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Are the impacts of carbon nanotubes enhanced in *Mytilus galloprovincialis* submitted to air exposure?

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A B S T R A C T

Intertidal species are frequently exposed to environmental changes associated with multiple stressors, which they must either avoid or tolerate by developing physiological and biochemical strategies. Some of the natural environmental changes are related with the tidal cycle which forces organisms to tolerate the differences between an aquatic and an aerial environment. Furthermore, in these environments, organisms are also subjected to pollutants from anthropogenic sources. The present study evaluated the impacts in *Mytilus galloprovincialis* exposed to multi-walled carbon nanotubes (0.01 mg/L MWCNTs) when continuously submersed or exposed to tides (5 h of low tide, 7 h of high tide) for 14 days. Our results demonstrated that mussels were physiologically and biochemically affected by MWCNTs, especially when exposed to tides. In fact, when only exposed to the carbon nanoparticles or only exposed to tides, the stress induced was not enough to activate mussels' antioxidant defenses which resulted in oxidative damage. However, when mussels were exposed to the combination of tides and MWCNTs increased metabolism was observed, associated with a possible higher production of reactive oxygen species (ROS), leading to a significant increase in the activities of antioxidant enzymes (superoxide dismutase, SOD and glutathione peroxide, GPx) and oxidized glutathione content (GSSG), preventing the occurrence of cellular damage, expressed as no lipid peroxidation (LPO) or protein carbonylation (PC). Therefore, organisms seemed to be able to tolerate MWCNTs and air exposure during tidal regime; however, the combination of both stressors induced higher oxidative stress. These findings indicate that the increasing presence of carbon nanoparticles in marine ecosystems can induce higher toxic impacts in intertidal organisms compared to organisms continuously submerged. Also, our results may indicate that air exposure can act as a cofounding factor on the assessment of different stressors in organisms living in coastal systems.

1. Introduction

Estuaries are ecologically and economically valuable ecosystems, presenting several essential ecological functions such as high biological productivity, hydrological regulation, biogeochemical cycling of metals and nutrients, as well as habitat and food source for wildlife (Caçador et al., 2007; Mitsch and Gosselink, 2015; Xiao and Li, 2004). However, these ecological functions are strongly influenced by physical and chemical disturbances from natural or anthropogenic sources, typical of transitional coastal ecosystems (Dauvin and Ruellet, 2009; Elliott and Quintino, 2007). Due to their nature, coastal systems, namely estuaries and coastal lagoons, represent one of the hardest environments to endure for the inhabiting organisms. Among the most stressful conditions to face, species that inhabit these areas are subjected to tides and a large variation of climatic conditions, such as temperature, salinity, as well as high desiccation risk and marked variation of oxygen availability between aquatic and aerial conditions (Davis, 1985; Freire et al., 2011; Horn et al., 1999; Underwood and Kromkamp, 1999). Furthermore, inputs of chemicals associated with industrial, domestic and agriculture activities from the surrounding areas are another disturbance that these organisms must cope with on a daily basis (Amiard-Triquet and Rainbow, 2009; Elliott et al., 2014).

In intertidal areas, as a consequence of air exposure, organisms may face prolonged hypoxia and/or anoxia. Although marine bivalves are among the most hypoxia-tolerant macrofauna (Abele et al., 2009; Gray et al., 2002), the impacts of air exposure on the physiological

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performance of several bivalves have already been observed (Almeida and Bainy, 2006; Altieri, 2006; Andrade et al., 2018; Chandurvelan et al., 2013; Letendre et al., 2008, 2011; Yin et al., 2017). It is already known that some bivalves, as the mussel *Mytilus galloprovincialis*, close their valves when exposed to air (Dowd and Somero, 2013; Nicastro et al., 2010). As a consequence, intertidal bivalves may face complete anoxia while closing their shells during low tides to avoid desiccation, although others may prevent anoxia by simply opening the valves for air gaping (Rivera-Ingraham et al., 2013). Different bivalve species have also showed induction of oxidative stress related to air exposure and reoxygenation. Studies demonstrated, for example, an increase on antioxidant defenses in the mussels *Perna perna* and *M. galloprovincialis* as a defense mechanism against oxidative stress during re-oxygenation (Almeida and Bainy, 2006; Andrade et al., 2018). A similar response was observed in specimens of the clam *Ruditapes philippinarum* daily exposed to rhythms of air exposure (Yin et al., 2017). Specimens of the mussel *M. edulis* demonstrated the generation of over-expression of several proteins involved especially in cytoskeleton, chaperoning, energy metabolism and transcriptional regulation after emergence (Letendre et al., 2011).

Estuaries are dynamic interface zones between water draining from inland river basis and oceans, and for this reason normally receive high concentrations of natural and anthropogenic materials (Amiard-Triquet and Rainbow, 2009; Müller et al., 1995; Lopes et al., 2011). Pollutants from different anthropogenic sources are increasing in marine ecosystems which can cause adverse effects (Fu et al., 2003; Maanan, 2008). This is the case of nanoparticles (NPs) which have been increasingly used in numerous applications (Renn and Roco, 2006) and, thereby, the increased introduction of these materials into the aquatic systems is expected to occur. Carbon-based NPs have a diversity of applications (Köhler et al., 2008; Muller and Nowack, 2008; Solarskaciuk et al., 2014; Vlasova et al., 2016; Wu et al., 2013). Among the most important carbon-based NPs are carbon nanotubes (CNTs) (Eckelman et al., 2012; Sanchez et al., 2012; Scown et al., 2010), recently detected in aquatic systems at predicted environmental concentrations (PECs) of approximately 0.001–1000 μg/L according to the most recent literature (see for example De Marchi et al., 2018b; Zhang et al., 2017). The toxic properties and impacts of CNTs on marine invertebrates are still limited, but the effects of different CNTs were already demonstrated, including biochemical and physiological alterations induced in the mussel *M. galloprovincialis* (Canesi et al., 2008, 2010), in the clam *R. philippinarum* (De Marchi et al., 2017c, 2018a), and in the polychaetes *Diopatra neopolitana* and *Hediste diversicolor* (De Marchi et al., 2017b). Nevertheless, the high surface to volume ratio and reactivity of CNTs make them highly dynamic in environmental systems and the resulting transformations of these carbon NPs under different environmental conditions (e.g. tidal exposure, with associated salinity and temperature shifts) will affect their fate, transport, and toxic properties (Velzeboer et al., 2013).

Among bivalves the mussel species *M. galloprovincialis* (Lamarck, 1819) is widely distributed across the globe, inhabiting infra littoral areas (FAO, 2016; Vazzana et al., 2016) being present on rocky areas, cliffs, boulders or substrates that are relatively movable and to which it adheres (FAO, 2016; Vazzana et al., 2016). In Portugal, this species exists along the entire coast (Mitchelmore et al., 1998) which is frequently exposed to tidal changes and, as a sedentary filter feeding organism, has the capacity to accumulate pollutants from the environment and reflect the imposed toxic impacts. Furthermore, bivalves are known to tolerate high concentrations of xenobiotics and provide a specific response to pollutants and, for these reasons, *M. galloprovincialis* has been widely used as a bioindicator species (Catsiki and Florou, 2006; Faggio et al., 2016; Kristan et al., 2014; Oliveira et al., 2017; Sureda et al., 2011). These organisms, present in a wave-exposure environment associated with rocky intertidal shores, appear to exhibit adaptive physiological, behavioral and morphological traits (Dowd et al., 2013; Sherratt and Mackenzie, 2016) such as the valve closure to protect from stressful conditions (Gazeau et al., 2013; Ishii et al., 2005; Poulain et al., 2011). However, little is still known about the physiological and biochemical effects of tidal changes in these organisms living under such environmental conditions. Furthermore, in the environment mussels are subjected to tidal changes which may act as a confounding factor when assessing the impacts induced by contaminants, such as CNTs exposure. In fact, when mussels are used in environmental monitoring programs and especially under laboratory conditions, their natural environment and its possible interactions with other stressors, namely pollutants, influencing their toxicity, is not considered. Within this context, the present study aimed to evaluate if physiological and biochemical alterations imposed by the presence of multi-walled CNTs (MWCNTs) were dependent on the submersion/tidal regime, to better understand the possible interactions of both conditions (contamination and exposure to air) in the physiological and biochemical performance of mussels.

2. Methodology

2.1. Sampling and experimental conditions

Mytilus galloprovincialis specimens were collected in September 2017 during low tide in an intertidal area at the Mira Channel (Ria de Aveiro, a coastal lagoon, northwestern Portugal). After sampling, the collected mussels were placed in aquaria for depuration and acclimation to laboratory conditions for 7 days. Artificial seawater (salinity 35 ± 1), made with artificial salt (Tropic Marin®SEA SALT from Tropic Marine Center) and deionized water, was used. During this period the organisms were maintained at 18 °C ± 1.0 °C and pH 8.0 ± 0.1, resembling estuarine conditions, and kept under continuous aeration using a 12 h light: 12 h dark photoperiod.

For the laboratory experiment, organisms were distributed into different aquaria (20 L seawater, salinity 35), with 6 individuals *per* aquarium and 3 aquaria *per* treatment (18 individuals *per* treatment). The treatments tested were: submersion without MWCNTs (Sub); submersion with 0.01 mg/L MWCNTs (Sub + MWCNTs); exposure to tidal simulation without MWCNTs (Tide); exposure to tidal simulation with 0.01 mg/L MWCNTs (Tide + MWCNTs). Aquaria were placed in a climatic room to maintain the temperature level at 18 ± 1.0 °C. For the tidal simulation, an automatic system that mimicked the estuary tidal regime typical of the habitat of this species (5 h of low tide and 7 h of high tide cycles) was developed and used.

The control temperature of 18 ± 1.0 °C was chosen considering the average temperature of the sampling area during September period (IPMA, 2017). The salinity of 35 was chosen considering salinity values at the sampling area located at the Mira Channel, one of the main branches of the Ria de Aveiro, connected to the ocean entrance (Lopes et al., 2007; Picado et al., 2010). The concentration of MWCNTs used was chosen taking into account previous works carried out by De Marchi et al. (2017b,c, 2018a) in the bivalve *R. philippinarum* and the polychaetes *D. neapolitana* and *H. diversicolor*, where 0.01 mg/L was the lowest concentration inducing observable physiological changes and following the predicted environmental concentrations (PECs) of CNTs in aqueous systems –. Functionalized MWCNTs were used in the present study to avoid the decrease of carbon content in the water column (due to its dispersion properties). It was already demonstrated that surface areas of CNTs containing carboxyl (COOH) groups are widely used as active sites for further functionalization which improves the solubility and biocompatibility of the material (Scheibe et al., 2010). A study conducted by Peng et al. (2009) investigated the precipitation of oxidized CNTs in water by salts. The results showed that CNTs concentration decreases slightly with aging time. CNTs concentration after 30 days aging was 85% of the initial CNTs concentration, which indicates that only 15% oxidized CNTs settled during 30 days. The stability of oxidized CNTs in water is probably related to the fact that the oxidation process introduces oxygen-containing groups on the CNTs