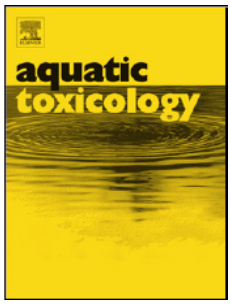

Title: Are the impacts of carbon nanotubes enhanced in *Mytilus galloprovincialis* submitted to air exposure?

Authors: Madalena Andrade, Lucia De Marchi, Carlo Pretti, Federica Chiellini, Andrea Morelli, Amadeu M.V.M. Soares, Rui Rocha, Etelvina Figueira, Rosa Freitas

PII: S0166-445X(18)30443-0
DOI: https://doi.org/10.1016/j.aquatox.2018.07.006
Reference: AQTOX 4979

To appear in: *Aquatic Toxicology*

Received date: 15-5-2018
Revised date: 7-7-2018
Accepted date: 9-7-2018

Please cite this article as: Andrade M, De Marchi L, Pretti C, Chiellini F, Morelli A, Soares AMVM, Rocha R, Figueira E, Freitas R, Are the impacts of carbon nanotubes enhanced in *Mytilus galloprovincialis* submitted to air exposure?, *Aquatic Toxicology* (2018), https://doi.org/10.1016/j.aquatox.2018.07.006

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

Madalena Andrade[a], Lucia De Marchi[a], Carlo Pretti[b], Federica Chiellini[c], Andrea Morelli[c], Amadeu M.V.M. Soares[a], Rui Rocha[a], Etelvina Figueira[a], Rosa Freitas[a]*

[a]Department of Biology & Center for Environmental and Marine Studies (CESAM), University of Aveiro 3810-193, Aveiro, Portugal

[b]Department of Veterinary Sciences, University of Pisa, San Piero a Grado, Pisa 56122, Italy

[c]Department of Chemistry and Industrial Chemistry, University of Pisa, Udr INSTM Pisa, Pisa 56126, Italy

*__Corresponding Author__: Rosa Freitas, Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

Highlights:

- *Mytilus galloprovincialis* was physiologically and biochemically affected by MWCNTs
- Greater impacts on mussel metabolism was induced by the exposure to tides
- Higher alterations were observed when organisms were exposed to MWCNTs and tides
- Mussels exposed to tides and MWCNTs avoided cellular damages activating antioxidant defenses

ABSTRACT

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 MWCNTs 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 higher production of reactive oxygen species (ROS), leading to a significant increase in the activities of antioxidant enzymes (superoxide dismutase, SOD and glutathione peroxidase, GPx) and oxidized glutathione content (GSSG), preventing the occurrence of cellular damage, with organisms showing 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.

Keywords: mussels, nanoparticles, oxidative stress, metabolism, tidal regime.

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, those 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 performance of several bivalves have already been observed (Almeida and Bainy, 2006, Andrade et al, 2018, Chandurvelan et al., 2013, Altieri, 2006; Letendre et al. 2008, 2011, Yin et al., 2017). It is 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 *Mytilus 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., 2016). 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., 2009; Matanan, 2008). This is the case of nanoparticles (NPs) which have been increasingly used in numerous applications including medicine, chemistry and electronics (Renn and Roco, 2006) and, thereby, the increased introduction of these materials into the aquatic systems is expected to occur. Among NPs, carbon-based NPs have a diversity of applications (Solarskaciuk et al., 2014; Vlasova et al., 2016; Wu et al., 2013; Muller and Nowack, 2008; Köhler et al., 2008). Among the most important carbon-based NPs are carbon nanotubes (CNTs) (Scown et al., 2010; Eckelman et al., 2012; Sanchez et al., 2012), 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 Zhang et al., 2017; De Marchi et al., 2018b). The toxic properties to 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, 2018), 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 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). This species 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 CNT 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 during low tide in an intertidal area at the Mira Channel (Ria de Aveiro, a coastal lagoon, northwestern Portugal), in September 2017. 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 18ºC ± 1.0 ºC 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 ºC. For the tidal simulation, an automatic system that mimicked the estuary tidal regime typical of the habitat of this species (5 hours of low tide and 7 hours of high tide cycles) was developed and used.

The control temperature of 18 ± 1.0 ºC was chosen considering the average temperature of the sampling area during September (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, 2017c, 2018) 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 (0.001-1000 µg/L). 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 and hydroxyl 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 CNT concentration decreases slightly with aging time. CNT concentration after 30 days aging was 85% of the initial CNT 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 CNT surface. These groups ionize in water and the oxygen-containing groups are negatively charged. In aqueous phase, the electrostatic repulsive forces between negative surface charges of the oxygen-containing groups may lead to stability of oxidized CNTs and the oxidized CNTs can form stable dispersion in water.

During the experimental period (14 days) organisms were fed three times *per* week with Algamac protein plus (10^7 cells/animal). After 7 days of the beginning of the experiment, seawater was renewed re-establishing seawater characteristics and MWCNT concentration. During the experimental period, water samples were taken immediately before seawater renewal to characterize MWCNTs in the water from aquaria exposed to this NP.

An experimental period of 14 days was chosen considering previous studies in mussels (Andrade et al., 2018; Hu et al., 2015; Huang et al., 2018; Letendre et al., 2011; Verlecar et al., 2007) which observed physiological changes during this period. At the end of the experimental period (14 days), the organisms were immediately frozen at -80 ºC until analysis with the exception of two organisms per aquarium which were immediately used for respiration rate determination.

2.2. MWNT characterization

The functionalized MWCNTs were produced via catalytic carbon vapor deposition (CCVD) process. These carbon nanoparticles were purchased from Times Nano: Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com and with manufacturer specifications of diameter 2-5 nm; length 10-

^{2}/g; amorphous carbon 8-10% and -COOH 3.86 wt%).

The concentration of MWCNTs used in this study (0.01 mg/L) was prepared from a stock solution of 50 mg/L concentration. For particle characterization, the average size distribution of MWCNT suspensions in seawater in each exposure condition was analyzed by dynamic light scattering (DLS), using a Delsa™ NanoC Particle Size Analyser (Beckman Coulter). Measurements were performed on 1 mL of suspension and each analysis was repeated three times.

The hydrodynamic radius and polydispersity index (PDI) of the analysed dispersions were calculated on three replicates of each sample collected after a week of the experimental period by using the cumulant method. Undetected colloidal material at the end of each measurement was indicated as Invalid data (I.d.).

2.3. Biological responses: physiological parameters

2.3.1. Respiration Rate

The respiration rate (RR) was measured at the end of the experimental period. Six *M. galloprovincialis* specimens *per* condition (2 individuals *per* aquarium/replicate, 6 individuals *per* treatment) were used to determine the respiration rate of organisms. Measurements were performed by simple static respirometry, filling the respirometric chambers with the same artificial seawater as used during the experimental period and two organisms of the same aquarium *per*

chamber. Organisms were put in the dark and oxygen concentrations were measured at each 15 min for 2 hours with an oxygen meter (model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow). Organisms were afterwards dried and weighed. The oxygen consumption rate was determined by calculating the differences between the oxygen content in the water before ($T_{initial}$ = 0h) and after (Tf_{inal} = 2h) the process. Respiration rate was expressed in *per* g dry tissue (DW) and an hour. Two blank controls (chambers with no organisms) were employed to correct the ambient oxygen depletion due to other factors than the respiration of organisms.

2.4. Biological responses: biochemical parameters

After the 14 days of the experimental period, shells of the frozen organisms (4 individuals *per* replicate, 12 individuals *per* treatment) were removed and the frozen whole soft tissue was pulverized with liquid nitrogen using a mortar and pestle. The homogenized tissue of each organism was then distributed in 0.5 g aliquots.

For each biochemical parameter, the extraction of the supernatant was accomplished with a specific buffer (see Andrade et al., 2018; De Marchi et al., 2017b; Mesquita et al., 2014) using a proportion of 1:2 (w/v). Tissue samples were homogenized during 1 min using a TissueLyser II (Qiagen), followed by a centrifugation for 20 min at 10.000 g and 4 ºC. The supernatants were then stored at -80 ºC or immediately used to determine: electron transport system (ETS) activity; glycogen (GLY) content; lipid peroxidation (LPO) and protein carbonylation (PC) levels; oxidized glutathione (GSSG) content and activity of antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxide, GPx). For the determination of each biochemical parameter, two replicates *per* sample were used.

2.4.1. Metabolic capacity

The ETS activity was measured based on the method of Packard (1971) and modifications by Coen and Janssen (1997). Absorbance was measured for 10 min at 490 nm with intervals of 25 s. The extinction coefficient of 15.900 $M^{1}cm^{-1}$ was used to calculate the amount of formazan formed and the results were expressed in nmol min *per* g of fresh weight (FW).

2.4.2. Energy reserves

The GLY content was quantified according to the sulfuric acid method (Dubois et al, 1956), using glucose standards. Absorbance was measured at 492 nm after 30 min incubation at room temperature. Results were expressed in mg *per* g of FW.

2.4.3. Oxidative damage

Levels of LPO were measured according to Ohkawa et al. (1979) with modifications referred by Carregosa et al. (2014b). Absorbance was measured at 535 0.156 mM^{-1} cm^{-1}), and the results expressed in nmol of MDA equivalents formed *per* g of FW.

The GSSG content was determined following the method described in Rahman et al. (2007), using GSSG as standard (0 results were *per* g FW.

The quantification of PC levels followed the DNPH alkaline method described by Mesquita et al., (2014). Absorbance was measured 0.022 mM^{-1} cm^{-1}) and the results were expressed in nmol of protein carbonyl groups formed *per* g of FW.

2.4.4. Antioxidant enzymes

The activity of SOD was determined following the method of Beauchamp and Fridovich (1971). The standard curve was generated with SOD standards (0.25-60 U/mL). Absorbance was measured at 560 nm after 20 min of incubation at room temperature. The SOD activity was

expressed in U *per* g of FW where one unit (U) of enzyme activity corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

The activity CAT was quantified based on the method of Johansson and Borg (1988). The standard curve was determined using formaldehyde standards (0-150 Absorbance was measured at 540 nm. CAT activity was also expressed in U *per* g of FW. In this case, one unit (U) is defined as the formation of 1 nmol formaldehyde *per* min.

The activity of GPx was quantified following Paglia and Valentine (1967). Absorbance was 0.00622 µM $^{-1}$ cm^{-1}) in 10 s intervals during 5 min. Results were expressed in U *per* g FW where one unit (U) represents the quantity of enzyme which catalyzes the conversion of 1 µmol nicotinamide adenine dinucleotide phosphate (NADPH) *per* min.

2.5. Data analysis

Due to a lack of homogeneity of variance, RR, ETS, GLY, LPO, PC, GSSG, SOD, CAT and GPx were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer 7) with a two-factor design: submersion condition (submersed (sub) or exposed to tide (tide)) as factor 1 and contamination condition (non-contaminated (ncont) or contaminated (MWCNTs)) as factor 2. PERMANOVA main test was performed to test the effect of submersion condition, contamination condition and the interaction between these two factors on each biomarker. PERMANOVA main tests were considered significant when $p < 0.05$ and followed by PERMANOVA pair-wise tests. Pair-wise tests were used to test the effect of contamination condition (ncont and MWCNTs) within each submersion condition and the effect of submersion condition (sub and tide) within each contamination condition. PERMANOVA pair-wise tests results are represented in figures with lower case letters and in the main text by p-values.

3. RESULTS

3.1. MWCNT characterization

The mean size (nm) and the polydispersity index (PDI) of simulated seawater samples collected from aquaria containing mussels (*Mytilus galloprovincialis*) were measured by dynamic light scattering (DLS). Results obtained by DLS analysis did not evidence the presence of dispersed materials in seawater samples collected from aquaria where organisms were subjected to tidal simulation, while samples kept submersed in water throughout the course of the experiments were found to be contaminated by micro-sized suspensions (Table 1).

3.2. Physiological parameters

3.2.1. Mortality

After 14 days of exposure, no mortality was observed in any tested treatment.

3.2.2. Respiration rate

Concerning respiration rate (RR), no significant differences were observed between contaminated and non-contaminated organisms either under submersion or tide exposure conditions (Figure 1). Comparing submersion and tide exposure conditions, non-contaminated and contaminated organisms submersed during the entire experiment tended to show lower RR values than organisms exposed to tides, but the difference was not statistically significant (Figure 1). No significant effect of the interaction between tide exposure and the presence of MWCNTs in the RR was observed (p=0.5709).

3.2. Biochemical parameters

3.2.1. Metabolic capacity

Concerning ETS activity, no significant differences were observed between non-contaminated and contaminated organisms either under submersion or tide exposure conditions (Figure 2A). Significantly lower ETS values were observed in mussels submersed for the entire experiment in comparison to mussels exposed to tides, both for non-contaminated and contaminated organisms (Figure 2A). The interaction between tides and MWCNTS showed no significant effects on the ETS activity (p=0.6504).

3.2.2. Energy reserves

Concerning GLY content, no significant differences were observed between contaminated and non-contaminated organisms either under submersion or tide exposure conditions (Figure 2B). Also, no significant differences were observed between submersion and tide exposure conditions, either for non-contaminated or contaminated organisms (Figure 2B). The interaction of both stressors (tides and MWCNTs) showed no significant effect on the GLY content (p=0.8487).

3.2.3. Oxidative damage

With regard to LPO levels, under submersion conditions, non-contaminated organisms presented significantly lower LPO values than contaminated organisms. When exposed to tides, significantly lower LPO levels were observed in contaminated organisms compared to non-contaminated mussels (Figure 3A). Comparing submersion and tide exposure conditions, non-contaminated mussels submersed during the entire experiment showed significantly lower LPO values than organisms exposed to tides. In the presence of MWCNTs, mussels submersed during the entire experiment showed significantly higher LPO values than organisms exposed to tides (Figure 3A). The interaction between tides and MWCNTs showed a significant effect on LPO levels (p=0.0234).

Concerning GSSG, no significant differences were observed between contaminated and non-contaminated organisms maintained under submersion conditions during the entire experiment. When exposed to tides significantly higher GSSG values were observed in the presence of MWCNTs (Figure 3B). Comparing submersion and tide exposure conditions, significant differences were only observed for contaminated mussels, with higher values in mussels exposed to tides in the presence of MWCNTs (Figure 3B). Nevertheless, the interaction between tides and MWCNTs showed no significant effect on GSSG content ($p=0.1524$).

Concerning PC, no significant differences were observed between contaminated and non-contaminated organisms always submersed or exposed to tides (Figure 3C). Comparing submersion and tide exposure conditions, no significant differences were observed either for non-contaminated or contaminated organisms (Figure 3C). No significant effects ($p=0.4293$) were observed on the PC resulting from the interaction between tides and MWCNTs.

3.2.4. Antioxidant enzymes

Concerning SOD activity under submersion conditions, no significant differences were observed between non-contaminated and contaminated organisms. When exposed to tides significantly higher SOD values were observed in contaminated compared to non-contaminated mussels (Figure 4A). Comparing submersion and tide exposure conditions, mussels in the absence of MWCNTs and submersed during the entire experiment showed significantly higher SOD values than organisms exposed to tides, while contaminated mussels exposed to tides showed significantly higher SOD values in comparison to contaminated organisms submersed the entire experimental period (Figure 4A). The interaction between tides and MWCNTs showed a significant effect ($p=0.0003$) on the SOD activity.

Regarding CAT activity, no significant differences were observed between contaminated and non-contaminated organisms always submersed or exposed to tides. Comparing submersion and tide exposure conditions, no significant differences were observed between non-contaminated and contaminated mussels (Figure 4B). No significant effects ($p=0.6662$) were observed due to the interaction between tides and MWCNTs.

Concerning GPx activity, no significant differences were observed between contaminated and non-contaminated organisms always submersed, while when exposed to tides significantly higher GPx values were observed in contaminated mussels compared to non-contaminated organisms (Figure 4C). Comparing submersion and tide exposure conditions, mussels submersed in the absence and presence of MWCNTs showed significantly lower GPx values than organisms exposed to tides (Figure 4C). The interaction between tides and MWCNTs showed no significant effects ($p=0.7146$) on the GPx activity.

4. DISCUSSION

The present study evaluated the physiological and biochemical performance of *M. galloprovincialis* when exposed to MWCNTs both under continuous submersion and exposed to tidal regime, aiming to understand if air exposure during simulated tidal regime would influence the toxic impacts induced by the NPs. This topic is of upmost importance as very little information is available on the impacts of air exposure in mussels, and especially when organisms are exposed to contaminants.

Physiological responses

Respiratory capacity

Respiration rate has been used in different intertidal organisms to assess the alterations induced by different stressors (De Marchi et al., 2017b; Gestoso et al., 2016; Freitas et al., 2017; Wang et al., 2015). Our results demonstrated that the exposure to MWCNTs did not change *M. galloprovincialis* respiratory capacity when compared to non-contaminated mussels, neither when individuals were exposed to tides nor when they were continuously submersed. Nevertheless, the increase of RR due to contaminant exposure has been demonstrated as a physiological adaptation-response (Pelexaas et al., 1988). In fact, this increase has been observed in different invertebrate species. Resgalla et al. (2010) obtained high respiration rates in the mussel *Perna perna* from contaminated areas. Nilin et al. (2012) showed high oxygen consumption and clearance rate in cockles from an Hg-contaminated area compared to cockles from a non-polluted area. However, similar to our results, De Marchi et al. (2017b) observed that the polychaetes *D. neapolitana* and *H. diversicolor* exposed to 0.01 mg/L of MWCNTs did not show any changes in RR, but at higher concentration (1.00 mg/L of MWCNTs) an increase of RR in *H. diversicolor* was observed. Therefore, the fact that in the present study the RR did not increase with contamination

may result from the low concentration of MWCNTs tested that was not enough to induce any changes on the respiratory capacity of mussels.

Our study also showed that RR slightly increased when the mussels were under the tidal regime, but the absence of significant changes compared to submersed mussels is probably due to the short experimental period tested. Yin et al. (2017) showed a significant increase in oxygen consumption in the clam *Ruditapes philippinarum* with the daily rhythms of air exposure (3h, 6h and 9h) followed by immersion, explaining that it was caused by the need to compensate for the oxygen debt resulting from the hypoxia caused by air exposure, when the clams where re-immersed.

Biochemical responses

Metabolic capacity and energy reserves

An indication of the metabolic status of organisms can be assessed by the determination of the electron transport system (ETS) activity, which allows an estimation of the energy consumption at the mitochondrial level (Coen and Janssen 1997). In the present study, mussels exposed to MWCNTs were able to maintain their metabolic capacity compared to non-contaminated organisms under both continuous submersion and tidal regime. The lack of significant differences in the ETS activity between contaminated and non-contaminated mussels can be associated to the similar respiratory capacity of mussels independently on the presence or absence of MWCNTs. These findings indicate that the concentration tested was not stressful enough to increase the metabolic activity of mussels to activate defense mechanisms or to inhibit metabolism, for example by valve closure to avoid contamination. Similarly, De Marchi et al. (2017c, 2018) observed no significant differences of ETS activity in *R. philippinarum* exposed for 28 days to the same nanoparticles and at the same concentration (0.01 mg/L).

Nevertheless, our results further revealed that mussels tended to increase the ETS activity when exposed to tides, in comparison to submersed mussels, which may result from re-immersion periods to which the mussels are subjected. Andrade et al. (2018) also demonstrated an increase of ETS activity in *M. galloprovincialis* under daily air exposure conditions (during 3h or 6h) followed by immersion. It is known that metabolic depression may occur under oxygen limitation (Guppy et al., 1994). However, little is still known about the physiological responses of mussels exposed to tidal regimes. The present findings point out that re-oxygenation after air exposure (during low tide simulation) induced high metabolic capacity in mussels, necessary to re-establish their physiological and biochemical performance after oxygen absence.

Also, the general condition of an organism can be assessed by the determination of the differences between the energy consumption and available energy reserves (Coen and Janssen 2003). The availability of energy reserves, such as GLY content, can be affected not just by chemical stressors, but also by general physiological stressors (Scott-Fordsmand and Weeks, 2000). Our results demonstrated no differences in GLY content between contaminated and non-contaminated mussels, indicating that either GLY was not the reserve used as a resource of energy to fuel the defense mechanisms of mussels when in the presence of MWCNTs or, most probably, the present results indicate that there was no need for an extra expenditure of energy reserves under contaminated conditions indicating that the concentration tested was not stressful enough to increase expenditure of energy reserves. De Marchi et al. (2018) got similar results in *R. philippinarum* exposed to the same MWCNT concentration (0.01 mg/L). Nevertheless, the same authors demonstrated a decrease of GLY content with an increase of MWCNT concentration (0.10 and 1.00 mg/L) which confirms the use of GLY only at higher MWCNT concentrations, highlighting that higher MWCNT concentrations may have greater impacts on the balance of bivalve energy reserves. In fact, different studies have demonstrated the expenditure of this energy reserve in invertebrates exposed to higher concentrations of various carbon

nanoparticles (De Marchi et al., 2017a; De Marchi et al, 2017b; De Marchi et al, 2017c). The preservation of GLY content observed in the present study could thus result from the low MWCNT concentration used and/or short exposure period that did not result in the increased expenditure of this reserve.

The present results further demonstrated that exposure to tides did not alter GLY content in comparison to submersed mussels. Similarly, Ivanina et al. (2011) did not observe differences in the GLY content in the oyster *Crassostrea virginica* exposed to hypoxic conditions for 2 weeks. Nevertheless, although GLY content was not quantified, Andrade et al. (2018) demonstrated that the lipid content decreased in mussels submitted to 6h of daily air exposure. In another study, Yin et al. (2017) demonstrated that *R. philippinarum* clams submitted to different daily air exposures used more energy than could be accumulated (shown by measuring lipid content and fatty acid composition): the net energy usage could be explained by an elevated aerobic metabolism during re-immersion periods. In the present study, although under tidal regime mussels showed increased metabolism, this activation was not reflected in the use of GLY content which, once again, may indicate that GLY was not used to fuel the defense mechanisms of mussels to fight against the stress caused by air exposure or this condition was not stressful enough to cause the net expenditure of energy reserves. Therefore, we may hypothesize that under tidal regimes mussels preferentially use lipids to fuel up their defense mechanisms.

Oxidative damage

With the abiotic changes in the environment, marine bivalves may be exposed to stressful conditions that can cause overproduction of reactive oxygen species (ROS) and, consequently, oxidative damage of the lipid membranes (Carregosa et al. 2014a; Freitas et al. 2016a; Liu et al. 2007; Lushchak 2011; Matozzo et al. 2012; Silva et al. 2005). When lipid membranes are attacked by ROS, lipid peroxidation (LPO) may occur, corresponding to the oxidation of the lipids into lipid hydroperoxides (Catalá 2009; Regoli and Giuliani 2014). The present results showed that LPO

levels increased in MWCNT-contaminated mussels submersed during the entire experiment, while when exposed to tides, contaminated mussels decreased their LPO in comparison to non-contaminated mussels. These results were accompanied by a significant increase on the GSSG content in contaminated mussels exposed to tides, evidencing that under this condition the organisms were experiencing oxidative stress. In fact, GSSG results from the oxidation of GSH, which participates in the antioxidant defense system as the most abundant cytosolic scavenger and neutralizing ROS directly, but also acts as a co-factor of antioxidant enzymes such as GPx, the activity of which, in fact, significantly increased in contaminated organisms exposed to tides. Thus, our findings showed that increased LPO levels in contaminated mussels under submersion conditions was not associated with increased GSSG content, which shows that ROS production was not high enough to either increase GSSG production or activate antioxidant enzymes activity leading to the oxidation of membrane lipids. The presence of the CNTs in submersion conditions, as demonstrated by DLS analysis, may lead to their possible uptake by these organisms with intracellular accumulation, enhancing possible oxidative degradation of lipids. In fact, aggregation of NMs may alter their biological effects by affecting ion release from the surface, their reactive surface area and the subsequent mode of cellular uptake of NMs together with the resultant biological responses in the organisms (Hotze et al., 2010). For the same MWCNT concentration (0.01mg/L), previous studies showed an increase of LPO in the clam *R. phillipinarum,* and the polychaetes *D. neapolitana* and *H. diversicolor* (De Marchi et al., 2017b, 2017c, 2018) after a 28 days exposure period. Likewise, Anisimova et al. (2005) demonstrated an increase of LPO levels in *C. grayanus* exposed to 12-14nm diameter MWCNTs (100mg/L) for 48h. De fact that MWCNTs were not detected in water from aquaria submitted to tides could explain the lower LPO levels observed at this condition, indicating that organisms were exposed to low contamination levels. However, higher GSSG content indicates that ROS production was extremely high under this condition inducing oxidation of GSH and thus the decrease of LPO resulted from the activation of antioxidant enzymes that also contributed to the elimination of ROS and, whereby the formation

of LPO was avoided. Therefore, our results highlight that although no MWCNTs were identified in water from Tides+MWCNT condition, mussels were exposed to these NPs, which caused cellular damages in organisms.

The present study further demonstrated that non-contaminated mussels increased their LPO levels when exposed to tides compared to submersion conditions but an opposite response was observed for contaminated mussels with higher LPO levels in mussels submersed during the entire experiment. These results demonstrated that in the absence of MWCNTs the exposure to air leads to cellular damages, because of the lack of activation of antioxidant defenses, which, in fact, shows that submersion was the least stressful condition to mussels. Similarly, Andrade et al. (2018) showed an increase of LPO in mussels exposed to daily cycles of 6h of air exposure. Likewise, in clams exposed to 3h, 6h and 9h of daily air exposure, Yin et al. (2017) observed a non-significant increase of LPO levels. For mussel *M. edulis,* exposed to anoxic conditions, Rivera-Ingraham et al. (2013) demonstrated an increase of ROS after reoxygenation and induction of LPO in the mantle. These authors suggested that the shell closure strategy during emersion as a typical behavior of intertidal bivalves to avoid oxidative stress during frequent anoxia-hyperoxia conditions. Furthermore, under anoxic conditions, ATP degrades in AMP, which is further converted to hypoxanthine. Hypoxanthine and xanthine are then oxidized upon reoxygenation, generating ROS (Jones 1986), and thus explaining the increase of LPO levels in mussels exposed to tides. On the other hand, when mussels were contaminated the decrease of LPO levels under tidal regime may indicate that the limited presence of MWCNTs, only during high tide periods, caused a less stressful condition or the combination of air exposure and MWCNT contamination induced higher oxidative stress compared to organisms submersed the entire experiment in the presence of these NPs which was surpassed by the activation of antioxidant enzymes that eliminated ROS and prevented the occurrence of LPO.

ROS can alter cellular functions through a reversible or irreversible post-translational modification (PTM) which may inactivate critical proteins (Sultan et al., 2018). In fact, ROS may promote the oxidation of proteins, a process known as protein carbonylation (PC) which constitutes the most common type of PTM triggered by oxidative stress (Cattaruzza and Hecker, 2008; Suzuki et al., 2010). In general, the present results showed no alteration on PC levels in mussels, with similar PC levels in non-contaminated and contaminated mussels exposed to submersion and tide conditions. Similarly, Marisa et al. (2016) showed no significant differences in either protein carbonyl content or LPO levels in *R. philippinarum* exposed to zinc oxide $_2$) for 7 days, putting in hypothesis that antioxidant defenses were enough to cope with the increase of oxidative damage and this way protect the cells.

Our results further demonstrated similar PC levels between submersed and tides exposure conditions both for non-contaminated and contaminated mussels. Rivera-Ingraham et al. (2013) observed that the mussel *M. edulis* maintained its protein carbonyl content after reoxygenation, although a burst of LPO was observed for the same condition. Thus, air exposure during low tide may not induce protein oxidation.

Antioxidant enzymes

Marine organisms can increase antioxidant defenses, including the activities of SOD and CAT enzymes, as a way to eliminate the excess of ROS produced under stressful conditions and to prevent the occurrence of LPO (Freitas et al. 2016b; Regoli and Giuliani, 2014; Velez et al., 2016). In addition to SOD and CAT, there is a third mechanism involving the antioxidant enzyme GPx, which reduces lipid hydroperoxides, by oxidizing GSH to oxide GSSG, the animals neutralize ROS directly (Regoli and Giuliani, 2014). Our results showed that under submersion conditions contaminated mussels had similar antioxidant enzyme activities compared to non-contaminated ones, which may explain increased LPO levels in mussels exposed to MWCNTs.

De Marchi et al. (2017a) showed for the polychaetes *D. neopolitana* and *H. diversicolor* at the same concentration of MWCNTs that neither CAT nor SOD activities changed, although LPO increased. Furthermore, De Marchi et al. (2018) observed an unchanged SOD and CAT antioxidant activity in *R. philippinarum* exposed to 0.01 mg/L of MWCNTs leading to LPO. For the same concentration of MWCNTs, De Marchi et al. (2018) did not observe any significant difference in GPx activity in *R. phillipinarum* between non-contaminated and contaminated conditions, being only significantly increased at higher MWCNT concentrations (0.10 mg/L and 1.00 mg/L). However, under tidal regime condition, contaminated organisms showed increased antioxidant enzyme activities compared to non-contaminated mussels which may result from higher ROS production, also associated with higher GSSG content at this condition that is related to higher GPx activity, leading to lower LPO levels at this condition. Therefore, it seems that higher stressful condition was generated by the combination of both stressors (air exposure and the presence of MWCNTs) which in turn leads to the highest activation of defense mechanisms that were able to prevent LPO and PC. Nevertheless, Letendre et al. (2011) showed no significant differences in CAT and Cu/Zn SOD activities in the mussel *M. edulis* submitted to an artificial tidal cycle for 14 days with and without PAHs.

Our results also revealed that exposure to tides resulted, in general, in higher antioxidant enzyme activities. These results demonstrated that mussels increase antioxidant defenses as an adaptation to high levels of ROS resulting from re-oxygenation typical of tidal cycles, which was especially noticed when mussels were contaminated. Andrade et al. (2018) observed an increase of SOD activity in non-contaminated mussels submitted to 3h and 6h of air exposure followed by immersion, showing the increase of antioxidant enzymes to deal with the formation of ROS originated during re-oxygenation. Likewise, Yin et al. (2017) demonstrated an increase of SOD activity in *R. philippinarum* under air exposure. Furthermore, the mussel *P. perna* under 4h of air exposure also demonstrated an increase of SOD activity (Almeida and Bainy, 2006). However, *P. perna* mussels showed non-significant differences of GPx activity in the digestive gland when

exposed to 4h of air exposure and re-immersed (Almeida and Bainy, 2006; Almeida et al., 2005). Also Letendre et al. (2011) observed a non-significant increase of GPx activity in *M. edulis* submitted to an artificial tidal cycle for 14 days. Therefore, increased antioxidant enzyme activity in mussels exposed to tides and especially under the combined effect of tides and MWCNTs indicate higher stress under this condition which was not accompanied by higher cellular damage due to the effective response of these defense mechanisms.

The present study demonstrated that, although

response for submersed organisms, contamination by MWCNTs may change physiological and biochemical performance of *M. galloprovincialis* by inducing alterations on the oxidative status of organisms, which was especially noticed when organisms were exposed to tides. The present findings revealed that mussels seemed to be able to tolerate oxidative stress caused by the high production of ROS induced by the contaminant and by exposure to air, being able to increase their metabolism to activate their defense mechanisms and, therefore, preventing cellular damages. Nevertheless, although being able to avoid cellular damages, the physiological and biochemical alterations induced in mussels exposed to tides and MWCNTs may have negative impacts on the physiological performance of organisms, including reproductive success and growth. Therefore, longer exposure periods should be tested in the future, as cellular damage and/or higher negative physiological injuries may possibly occur.

R sa Freitas benefited from a Research position funded by Integrated Programme of SR&

-01-0145-FEDER-000018), co-funded by Centro 2020 program, Portugal 2020, European Union, through the European Regional

Development Fund. Thanks are also due, for the financial support to CESAM (UID/AMB/50017), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. This work was also financially supported by the project BISPECIAl: BIvalveS under Polluted Environment and ClImate chAnge (POCI-01-0145-FEDER-028425) funded by FEDER, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES.

6. REFERENCES

Abele, D., Brey, T., Philipp, E.E.R., 2009. Bivalve models of aging and the determination of molluscan lifespans. Exp. Gerontol. 44, 307 315. http://doi.org/10.1016/j.exger.2009.02.012.

Almeida, E.A., Bainy, A.C.D., 2006. Effects of Aerial Exposure on Antioxidant Defenses in the Brown Mussel *Perna perna*. Braz. arch. biol. technol. 49, 225-229. http://doi.org/10.1590/S1516-89132006000300007.

Almeida, E.A., Bainy, A.C.D., Dafre, A.L., Gomes, O.F., Medeiros, M.H.G., Mascio, P.D., 2005. Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed. J. Exp. Mar. Biol. Ecol. 318, 21-30. http://doi.org/10.1016/j.jembe.2004.12.007.

Altieri, A.H., 2006. Inducible variation in hypoxia tolerance across the intertidal subtidal distribution of the blue mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser. 325: 295-300. http://doi.org/10.3354/meps325295.

Amiard Triquet, C., Rainbow, P.S., 2009. Environmental Assessment of Estuarine Ecosystems. Taylor & Francis Group, New York. Andrade, M.,, Soares, A., Figueira, E., Freitas, R., 2018. Biochemical changes in mussels submitted to different time periods of air exposure. Environ. Sci. Pollut. Res. 9, 8903 8913. https://doi.org/10.1007/s11356-017-1123-7.

Andrade, M., Soares, A., Figueira, E., Freitas, R., 2018. Biochemical changes in mussels submitted to different time periods of air exposure. Environ. Sci. Pollut. Res. 25(9), 8903-8913. https://doi.org/10.1007/s11356-017-1123-7.

Anisimova, A.A., Chaika, V.V., Kuznetsov, V.L., Gelokhvast, K.S., 2015. Study of the influence of multiwalled carbon nanotubes (12 14 nm) on the main target tissues of the bivalve *Modiolus modiolus*. Nanotechnol. Russ. 10, 278 287. https://doi.org/10.1134/S1995078015020020.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276 287.http://doi.org/10.1016/0003-2697(71)90370-8.

Caçador, I., Costa, A.L., Vale, C., 2007. Nitrogen sequestration capacity of two salt marshes from the Tagus estuary. Hydrobiologia 587, 137-145. https://doi.org/10.1023/B:WAFO.0000028388.84544.ce.

Canesi, L., Ciacci, C., Betti, M., Fabbri, R., Canonico, B., Fantinati, A., Marcomini, A., Pojana, G., 2008. Immunotoxicity of carbon black nanoparticles to blue mussel hemocytes. Environ. Int., 34, 1114-1119. https://doi.org/10.1016/j.envint.2008.04.002.

Canesi, L., Fabbri, R., Gallo, G., Vallotto, D., Marcomini, A., Pojana, G., 2010. Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (Nano carbon black, C60 fullerene, Nano-TiO2Nano-SiO2). Aquat. Toxicol. 100, 168-177. https://doi.org/10.1016/j.aquatox.2010.04.009.

Carregosa, V., Figueira, E., Gil, A.M., Pereira, S., Pinto, J., Soares, A.M.V.M., Freitas, R., 2014a. Tolerance of *Venerupis philippinarum* to salinity: osmotic and metabolic aspects. Compar. Biochem. Physiol. A Mol. Integr. Physiol. 171, 36 43. https://doi.org/10.1016/j.cbpa.2014.02.009.

Carregosa, V., Velez, C., Soares, A.M.V.M., Figueira, E., Freitas, R., 2014b. Physiological and biochemical responses of three Veneridae clams exposed to salinity changes. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 177 178, 1 9. https://doi.org/10.1016/j.cbpb.2014.08.001.

Catalá, A., 2009. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. Chem. Phys. Lipids 157, 1 11, http://doi.org/10.1016/j.chemphyslip.2008.09.004.

Catsiki, V.A., Florou, H., 2006. Study on the behavior of the heavy metals Cu, Cr, Ni, Zn, Fe, Mn and 137Cs in an estuarine ecosystem using *Mytilus galloprovincialis* as a bioindicator species J. Environ. Radioact. 86, 31-44. https://doi.org/10.1016/j.jenvrad.2005.07.005.

Cattaruzza, M., Hecker, M., 2008. Protein carbonylation and decarboylation: a new twist to the complex response of vascular cells to oxidative stress. Circ. Res., 102, 273-274.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013. Field-to-laboratory transport protocol impacts subsequent physiological biomarker response in the marine mussel, Perna canaliculus. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 164, 84-90. https://doi.org/10.1016/j.cbpa.2012.10.011.

Coen, W.M.D., Janssen, C.R, 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristic. Environ. Toxicol. Chem. 22, 1632 1641. http://doi.org/10.1002/etc.5620220727.

Coen, W.M.D., Janssen, C.R., 1997. The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress Recovery 6, 43 55. http://doi.org/10.1023/A:1008228517993.

Dauvin, J.-C., Ruellet, T, 2009. The estuarine quality paradox: is it possible to define an ecological quality status for specific modified and naturally stressed estuarine ecosystems? Mar. Pollut. Bull. 59, 38 47. http://doi.org/10.1016/j.marpolbul.2008.11.008.

Davis, R.A.(Ed.), 1985. Coastal Sedimentary Environments. Springer New York, New York, NY.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini. F., Morelli, A., Soares, A.M.V.M, Freitas, R., 2017a. The impacts seawater acidification on *Ruditapes philippinarum* sensitivity to carbon nanoparticles exposure. Environ. Sci. Nano 4, 1692-1704. https://doi.org/10.1039/c7en00335h.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini. F., Soares, A.M.V.M, Freitas, R., 2017b. Physiological and biochemical responses of two keystone polychaete species: *Diopatra neapolitana* and *Hediste diversicolor* to Multi-walled carbon nanotubes. Environ. Res. 154, 126-138. https://doi.org/10.1016/j.envres.2016.12.018.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini. F., Soares, A.M.V.M, Freitas, R., 2017c. The impacts of emergent pollutants on *Ruditapes philippinarum*: biochemical responses to carbon nanoparticles exposure. Aquat. Toxicol. 187, 38-47. https://doi.org/10.1016/j.aquatox.2017.03.010.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini. F., Morelli, A., Soares, A.M.V.M, Freitas, R., 2018a. Effects of multi-walled carbon nanotube materials on *Ruditapes philippinarum* under climate change: The case of salinity shifts. Aquat. Toxicol. 199, 199-211. https://doi.org/10.1016/j.aquatox.2018.04.001.

De Marchi, L., Pretti, C., Gabriel, B., Marques, P. A. A. P., Freitas, R., Neto, V., 2018b. An overview of graphene materials: Properties, applications and toxicity on aquatic environments. Sci. Total Environ. 631 632, 1440 1456. https://doi.org/10.1016/j.scitotenv.2018.03.132.

Dowd, W.W., Felton, C.A., Heymann, H.M., Kost, L.E., Somero, G.N., 2013. Food availability, more than body temperature, drives correlated shifts in ATP-generating and antioxidant enzyme capacities in a population of intertidal mussels (*Mytilus californianus*). Journ. Exper. Mar. Biol. Ecol. 449, 171-185. http://doi.org/10.1016/j.jembe.2013.09.020.

Dowd, W.W., Somero, G.N., 2013. Behavior and survival of Mytilus congeners following episodes of elevated body temperature in air and seawater. J. Exp. Biol. 216, 502-514. http://doi.org/10.1242/jeb.076620.

Dubois, M.K., Gilles, A., Hamilton, J.K., Rebers, P.A., Sith, F., 1956. Calorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356. https://doi.org/10.1021/ac60111a017.

Eckelman, M.J., Mauter, M.S., Isaacs, J.A., Elimelech, M., 2012. New perspectives on nanomaterial aquatic ecotoxicity: production impacts exceed direct exposure impacts for carbon nanotoubes. Environ. Sci. Technol. 46, 2902-2910. https://doi.org/10.1021/es203409a.

Elliott, M., Cutts, N.D., Trono, A., 2014. A typology of marine and estuarine hazards and risks as vectors of change: A review for vulnerable coasts and their management. Ocean Coast Manag. 93, 88-99. https://doi.org/10.1016/j.ocecoaman.2014.03.014.

Elliott, M., Quintino, V., 2007. The estuarine quality paradox, environmental homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed area. Mar. Pollut. Bull. 54, 640-645. http://doi.org/10.1016/j.marpolbul.2007.02.003.

Faggio, C., Pagano, M., Alampi, R., Vazzana, I., Felice, M.R., 2016. Cytotoxicity, haemolymphatic parameters, and oxidative stress following exposure to sub-lethal concentrations of quaternium-15 in *Mytilus galloprovincialis*. Aquat. Toxicol. 180, 258-265. https://doi.org/10.1016/j.marpolbul.2013.05.004.

FAO, Food and Agriculture Organization of the United Nations, 2016. *Mytilus galloprovincialis* (Lamarck ,1819). Fisheries Department publications. Publications pages. In: FAO Fisheries and Aquaculture Department [online]. Rome.

Freire, C.A., Welker, A.F., Storey, J.M., Storey, K.B., Hermes-Lima, M., 2011. Oxidative Stress in Estuarine and Intertidal Environments (Temperate and Tropical). Oxidat. Str. Aquat. Ecosyst. John Wiley & Sons, New York, 41 57.

Freitas, R., De Marchi, L., Moreira, A., Pestana, J.L.T., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2017. Physiological and biochemical impacts induced by mercury pollution and seawater acidification in *Hediste diversicolor.* Sci. Total Environ. 595, 691-701. http://doi.org/10.1016/j.scitotenv.2017.04.005

Freitas, R., Pires, A., Velez, C., Almeida, A., Moreira, A., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2016a. Effects of seawater acidification on *Diopatra neapolitana* (Polychaete, Onuphidae): biochemical and regenerative capacity responses. Ecol. Indic. 60:152 161. https://doi.org/10.1016/j.ecolind.2015.06.032

Freitas, R., Salamanca, L., Velez, C., Wrona, F.J., Soares, A.M.V.M, Etelvina Figueira, E., 2016b. Multiple stressors in estuarine waters: Effects of arsenic and salinity on *Ruditapes philippinarum*. Sci. Total Environ. 541, 1106-1114. http://doi.org/10.1016/j.scitotenv.2015.09.149.

Fu, J., Mai, B., Sheng, G., Zhang, G., Wang, X., Peng, P., Xiao, X., Ran, R., Cheng, F., Peng,X., Wang, Z., Tang, U.W., 2003. Persistent organic pollutants in environment of the Pearl river delta, China: an overview. Chemosp. 52, 1411 1422. http://doi.org/10.1016/S0045-6535(03)00477-6.

H., Ross, P.M., 2013. Impacts of ocean acidification on marine shelled molluscs. Mar. Biol. 160, 2207 2245, http://doi.org/10.1007/s00227-013-2219-3.

Gestoso, I., Arenas, F., Olabarria, C., 2016. Ecological interactions modulate responses of two intertidal mussel species to changes in temperature and pH. J. Exp. Mar. Biol. Ecol. 474, 116-125. https://doi.org/10.1016/j.jembe.2015.10.006.

Gomes, T., Pereira, C.G., Cardoso, C., Bebianno, M.J., 2013. Differential protein expression in mussels *Mytilus galloprovincialis* exposed to nano and ionic Ag. Aquat. Toxicol, 136 137, 79-90. https://doi.org/10.1016/j.aquatox.2013.03.021.

Guppy, M., Fuery, C., Flanigan, J., 1994. Biochemical principles of metabolic depression. Comp. Biochem. Physiol. B, 109:175-189. https://doi.org/10.1016/0305-0491(94)90001-9.

Horn, M.H., Martin, K.L.M., Chotkowski, M.A., 1999. Intertidal fishes: life in two worlds. Elsevier, Amsterdam, 1 399.

Hotze, E. M., Phenrat, T., Lowry, G. V., 2010. Nanoparticle aggregation: challenges to understanding transport and reactivity in the environment. J. Environ. Qual. 39(6), 1909. https://doi.org/10.2134/jeq2009.0462.

Li, M., Li, L., Sui, Y., Li, J., Wang, Y., Lu, W., Dupont, S., 2015. Effect of pH and temperature on antioxidant responses of the thick shell mussel *Mytilus coruscus*. Fish Shellfish Immunol. 46, 572-583. https://doi.org/10.1016/j.fsi.2015.07.025.

Huang, X., Liu, Z., Xie, Z., Dupont, S., Huang, W., Wu, F., Kong, H., Liu, L., Sui, Y., Lin, D., Lu, W., Hu, M., Wang, Y., 2018. Oxidative stress induced by titanium dioxide nanoparticles increases under seawater acidification in the thick shell mussel *Mytilus coruscus*, Mar. Environ. Res. 137, 49-59. https://doi.org/10.1016/j.marenvres.2018.02.029.

IPMA, 2017. (Portuguese Institute for Sea and Atmosphere (IPMA)). https://www.ipma.pt/en/maritima/sat-sst/index-8days.jsp?area=zona2.

Ishii, R., Sekiguchi, H., Jinnai, Y., 2005. Vertical distributions of larvae of the clam *Ruditapes philippinarum* and the striped horse mussel *Musculista senhousia* in Eastern Ariake Bay, Southern Japan. J. Oceanogr. 61, 973-978. http://doi.org/10.1007/s10872-006-0013-2.

Ivanina, A.V., Froelich, B., Williams, T., Sokolov, E.P., Oliver, J.D., Sokolova, I.M., 2011. Interactive effects of cadmium and hypoxia on metabolic responses and bacterial loads of eastern oysters *Crassostrea virginica* Gmelin. Chemosphere 82, 377-389. https://doi.org/10.1016/j.chemosphere.2010.09.075.

Johansson, L.H., Borg, L.A.H., 1988. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal. Biochem. 174, 331 336. http://doi.org/10.1016/0003-2697(88)90554-4.

Jones, D.P.,1986. Renal metabolism during normoxia, hypoxia, and ischemic injury. An. Rev. Physiol. 48, 33-50. https://doi.org/10.1146/annurev.ph.48.030186.000341.

Köhler, A.R., Som, C., Helland, A., Gottschalk, F., 2008. Studying the potential release of carbon nanotubes throughout the application life cycle. J. Clean. Prod. 16, 927-937. https://doi.org/10.1016/j.jclepro.2007.04.007.

Assessment of pollution level using as a bioindicator species: The case of the Gulf of Trieste. Mar. Pollut. Bull. 89, 455-463. http://doi.org/10.1016/j.marpolbul.2014.09.046.

Letendre, J., Chouquet, B., Rocher, B., Manduzio, H., Leboulenger, F., Durand, F., 2008. Differential pattern of Cu/Zn superoxide dismutase isoforms in relation to tidal spatio-temporal changes in the blue mussel *Mytilus edulis*. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 148(3), 211 216. https://doi.org/10.1016/j.cbpc.2008.05.012.

Letendre, J., Dupont-Rouzeyrol, M., Hamuet, A., Durand, F., Budzinski, H., Chan, P., Vaudry, D., Rocher, B., 2011. Impact of toxicant exposure on the proteomic response to intertidal condition in *Mytilus edulis*. Comp. Biochem. Physiol. Part D Genomics Proteomics, 6(4), 357-369. https://doi.org/10.1016/j.cbd.2011.08.002.

Liu, C., Liang, C., Lin, K., Jang, C., Wang, S., Huang, Y., Hsueh, Y. 2007. Bioaccumulation of arsenic compounds in aquacultural clams (*Meretrix lusoria*) and assessment of potential carcinogenic risks to human health by ingestion. Chemosphere 69, 128 134. https://doi.org/10.1016/j.chemosphere.2007.04.038.

Lopes, C.B., Pereira, M.E., Vale, C., Lillebø, I., Pardal, M.A., Duarte, A.C., 2007. Assessment of spatial environmental quality status in Ria de Aveiro (Portugal). Sci. Mar. 71, 293-304.

Lopes, C.L., Silva, P.A., Rocha, A., Dias, J.M., 2011. Sensitivity analysis of Ria de Aveiro hydro-morphodynamics to the sea level rise integration period. Journ. Coast. Res. 64, 230 234. https://doi.org/10.3989/scimar.2007.71n2293.

Lushchak, VI., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13 30. https://doi.org/10.1016/j.aquatox.2010.10.006.

Maanan, M., 2008. Heavy metal concentrations in marine molluscs from the Moroccan coastal region. Environ. Pollut. 153 (1), 176 183. http://doi.org/10.1016/j.envpol.2007.07.024.

Marisa, I., Matozzo, V., Munari, M., Binelli, A., Parolini, M., Martucci, A., 2016. In vivo exposure of the marine clam *Ruditapes philippinarum* to zinc oxide nanoparticles: responses in gills, digestive gland and haemolymph. Environ. Sci. Pollut. Res. 23, 15275. https://doi.org/10.1007/s11356-016-6696-5.

Matozzo, V., Binelli, A., Parolini, M., Previato, M., Masiero, L., Finos, L., Bressan, M.,Marin, M.G., 2012. Biomarker responses in the clam Ruditapes philippinarum and contamination levels in sediments from seaward and landward sites in the lagoon of Venice. Ecol. Indic. 19, 191 205. http://doi.org/10.1016/j.ecolind.2011.06.020.

Mesquita, C.S., Oliveira, R., Bento, F., Geraldo, D., Rodrigues, J.V., Marcos, J.C., 2014. Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. Anal. Biochem. 458, 69-71. https://doi.org/10.1016/j.ab.2014.04.034.

Mitchelmore, C.L., Birmelin, C., Chipman. J.K., Livingstone, D.R., 1998. Evidence for cytochrome P-450 catalysis and free radical involvement in the production of DNA strand

breaks by benzo[a]pyrene and nitroaromatics in mussel (*Mytilus edulis*) digestive glands. Aquat. Toxicol. 41, 193-212. http://doi.org/10.1016/S0166-445X(97)00083-0.

Mitsch, W.J., Gosselink. J.G., 2015. Wetlands (fifth ed,). John Wiley & Sons, Inc., Hoboken, New Jersey.

Müller, A.M.F., Makropoulos, V., Bolt, H.M., 1995. Toxicological aspects of oestrogenmimetic xenobiotics present in the environment. Toxicol. Environ. News 2, 68-73.

Muller, N., Nowack, B., 2008. Exposure modeling of engineered nanoparticles in the environment. Environ. Sci. Technol. 41, 4447-4453. http://doi.org/10.1021/es7029637.

Nicastro, K., Zardi, G., McQuaid, C., Stephens, L., Radloff, S., Blatch, G.L., 2010. The role of gaping behaviour in habitat partitioning between coexisting intertidal mussels. BMC Ecol. 10, 17. https://doi.org/10.1186/1472-6785-10-17.

Nilin, J., Pestana, J.L.T., Ferreira, N.G., Loureiro, S., Costa-Lotufo, L.V., Soares, A.M.V.M., 2012. Physiological responses of the European cockle *Cerastoderma edule* (Bivalvia: Cardidae) as indicators of coastal lagoon pollution. Sci. Total Environ. 435–436, 44–52. http://doi.org/10.1016/j.scitotenv.2012.06.107.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituricacid reaction. Anal. Biochem. 95, 351–358. http://doi.org/10.1016/0003-2697(79)90738-3.

Oliveira, P., Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Wrona, F.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2017. Physiological and biochemical alterations

induced in the mussel *Mytilus galloprovincialis* after short and long-term exposure to carbamazepine. Water Res. 117, 102-114. https://doi.org/10.1016/j.watres.2017.03.052.

Packard, T.T., 1971. The measurement of respiratory electron-transport activity in marine phytoplankton. J. Mar. Res. 29, 235-244.

Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70 (1), 158 169. https://doi.org/10.1002/etc.710.

Peng, X., Jia, J., Gong, X., Luan, Z., Fan, B., 2009. Aqueous stability of oxidized carbon nanotubes and the precipitation by salts. J. Hazard. Mater. 165(1-3), 1239-1242. https://doi.org/10.1016/j.jhazmat.2008.10.049.

Picado, A., Dias, J.M., Fortunato, A., 2010. Tidal changes in estuarine systems induced by local geomorphologic modifications. Cont. Shelf. Res. 30 (17), 1854-1864. https://doi.org/10.1016/j.csr.2010.08.012.

Poulain, C., Lorrain, A., Elye-Sainte-Marie, J., Amice, E., Morize, E., Paulet, Y-M., 2011. An environmentally induced tidal periodicity of microgrowth increment formation in subtidal populations of the clam *Ruditapes philippinarum*. J. Exp. Mar. Biol. Ecol. 397, 58 64. http://doi.org/10.1016/j.jembe.2010.11.001.

Rahman, I., Kodel, A., Biswas, S.K., 2007. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat. Protoc., 1, 3159-3165. https://doi.org/10.1038/nprot.2006.378.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106 117, https://doi.org/10.1016/j.marenvres.2013.07.006.

Relexans, J.C., Meybeck, M., Billen, G., Brugeaille, M., Etcheber, H., Somville, M., 1988. Algal and microbial processes involved in particulate organic matter dynamics in the Loire estuary. Estuar. Coast. Shelf Sci. 27:625 644. http://doi.org/10.1016/02727714(88)90072-8.

Renn, O., Roco, M.C., 2006. Nanotechnology and the need for risk governance. J. Nanopart. Res. 8, 153-191. http://doi.org/10.1007/s11051-006-9092-7.

Resgalla, C., Radetski, C.M., Schettini, C.A.F., 2010. Physiological energetics of the brown mussel *Perna perna* (L.) transplanted in the Itajaì-Açu river mouth, Southern Brazil. Ecotoxicology 19, 383 390. http://doi.org/10.1007/s10646-009-0422-2.

Rivera-Ingraham, G.A., Rocchetta, I., Meyer, S., Abele, D., 2013. Oxygen radical formation in anoxic transgression and anoxiareoxygenation: Foe or phantom? Experiments with a hypoxia tolerant bivalve. Mar. Environ. Res. 92, 110-119. http://doi.org/10.1016/j.marenvres.2013.09.007.

Sanchez, V.C., Jachak, A., Hurt, R.H., Kane, A.B., 2012. Biological interactions of graphene-family nanomaterials an interdisciplinary review. Chem. Res. Toxicol. 15-34. https://doi.org/10.1021/tx200339h.

Scheibe, B., Borowiak-Palen, E., Kalenczuk, R. J., 2010. Oxidation and reduction of multiwalled carbon nanotubes preparation and characterization. Mater. Charact. 61(2), 185-191. https://doi.org/10.1016/j.matchar.2009.11.008.

Scott-Fordsmand, J.J., Weeks, J.M., 2000. Biomarkers in earthworms. Rev. Environ. Contam. Toxicol. 165, 117 159. http://doi.org/10.1007/978-1-4612-1172-3_3.

Scown, T.M., van Aerle, R., Tyler, C.R., 2010. Review: do engineered nanoparticles pose a significant threat to the aquatic environment? Crit. Rev. Toxicol. 40, 653-670. https://doi.org/10.3109/10408444.2010.494174.

Sherratt, J.A., Mackenzie, J.J., 2016. How does tidal flows affect pattern formation in mussel beds? J. Theoret. Biol. 406, 83-92. http://doi.org/10.1016/j.jtbi.2016.06.025.

Silva, A.Z., Zanette, J., Ferreira, J.F., Guzenski, J., Marques, M.R.F.,Bainy, A.C.D., 2005. Effects of salinity on biomarker responses in *Crassostrea rhizophorae* (Mollusca, Bivalvia) exposed to diesel oil. Ecotoxicol. Environ. Saf. 62, 376 382. http://doi.org/10.1016/j.ecoenv.2004.12.00[illegible]

Skolimowski, J., Burda, K., C. Bartosz, 2014. Effect of functionalized and non-functionalized nanodiamond on the morphology and activities of antioxidant enzymes of lung epithelial cells (A549). Chem. Biol. Interact. 222, 135-147. https://doi.org/10.1016/j.cbi.2014.10.003.

Sultan, C.S., Stackel, A., Stank, A., Fleming, T., Fedorova, M., Hoffmann, R., Wade, R.C., Hecker, M., Wagner, A.H., 2018. Impact of carbonylation on glutathione peroxidase-1 activity in human hyperglycemic endothelial cells, Redox Biol. 16, 113-122. https://doi.org/10.1016/j.redox.2018.02.018.

Sun, T.Y., Bornhöft, N.A., Hungerbühler, K., Nowack, B., 2016. Dynamic probabilistic modeling of environmental emissions of engineered nanomaterials. Environ. Sci. Technol. 50 (9), 4701-4711. https://doi.org/10.1021/acs.est.5b05828.

Sureda, A., Box, A., Tejada, S., Blanco, A., Caixach, J., Deudero, S., 2011. Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). Aquat. Toxicol. 101, 540-549. https://doi.org/10.1016/j.aquatox.2010.12.011.

Suzuki, Y.J., Carini, M., Butterfield, D.A., 2010. Protein Carbonylation. Antioxid. Redox Signal 12(3), 323-325. https://doi.org/10.1089/ars.2009.2887.

Underwood, G.J.C., Kromkamp, J., 1999. Primary Production by Phytoplankton and Microphytobenthos in Estuaries, in: Advances in Ecological Research: Estuaries. 29, 93-139.

Vazzana, M., Celi, M., Maricchiolo,G., Genovese, L., Corrias, V., Quinci, E.M., Vincenzi, G., Maccarrone, V., Cammilleri, G., Mazzola, S., Buscaino, G., Filiciotto, F., 2016. Are mussels able to distinguish underwater sounds? Assessment of the reactions of *Mytilus galloprovincialis* after exposure to lab-generated acoustic signals. Compar. Biochem. Physiol. A: Mol. Integr. Physiol. 201, 61-70, http://doi.org/10.1016/j.cbpa.2016.06.029.

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. Combined effects of seawater acidification and salinity changes in *Ruditapes philippinarum*. Aquat. Toxicol. 176, 141-150. http://doi.org/10.1016/j.aquatox.2016.04.016.

Velzeboer, I., Peeters, E. T. H. M., Koelmans, A. A., 2013. Multiwalled carbon nanotubes at environmentally relevant concentrations affect the composition of benthic communities. Environ. Sci. Technol. 47(13), 7475-7482. http://doi.org/10.1021/es400777j.

Verlecar, X.N, Jena, K.B, Chainy, G.B.N., 2007. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. Chem.-Biol. Interact. 167 (3), 219-226. https://doi.org/10.1016/j.cbi.2007.01.018.

Vlasova, I.I., Kapralov, A.A., Michael, Z.P., Burkert, S.C., Shurin, M.R., Star, A., Shvedova, A.A, Kagan, V.E., 2016. Enzymatic oxidative biodegradation of nanoparticles: mechanisms, significance and applications. Toxicol. Appl. Pharmacol. 299, 58-69. https://doi.org/10.1016/j.taap.2016.01.002.

Wang, Y., Li, L., Hu, M., Lu, W., 2015. Physiological energetics of the thick shellmussel *Mytilus coruscus* exposed to seawater acidification and thermal stress. Sci. Total Environ. 514, 261 272. http://doi.org/10.1016/j.scitotenv.2015.01.092.

Wu, Q., Yin, L., Li, X., Tang, M., Zhang, T., Wang, D., 2013. Contributions of altered permeability of intestinal barrier and defecation behavior to toxicity formation from graphene oxide in nematode *Caenorhabditis elegans*. Nanoscale, 5, 9934-9943. https://doi.org/10.1039/c3nr02084c.

Xiao, D.N., Li, X.Z., 2004. Ecological and Environmental Function of Wetland Landscape in the Liaohe Delta, in: Wetlands Ecosystems in Asia. Elsevier, 35 46. https://doi.org/10.1016/B978-044451691-6/50006-5.

Yin, X., Chen, P., Chen, H., Jin, W., Yan, X., 2017. Physiological performance of the intertidal Manila clam (*Ruditapes philippinarum*) to long-term daily rhythms of air exposure. Sci. Rep. 7, 41648. https://doi.org/10.1038/srep41648.

Zhang, P., Selck, H., Tangaa, S. R., Pang, C., Zhao, B., 2017. Bioaccumulation and effects of sediment-associated gold- and graphene oxide nanoparticles on *Tubifex tubifex*. J. Environ. Sci. (China) 51, 138–145. https://doi.org/10.1016/j.jes.2016.08.015.

Zhu, X., Zhou, J., Cai, Z., 2011. The toxicity and oxidative stress of TiO2 nanoparticles in marine abalone (*Haliotis diversicolor* supertexta). Mar. Pollut. Bull. 63 (5–12), 334–338. https://doi.org/10.1016/j.marpolbul.2011.03.006.

Figures:

Figure 1. Respiration Rate (RR) in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+MWCNTs, Tide, Tide+MWCNTs) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tide. Continuous line represents the absence of MWCNTs while dashed line represents the presence of MWCNTs. Different letters represent significant differences (p 0.05) among conditions.

Figure 2. A: Electron transport system activity (ETS); B: Glycogen (GLY) content, in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+MWCNTs, Tide, Tide+MWCNTs) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tide. Continuous line represents the absence of MWCNTs while dashed line represents the presence of MWCNTs. Different letters represent significant differences (p 0.05) among conditions.

Figure 3. A: Lipid peroxidation (LPO) levels; B: Oxidized glutathione (GSSG) content; C: Protein carbonylation (PC) levels in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+MWCNTs, Tide, Tide+MWCNTs) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tide. Continuous line

represents the absence of MWCNTs while dashed line represents the presence of MWCNTs. Different letters represent significant differences (p 0.05) among conditions.

Figure 4. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione peroxidase (GPx) activity in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+MWCNTs, Tide, Tide+MWCNTs) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tide. Continuous line represents the absence of MWCNTs while dashed line represents the presence of MWCNTs. Different letters represent significant differences (p 0.05) among conditions.

Figures:

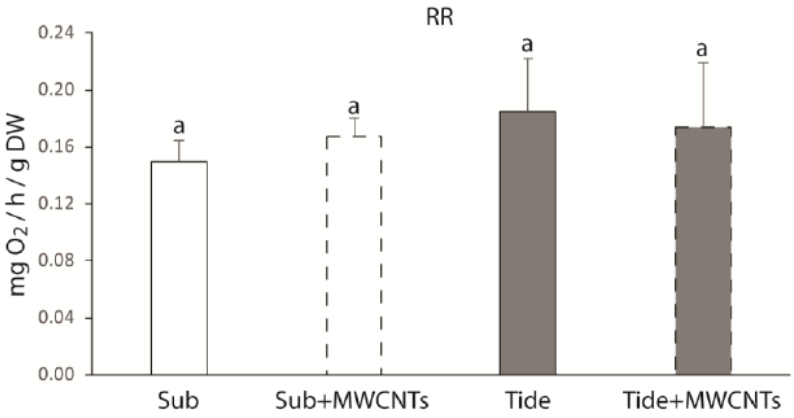

Figure 1

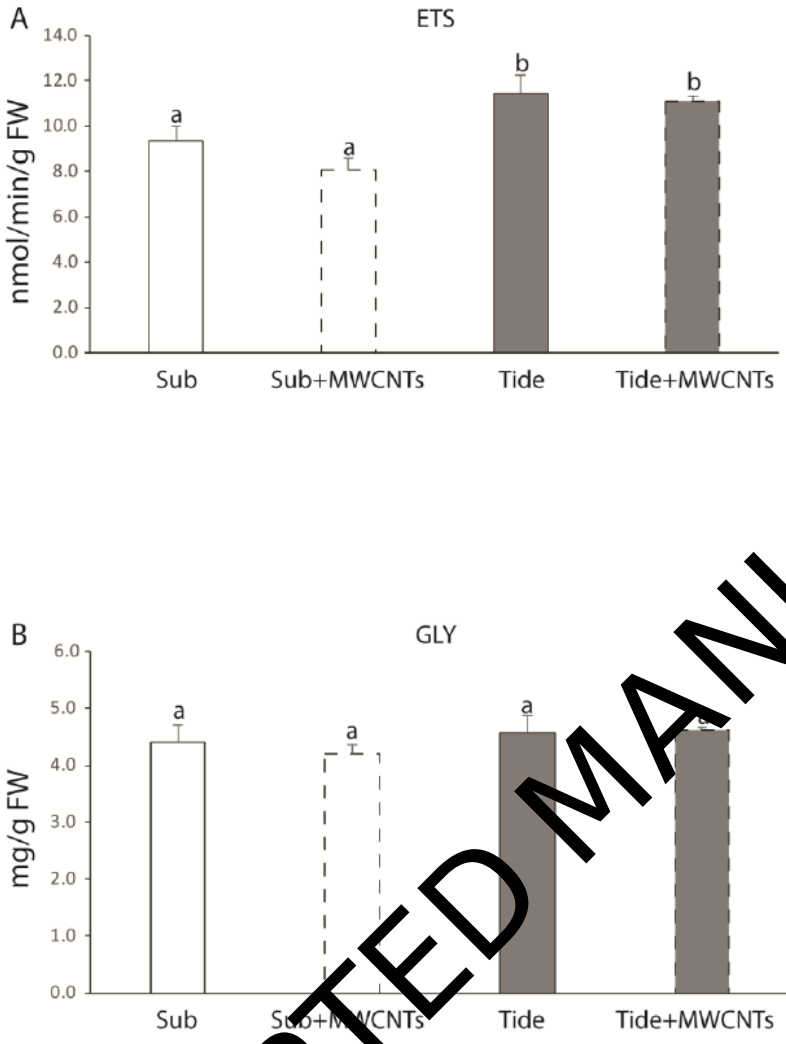

Figure 2

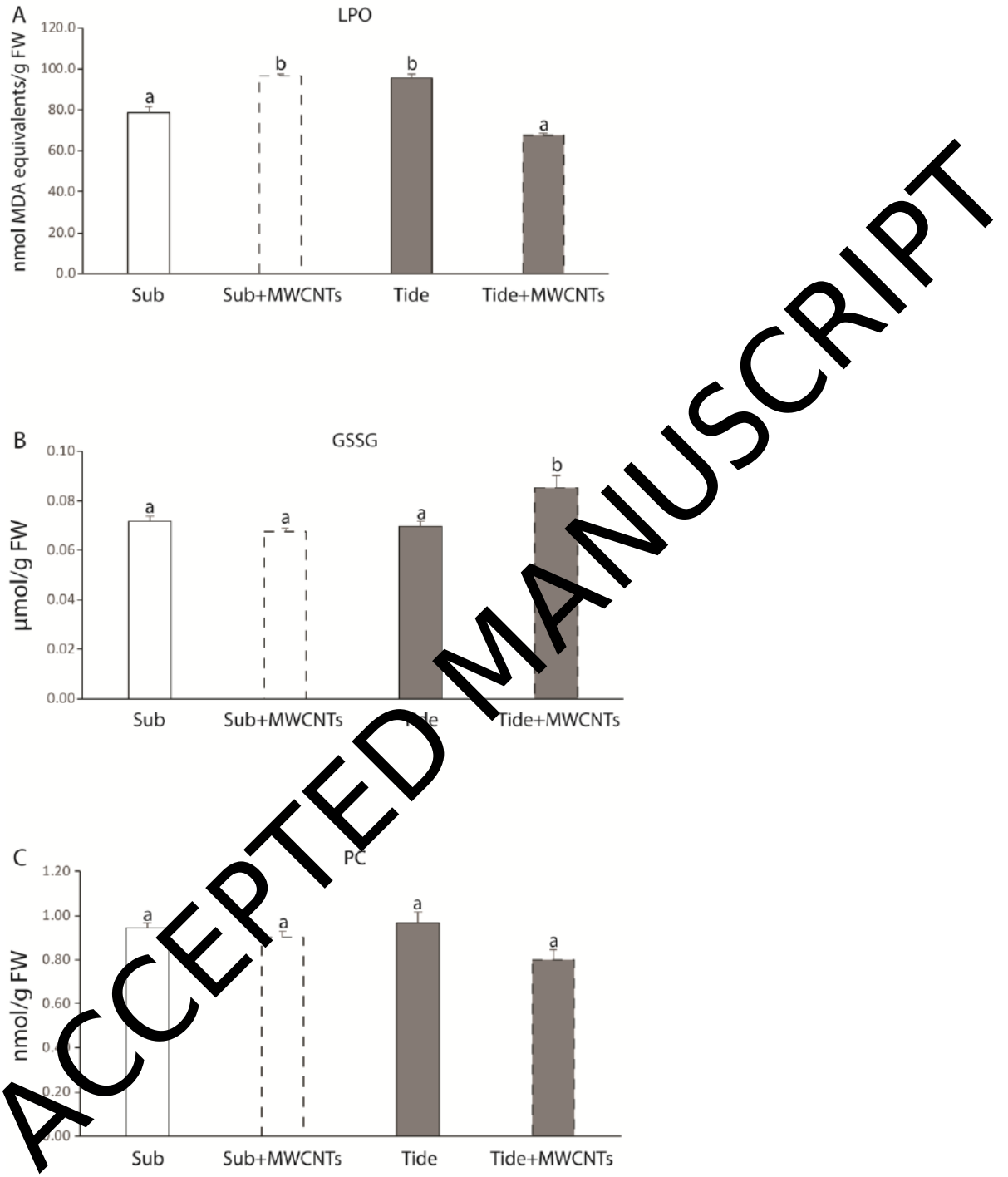

Figure 3

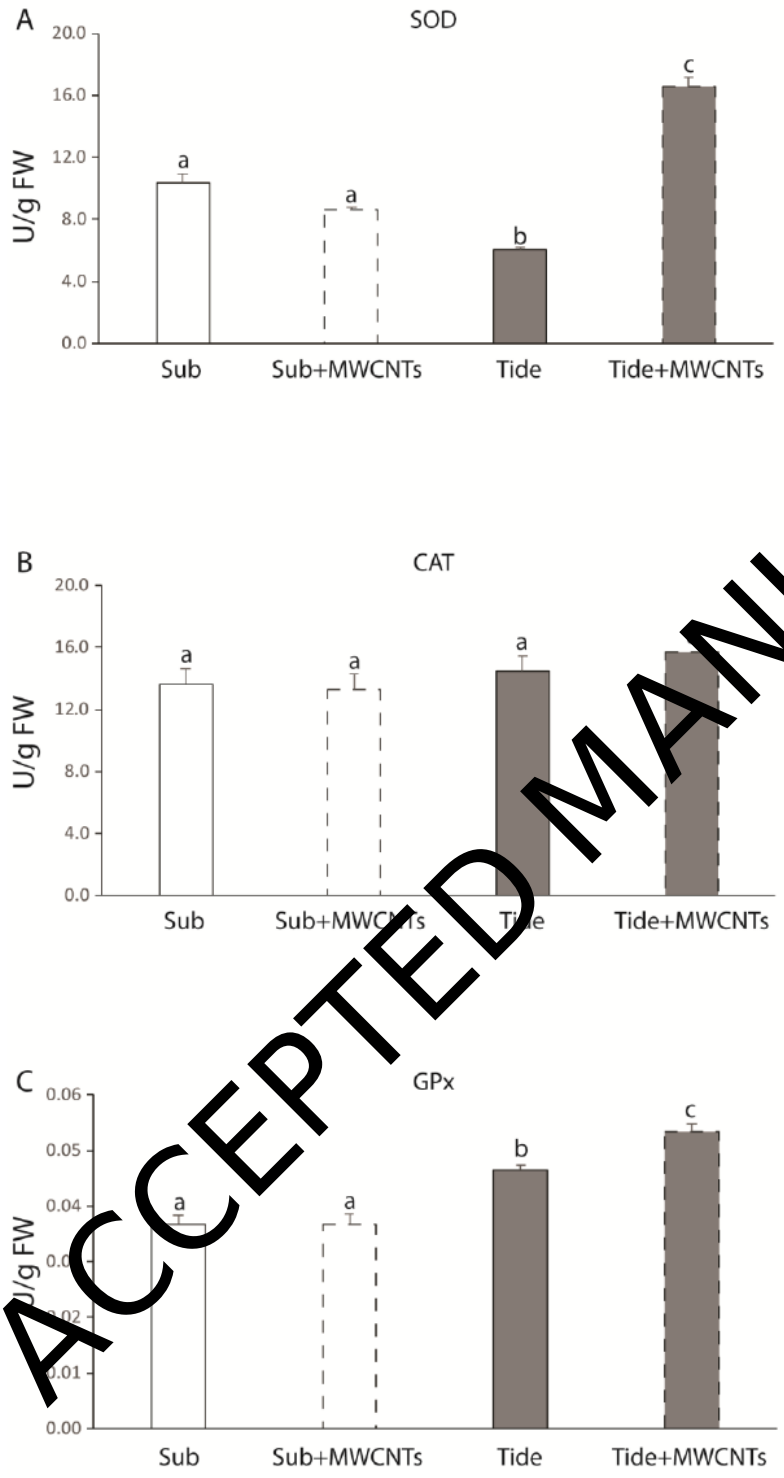

Figure 4

Table:

Table 1. Mean Dynamic light scattering (DLS) data of size (nm) and mean polydispersity index (PDI) of water samples from different exposure conditions (Sub+MWCNTs and Tide+MWCNTs) each condition).

Exposure Condition	*Size (nm)*	*PDI*
Sub+MWCNTs	1109.5	0.794
Tide+MWCNTs	n.d	-