to GSSG (Lawrence and Burk, 1976). A yellow product which had absorbance at 412 nm could be formed as GSH reacted with dithiobisnitrobenzoic acid. One U of GPx activity was defined as the amount that reduced the level of GSH by 1 mmol/L in 1 min per milligram of protein and the enzyme activity was expressed as U/mg protein. The GST activity was determined according to a method of Habig et al. (1974), using 2 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 2 mM reduced glutathione in 0.1 M potassium phosphate buffer, pH 7.0. The absorbance was monitored for 2 min at 30 °C at 340 nm for a better detection in the microplate reader. One U of GST activity is the amount of enzyme which catalyzes the conjugation of 1 mM of substrate per minute. Levels of GSH were measured based on the method of Ringwood et al. (1999) by reading the optical density of the yellow substance formed when 5,5′-dithio-2-nitrobenzoic acid is reduced by GSH at 412 nm. GSH content in the extract was determined as nmol/mg protein by a standard curve generated with GSH at diverse concentrations. MDA was evaluated with the thiobarbituric acid reactive substances (TBARS). The TBARS formed was measured in a microplate reader at 532 nm and quantified as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane as the standard (Ohkawa et al., 1979). MDA contents were expressed as nmol TBARS/mg protein/min.

2.4. Statistical analyses

Using SPSS 18.0, data were tested for normality using the Shapiro-Wilk's test and homogeneity of variance using the Levene's test. Arcsine