Universidade de Aveiro Departamento de Biologia
Ano 2018

Maria Madalena da Silva Andrade

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

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Rosa de Fátima Lopes de Freitas (Investigadora pós-doutorada e professora auxiliar do Departamento de Biologia & Centro de Estudos do Ambiente e do Mar) e co-orientação da Doutora Etelvina Maria de Almeida Paula Figueira (Professora do Departamento de Biologia da Universidade de Aveiro).

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presidente

Doutora Isabel Maria Cunha Antunes Lopes
Investigadora principal do centro de estudos do ambiente e do mar (CESAM) da Universidade de Aveiro

Doutora Georgina Alexandra Rivera-Ingraham
Investigadora de Pós-Doutoramento do centro de investigação da Biodiversidade Marinha, Exploração e Conservação (MARBEC) da Universidade de Montpellier (França)

Doutora Rosa de Fátima Lopes de Freitas
Investigadora pós-doutorada e professora auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro

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palavras-chave

Mexilhões, nanoparticulas, aumento da temperatura, stress oxidativo, metabolismo, regime de marés.

resumo

Espécies intertidais estão frequentemente expostas a mudanças ambientais associadas a múltiplos stresses, dos quais estas devem evitar ou tolerar desenvolvendo certas estratégias. Algumas das mudanças naturais estão ligadas ao ciclo de maré, do qual organismos devem tolerar as diferenças entre o ambiente aquático e o ambiente aéreo. Para além disso, estes organismos estão também sujeitos ao aquecimento global e consequentemente, ao risco de dessecação especialmente sob ambiente aéreo. Ademais, a exposição a poluentes de fontes antropogénicas é um outro stress diário com que os organismos devem lidar. O presente estudo avaliou os impactos em *Mytilus galloprovincialis* expostos a diferentes temperaturas (18ºC e 21ºC) ou a nanopartículas de carbono (0.01mg/L MWCNT) quando continuadamente submersos ou expostos a marés (5h de maré baixa, 7h de maré alta) por 14 dias. Os resultados evidenciaram que os mexilhões foram fisiologicamente e bioquimicamente afetados pelo aumento da temperatura ou exposição de MWCNTs, especialmente quando também expostos a marés. Quando só expostos ao aumento da temperatura, o stress induzido foi o suficiente para ativar as defesas antioxidantes dos mexilhões gastando reservas de energia e evitando danos oxidativos. Quando só expostos a MWCNTs ou só a marés, o stress induzido não foi suficiente para induzir as defesas antioxidantes dos mexilhões resultando em danos oxidativos. Contudo, a combinação de marés e temperatura, resultou numa alta produção de espécies reativas de oxigénio (ROS), a qual levou a um decréscimo significativo no teor de lípidos (LIP), a uma ativação das defesas antioxidantes (superóxido dismutase, SOD e glutationa peroxidase, GPx) e ao aumento da glutationa oxidada (GSSG), contudo os organismos não foram capazes de prevenir os danos celulares demonstrando um aumento de peroxidação lipídica (LPO). Desta forma, a combinação do aumento da temperatura e da exposição ao ar durante marés baixas demonstrou induzir maior stress oxidativo. Quando expostos à combinação de marés e MWCNTs, os mexilhões demonstraram uma alta produção de ROS, associada ao aumento do metabolismo, o qual levou ao aumento significativo de defesas antioxidantes (SOD, GPx) e de GSSG, e desta forma os organismos foram capazes de prevenir os danos celulares não demonstrando LPO ou cabonilação proteica (PC). Desta forma, os organismos pareceram ser capazes de tolerar MWCNTs e exposição ao ar durante marés baixas, contudo a combinação de ambos os stressores demonstrou induzir maior stress oxidativo.

Estes resultados indicam que o aumento do aquecimento global e da presença de nanopartículas de carbono em ecossistemas marinhos poderá induzir impactos subletais e mais tóxicos em organismos intertidais quando comparados a organismos que estão continuadamente submersos no ecossistema marinho. Além do mais, os resultados também indicaram que a exposição ao ar poderá influenciar a avaliação de diferentes stressores em organismos a viver em sistemas costeiros.

keywords

Mussels, nanoparticles, increased temperature, oxidative stress, metabolism, tidal regime.

abstract

Intertidal species are frequently exposed to environmental changes associated with multiple stressors to which they must avoid or tolerate by developing certain strategies. Some of the natural environmental changes are correlated with the tidal cycle which forces organisms to tolerate the differences between an aquatic and an aerial environment. Apart of these differences, intertidal mussels are also subjected to global warming and consequently, the risk of desiccation especially under aerial environment. Furthermore, pollutants exposure from anthropogenic sources is another daily stress that organisms must cope with it. The present study evaluated the impacts in *Mytilus galloprovincialis* exposed to different temperatures (18ºC and 21ºC) or carbon nanoparticles (0.01 mg/L MWCNT) when continuously submersed or exposed to tides (5 h of low tide, 7 h of high tide) for 14 days. Results evidenced that mussels were physiological and biochemical affected by increased temperature or MWCNTs exposure, especially when exposed to tides. When only exposed to increased temperature, the stress induced was enough to activate mussels' antioxidant defenses by spending energy reserves and avoid oxidative damage. 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. Nevertheless, the combination of tides and temperature, resulted into high production of reactive oxygen species (ROS), which lead to a significant decrease of lipids (LIP) content, activation of antioxidant defenses (superoxide dismutase, SOD and glutathione peroxide, GPx) and increase of oxidized glutathione (GSSG), yet organisms couldn't prevent cellular damage, showing an increase of lipid peroxidation (LPO). Therefore, the combination of increased temperature and air exposure during ebb tides demonstrated to induce higher oxidative stress. Similarly, when mussels were exposed to the combination of tides and MWCNTs, resulted into high production of reactive oxygen species (ROS), associated with an increase of metabolism, which lead to a significant increase of antioxidant defenses (superoxide dismutase, SOD and glutathione peroxide, GPx) and oxidized glutathione (GSSG), and thus organisms were able to prevent cellular damage, showing no lipid peroxidation (LPO) or protein carbonylation (PC) levels. Therefore, organisms seemed to be able to tolerate MWCNTS and air exposure during ebb tides, however the combination of both stressors demonstrated to induce higher oxidative stress.

These findings indicate that the increasing global warming and the increasing presence of carbon nanoparticles in marine ecosystems can induce sub-lethal and higher toxic impacts, respectively, in intertidal organisms compared to organisms continuously submersed in marine ecosystems. Furthermore, 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.

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List of Abbreviations

GHG – greenhouse gases

NPs – nanoparticles

CNPs – carbon-based nanoparticles

CNTs – carbon nanotubes

PECs – predicted environmental concentrations

SWCNTs – single-walled carbon nanotubes

MWCNTs – multi-walled carbon nanotubes

MWCNT-COOH – functionalized/oxidized MWCNT

NCB – nano carbon black

C60 – C60-fullerene

nTiO_2 – nano-titatinum

n-SiO_2 – nanosilica

ROS – reactive oxygen species

SOD – superoxide dismutase

CAT – catalase

LPO – lipid peroxidation

GPx – glutathione peroxidase

GSH – reduced glutathione

GSSG – oxidized glutathione

LIP – total lipid

GLY – glycogen

PC – protein carbonylation

ETS – electron transport system

CI – condition index

RR – respiration rate

DW – dry weight

FW – fresh weight

Chapter 1

General Introduction

1.1. Estuaries and associated environmental alterations

Coastal systems are dynamic and frequently subjected to natural and anthropogenic changes. In fact, being commonly defined as the interface or transition between sea and land (FAO, 1998), coastal areas are exposed to a wide variety of environmental factors affecting them. In particular, estuaries are often semi-closed coastal systems with a restricted opening to the sea and are highly related to the tidal environment. The tidal cycles, being semidiurnal, diurnal, weekly, fortnightly, equinoctial and annual, along with the processes caused by the interaction of freshwater and seawater, the wind, rainfall, evaporation and other oceanic events such as upwelling, currents and storms with the spatially and temporally varying bathymetry and geomorphology, result in the fact that estuaries have never a steady state on its hydrodynamic regimes (Dyer, 1997; Prandle, 2009).

Estuaries also provide habitats and food source to a wealth of wildlife within the intertidal and subtidal zones, making these ecosystems extremely ecological and economical valuables. In fact, these are some of the several essential ecological functions of estuaries in combination with others such as high biological productivity, hydrological regulation and the biogeochemical cycling of metals and nutrients (Caçador et al., 2007; Mitsch and Gosselink, 2015; Xiao and Li, 2004). However, estuaries are also important human and industry settlement places, being therefore subjected to increasing environmental stress (Haslett, 2008).

The estuarine ecological functions are highly dependent on physical and chemical disturbances from natural to anthropogenic sources, typical of transitional coastal ecosystems (Dauvin and Ruellet, 2009; Elliott and Quintino, 2007). Therefore, and due to their nature, coastal systems such as estuaries, represent one of the hardest environments to endure for 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 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 is another disturbance that these organisms must cope with in a daily basis (Amiard-Triquet and Rainbow, 2009, Elliott et al., 2014).

1.1.1. Tidal regime

Intertidal organisms are daily subjected to air exposure due to a tidal regime typical of coastal systems. At the sea, tides are a sequence of sinusoidal, tidal harmonic components which differ for every location. Depending on their sequence, tides are giving different nominations such as flood tide during rising tide, ebb tide during falling tide and slack water during intermediate periods (Wolanski and Elliot, 2015). However, in estuaries tides are distorted as they propagate these coastal systems being the point at which they are no longer observable called the tidal intrusion limit or tidal limit (Prandle, 2009). Moreover, tides are often asymmetric in these coastal areas since ebbs and flood tides may differ in duration. This asymmetry is controlled principally by the bathymetry, bottom friction and the river inflow of the estuary, being considered a function of its hydromorphology (Wolanski and Elliot, 2015).

In intertidal conditions, organisms may have to avoid or withstand exposure to desiccation, osmotic stress, temperature stress and UV radiation, in addition to exposure to air during ebb tides (Rawlings, 1999). Tidal regime may also act as a stressor by frequent re-immergence in intertidal organisms. Furthermore, as a consequence of air exposure, organisms may also face prolonged hypoxia or/and anoxia conditions, which may bring negative impacts to these organisms (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 known that organisms may exhibit structural, behavioral and physiological adaptations in response to these stressors. In fact, in these intertidal habitats, it is visible a distribution and vertical zonation of intertidal communities affected by the temperature gradients, desiccation and oxygen availability, especially for sessile and sedentary organisms (Menconi et al., 1999; Newell, 1979; Wethey, 1983; Zandee et al., 1986). Different organisms have also shown physiological responses such as increased thermal resistance (Sokolova et al., 2000), heat stability of key metabolic enzymes (Sokolova et al., 2003), and stress-induced expression of heat stress proteins (Tomanek and Somero, 2000). During ebb tides, organisms as bivalves have been demonstrating behavioral responses as closing their valves undergoing anaerobic metabolism or exhibiting alternate valve closure and using valve gaping allowing the maintenance of aerobic respiration under hypoxia conditions (Famme and Kofoed, 1980; Widdows et al., 1979).

Although some of the intertidal organisms' responses to the tidal regimes are well documented, the biochemical and physiological impacts induced by re-immersion in organisms

in this type of environment, especially under laboratory conditions, are still even scarcer. Furthermore, scientific literature about the consequences of this frequent exposure to air in combination with other abiotic factors such as temperature differences due to climate change and anthropogenic contamination are still scarce.

1.1.2. Climate change: temperature increase

Due to climate change, intertidal organisms may face environmental stresses in addition to air exposure during tidal regimes. Furthermore, daily and seasonal environmental changes during the tidal regime such as temperature, salinity and pH differences may be enhanced with the global climate change. Its is known in fact that climate change is influencing global temperature, oceans' pH, salinity and other factors such as rising sea levels, which in consequence influences coastal systems and the organisms present in these ecosystems (IPCC, 2014; Letcher, 2009). In particular, the marine organisms such as intertidal organisms, are frequently exposed to natural changes of these factors presenting high tolerance to them in a wide range of values. These organisms are subjected to extreme temperatures resulting in frequent fluctuations of over 30ºC in their bodies temperature (Helmuth et al., 2003). Furthermore, intertidal organisms also live close to their physiological tolerance limits being subjected to other stressors such as desiccation, rapid fluctuations in salinity, oxygen availability and nutrients levels (Burnett, 1997; Davenport and Macalister, 1996; Foster, 1971; Hutchins, 1947; Li and Brawley, 2004; Wolanski and Elliott, 2015). In this way, with the enhanced stressors due to climate change, organisms' tolerance level may be exceeded with negative impacts at different biological levels.

In particular, as a consequence of global climate change, the increase in temperature may cause deleterious effects in intertidal organisms (IPCC, 2007). It is known that the cumulative emissions of anthropogenic greenhouse gases (GHG) such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) in the last decades driven by economic and population growth are extremely correlated to the global warming observed since the mid-20th century (IPCC, 2014). In fact, these GHGs absorb long-wave infrared radiation emitted from the earth's surface, reflecting back afterwards and trapping the energy associated within the atmosphere, consequently increasing earth's surface temperature in comparison to when the GHGs were low (Reddy and DeLaune, 2008). The predicted continuous increase of mainly

atmospheric CO_2 until the end of the 21st century is considered one of the most important factors contributing to global warming (1.0-4.0ºC) and consequently, to an increase of the global mean air and ocean temperatures (IPCC, 2007). As a consequence, the glacial melting and thermal expansion of oceans will increase the sea level, thus flooding of coastal regions as estuaries and shifting the wetland areas in direction to landward (Reddy and DeLaune, 2008).

Intertidal organisms that are exposed to increased temperatures associated with aerial exposure may be subjected to extreme desiccation events (IPCC, 2001). Different studies have already demonstrated that temperature exceeding the organisms' thermal tolerance range can cause physiological and molecular perturbations, as in the individuals' growth and reproduction (Pörtner and Knust, 2007; Boukadida et al., 2016) and, in addition, the decrease of aerobic capacity, metabolic rate and respiratory capacity (Jansen et al., 2009; Pörtner et al., 2005, 2010; Velez et al., 2017). It is also known that extreme desiccation events, not only can have sublethal and lethal consequences at the populations' phenotypic traits (Jones & Boulding, 1999) but, as well, at the individuals' physiology (Helmuth & Hofmann, 2001; Tsuchiya, 1983; Silva et al., 2005b). Furthermore, warming can also enhance reactive oxygen species (ROS) production in the cells (Kefaloyianni et al., 2005; Verlecar et al., 2007), leading to oxidative stress.

1.1.3. Emerging contaminants: nanoparticles in the environment

Pollution may be defined as the result of adding a xenobiotic material in an ecosystem where it brings biological effects and often a reduction in the health of the system (McLusky and Elliot, 2004). The reduction in health may be manifested in different biological levels, from cell to individual, and consequently population, community and ecosystem, with the effect of the xenobiotic moving though each level unless the system can absorb it or there is a survival of one component (i.e. cell, individual, population, etc) without its functions being debilitated (Tett et al., 2013). Estuaries are dynamic interface zones between water draining from inland river basins 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). Furthermore, xenobiotics from different anthropogenic sources are increasing in marine ecosystems which can cause adverse effects (Fu et al., 2003; Maanan, 2008).

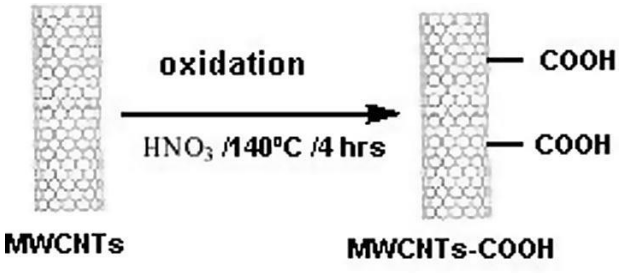
An emergent field in the industry and science that has been increasing in the last years is the nanotechnology. In fact, nanoparticles (NPs) have been increasingly used in numerous applications including medicine, chemistry and electronics (Renn and Roco, 2006) and, thereby, the increased introduction of these materials into the aquatic systems is expected to occur. Nanomaterials, as NPs may be defined as a natural, incidental or manufactured material with dimensions between 1 and 100 nm containing 50% or more particles in an unbound, aggregated or agglomerated state (EC, 2011). Due to their unique physical and chemical properties there has been an increase of production and research on NPs at a global level. However, besides anthropogenic sources, NPs are also naturally released in the environment by natural sources (e.g. volcanos and forest fires) (Mottier et al., 2017).

Among NPs, carbon-based NPs (CNPs) present a diversity of applications (Solarskaciuk et al., 2014; Vlasova et al., 2016; Wu et al., 2013; Muller and Nowack, 2008; Köhler et al., 2008). In fact, these CNPs are already been used in daily life products such as paints, energy storage and waste water treatment being very likely released to the environment (Keller et al. 2013, Mitrano et al., 2015; Petersen et al., 2011). Among the most important CNPs 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 reported from the most recent literature (see for example Zhang et al., 2017; De Marchi et al., 2018b).

CNTs are constituted by hollow graphene cylinders which can be divided in single-walled (SWCNTs) with 0.7 to 3nm of diameter, and multi-walled (MWCNT) with 10 to 25nm of diameter (Baughman et al., 2002). In the aquatic environment CNTs can be easily accumulated by the aquatic biota through body surface, digestive and respiratory system (Jackson et al., 2013) due to their hydrophobic and non-biogradable nature, making the dispersion of CNTs in the water rather difficult (Donaldson et al., 1998). Because of their property to easily aggregate in solution, particularly in saltwater (Kataoka et al., 2016), CNTs are functionalized through chemical modifications such as amidation and esterification of the nanotube-bound carboxy acids making them more dispersible (Sun et al., 2002). The nanotubes dispersibility is increased by breaking the nanotubes bundles essential to solubility (Sun et al., 2002) and by eliminating the functional groups on nanotubes surface (Shahnawaz et al., 2017). In fact, inserting polar groups as carboxyl groups (-COOH) in the chemical functionalization of CNTs (**Figure 1.1.** A), is one of the most used approaches to produce better dispersibility of CNTs in

aqueous media (**Figure 1.1.** B) (Shahnawaz et al., 2017). CNTs with higher water dispersible characteristics have shown to induce higher levels of toxicity to biological systems (Arndt et al., 2013; Kataoka et al., 2016). Although the use of CNTs is increasing at global levels, their biochemical and physiological impacts towards aquatic organisms are still very limited.

A



B

Figure 1.1. A- Surface functionalization of MWCNT surface; B- Solubility of pristine MWCNT and MWCNT-COOH in different solvents (from left to right, deionized water, dichloromethane, methanol, and hexane) (Adapted from Salam et al., 2017).

1.2. Marine bivalves as bioindicators

Bivalves are easily identified by their two shell valves connected by an elastic ligament. The shell is mainly composed by calcium carbonate ($CaCO_3$) where this mineral component represents more than 95% of the shell weight (Gosling, 2015). The vast majority of bivalves are suspension- or filter-feeding organisms where their gills, with several ciliary tracts, are used to remove suspended particles in the water that was before pumped through the mantle cavity. Most bivalves have sessile adult lives which, such as oysters and mussels, attach themselves to hard surfaces using byssal threads making them sessile epifaunal bivalves (Gosling, 2015).

The sessile and feeding condition of the majority of the bivalves, leads to the constant exposure of most environmental and biological stressors, reflecting site-specific conditions. In fact, bivalves are regularly subject to abiotic stressful conditions, such as variations in temperature, salinity and oxygen availability, alterations on food availability and in the quality of the surrounded environment (Almeida et al. 2007; Davis 1985; Gagné et al., 2006; Horn et al., 1999). It is known that these abiotic factors may affect marine bivalve species (Carregosa et al., 2014b; Parker et al 2013). Furthermore, bivalves are known to tolerate high concentrations of xenobiotics, however their ability to filter high quantities of water either for feeding or respiration may result in ecotoxicological effects resulted in any xenobiotic dissolved or suspended in the water column and thus, providing a strong and specific response to these pollutants (McEneff et al., 2013). In addition to these factors, bivalves are also organisms with a high abundance and widespread distribution which makes them excellent bioindicator organisms (Catsiki and Florou, 2006; Faggio et al., 2016; Hamze-Chaffai, 2014; Kristan et al., 2014; McEneff et al., 2014; Oliveira et al., 2017). A bioindicator species may be defined as a species or group of species that can reflect and/or reveal the impacts of environmental changes on a population, community or ecosystem (Hamze-Chaffai, 2014).

Because of their habitat characteristics, bivalves present a natural wide range of tolerance to abiotic factors, however under climate changes the limit of abiotic tolerance can be reached (Bielen et al., 2016; Rodrigues et el., 2015). For the assessment of the effects of different abiotic factors such as temperature, salinity and pH on marine invertebrates, bivalves have been recurrently used as model organisms (Anestis et al., 2007; Carregosa et al., 2014b; Dickinson et al., 2012).

1.2.1. Responses of bivalves to air exposure

In intertidal areas, as a consequence of air exposure, organisms may face prolonged hypoxia and/or anoxia conditions. 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.

Different bivalves already showed an 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 reoxygenation (Almeida and Bainy, 2006, Andrade et al., 2018). The same biochemical response was observed in *Ruditapes philippinarum* clams daily exposed to rhythms of air (Yin et al., 2017). Furthermore, in the same study, clams showed lower survival and growth when exposed to increased duration of air exposure (from 0h, 3h, 6h to 9h) after 60 days. The mussels *Mytilus edulis* demonstrated over-expression of proteins specially involved in cytoskeleton, chaperoning, energetic metabolisms and transcription regulation after emerged conditions (Letendre et al., 2011). For the same species, differences in hypoxia tolerance between mussels transplanted in the upper intertidal and the mussels transplanted in the subtidal portion of their natural depth distribution were observed (Altieri, 2006).

It is known that some bivalves, 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 at ebb tides to avoid from suffering desiccation, although others may prevent anoxia by simply opening the valves for air gaping as a behavioral adaptation (Rivera-Ingraham et al., 2013).

1.2.2. Responses of bivalves to warmer temperature

The thermal tolerance under changing temperatures have been the focus of many studies (Pörtner and Knust, 2007). Under warmer temperatures, bivalves tend to increase feeding and metabolic rates, leading to an increase in growth and reproduction within the species thermal optimum range with the existence of sufficient food and oxygen (Filgueira et al., 2016). However, with the global warming these conditions may not be obtained and the increase in temperature may negatively affect shellfish.

Studies have been demonstrating that exceeding organisms' thermal tolerance range may cause physiological and molecular perturbations. In *M. galloprovincialis* the increased of temperature has displayed an increase of the number in abnormal larvae demonstrating a possible impairment of this mussel's reproduction due to increased temperature (Boukadida et al., 2016). Different bivalves have been demonstrating decreased aerobic capacity, metabolic rate and respiratory capacity under increased temperatures (Jansen et al., 2009; Pörtner et al., 2005; Velez et al., 2017)

The induction of oxidative stress has also been observed in different bivalves exposed to warming conditions. *M. galloprovincialis* mussels showed an increase of gene expression levels related to antioxidant enzymes and metallothioneins when exposed to heat stress (Banni et al., 2014). In the same organism, significant variations in the immune system were also observed due to increased temperature (Nardi et al., 2017). The clams *Ruditapes decussatus* and *R. philippinarum* displayed an induction of electron transport system and antioxidant enzyme activities, as well as the expression of a chaperone function related gene at an increased temperature of 21ºC (Velez et al., 2017). However, *R. decussatus* presented a different behavior at 25ºC where results suggested valve closure as a behavioral strategy as well as down-regulating in the expression of genes related with mitochondrial metabolism and chaperone function, while at same temperature, *R. philipinarum* increased electron transport system and antioxidant enzyme activities, as well the expression of genes related to apoptosis and molecular chaperone (Velez et al., 2017). *M. coruscus* mussels displayed an increase of enzymatic activity such as superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, acid phosphatase, alkaline phosphatase and glutamic-pyruvic transaminase with increased temperature (Hu et al., 2015).

1.2.3. Responses of bivalves to nanoparticles

Being emergent pollutants the presence of NPs in the aquatic environmental has been increasing in the last years. In terms of nanoparticle toxicology, it has been suggested that suspension-feeding invertebrates as bivalves mollusks may represent an unique target group (Moore, 2006). In fact, these organisms have developed processes as endocytosis and phagocytosis for the cellular internalization on NPs and microparticles, which are crucial in physiological functions as intra-cellular digestion and cellular immunity (Canesi et al., 2010b).

In marine bivalves, the effects of different NPs were already demonstrated. Nano carbon black (NCB) exposure in *M. galloprovincialis* mussels was shown to induce inflammatory processes (Canesi et al., 2008). In the same organism, exposure to NCB, C60-fullerene (C60), nano-titatinum ($nTiO_2$) and nanosilica ($n\text{-}SiO_2$), increased the activation of the antioxidant enzyme catalase, with $n\text{-}TiO_2$ also increasing glutathione transferase (Canesi et al., 2010a). Exposure to aqueous suspensions of carbon nanoparticles (C60) in these mussels also demonstrated to induce cytotoxicity in circulating phagocytic hemocytes, which is a crucial component of the mussels' immune system (Moore et al., 2009). *M. edulis* mussels exposed to Fe_2O_3 nanoparticles showed to suffer impairment of lysosomal stability in circulating blood cells and lipid peroxidation (Kádár et al., 2010). Furthermore, exposure to MWCNTs in the clam *R. philippinarum* revealed altered energy-related responses, with differences in the metabolic capacity and energy reserves. In addition, this specimen also suffered oxidative stress and neurotoxicity (De Marchi et al., 2017c, 2018a).

1.3. Objectives

In the environment mussels are subjected to tidal changes which may act as a confounding factor when assessing the impacts induced in these organisms by pollutants, such as CNTs, and abiotic changes, as temperature increase. In fact, when mussels are used in environmental monitoring programs and specially under laboratory conditions, it is not considered their natural environment and the possible derived implications to assess the impacts of other stressors. Moreover, it's possible that the tidal changes may alter organisms' tolerance and responses to contaminants and abiotic factors, as air temperature and CNTs. Within this context, the present study aimed to evaluate if physiological and biochemical alterations imposed by the presence of multi-walled CNTs (MWCNTs) or increased temperature were dependent on the submersion/tidal regime, to better understand the possible interactions of exposure to air with warming (**Figure 1.2.** A) or MWCNTs exposure (**Figure 1.2.** B) in mussels' performance and tolerance to them.

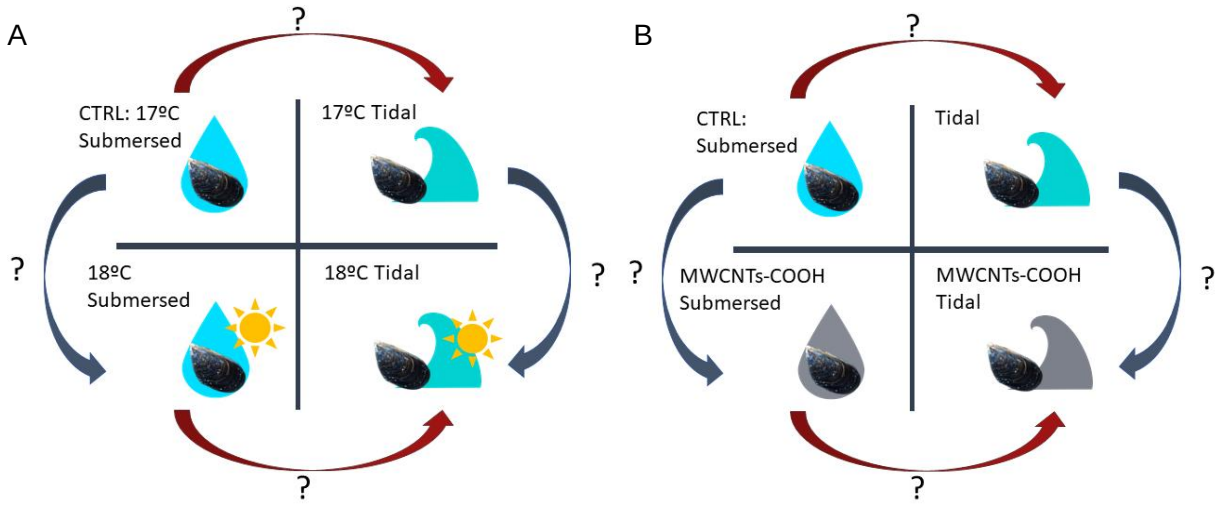


Figure 1.2. Graphical representation of possible interactions between: A – Air exposure and different temperatures; B – Air exposure and MWCNTs.

Chapter 2

Material and Methods

2.1. Test organisms

Among bivalves, mussels have been extensively used to assess environmental contamination. Among mussels, *M. galloprovincialis* (Lamarck, 1819) is widely distributed from temperate to subarctic coasts across the globe, inhabiting generally infra littoral areas from the top of the intertidal zone to depths of a few meters (FAO, 2016; Vazzana et al., 2016). In Portugal, this species exists along the entire coast as well in the northern areas of the Iberian Peninsula (Mitchelmore et al., 1998), being present on rocky areas, cliffs, boulders or substrates that are relatively movable (ropes) and to which it adheres (FAO, 2016; Vazzana et al., 2016). Although being native in Portugal, this species was introduced in various locations across the globe most likely due to its association with large shipping ports being transported via ballast water or hull fouling, were it colonized and formed naturalized populations (Branch and Steffani, 2004).

Although considered an invasive species in some locations *M. galloprovincialis* may also be ecological and economic relevant. In fact, this mussel can improve water quality thought the filtration of particles and excess of nitrogen in aquatic environment (Shumway et al., 2003). The invasion of this species has also benefit a near-threatened bird species, the African black oystercatcher *Haemotopus moquini*, which switched its diet to this mussel and increased its food availability this way (Hockey and Schurink, 1992). There's a high wordwide annual production of *M. galloprovincialis*, estimated to around 116 269 tonnes in 2014 (FAO, 2016) and aquaculture facilities for this production can also provide refuge to fish or even function as a nursery ground for juvenile fish and crustaceans (Shumway et al., 2003).

M. galloprovincialis is frequently exposed to tidal changes and, as a sedentary filter feeding organism, has also 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 strong and 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 to rocky intertidal shores, appear to exhibit adaptive physiological, behavioral and morphological traits (Dowd et al., 2013; Sherratt and Mackenzie, 2016) such as the valves closure to protect from stressful conditions (Gazeau et al., 2013; Ishii et al., 2005; Poulain et al., 2011).

2.2. Sampling area

In the present study, *M. galloprovincialis* specimens were collected in an intertidal area at the Mira Channel in the Ria de Aveiro (**Figure 2.1.**). Ria de Aveiro is a shallow coastal lagoon in the Northwest of Portugal and represents one of the most important estuarine systems in the country. Between low and high spring tides, the Ria de Aveiro comprises an area between 66 and 83 km^2 being 45 km long, 10 km wide and connected to the ocean by a single inlet (Dias and Lopes, 2006). Furthermore, its characterized by a very complex geometry of distinct intertidal areas, such as mud flats and salt marshes, and a web of narrow channels (Dias et al., 2000; Dias and Picado, 2011). Connected to the ocean entrance, there are four main branches: Mira, S.Jacinto, Ílhavo and Espinheiro channels (Picado et al., 2010). Among them, the Mira channel is considered the least impacted channel (Castro et al., 2006), while Estarreja channel together with Laranjo Bay are considered the most impacted areas due to substantial contamination in bottom sediments (Pereira et al., 2008).

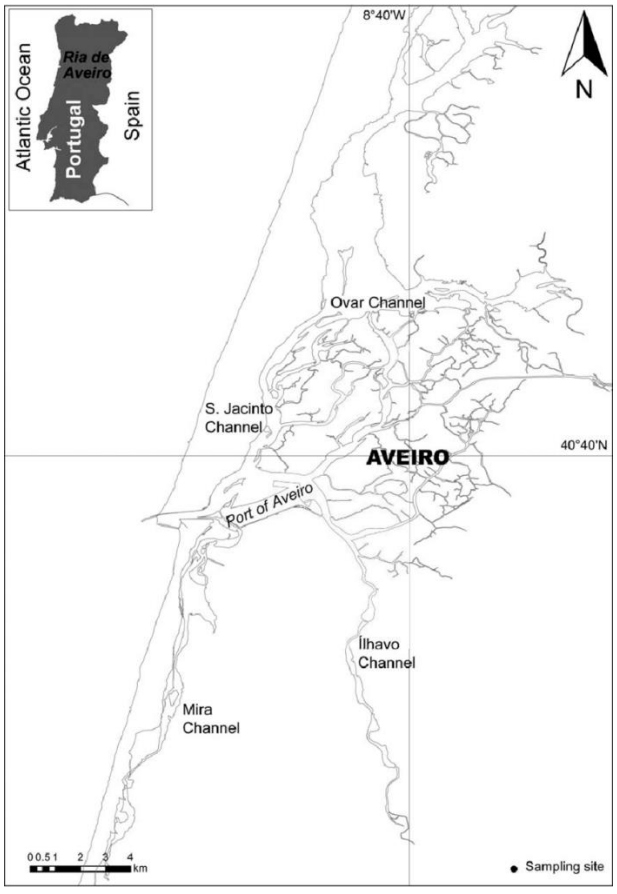


Figure 2.1. Study area: Ria de Aveiro.

The hydrology of Ria de Aveiro is tidal dominated (Dias et al., 1999) where the strongest currents reaching values higher than 2 m/s are predominated at the inlet channel (Vaz et al., 2009). In fact, the water circulation is dominated by the seawater input (70 x 10^6 m^3 in spring tides) rather than by the input of freshwater (1.8 x 10^6 m^3 per tidal circle) (Moreira et al., 1993). The tidal inputs are effectuated from the navigation channel (Dias et al., 1999) and fresh water discharges ranging between 1.0 to 61.3 m^3/s from rivers and streams that flow into Ria de Aveiro, being the most notorious, Rio Vouga, Antuâ, Fontão and Boco (Dias et al., 2003; Santos et al., 2014). It is also notorious a seasonal and spatial gradient of salinity in this lagoon (Dias et al., 2011). With the combined effects of the freshwater and tidal inputs, the longitudinal gradient of salinity can go from 0 near the river entrances to about 36 at the inlet channel (Lopes et al., 2007).

The Ria de Aveiro can also be considered one of the most important coastal systems of Portugal in a conservationist point of view. This lagoon presents a large number of characteristics and biotopes that makes life ideal to a large number of different species, such as salt marshes (Sousa et al., 2017a), seagrass meadows of *Zostera noltei* (Azebedo et al., 2013; Sousa et al., 2017b) and dune systems (Lopes et al., 2007). In the case of the benthic populations distribution, such as the species used in this work, there is a high correlation with to the lagoon hydrodynamics and salinity gradients (Rodrigues et al., 2011).

2.3. Experimental conditions

After sampling, the collected mussels in Mira channel 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. In order to resemble estuarine conditions, organisms were maintained during this period at 18ºC ± 1.0 ºC and pH 8.0 ± 0.1, and kept under continuous aeration during a 12 h light: 12 h dark photoperiod.

For the laboratory experiment, organisms were distributed into different aquaria (20 L seawater, salinity 35), with six individuals per aquarium and three aquaria per treatment. The treatments tested were divided in three experimental setups. For the first experimental setup, submersed under control temperature (Sub) and exposure to tides simulation under control

temperature (Tide) treatments were considered. For the second experimental setup, submersed under increased temperature (Sub+Temp) and exposure to tides simulation under increased temperature (Tide+Temp) treatments were considered, using the first experimental setup as control treatments. For the third experimental setup, submersed with MWCNTs 0.01 mg/L at control temperature (Sub+MWCNTs) and exposed to tidal simulation with MWCNTs 0.01 mg/L at control temperature (Tide+MWCNTs) treatments were considered, using the first experimental setup as control treatments as well. Aquaria were placed in two different climatic rooms to maintain the temperature levels at 18 ± 1.0 ºC (control temperature) and 21 ± 1.0 ºC (increased temperature). For the tidal simulation, an automatic system that mimicked the estuary tidal regime typical of this species habitat (5 hours of low tide and 7 hours of high tide cycles) was developed and used (**Figure 2.2.**). **Figure 2.3**. illustrates the experimental setup performed in this work.

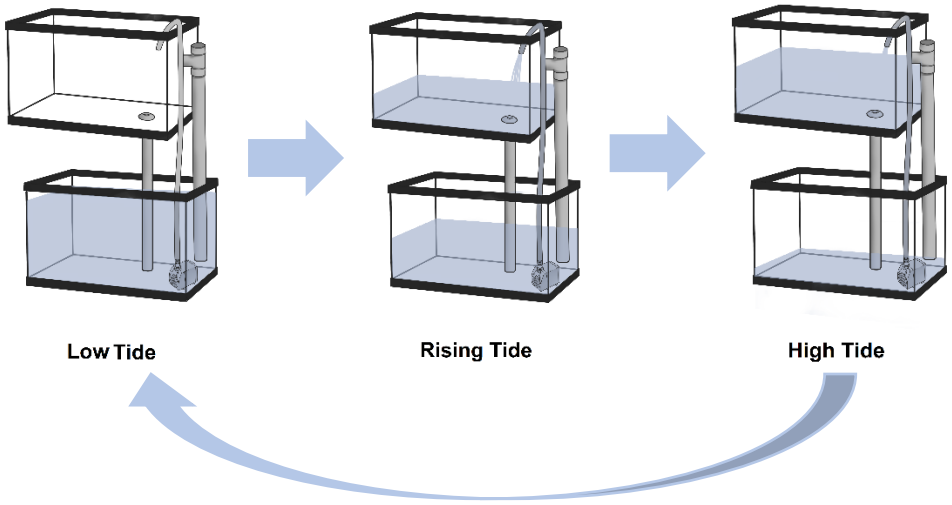


Figure 2.2. Experimental design of the automatic system developed to simulate tidal regime.

Figure 2.3. Experimental setup in one climatic room. Aquaria on the left were linked to a timer to simulate tidal regime while on the right organisms were always kept submersed, half of the aquariums were contaminated with MWCNTS.

The control temperature of 18 ± 1.0 ºC was chosen considering the average temperature of the sampling area during September (IPMA, 2017). To simulate warming conditions, temperature of 21 ºC was selected taking in account the annual range of average temperatures (13.4-22.9 ºC) for *M. galloprovincialis* habitats in the Ria de Aveiro (Coelho et al., 2014; Santos et al., 2009; Velez et al., 2015) and considering the predicted increase of temperature from 1.0 ºC to 4.0 ºC (IPCC, 2007).

The concentration of MWCNTs used was chosen taking in account previous works carried out by De Marchi et al. (2017b, 2017c, 2018) in the clam *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).

During the experimental period (14 days) organisms were fed three times per week with Algamac protein plus (107 cells/animal). After 7 days of beginning of the experiment, seawater was renewed re-establishing seawater characteristics, including salinity, temperature, pH and MWCNTs concentration. An experimental period of 14 days was chosen taking in account

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 after 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. After each week, water samples were taken right before of seawater renewal to characterize MWCNTs in the water column.

2.4. MWCNTs 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 manufacturer specifications of: diameter 2-5 nm; length 10-30 μm; carbon purity 98%; surface area 400m2/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 particles characterization, the average size distribution of MWCNTs suspensions in seawater in each exposure condition was analyzed by dynamic light scattering (DLS), using a DelsaTM NanoC Particle Size Analyser (Beckman Coulter). Measurements were performed on 1 mL of suspension measurements 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.).

2.5. Biological responses

Physiological indicators are used to study the existence of seasonal metabolic variations normally attributed to the changes in environmental parameters as environmental stressors and the physiological status (Livingstone, 2001; Schiedek et al., 2006; Schmidt et al.,

2013). Respiration rate (RR) may also be used to assess the alterations induced by different stressors in organisms (Gestoso et al., 2016; Freitas et al., 2017; Wang et al., 2015). Furthermore, the condition index (CI) is the index most frequently used which provides information on both the physiological status and the growth of organisms (Andral et al., 2004).

The metabolic capacity of marine organisms and energy reserves can further provide information on the organisms' physiological status. In this respect, the energy production at the mitochondrial level such as the electron transport activity (ETS) and energy reserves for metabolism such as glycogen (GLY) and total lipid (LIP) content and can be estimated, giving thus an indication of organisms' metabolic capacity (Coen and Janssen, 1997, 2003).

When organisms are exposed to natural and anthropogenic stressors, the production of reactive oxygen species (ROS) may affect especially lipids, proteins, carbohydrates and nucleic acids (Freitas et al., 2016a). When organisms are then exposed to oxidative stress, a cascade of defensive reactions is activated in order to cope with the ROS overproduction. This process attempts to protect the cells and tissues avoiding severe and generalized pro-oxidative damage (Correia et al., 2016). Some biomarkers related to oxidative stress and oxidative damage can then be determined for the assessment of the contaminant and abiotic stressors in the organism. When cells' defense mechanisms fail or are insufficient, the lipids attacked by radicals and ROS, initiate an autocatalytic oxidative process known as lipid peroxidation (LPO) were lipids are oxidized generating lipid hydroperoxides (Catalá, 2009; Freitas et al, 2016a; Regoli and Giuliani, 2014). There is an antioxidant enzymatic mechanism involving glutathione peroxidase (GPx), which reduces lipid hydroperoxides, with the consequent oxidation of reduced glutathione (GSH) to oxidize glutathione (GSSG) and thus neutralize directly ROS. GSH can also be a cofactor of glutathione-dependent enzymes (Regoli and Giuliani, 2014). Another process that can be promoted by ROS, is the protein carbonylation (PC) were, similar to LPO, proteins are oxidized (Suzuki et al., 2010). However, the first mechanism acting at the enzymatic defense level, and to be used as a biomarker, is the superoxide dismutase (SOD), catalyzing the dismutation of superoxide (O_2^-) in oxygen and hydrogen peroxide (McCord and Fridovich, 1969). However, the formed hydrogen peroxide is also a harmful sub-product which is needed to be eliminated and degraded from the organism. Thus, a second enzymatic mechanism, catalase (CAT), is activated to avoid more damage in the cells and tissues. This last mechanism is involved in the conversion of hydrogen peroxide in water (Aebi, 1984).

2.5.1. Physiological parameters

2.5.1.1. Respiration Rate

Paragraph should be change to: “After 14 days of exposure, respiration rate (RR) was measured in six mussels per condition (two per aquarium/replicate). Measurements were performed by simple static respirometry, using two organisms of the same aquarium per respirometric chamber. Each of these chambers, which were equipped with an oxygen sensor spot glued to its inner wall using silicon paste, was filled with the same seawater used during the experimental period. Organisms were placed in these chambers under dark and fully-oxygenated concentrations where they were allowed to acclimate for 30 min to avoid the influence of manipulation on RR. After this period, chambers were filled to their maximum capacity (1L) to avoid the formation of air bubbles and were then air-tight sealed. Measurements started in fully oxygenated medium and RR was recorded as a function of declining O_2 concentration (mg/L) over time every 15 min during 2h, with a multi-channel fiber optic oxygen meter (Multi channel oxygen meter, PreSens GmbH, Regensburg, Germany) for simultaneous read-outs. Data were recorded using the software PreSens Measurement Studio 2. Twenty-two measurements were carried out at a time (including a blank, i.e. chamber containing no organisms to account for background respiration). Organisms were posteriorly dried and weighed. Respiration rate was expressed in mg O_2 consumed per h per g dry weight (DW).”

2.5.1.2. Condition Index

The CI was calculated considering that this parameter can give an indication of the general physiological status of the animals. After the 14 days of the experimental period, the soft tissues of six frozen organisms per condition, previously used for RR determination, were carefully separated from the shells. Both shells and tissues were put in an oven at 60 ºC for 48 h (**Figure 2.4.**). After this period, the dry soft tissues and shells were weighed and CI calculated. Following Matozzo et al. (2012), CI values were expressed as the ratio between the DW of softs tissues and the DW of shell x 100. The dry tissue was stored and used for lipid quantification.

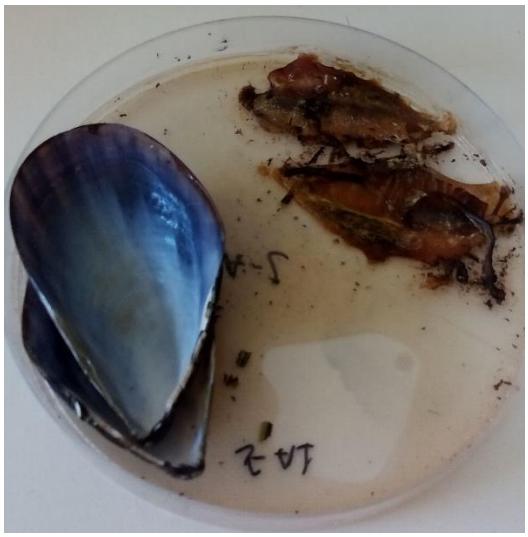


Figure 2.4. Mussels' dry shells and tissue after being carefully separated and put in an oven.

2.5.2. Biochemical parameters

After the 14 days of the experimental period, the soft tissue of six frozen organisms per condition (used previously for the CI and RR) were carefully separated from the shells to determinate mussels' LIP content. Both shells and tissues were dried in an oven at 50ºC for 48h. After this period, dry tissue was homogenized with a mortar and a pestle, divided in 0.5 g aliquots and stored for further LIP quantification (**Figure 2.5.**).

Figure 2.5. Homogenization process of mussels' dry tissue.

For all other parameters except LIP, shells of the frozen organisms (four per aquarium/replicate, twelve per condition) were removed and the frozen whole soft tissue was homogenized using a mortar and pestle with liquid nitrogen. The homogenized tissue of each organism was distributed in 0.5 g aliquots (**Figure 2.6.**).

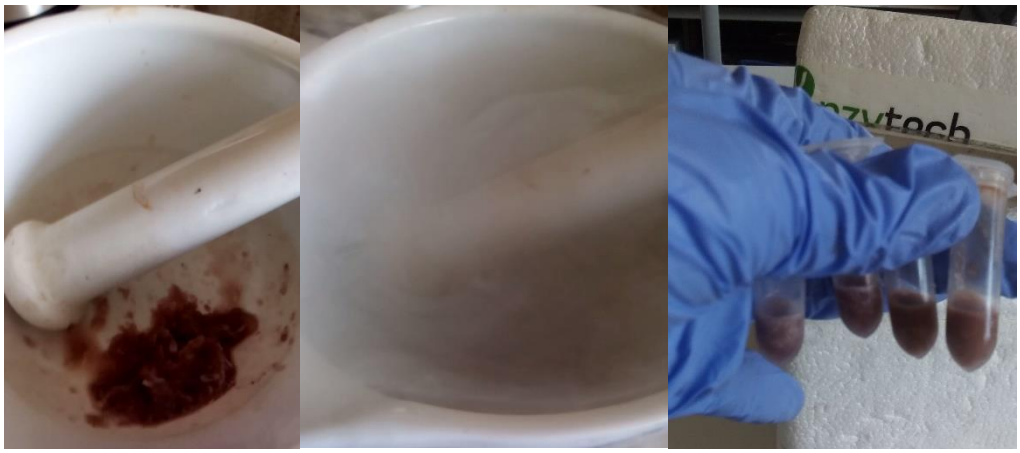


Figure 2.6. Homogenization process of mussels' frozen tissue with liquid nitrogen.

For each biochemical parameter, a specific buffer was used in the extraction of the supernatant using a proportion of 1:2 (w/v). Firstly, the tissue sample were homogenized using a TissueLyser II (Qiagen) during 1 min, after which they were centrifuged 20 min at 10,000 g or 3,000 g depending on the biomarker at 4 ºC. Supernatants were stored at -80ºC or immediately used to determine: ETS activity; GLY content; LIP content; LPO levels; GSSG content; PC levels; and activity of antioxidant enzymes (SOD, CAT, GPx). Two replicates per sample were used for the determination of each biochemical parameter.

2.5.2.1. Metabolic capacity

The ETS activity was measured based on the method of King and Packard (1975) and modifications by Coen and Janssen (1997). The supernatant extraction was done with 0.1 M Tris-HCL pH 8.5, 15%(w/v) PVP, 153 mM magnesium sulfate (MhSO4) and 0.2%(v/v) Triton X-100. 35.7 µL of supernatant was incubated on a microplate with 107 µL of buffered substrate

solution (0.13 M Tris-HCl, 0.3% (v/v) Triton X-100, pH 8.5) and 35.7 µL of NAD(P)H (1.7 mM NADH and 250 µM NADPH). The reaction started after 71.4 µL of 8 mM p-IodoNitroTetrazolium were added. Absorbance measurement was performed during 10 min at 490 nm with intervals of 25 s and the extinction coefficient of 15.900 $M^{-1}cm^{-1}$ was used to calculate the amount of formazan formed. Results were expressed in nmol min per g fresh weight (FW).

2.5.2.2. Energy reserves

The GLY content was measured according to the sulfuric acid method (Dubois et al, 1956), using glucose standards. The supernatants were extracted with sodium phosphate buffer, pH 7.0 (50 mM sodium dihydrogen phosphate monohydrate, 50 mM, disodium hydrogen phosphate dehydrate, 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinvlpyrrolidone (PVP); 1 nM dithiothreitol (DDT)). For the GLY determination, 10 µL of the each sample was used. To every sample, 100 µL of phenol (5%) and 600 µL of sulfuric acid (H_2SO_4, 96%) were added. The standard curved was generated with GLY standards (0 mg to 5 mg/L). Absorbance measurement was executed at 492 nm after samples being incubated for 30 min at room temperature. The results were expressed in mg per g of FW.

The LIP content was determined following the methods developed by Folch et al. (1957) and Cheng et al. (2011). To each 10-15 mg sample of dry tissue, 5 mL of a mixture of methanol/chloroform (2 chloroform:1 methanol) was added and vortexed. The samples were then centrifuged for 10 min at 3500 rpm and 10 ºC and a standard curve was determined using cholesterol standards (0–100%). Tubes with 1 mL of each sample were left at 50 ºC until the next day. 1 mL of sulfuric acid (95%) was then added to each tube, agitated and left for 10 min at 100 ºC, being vortexed after this procedure. 5 mL of vanillin solution were mixed with 200 µL of each sample. After 1 h of color development in the dark at the room temperature, the absorbance was measured at 520 nm. Results were expressed in percentage per mg DW.

2.5.2.3. Oxidative damage

LPO levels were quantified following the method described in Ohkawa et al. (1979) with modifications referred by Carregosa et al. (2014b). Supernatants were extracted using 20% (v/v) trichloroacetic acid (TCA). 50 µL of supernatant were prepared on microtubes with 200 µL of TBA (0.5% thiobarbituric acid in 20% (v/v) TCA) and with 150 µL of TCA each. All samples were incubated at 96 ºC for 25 min and the reaction was stopped by transferring those samples to ice (**Figure 2.7.**). After 300 µL of each sample were transferred to a microplate the absorbance was measured at 535 nm (ε=156 mM^{-1} cm^{-1}) and results expressed in nmol of MDA equivalents formed per g FW.

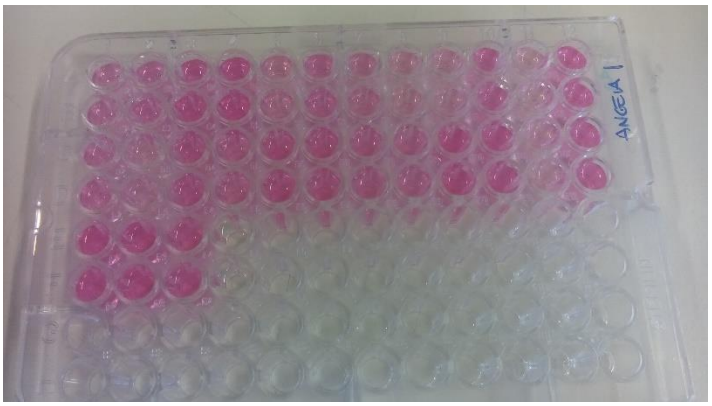


Figure 2.7. LPO colored samples after the reaction being stopped.

The quantification of GSSG content was measured according to Rahman et al. (2007), using GSSG as standards (0–90 µmol/L). Extraction was performed using 0.6% sulfosalicylic acid in potassium phosphate buffer (100 mM dipotassium phosphate; 5 mM EDTA; 0.1% (v/v) Triton X-100; pH7.5). Absorbance measurement was realized at 412 nm with the results being expressed in µmol per FW.

PC levels were measured according to the DNPH alkaline method described by Mesquita et al. (2014). The supernatant was extracted with sodium phosphate buffer, pH 7.0 (50 mM sodium dihydrogen phosphate monohydrate, 50 mM, disodium hydrogen phosphate dehydrate, 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinvlpyrrolidone (PVP); 1 nM dithiothreitol (DDT)). For PC

determination, 120 µL of sample were added to a microplate followed by 120 µL of DNPH (10 mM in 2M HCl). After incubating for 10 min at room temperature, 60 µL of NaOH (6M) were added at each well (**Figure 2.8.**), being incubated again during another 10 min. Absorbance measurement was performed at 450 nm (ε=22 mM^{-1} cm^{-1}) with the results being expressed in nmol of protein carbonyls groups formed per g FW.

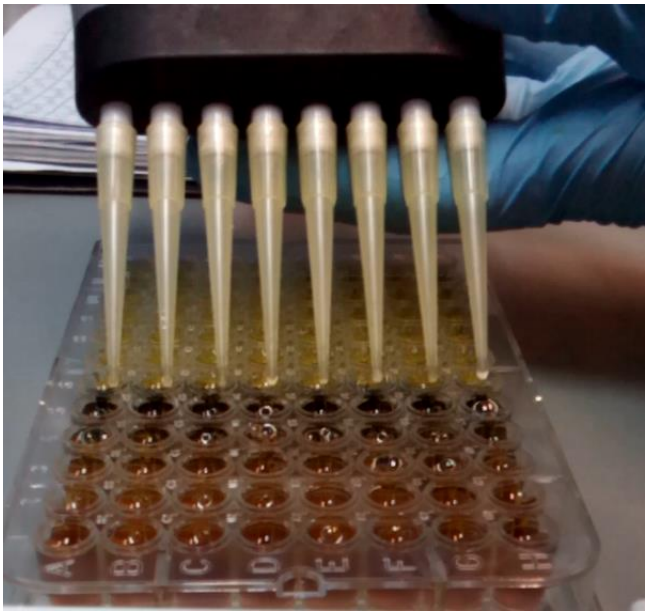


Figure 2.8. Samples with DNPH changing color by reacting with NaOH.

2.5.2.4. Antioxidant enzymes

The activity of SOD was quantified based on the method of Beauchamp and Fridovich (1971). The supernatant extraction was performed with sodium phosphate buffer, pH 7.0 (the same used for PC determination). SOD standards (0.25-60 U/mL) were used to generate the standard curve. For SOD determination, 25 µL of each sample was incubated on a microplate, during 20 min at room temperature, with 25 µL of xanthine oxidase 56,1 mU/mL and 250 µL of reaction buffer with nitroblue tetrazolium (Tris-HCl 50 mM, pH 8.0; diethylene triamine pentaacetic acid (DTPA) 0.1 mM; hypoxanthine 0.1 mM; nitroblue tetrazolium (NBT) 68.4 µM). Standards (25 µL) were incubated with 25 µL of extraction buffer, 225 µL of reaction buffer with NBT and 25 µL of xanthine oxidase. After incubation, absorbance was measured at 560 nm.

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

The activity of CAT was quantified following Johansson and Borg (1988). Supernatants were also extracted using sodium phosphate buffer, pH 7.0 (the same used for PC and SOD quantification). Formaldehyde standards (0-150 µM) were using to perform the standard curve. 25 µL of each sample and standard were incubated on a microplate with 125 µL of potassium phosphate buffer 50 mM (pH 7.0), 37.5 µL of methanol and 25 µL of hydrogen peroxide 32.28 mM. The microplate was incubated for 20 min in a shaker at room temperature to perform the reaction. 37.5 µL of KOH and 37.5 µL of Purpald 34.2 mM were added to stop the reaction, and the microplate was incubated in the shaker another 10 min. At the end of this procedure, 12.5 µL of potassium periodate 65.2 mM was added and it was again incubated in the shaker for a period of 5 min. Absorbance measurement was executed at 540 nm and results expressed in U per g FW. One unit (U) is defined as the formation of 1 nmol formaldehyde per min in this case.

The activity of GPx was determined following the method of Paglia and Valentine (1967). Supernatants extraction was realized using sodium phosphate buffer, pH 7.0 (the same used for SOD and CAT quantification). For GPx determination, 30 µL of sample were incubated with 112.5 µL of dilution buffer (50 mM Tris-HCl pH7,6; 5 mM EDTA), 60 µL of GSH 5 mM, 45 µL of cumene hydroperoxide 2 mM and 30 µL of glutathione reductase 2.5 U/mL. 22.5 µL of NADPH were added to start the reaction, and after being homogenized for some seconds, absorbance measurement was performed at 340 nm (ε=0.00522 $\mu M^{-1} cm^{-1}$) during 5 min in 10 s intervals. Results were expressed in U per g FW where one unit (U) corresponds to the quantity of enzyme which catalyzes the conversion of 1 µmol nicotinamide adenine dinucleotide phosphate (NADPH) per min.

2.6. Data analysis

The data obtained was divided in three experimental setups to better understand the differences between among conditions. In the first one only submersion and tidal conditions at control temperature were considered. In the second one only submersion and temperature conditions were considered, while for the third only submersion and MWCNTs exposure

conditions were examined. Since the data for submersion condition (first experimental setup) is common for both second and third experimental setups, differences between this condition at control temperature or for organisms non-contaminated are the same.

For the first experimental setup, the biochemical data (RR, CI, ETS, GLY, LIP, LPO, GSSG, PC, SOD, CAT and GPx) obtained from the submersion at control temperature and exposure to tides at control temperature conditions was submitted to hypothesis testing using permutational multivariate analysis of variance, employing the PERMANOVA add-on in PRIMER v7 (Anderson et al., 2008). The null hypothesis tested was: no significant differences exist between conditions (sub vs tide). For this hypothesis, significance levels ($p \leq 0.05$) among conditions are presented in figures with different letters while the same letters represent no significant differences among conditions.

For the second experimental setup, due to a lack of homogeneity of variance, RR, CI, ETS, GLY, LIP, LPO, PC, GSSG, SOD, CAT and GPx were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7) with a two factors design: submersion condition (submersed (sub) and exposed to tide (tide)) as factor 1 and temperature condition (control (ctrl) and increased temperature (temp)) as factor 2. PERMANOVA main test was performed to test the effect of submersed condition, temperature condition and the interaction between these two factors on each biomarker. PERMANOVA main tests were considered significant when $p \leq 0.05$ and followed by PERMANOVA pair-wise tests. Pair-wise tests were used to test the effect of temperature condition (ctrl and temp) within each submersion condition and the effect of submersion condition (sub and tide) within each temperature condition.

For the third experimental setup, due to a lack of homogeneity of variance as well, RR, CI, ETS, GLY, LIP, LPO, PC, GSSG, SOD, CAT and GPx were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7) with a two factors design: submersion condition (submersed (sub) and exposed to tide (tide)) as factor 1 and contaminated condition (non-contaminated (ncont) and contaminated (MWCNTs)) as factor 2. PERMANOVA main test was performed to test the effect of submersed condition, contaminated condition and the interaction between these two factors on each biomarker. PERMANOVA main tests were considered significant when $p \leq 0.05$ and followed by PERMANOVA pair-wise tests. Pair-wise tests were used to test the effect of contaminated

condition (ncont and MWCNTs) within each submersion condition and the effect of submersion condition (sub and tide) within each contaminated condition.

PERMANOVA pair-wise tests results for both second and third experimental setups are represented in figures with lower case letters and in the main text by p-values.

Chapter 3

Results

The following results were used in two papers already submitted for publication:

Andrade, M., De Marchi, L., Soares, A.M.V.M, Rocha, R., Figueira, E., Freitas, R., 2018. Are the effects induced by increased temperature enhanced in *Mytilus galloprovincialis* submitted to air exposure? In Science of Total Environmental.

Andrade, M., De Marchi, L., Pretti, C., Chiellini, F., Morelli, A., Soares, A.M.V.M, Rocha, R., Figueira, E., Freitas, R., 2018. Are the impacts of carbon nanotubes enhanced in *Mytilus galloprovincialis* submitted to air exposure? In Aquatic Toxicicology.

3.1. MWCNTs 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 (T**able 1**).

Table 3.1. Mean Dynamic light scattering (DLS) data of size (nm) and mean polydispersity index (PDI) of aquarium samples exposed to different exposure conditions. n.d.: "no data" (invalid data (I.d.) results in 3 out of 5 samples of each condition).

Exposure Condition	*Size (nm)*	*PDI*
Sub Xeno Temp CTL	1109.5	0.794
Tide Xeno Temp CTL	n.d	-

3.2. Air Exposure: physiological parameters

3.2.1. Mortality

After the experimental period of 14 days, both tide exposure and always submersed conditions did not induce mortality indicating that the effects were sublethal.

3.2.2. Respiration rate

Concerning RR, results demonstrated higher but not statistically different values in mussels exposed to tides in comparison to organisms submersed during the entire experiment (**Figure 3.1.A**).

3.2.3. Condition index

The results obtained for CI indicated that no significant differences were observed between mussels *M. galloprovincialis* submersed during the entire experiment and organisms exposed to tidal regime (**Figure 3.1.B**).

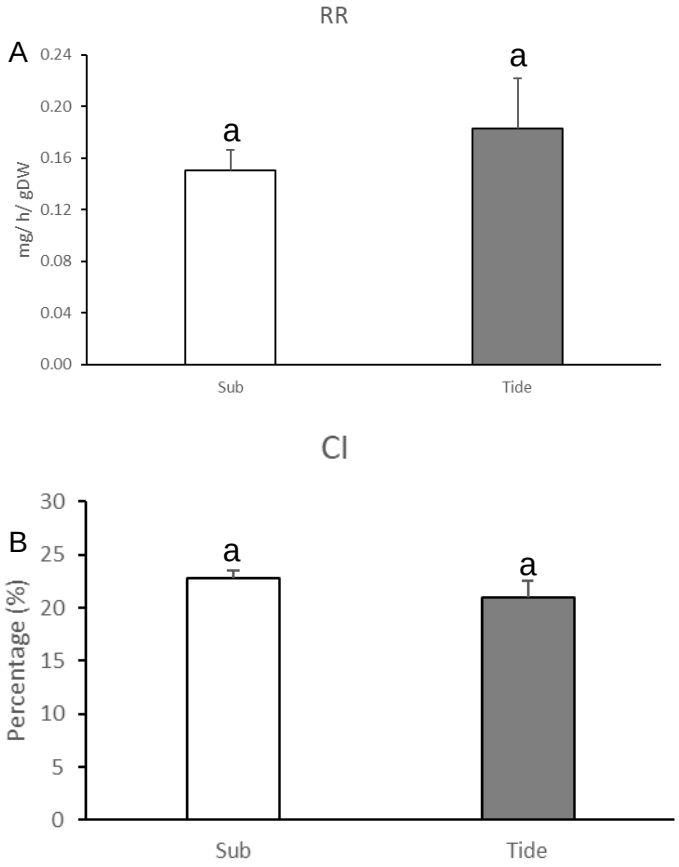


Figure 3.1. A: Respiration Rate (RR); B: Condition Index (CI), in *Mytilus galloprovincialis* always submersed (Sub) and exposed to tides (Tide) conditions for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides. Different letters represent significant differences ($p \leq 0.05$) between conditions.

3.3. Air exposure: biochemical parameters

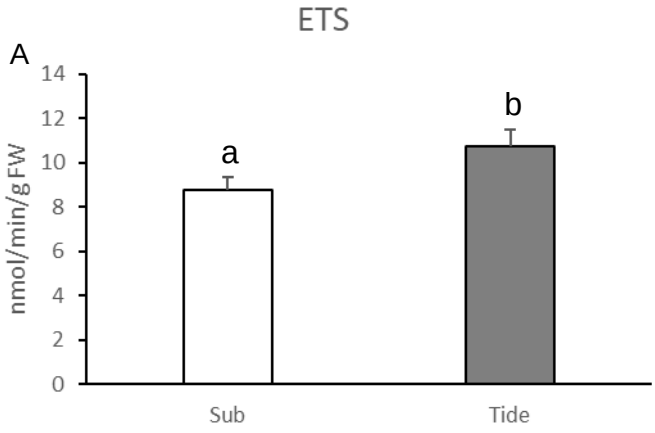
3.3.1. Metabolic capacity

The ETS activity results demonstrated significantly lower values in organisms submersed during the entire experiment in comparison to mussels exposed to tides (**Figure 3.2.A**).

3.3.2. Energy reserves

Concerning GLY content results showed no significant differences between organisms submersed during the entire experiment and organisms exposed to tides (**Figure 3.2.B**).

Regarding LIP content results also indicated no significant differences between organisms submersed during the entire experiment and organisms exposed to tides (**Figure 3.2.C**).



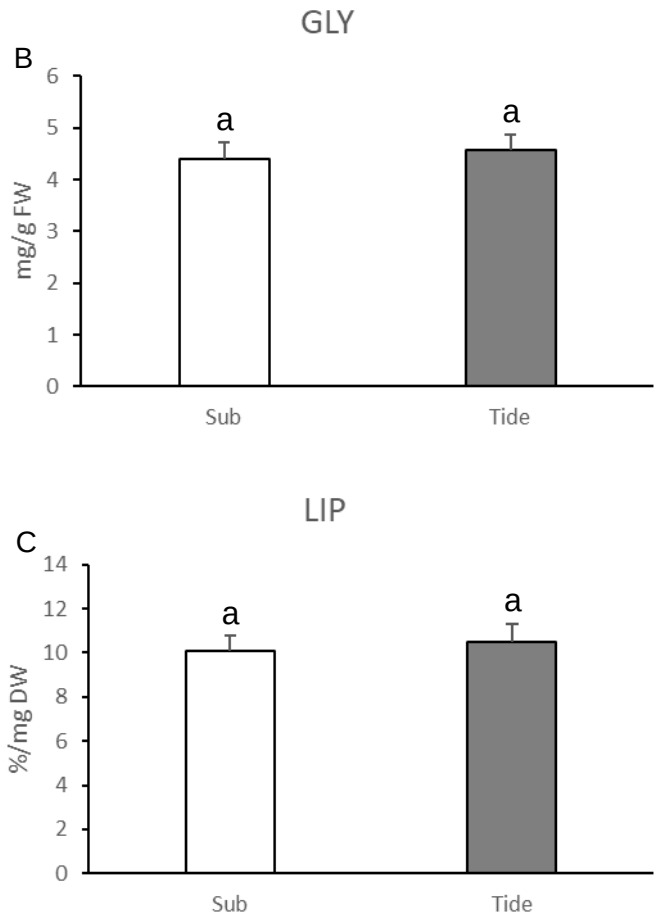


Figure 3.2. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Lipids (LIP) content, in *Mytilus galloprovincialis* always submersed (Sub) and exposed to tides (Tide) conditions for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides. Different letters represent significant differences ($p \leq 0.05$) between conditions.

3.3.3. Oxidative damage

Relatively to LPO levels, results demonstrated significantly lower values in organisms submersed during the entire experiment in comparison to mussels exposed to tides (**Figure 3.3.A**).

GSSG results indicated no significant differences between organisms submersed during the entire experiment and organisms exposed to tides (**Figure 3.3.B**).

The PC results also indicated no significant differences between organisms submersed during the entire experiment and organisms exposed to tides (**Figure 3C**).

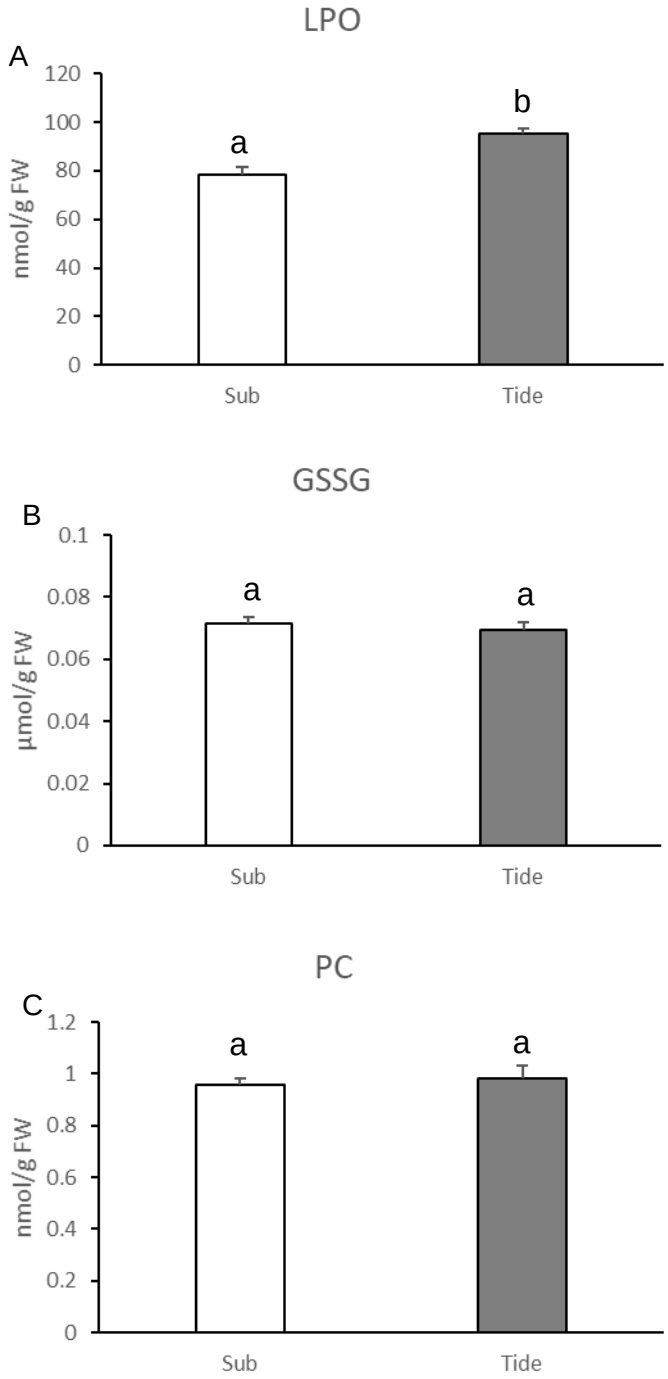


Figure 3.3. A: Lipid peroxidation (LPO) levels: B: Oxidized glutathione (GSSG) content; C: Protein carbonylation (PC) levels, in *Mytilus galloprovincialis* always submersed (Sub) and exposed to

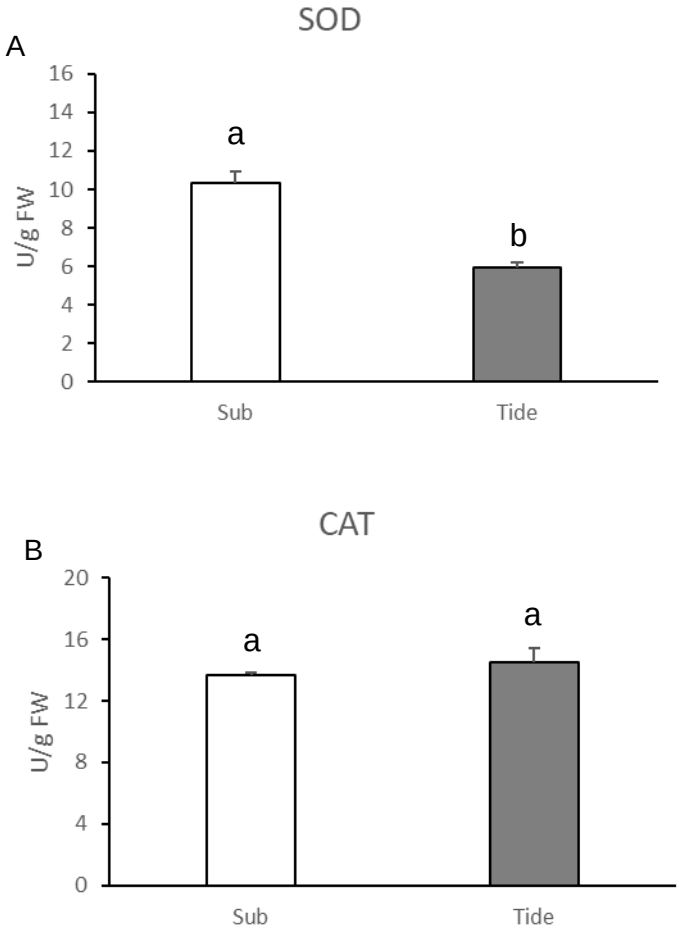
tides (Tide) conditions for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides. Different letters represent significant differences ($p \leq 0.05$) between conditions.

3.3.4. Antioxidant enzymes

The activity of SOD showed significantly higher values for organisms submersed during the entire experiment in comparison to mussels exposed to tides (**Figure 3.4.A**).

Regarding CAT activity results indicated no significant differences between organisms submersed during the entire experiment and organisms exposed to tides (**Figure 3.4.B**).

The activity of GPx showed significantly lower values for organisms submersed during the entire experiment in comparison to mussels exposed to tides (**Figure 3.5.B**).



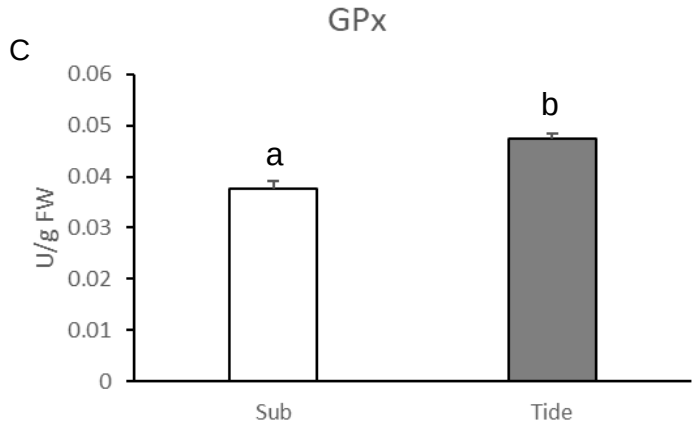


Figure 3.4. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione peroxidase (GPx) activity, in *Mytilus galloprovincialis* always submersed (Sub) and exposed to tides (Tide) conditions for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides. Different letters represent significant differences ($p \leq 0.05$) between conditions.

3.4. Temperature increase and air exposure: physiological parameters

3.4.1. Mortality

After the experimental period of 14 days, all treatments tested did not induce mortality indicating that increased temperature with or without air exposure the effects were sublethal.

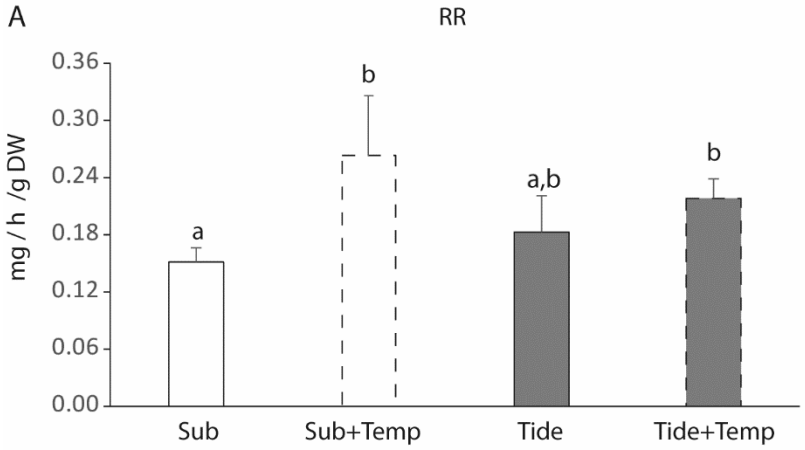
3.4.2. Respiration Rate

In mussels submersed during the entire experiment significantly lower RR values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Sub vs Sub+Temp). For mussels exposed to tides, no significant differences were observed between organisms exposed to control and to increased temperatures (Tide vs Tide+Temp) (**Figure 3.5.A**). Comparing submersion and tides exposure conditions, at the control temperature lower but not statistically different values were found in mussels exposed to submersion during the entire experiment (Sub vs Tide). For the increased temperature, no significant differences were observed between mussels submersed during the entire experiment and mussels exposed to tides (Sub+Temp vs Tide+Temp) (**Figure 3.5.A**).

The interaction between tides exposure and the increased temperature showed no significant effects on the RR (p=0.3914).

3.4.3. Condition Index

Concerning CI values and comparing both temperatures (control and increased temperature), both for organisms submersed during the entire experiment (Sub vs SubTemp) and organisms exposed to tidal regime (Tide vs TideTemp) no significant differences were observed (**Figure 3.5.B**). Comparing submersion and tides exposure conditions, no significant differences were observed for organisms under control temperature (Sub vs Tide). For the increased temperature, although with no statistically differences, higher CI values were observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (SubTemp vs TideTemp) (**Figure 3.5.B**). No significant effect of the interaction between tides exposure and the increased temperature on the CI was observed (p=0.9243).



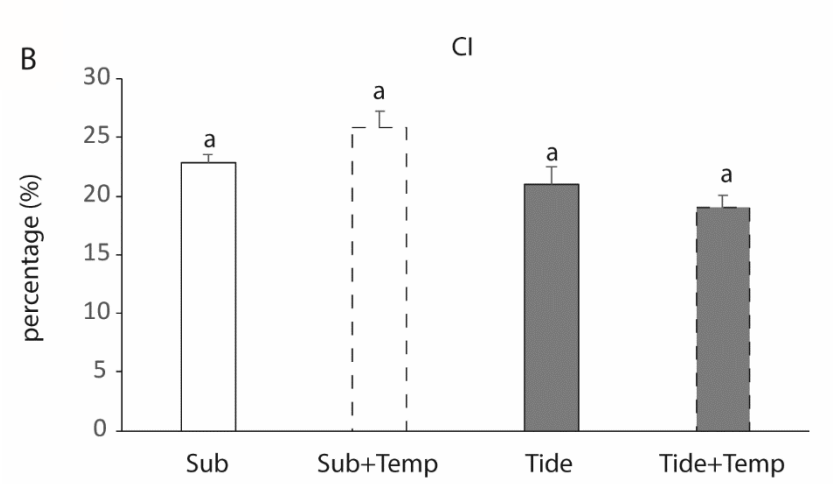


Figure 3.5. A: Respiration Rate (RR); B: Condition Index (CI), in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+Temp, Tide, Tide+Temp) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides. Continuous line represent control temperature while dashed line represent increased temperature. Different letters represent significant differences ($p \leq 0.05$) among conditions.

3.5. Temperature increase and air exposure: biochemical parameters

3.5.1. Metabolic capacity

Concerning ETS activity and comparing both temperatures (control and increased temperature), for organisms submersed during the entire experiment no significant differences were observed between mussels exposed to control and increased temperature (Sub vs Sub+Temp). For mussels exposed to tides, significantly higher values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (**Figure 3.6.A**). Comparing submersion and tide exposure conditions, at the control temperature significantly lower ETS values were observed in organisms submersed during the entire experiment in comparison to mussels exposed to tides (Sub vs Tide). For the increased temperature, no significant differences were observed

between mussels submersed during the entire experiment and mussels exposed to tides (Sub+Temp vs Tide+Temp) (**Figure 3.6.A**). The interaction between tides exposure and the increased temperature showed no significant effects on the ETS activity (p=0.312).

3.5.2. Energy reserves

Regarding GLY content and comparing both temperatures (control and increased temperature), significant lower GLY values were observed in organisms exposed to increased temperature for both organisms submersed and exposed to tides (Sub vs Sub+Temp, Tide vs Tide+Temp) (**Figure 3.6.B**). Comparing submersion and tide exposure conditions, no significant differences were observed both for organisms under control (Sub vs Tide) and increased (Sub+Temp vs Tide+Temp) temperatures (**Figure 3.6.B**). The interaction of both stressors (tides and increased temperature) showed no significant effect on the GLY content (p=0.6155).

For the LIP content and comparing both temperatures (control and increased temperature), for organisms submersed during the entire experiment no significant differences were observed (Sub vs Sub+Temp). For mussels exposed to tides, significantly higher values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (**Figure 3.6.C**). Comparing submersion and tide exposure conditions, no significant differences were observed for organisms under control temperature (Sub vs Tide). For the increased temperature, significant higher LIP values were observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (Sub+Temp vs Tide+Temp) (**Figure 3.6.C**). The interaction of both stressors (tides and increased temperature) showed significant effects on the LIP content (p=0.0065).

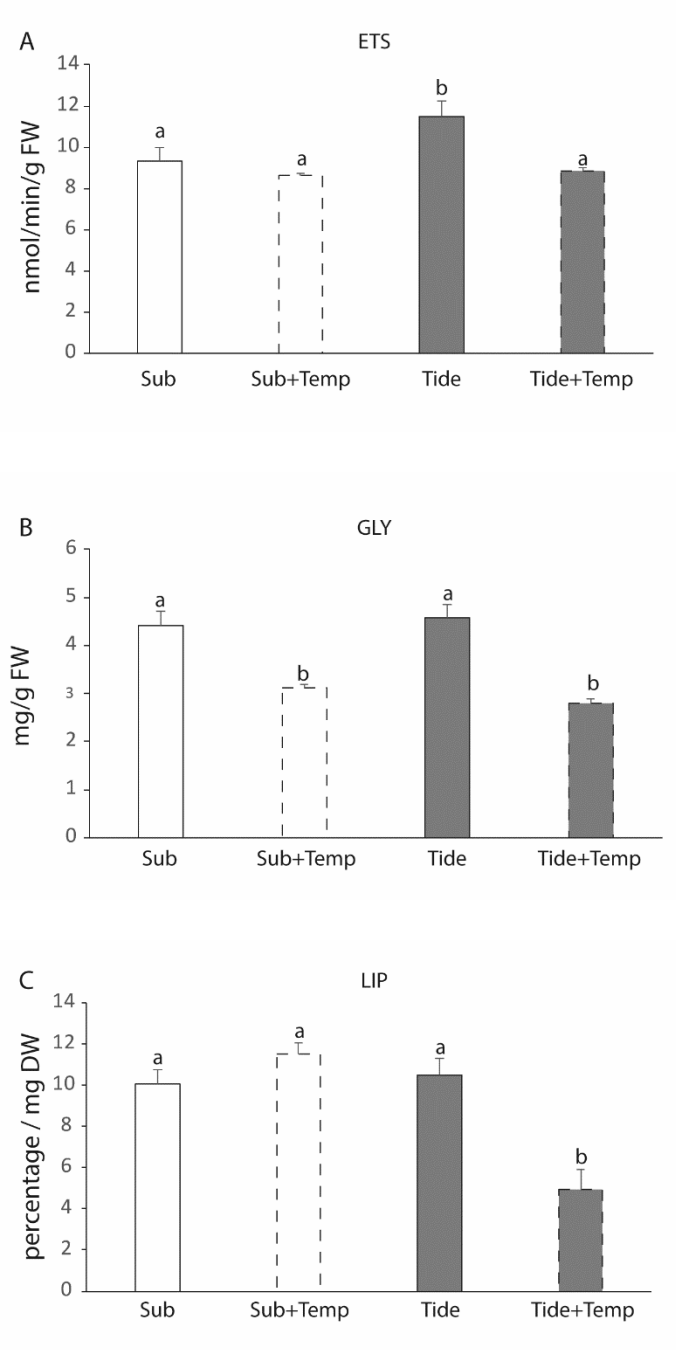


Figure 3.6. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Lipids (LIP) content, in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+Temp, Tide, Tide+Temp) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides.

Continuous line represent control temperature while dashed line represent increased temperature. Different letters represent significant differences (p≤0.05) among conditions.

3.5.3. Oxidative damage

Relatively to LPO levels and comparing both temperatures (control and increased temperature), significantly lower LPO values were observed in organisms exposed to increased temperature for both organisms submersed and exposed to tides (Sub vs Sub+Temp, Tide vs Tide+Temp) (**Figure 3.7.A**). Comparing submersion and tide exposure conditions, significant lower LPO values were observed for organisms submersed during the entire experiment in comparison to mussels exposed to tides for both control (Sub vs Tide) and increased (Sub+Temp vs Tide+Temp) temperatures (**Figure 3.7.A**). The interaction of both stressors (tides and increased temperature) showed no significant effect on the LPO levels (p=0.3795).

Concerning GSSG and comparing both temperatures (control and increased temperature), for organisms submersed during the entire experiment no significant differences were observed between organisms exposed to control and increased temperatures (Sub vs Sub+Temp). For mussels exposed to tides, significantly lower values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (**Figure 3.7.B**). Comparing submersion and tide exposure conditions, no significant differences were observed for organisms under control temperature (Sub vs Tide). For the increased temperature, significantly lower GSSG values were observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (Sub+Tide vs Tide+temp) (**Figure 3.7.B**). The interaction between tides exposure and the increased temperature showed no significant effects on the GSSG content (p=0.2163).

Concerning PC and comparing both temperatures (control and increased temperature), significantly lower PC values were observed in organisms exposed to increased temperature for both organisms submersed during the entire experiment (Sub vs Sub+Temp) as well as for organisms exposed to tides (Tide vs Tide+Temp) (**Figure 3.7.C**). Comparing submersion and tide exposure conditions, no significant differences were observed for organisms under both control and increased temperatures (Sub vs Tide, Sub+temp vs Tide+Temp) (**Figure 3.7C.**). The interaction of both stressors (tides and increased temperature) showed no significant effect on the PC levels (p=0.7076).

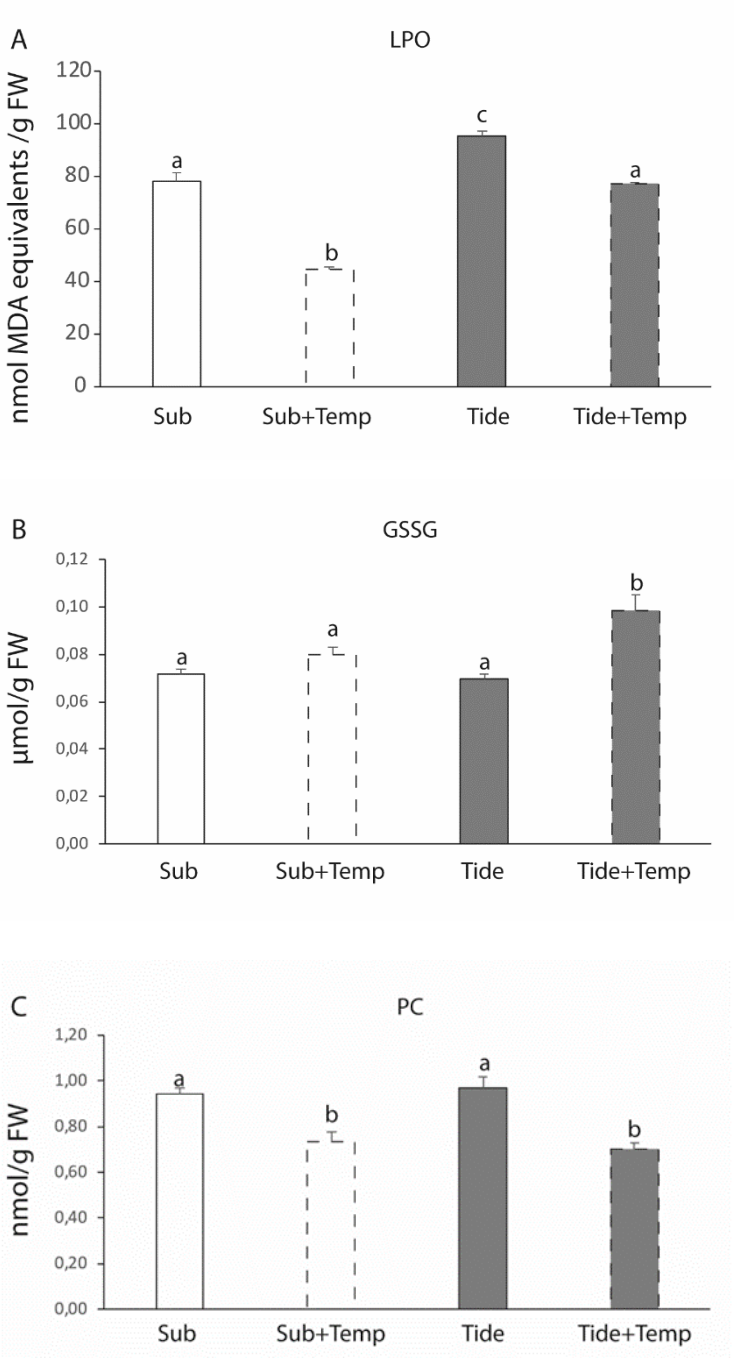


Figure 3.7. A: Lipid peroxidation (LPO) levels: B: Oxidized glutathione (GSSG) content; C: Protein carbonylation (PC) levels, in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+Temp, Tide, Tide+Temp) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides.

Continuous line represent control temperature while dashed line represent increased temperature. Different letters represent significant differences ($p \leq 0.05$) among conditions.

3.5.4. Antioxidant enzymes

Concerning SOD activity and comparing both temperatures (control and increased temperature), for organisms submersed during the entire experiment no significant differences were observed between organisms exposed to control and increased temperature (Sub vs Sub+Temp). For mussels exposed to tides, significantly lower values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (**Figure 3.8.A**). Comparing submersion and tide exposure conditions, at the control temperature significantly higher SOD values were observed for organisms submersed during the entire experiment in comparison to mussels exposed to tides (Sub vs Tide). For the increased temperature, significantly lower SOD values were observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (Sub+Temp vs Tide+Temp) (**Figure 3.8.A**). The interaction between tides exposure and the increased temperature showed significant effects on the SOD activity ($p=0.0011$).

Regarding CAT activity and comparing both temperatures (control and increased temperature), no significant differences were observed for both organisms submersed and exposed to tides (Sub vs Sub+Temp, Tide vs Tide+Temo) (**Figure 3.8.B**). Comparing submersion and tide exposure conditions, no significant differences were observed for organisms both under control and increased temperatures (Sub vs Tide, Sub+Temp vs Tide+Temp) (**Figure 3.8.B**). The interaction between tides exposure and the increased temperature showed no significant effects on the CAT activity ($p=0.9979$).

In what regards the activity of GPx and comparing both temperatures (control and increased temperature), significantly higher GPx values were observed in organisms exposed to increased temperature both for organisms submersed and exposed to tides (Sub vs Sub+Temp, Tide vs Tide+Temp) (**Figure 3.8.C**). Comparing submersion and tide exposure conditions, significantly lower GPx values were observed for organisms submersed during the entire experiment in comparison to mussels exposed to tides both for control temperature and increased temperature (Sub vs Tide, Sub+Temp vs Tide+Temp) (**Figure 3.8.C**). The interaction

of both stressors (tides and increased temperature) showed no significant effect on the GPx activity (p=0.7171).

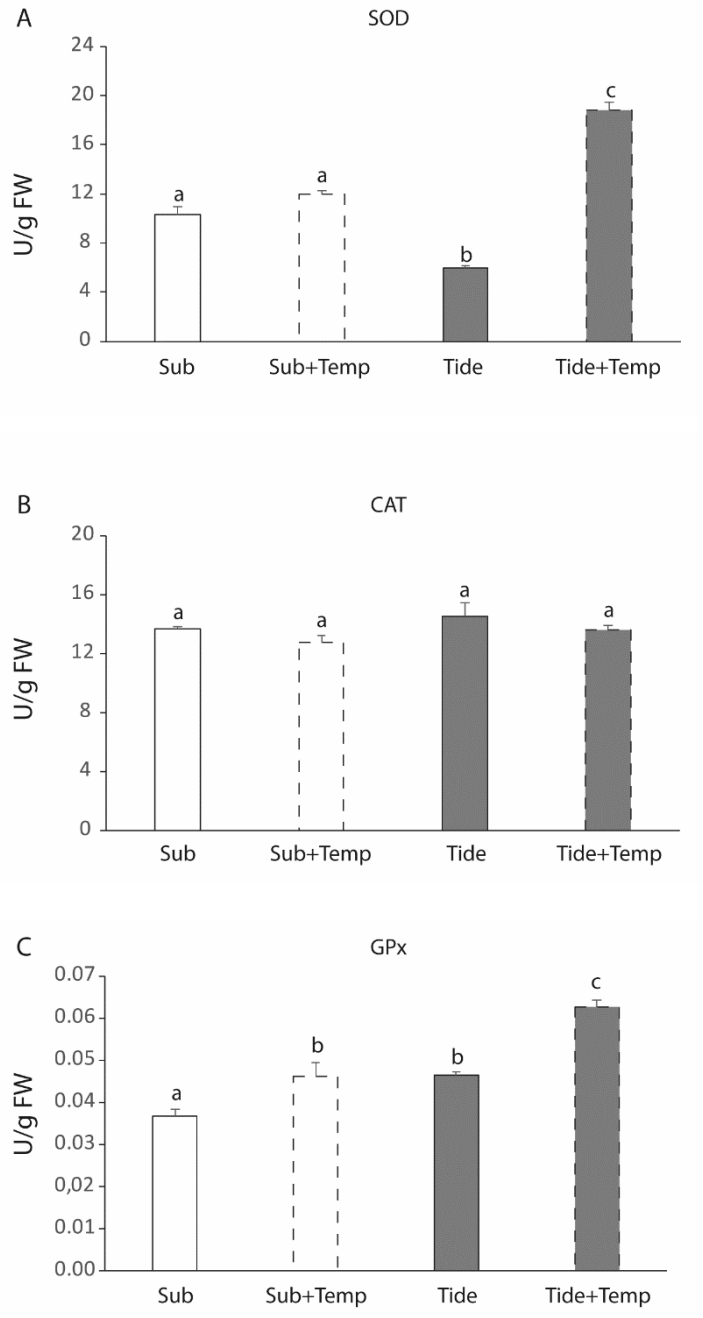


Figure 3.8. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione peroxidase (GPx) activity, in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+Temp, Tide, Tide+Temp) for 14 days. Results are the means + standard errors. White bars represent organisms submersed during the entire experiment while gray bars represent organisms exposed to tides.

Continuous line represent control temperature while dashed line represent increased temperature. Different letters represent significant differences (p≤0.05) among conditions.

3.6. MWCNTs and air exposure: physiological parameters

3.6.1. Mortality

After the experimental period of 14 days, all treatments tested did not induce mortality indicating that MWCNTs exposure with or without air exposure effects were sublethal.

3.6.2. Respiration Rate

Concerning RR, no significant differences were observed between contaminated and non-contaminated organisms both under submersion (Sub vs SubMWCNTs) and tides exposure conditions (Tide vs TideMWCNTs) (**Figure 3.9.A**). Comparing submersion and tide exposure conditions, non-contaminated and contaminated organisms (submersed during the entire experiment showed lower, but not statistically significant, RR values than organisms exposed to tides (Sub vs Tide, SubMWCNTs vs Tide MWCNTs) (**Figure 3.9.A**). No significant effect of the interaction between tides exposure and the presence of MWCNTs on the RR was observed (p=0.5709).

3.6.3. Condition Index

Concerning CI values, no significant differences were observed between contaminated and non-contaminated organisms both under submersion (Sub vs SubMWCNTs) and tides exposure conditions (Tide vs TideMWCNTs) (**Figure 3.9.B**). Comparing submersion and tides exposure conditions, no significant differences were observed both for non-contaminated and contaminated organisms (Sub vs Tide, SubMWCNTs vs Tide MWCNTs) (**Figure 3.9.B**). No significant effect of the interaction between tides exposure and the presence of MWCNTs on the CI was observed (p=0.4411).

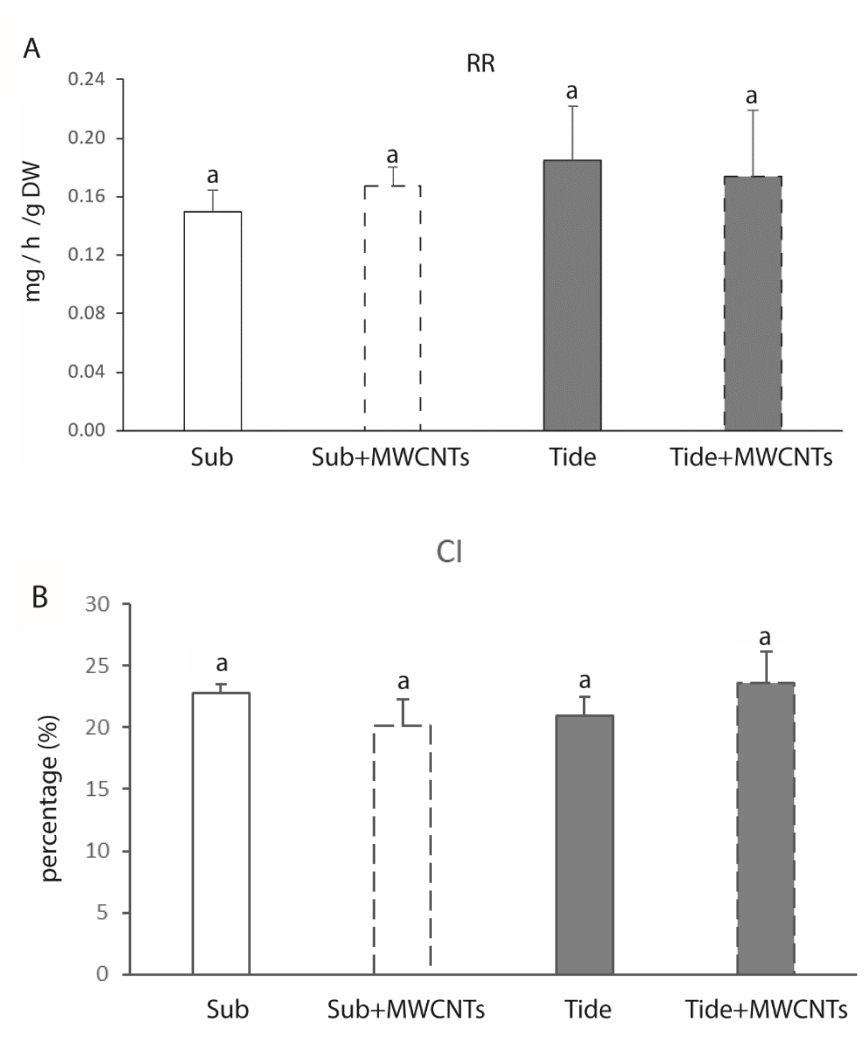


Figure 3.9. A: Respiration Rate (RR); B: Condition Index (CI), in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+MWCNTs, Tide, Tide+MWCNTs) for 14 days. Results are means + standard errors. Different letters represent significant differences ($p \leq 0.05$) among conditions.

3.7. MWCNTs and air exposure: biochemical parameters

3.7.1. Metabolic capacity

Concerning ETS activity, no significant differences were observed between non-contaminated and contaminated organisms both under submersion and tides exposure conditions (Sub vs SubMWCNTs, Tide vs TideMWCNTs) (**Figure 3.10.A**). Significantly lower ETS values were observed in mussels submersed during the entire experiment in comparison

to mussels exposed to tides, both for non-contaminated and contaminated organisms (Sub vs Tide, SubMWCNTs vs Tide MWCNTs) (**Figure 3.10.A**). The interaction between tides and MWCNTS showed no significant effects on the ETS activity (p=0.6504).

3.7.2. Energy reserves

For the GLY content, no significant differences were observed between contaminated and non-contaminated organisms both under submersion and tides exposure conditions (Sub vs SubMWCNTs, Tide vs TideMWCNTs) (**Figure 3.10.B**). Also, no significant differences were observed between submersion and tides exposure conditions, both for non-contaminated and contaminated organisms (Sub vs SubMWCNTs, Tide vs TideMWCNTs) (**Figure 3.10.B**). The interaction of both stressors (tides and MWCNTs) showed no significant effect on the GLY content (p=0.8487).

For the LIP content, no significant differences were observed as well between contaminated and non-contaminated organisms both under submersion and tides exposure conditions (Sub vs SubMWCNTs, Tide vs TideMWCNTs) (**Figure 3.10.C**). Also, no significant differences were observed between submersion and tides exposure conditions, both for non-contaminated and contaminated organisms (Sub vs SubMWCNTs, Tide vs TideMWCNTs) (**Figure 3.10.B**). The interaction of both stressors (tides and MWCNTs) showed no significant effect on the LIP content (p=0.9343).

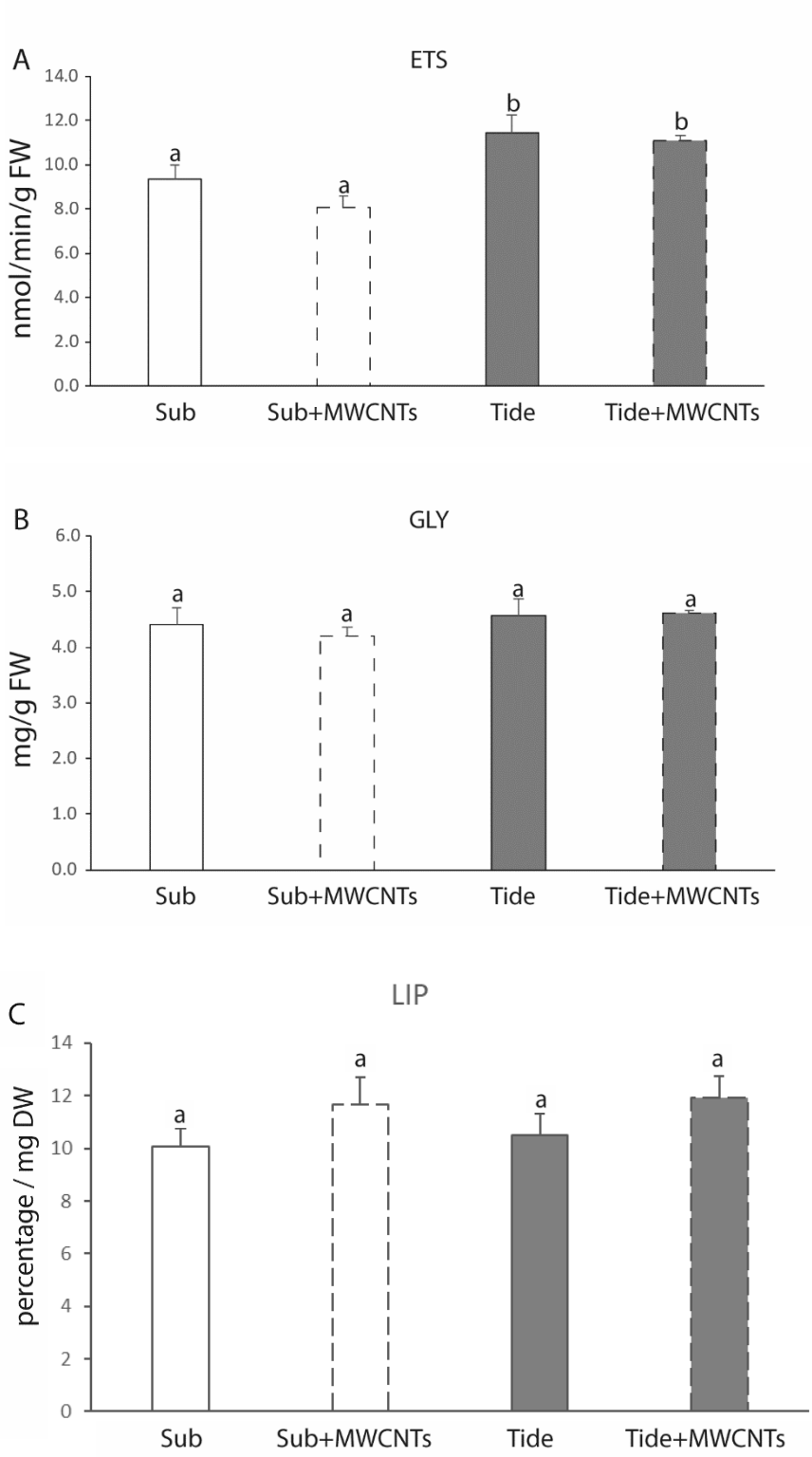


Figure 3.10. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Total lipid (LIP) content, in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+MWCNTs, Tide,

Tide+MWCNTs) for 14 days. Results are means + standard errors. Different letters represent significant differences (p≤0.05) among conditions.

3.7.3. Oxidative damage

Relatively to LPO levels, under submersion conditions, non-contaminated organisms presented significantly lower LPO values than contaminated organisms (Sub vs SubMWCNTs). When exposed to tides, significantly lower LPO levels were observed in contaminated organisms compared to non-contaminated mussels (Tide vs TideMWCNTs) (**Figure 3.11.A**). Comparing submersion and tides exposure conditions, non-contaminated mussels submersed during the entire experiment showed significantly lower LPO values than organisms exposed to tides (Sub vs Tide). In the presence of MWCNTs, mussels submersed during the entire experiment showed significantly higher LPO values than organisms exposed to tides (SubMWCNTs vs Tide MWCNTs) (**Figure 3.11.A**). 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 (Sub vs SubMWCNTs). When exposed to tides significantly higher GSSG values were observed in the presence of MWCNTs (Tide vs TideMWCNTs) (**Figure 3.11.B**). Comparing submersion and tides exposure conditions, significant differences were only observed for contaminated mussels (SubMWCNTs vs Tide MWCNTs) (**Figure 3.11.B**). Nevertheless, the interaction between tides and MWCNTs showed no significant effect on GSSG content (p=0.1524).

Regarding PC, no significant differences were observed between contaminated and non-contaminated organisms always submersed or exposed to tides (Sub vs SubMWCNTs, Tide vs TideMWCNTs) (**Figure 3.11.C**). Comparing submersion and tides exposure conditions, no significant differences were observed both for non-contaminated and contaminated organisms (Sub vs Tide, SubMWCNTs vs Tide MWCNTs) (**Figure 3.11.C**). No significant effects (p=0.4293) were observed on the PC resulting from the interaction between tides and MWCNTs.

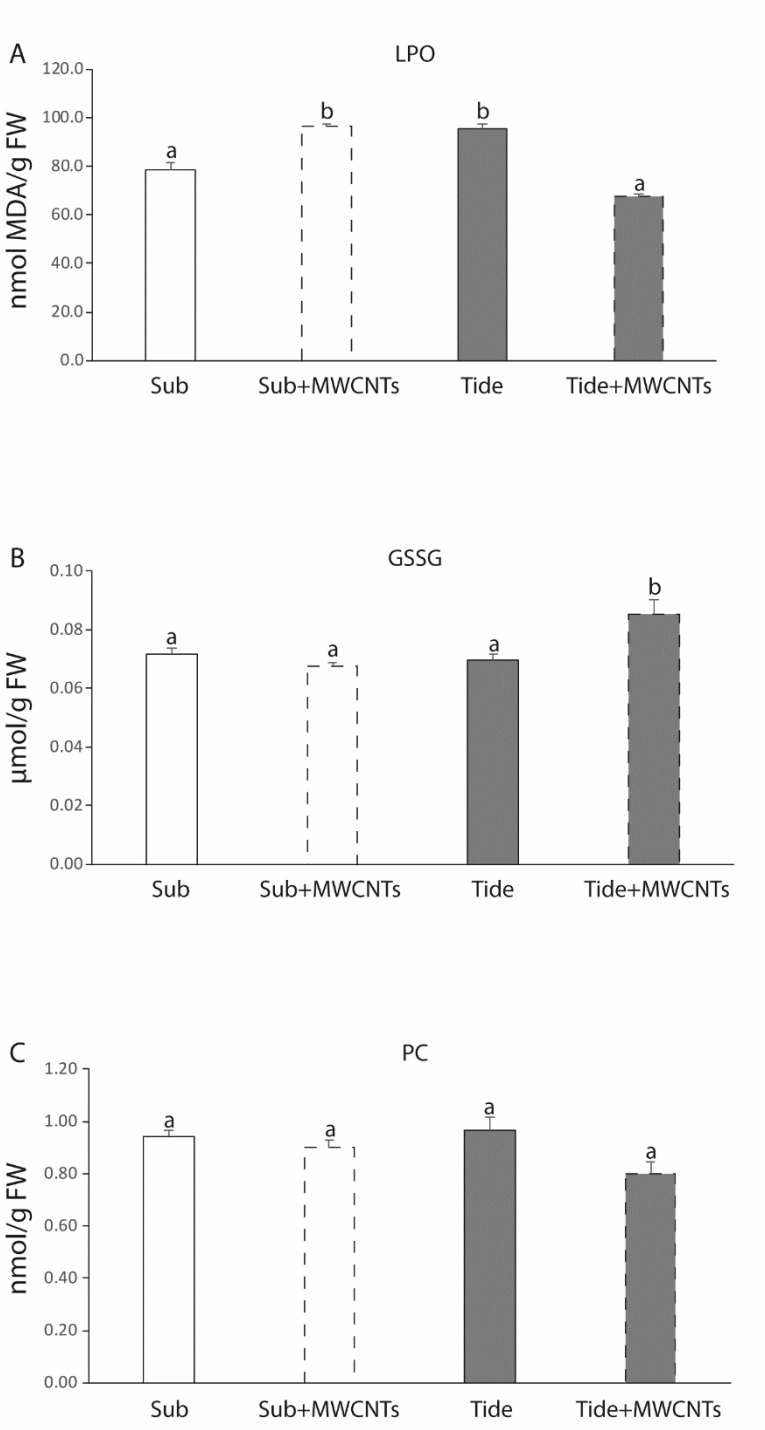


Figure 3.11. A: Lipid peroxidation (LPO) levels: B: Oxidized glutathione (GSSG) content; C: Protein carbonylation (PC) levels, in *Mytilus galloprovincialis* exposed to different conditions (Sub, Sub+MWCNTs, Tide, Tide+MWCNTs) for 14 days. Results are means + standard errors. Different letters represent significant differences ($p \leq 0.05$) among conditions.

3.7.4. Antioxidant enzymes

Concerning SOD activity, under submersion conditions, no significant differences were observed between non-contaminated and contaminated organisms (Sub vs SubMWCNTs). When exposed to tides significantly higher SOD values were observed in contaminated compared to non-contaminated mussels (Tide vs TideMWCNTs) (**Figure 3.12.A**). 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 (Sub vs Tide), while contaminated mussels exposed to tides showed significantly higher SOD values in comparison to contaminated organisms submersed the entire experimental period (SubMWCNTs vs Tide MWCNTs). (**Figure 3.12.A**). 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 (Sub vs SubMWCNTs, Tide vs TideMWCNTs) (**Figure 3.12.B**). Comparing submersion and tides exposure conditions, no significant differences were observed both for non-contaminated and contaminated mussels (Sub vs Tide, SubMWCNTs vs Tide MWCNTs) (**Figure 3.12.B**). No significant effects ($p=0.6662$) were observed due to the interaction between tides and MWCNTs.

For the activity of GPx, no significant differences were observed between contaminated and non-contaminated organisms always submersed (Sub vs SubMWCNTs), while when exposed to tides significantly higher GPx values were observed in contaminated mussels compared to non-contaminated organisms (Tide vs TideMWCNTs) (**Figure 3.12.C**). Comparing submersion and tides exposure conditions, mussels submersed in the absence and presence of MWCNTs showed significantly lower GPx values than organisms exposed to tides (Sub vs Tide, SubMWCNTs vs Tide MWCNTs) (**Figure 3.12.C**). The interaction between tides and MWCNTs showed no significant effects ($p=0.7146$) on the GPx activity.

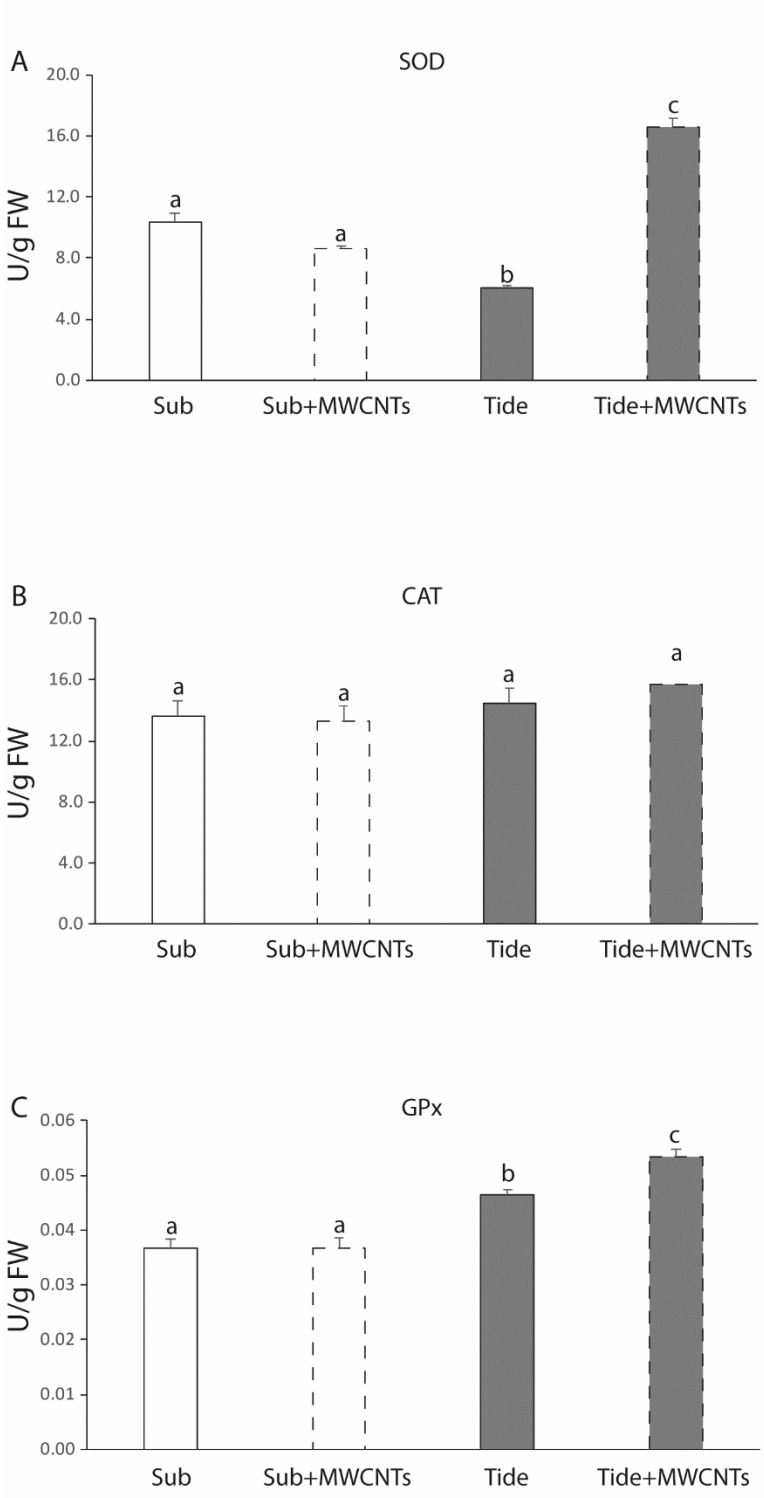


Figure 3.12. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione peroxidase (GPx) activity, in *Mytilus galloprovincialis* exposed to different conditions (Sub,

Sub+MWCNTs, Tide, Tide+MWCNTs) for 14 days. Results are means + standard errors. Different letters represent significant differences ($p<0.05$) among conditions.

Chapter 4

Discussion

4.1. Contextualization

Intertidal organisms such as *Mytilus galloprovincialis* are exposed to a combination of stressors due to their spatial localization. Previous studies revealed different adaptive behavior of organisms normally when in an environment of low tide, such as the clam *Ruditapes philippinarum* which closes the valves for protection against low salinities and pH impacts (Gazeau et al., 2013; Ishii et al., 2005; Poulain et al., 2011). However, the biochemical impacts induced by emersion/immersion in organisms from this type of environment are still not well known, as well as the possible confounding factor that tidal regime may have when assessing the impacts of other environmental stressors.

One of the many stressful changes that intertidal organisms may suffer, is the increase of water and air temperature due to global warming, which may exceed organisms' thermal tolerance and cause physiological and biochemical perturbations. It is also important to recognize the biochemical and physiological effects of new emerging contaminants. In fact, effects in marine organisms of contaminants, such as CNTs are still not well known showing this way the need to study them.

Thus, the present study evaluated the physiological and biochemical performance of *M. galloprovincialis* specimens when exposed to increased temperature or MWCNTs both under continuous submersion and exposed to tidal regime, aiming to understand if effects due to warming conditions or due to nanoparticles exposure would be enhanced in organisms exposed to air during ebb tides.

4.2. Air exposure: physiological responses

4.2.1. Respiration rate

RR has been used to assess the alterations induced by different stressors in intertidal organisms (Gestoso et al., 2016; Freitas et al., 2017; Wang et al., 2015). The present results indicate that mussels tended to increase their RR when exposed to tides in comparison to organisms submersed the entire experiment. The exposure to tides, with re-oxygenation periods, may require from organisms a high metabolic capacity (Andrade et al., 2018), which can result in high RR. Yin et al. (2017) showed in the clam *Ruditapes philippinarum* a significant

increase on oxygen consumption with the daily rhythms of air exposure (3h, 6h and 9h) followed by immersion, suggesting that after hypoxia clams needed to compensate the oxygen debt caused during re-immersion.

4.2.2. Condition index

One of the physiological parameters used to define organisms' general health status is the CI (Lucas and Beninger, 1985). The findings obtained in the present study demonstrated that organisms submersed during the entire experiment or exposed to tides presented no differences in terms of CI. These results may indicate that the conditions tested were not stressful enough to result into losses in weight and differences in terms of CI, but may also indicate an adaptive behavior of this species to withstand stressful conditions common for an intertidal environment such as anoxic submission cycles. However, the short experimental period may be not enough to observe significant differences between conditions.

4.3. Air exposure: biological responses

4.3.1. Metabolic capacity and energy reserves

Electron transport system (ETS) activity has been used to estimate the energy consumption at the mitochondrial level and thus obtain an indication of organisms' metabolic status (Coen and Janssen, 1997; Berridge et al., 2005; Fanslow et al., 2001; García-Martín et al., 2014). Results revealed that mussels under control temperature increased their ETS activity when exposed to tides, in comparison to submersed mussels, which may result from re-immersion periods at which the mussels are subjected. The response of mussels in terms of metabolic activity is well correlated with results of RR. Similarly, Andrade et al. (2018) showed, in *M. galloprovincialis* under daily air exposure conditions (3h or 6h) followed by immersion, an increase of ETS activity. It is known that metabolic depression may occur under oxygen limitation (Guppy et al., 1994), however little is still known about the physiological effects in mussels exposed to tidal regimes. These findings may indicate that re-oxygenation after ebb tide induced high metabolic capacity in mussels, probably to, after suffering oxygen absence, re-establish their physiological and biochemical performance.

The availability of energy reserves, as the case of GLY and LIP content, can be affected by general physiological stressors (Scott-Fordsmand and Weeks, 2000). The present results demonstrated that exposure to tides did not alter GLY and LIP 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 hypoxia conditions for 2 weeks. Nevertheless, although GLY content was not quantified, Andrade et al. (2018) demonstrated that the LIP content decreased in mussels submitted to 6h of daily air exposure for 14 days. In another study, Yin et al. (2017) demonstrated that *R. philippinarum* clams submitted to different daily air exposures spent energy (by measuring lipid content and fatty acid composition) 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 and LIP content which, may indicate that GLY and LIP were not used to fuel mussels defense mechanisms to fight against the stress caused by air exposure or this condition was not stressful enough to originate the expenditure of energy reserves.

4.3.2. Oxidative stress

Abiotic changes may cause overproduction of reactive oxygen species (ROS) in marine bivalves which may, therefore, cause oxidative damage of the lipids' membranes (Carregosa et al., 2014a; Freitas et al., 2016b; Liu et al., 2007; Lushchak, 2011; Matozzo et al., 2012; Silva et al., 2005a). The present study demonstrated that mussels exposed to tides increased their LPO levels. These results demonstrated that submersion was the least stressful condition to mussels since exposure to air led to cellular damages, probably due to the lack of activation of antioxidant defenses. The high LPO levels in organisms exposed to tides may also result from higher ROS production due to increased ETS activity at these conditions. Studies have shown that the mitochondrial electron system activity is one of the major cellular generators of ROS (Loschen et al., 1971; Boveris et al., 1972; Chance et al., 1979). Likewise, in mussels exposed to daily cycles of 6h of air exposure Andrade et al. (2018) observed an increase of LPO in mussels. In *M. edulis*, Rivera-Ingraham et al. (2013) demonstrated an increase of ROS with an induction of LPO in the mantle after mussels being exposed to anoxic conditions and reoxygenation. These authors suggested that during ebb tide emersion intertidal bivalves used

a shell closure strategy to avoid oxidative stress during usual anoxia-hyperoxia conditions. In addition, ATP degrades in AMP under anoxic conditions, being degraded in hypoxanthine. Upon reoxygenation, hypoxanthine and xanthine are oxidized and generate ROS (Jones 1986), explaining in this way the increase of LPO levels in mussels exposed to tides. Nevertheless, Yin et al. (2017) showed a non-significant increase of LPO levels in clams exposed to daily cycles of 3h, 6h and 9h of air exposure.

Cellular functions can be modified by ROS through a reversible or irreversible post-translational modification (PTM) which may inactivate critical proteins (Sultan et al., 2018). In fact, a process that constitutes the most common type of PTM triggered by oxidative stress, protein carbonylation (PC), may be promoted by ROS though the oxidation of proteins (Cattaruzza and Hecker, 2008; Suzuki et al., 2010). Cellular functions can be modified by ROS through a reversible or irreversible post-translational modification (PTM) which may inactivate critical proteins (Sultan et al., 2018). Results demonstrated similar PC levels between submersed and tides exposure conditions for mussels. In accordance to our results, Rivera-Ingraham et al. (2013) demonstrated that mussels *M. edulis* maintained their protein carbonyls content after reoxygenation observing a burst of LPO in the same condition. Furthermore, Ivanina and Sokolova (2016) also observed no differences in carbonyls levels in the clam *Mercenaria mercenaria* exposed to anoxia or hypoxia and later re-oxygenated. Thus, our results may indicate that air exposure during ebb tide did not produce enough stress to generate proteins oxidation in mussels.

4.3.3. Antioxidant enzymes

When under stressful conditions, marine organisms may increase antioxidant defenses, as the activity of SOD and CAT enzymes, eliminating the excess of ROS produced and prevent LPOs' occurrence (Freitas et al., 2016c; Regoli and Giuliani, 2014; Velez et al., 2016). There is another mechanism involving the antioxidant enzyme GPx which by oxidizing GSH to GSSG reduces lipid hydroperoxides, by neutralizing ROS directly (Regoli and Giuliani, 2014). Results revealed that there was an increase of GPx activity when organisms were exposed to tides, although SOD activity decreased and CAT activity was maintained. These results

demonstrated that although there was an increase of GPx, there was an overall maintenance of the antioxidant defenses and those seemed to not be enough to prevent LPO in the cells. However, different results were obtained by other authors. Yin et al. (2017) demonstrated an increase of SOD activity in *R. philippinarum* after daily exposure to air. Additionally, Andrade et al. (2018) observed in *M. galloprovincialis* submitted to 3h and 6h of air exposure followed by immersion an increase of SOD activity, showing the increase of antioxidant enzymes activity to neutralize ROS originated during re-oxygenation. Likewise, Almeida and Bainy (2006) showed in mussels *P. perna* under 4h of air exposure an increase of SOD activity. However, non-significant differences of GPx activity in the digestive gland of *P. perna* mussels where demonstrated after being exposed to 4h of air exposure and re-immersed (Almeida and Bainy, 2006; Almeida et al., 2005). Furthermore, a non-significant increase of GPx activity in *M. edulis* was observed by Letendre et al. (2011) after being submitted to a simulated tidal cycle during 14 days.

4.4. Temperature increase and air exposure: physiological responses

4.4.1. Respiration rate

The present findings may indicate that the increase of temperature at 21 ºC is a moderate stressful condition, since at moderate levels of temperature metabolic rate may increase, decreasing under severe stress (Gestoso et al., 2016, Kiibus and Kautsky, 1996). Similarly, previous studies demonstrated that RR of bivalves is dependent on temperature (Bayne and Newell, 1983; Griffiths and Griffiths, 1987). Resgalla Jr. et al. (2007) observed in *Perna perna* mussels an increase of RR with the increase of temperature (15, 20, 25 and 30 ºC) both after acute and chronic exposures. Gestoso et al. (2016) observed an enhanced RR with increasing temperature of 21 ºC in *M. galloprovincialis*. Likewise, in mussels *M. galloprovincialis*, Jansen et al. (2009) demonstrated an overall increase of RR with the increase of temperature (17, 24, 27, 31 and 34ºC). Therefore, in the present study the RR showed to be highly correctly with the increase of temperature, being more remarkable in organisms always submersed. Compared to the results obtained only during air exposure, under tidal exposure the effect of temperature was less evident probably because under severe stress (tides+temp), organisms may have employed adaptative mechanisms as valves closure to avoid oxidative

damage with the higher oxidative stress and/or dissection effects of warmer temperature during aerial exposure.

4.4.2. Condition index

Previous studies showed that this physiological parameter was not only affected by reproductive cycles, but also by environmental factors (e.g. salinity, temperature and environmental contaminants) (Azpeitia et al., 2016; Çelic et al., 2012; Matozzo et al., 2012). Results have demonstrated that independently on the temperature of exposure, organisms submersed during the entire experiment or exposed to tides presented no differences in terms of CI. Similarly, studies conducted by Gestoso et al. (2016) showed no significant differences on *M. galloprovincialis* exposed to an increased temperature of 21 ºC for 22 days. Thus, our results may indicate that the combination of both stressors was not stressful enough to result into losses in weight and differences in terms of CI. Apart of the adaptive behavior of this species to withstand stressful conditions common for an intertidal environment such as anoxic submission cycles suggested before, this species may also be able to tolerate the exposure of different temperatures. However, as indicated before, the short experimental period may also not have been enough to observe significant differences between conditions.

4.5. Temperature increase and air exposure: biological responses

4.5.1. Metabolic capacity and energy reserves

In the present study, submersed mussels exposed to increased temperature were able to maintain their ETS activity, indicating that although increasing their RR under warming conditions mussels were able to avoid the increase of their ETS activity. Nevertheless, when submitted to tidal regime mussels under increased temperatures tended to present lower metabolic capacity then mussels exposed to control temperature, with values similar to mussels submersed at control temperature. The fact that organisms under warming conditions, both submersed the entire experiment or exposed to tidal regime, were able to maintain their metabolic capacity similar to organisms maintained at control temperature may indicate that under warming conditions organisms are able to prevent the increase of their metabolic

capacity by activating behavioral adaptations as valves closure (Anestis et al., 2007; Gosling, 2003). In fact, Anestis et al. (2007) demonstrated that *M. galloprovincialis* exposed to warming at 24 °C lead mussels to keep their valves closed for long periods and caused metabolic depression. Coppola et al. (2017, 2018) also observed a significant decrease of ETS activity in *M. galloprovincialis* exposed to increased temperature (21ºC) and submersed for 14 and especially 28 days. Besides the results obtained only during air exposure, the present findings further revealed that under warming conditions organisms submersed or exposed to tides presented similar metabolic capacity. As observed before, although organisms under control temperature exposed to tides increased their ETS activity, under increased temperature organisms may deploy adaptative behaviors, as valve closure, to avoid increased stress from the combination of tide exposure and increased temperature and thus were able to maintain their metabolic capacity.

Results demonstrated a decrease of energy reserves when mussels were exposed to increased temperature, especially when organisms were exposed to tides. Under increased temperature, mussels demonstrated a need for an extra expenditure of energy reserves which may reveal that the temperature tested was stressful enough to increase expenditure of energy reserves probably associated with its use in defense mechanisms (as antioxidant enzymes) or/and repair of damaged cellular structures through the renewal of damaged LIP. Nevertheless, previously published studies revealed an increase of energy reserves in *M. galloprovincialis* with the increase of temperature (Coppola et al., 2017, 2018). However, the expenditure of energy reserves in bivalves under different stressful conditions has been observed as well. Velez et al. (2016) observed that clams *Ruditapes philippinarum* started to mobilize GLY as a source of energy when under salinity stressful conditions. Dickinson et al. (2012) demonstrated that the oyster *Crassostrea virginica* at salinity and hypercapnic stressful conditions revealed a partial depletion of GLY and LIP reserves. Results further demonstrated that mussels exposed to tides started to mobilize LIP content at the increased temperature. This mobilization of LIP content may be associated with the repair of damaged cellular structures through the renewal of damaged LIP, due to increased LPO under this condition. In mussels submitted to 6h of daily air exposure Andrade et al. (2018) demonstrated that the LIP content decreased. In another study with *R. philippinarum* clams, Yin et al. (2017) demonstrated expenditure of energy (by measuring lipid content and fatty acid composition)

when submitted to different daily air exposures explained by an elevated aerobic metabolism during re-immersion periods. Besides the results obtained only during air exposure, the combination of tidal regime and increased temperature exposure showed the activation of LIP content as a reserve energy to probably fuel mussels defense mechanisms to fight against the stress caused by this condition beyond the repair of damaged lipids due to the increase of ROS. Therefore, the tidal regime and increased temperature exposure may be the condition most stressful in terms of energy reserves expenditure.

4.5.2. Oxidative stress

The present results showed that, when exposed to increased temperature, LPO levels decreased in mussels both submersed during the entire experiment and exposed to tides in comparison to organisms from the control temperature. These results were accompanied by an increase on the GSSG content, especially noticed in mussels exposed to tides, evidencing that organisms were experiencing oxidative stress as GSSG results from the oxidation of reduced glutathione (GSH), the most abundant cytosolic scavenger participating in the antioxidant defense system, neutralizing directly ROS (Regoli and Giuliani, 2014). Furthermore, GSH also acts as a co-factor of other antioxidant enzymes such as GPx, which in fact significantly increased in organisms under increased temperature and/or exposed to tides. The decrease of LPO levels in mussels exposed to warming conditions may thus indicate that the excess of ROS produced was eliminated by enzymatic and non-enzymatic antioxidant mechanisms that prevented the occurrence of cellular damages. Furthermore, the highest LPO decrease observed in mussels under warming conditions and submersed compared to organisms exposed to tides may indicate that higher ROS production occurred in mussels exposed to tides which were not eliminated at the same rate by antioxidant defenses. Nevertheless, for the same increased temperature (21 ºC), Coppola et al. (2017, 2018) observed an increase of LPO but a decrease of GSH/GSSG in *M. galloprovincialis* after 28 days of exposure, which may indicate oxidative stress as GSSG increased in comparison to GSH but the antioxidant defenses were not able to prevent the occurrence of LPO. Nardi et al. (2017) showed no differences in LPO levels with a slight but no significative increase of total GSH, in the digestive gland and gills of *M. galloprovincialis* exposed 4 weeks to warming (25 ºC) compared to control (20 ºC). In the same organism, Kamel et al. (2012) observed no

differences in LPO when mussels were exposed for 7 days to 20 ºC, increasing significantly LPO levels at 22 ºC. The present study further demonstrated that mussels exposed to tides and increased temperature, increased their LPO levels similarly to the ones from control temperature. Demonstrating once again that submersion was the least stressful condition to mussels since exposure to air led to cellular damages, although antioxidant defenses seemed to be activated under tidal exposure.

Results showed a decrease of PC levels in mussels when exposed to increased temperature in both submersed and exposed to tides conditions. Similar to the decrease of LPO observed in the same conditions, mussels exposed to increased temperature, seemed to be able to avoid the carbonylation of proteins by activating GSSG and/or antioxidant enzymes activity due to high ROS production under this condition. Paital and Chainy (2013) observed an increase of carbonyls in the muscle of the mud crab *Scylla serrata* collected in summer compared to organisms captured during winter, although overall the difference wasn't significant in the gills and hepatopancreas of these organisms. Results further demonstrated similar PC levels between submersed and tides exposure conditions for mussels under increased temperatures, similarly to what was observed to the organisms under control temperature. Thus, our results may indicate that air exposure during ebb tide for both temperatures did not produce enough stress to generate proteins oxidation in mussels.

4.5.3. Antioxidant enzymes

Results showed that under submersion conditions mussels were able to activate their antioxidant defenses, which could explain the decrease of LPO and PC levels in mussels exposed to this condition. Furthermore, under tidal regimes, mussels submitted to increased temperature showed the greatest increase on antioxidant enzymes activity which may result from higher ROS production, also associated to higher GSSG content and which led to lower LPO and PC levels under this condition. Hence, the combination of both stressors (air exposure and increased temperature) seems to be the most stressful condition which led to higher enzymatic activity, which was able to prevent higher cellular damage. Coppola et al. (2017) showed that when exposed to an increased temperature (21 ºC) *M. galloprovincialis* increased

SOD activity after 28 days of exposure. Kamel et al. (2012) demonstrated in the digestive gland of *M. galloprovincialis* no significant differences of CAT activity and LPO under 20 ºC, however both CAT and LPO increased under 22 ºC. With the exception of GPx activity, Hu et al. (2015) showed an increase of SOD and CAT activities in the mussel *Mytilus coruscus*. Thus, our findings clearly suggest that the interaction of air exposure and warming conditions greatly increased the antioxidant defenses to avoid oxidative damage, suggesting a higher oxidative stress in organisms under this condition. Similar to the results obtained only during air exposure, the present findings also revealed that in general higher antioxidant enzymes activities were shown in organisms exposed to tides. These results demonstrated an adaptation to high levels of ROS produced during re-oxygenation typical of tidal changes that mussels are subject, by increasing the antioxidant defenses especially in mussels exposed to an increased temperature. However, these increase of antioxidant defenses seemed to not be enough to prevent LPO in the cells. Therefore, mussels exposed to tides and especially under the combined effect of tides and increased temperature showed higher oxidative stress, since this increase was accompanied by higher cellular damage demonstrating that the defense mechanisms under these stressful conditions weren't enough to cope with the oxidative stress.

4.6. MWCNTs and air exposure: physiological responses

4.6.1. Respiration rate

Results demonstrated that the exposure to MWCNTs did not change *M. galloprovincialis* respiratory capacity when compared to non-contaminated mussels, both when individuals were exposed to tides or continuously submersed. Nevertheless, the increase of RR due to contaminants exposure has been demonstrated as a physiological adaptation-response (Relexans 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, similarly to the present findings, De Marchi et al. (2017b) observed that the polychaetes *D. neopolitana* 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.

4.6.2. Condition Index

As mentioned before, physiological parameters may be affected by environmental factors such as environmental contaminants (Azpeitia et al., 2016). Results have demonstrated that independently of the submersion condition, non-contaminated and contaminated organisms presented no differences in terms of CI. Thus, our results may indicate that the combination of both stressors was not stressful enough to result in differences in terms of CI. Similar to the results observed before for air exposure with or without temperature, of this species may be able to withstand stressful conditions common for an intertidal environment as an adaptive behavior such as air and contaminants exposure. However, as suggested before, the short experimental period may as well not have been enough to observe significant differences between conditions.

4.7. MWCNTs and air exposure: biological responses

4.7.1. Metabolic capacity and energy reserves

In the present study, mussels exposed to MWCNTs were able to maintain their metabolic capacity compared to non-contaminated organisms, both under continuous submersion and tidal regime. The lack of significant differences in the ETS activity between contaminated and non-contaminated mussels may be associated to the similar respiratory capacity of mussels independently on the presence or absence of MWCNTs. These findings may thus indicate that the concentration tested was not stressful enough to alter mussels' metabolic activity. Similarly, De Marchi et al. (2017c, 2018) observed no significant differences of ETS activity in *R. philippinarum* exposed to the same nanoparticles and at the same concentration level (0.01 mg/L). Similarly to the results obtained only during air exposure, the present findings further revealed that contaminated mussels also tended to increase the ETS activity when exposed to tides, in comparison to submersed contaminated mussels. The present findings thus reinforce once more that re-oxygenation after ebb tide may have induced

high metabolic capacity in mussels to re-establish their physiological and biochemical performance after oxygen absence.

Results demonstrated however no differences in GLY and LIP content between contaminated and non-contaminated mussels, indicating that these reserves were not used as a resource of energy to fuel mussels defense mechanisms when in the presence of MWCNTs or, most probably, the present results may indicate that there was no need for an extra expenditure of energy reserves under contaminated conditions indicating that the concentration level tested was not stressful enough to increase expenditure of energy reserves. De Marchi et al. (2018) observed similar results in *R. philippinarum* exposed to the same MWCNTs 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 may confirm the use of GLY only at higher MWCNTs concentrations, highlighting that higher MWCNTs concentrations may have greater impacts on bivalves' energy reserves balance. In fact, different studies have been demonstrating 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 and LIP content observed in the present study could thus result from the low MWCNT concentration used that did not result in the expenditure of this reserve.

4.7.2. Oxidative stress

The present results showed that LPO levels increased in MWCNTs 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 besides acting as a co-factor of antioxidant enzymes such as GPx, also participates in the antioxidant defense system as the most abundant cytosolic scavenger and neutralizing ROS directly. Thus, our findings showed that increased LPO levels in contaminated mussels under submersion conditions was not

associated to increased GSSG content, which may indicate that ROS production was not extremely high to increase GSSG production neither to 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 a 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 consequence mode of cellular uptake of NMs together with subsequent biological responses in the organisms (Hotze et al., 2010). For the same MWCNTs concentration (0.01mg/L), previous studies showed an increase of LPO in the clam *R. phillipinarum* and the polychaetes *D. neopolitana* 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. However, when mussels were exposed to tides lower LPO levels and higher GSSG content in contaminated mussels occurred which can indicate that ROS production was extremely high under this condition inducing the oxidation of GSH and the decrease of LPO resulted from the activation of antioxidant enzymes that also contributed to the elimination of ROS and avoided the occurrence of LPO. As observed by results from only air exposure condition, 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. As explained before, these results demonstrated that when in the absence of MWCNTs the exposure to air lead to cellular damages, probably due to the lack of activation of antioxidant defenses, showing that submersion was the least stressful condition to mussels. 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, originated a less stressful condition or the combination of air exposure and MWCNTs contamination induced higher oxidative stress compared to organisms submersed the entire experiment in the presence of these nanoparticles which was surpassed by the activation of antioxidant enzymes that eliminated ROS and prevented the occurrence of LPO.

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 tides

conditions. Similarly, Marisa et al. (2016) showed no significant differences of protein carbonyl content neither LPO levels in *R. philippinarum* exposed during 7days to zinc oxide nanoparticles (1 µg/L nZnO, 10 µg/L nZnO, 10 µg/L ZnCl2), putting in hypothesis that antioxidant defenses were enough to cope with the increase of oxidative damage and this way protect the cells. Results further demonstrated similar PC levels between submersed and tides exposure conditions both for non-contaminated and contaminated mussels. Demonstrating once again that air exposure during ebb tide may not induce proteins oxidation.

4.7.3. Antioxidant enzymes

Our results showed that under submersion conditions contaminated mussels presented similar antioxidant enzymes activity compared to non-contaminated ones, which may explain higher LPO levels in mussels exposed to MWCNTs. De Marchi et al. (2017a) showed in polychaetes *D. neopolitana* and *H. diversicolor*, at the same concentration of MWCNTs, that both CAT and SOD activities didn't change 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 ativity in *R. phillipinarum*, being only significantly increased at higher concentrations (0.10 mg/L and 1.00 mg/L). However, under tidal regime condition, contaminated organisms (Tide+MWCNTs) showed increased antioxidant enzymes activity compared to non-contaminated mussels (Tide) which may result from higher ROS production, also associated to higher GSSG content at this condition, leading to lower LPO levels at this condition (Tide+MWCNTs). 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 lead to the 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 during 14 days with and without PAHs. Results also revealed that exposure to tides originated, in general, higher antioxidant enzymes 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. Therefore, higher antioxidant enzymes activity in mussels exposed to tides and

especially under the combined effect of tides and MWCNTs may indicate higher stress under this condition which was not accompanied by higher cellular damage due to the effective response of these defense mechanisms.

Chapter 5

Conclusions

5.1. Conclusions

Bivalves are economically and ecologically relevant organisms which can provide information about the physiological status of their population and of the environment where they are present. For this reason, ecotoxicological studies, as the one performed here, may provide relevant information about the biochemical and physiological effects of present and future stressors, by assessing organisms sub cellular responses. However, what is not considered in many cases is the effects of the interaction with natural abiotic changes (salinity, temperature, tide) with environmental stressors related to climate change and another anthropogenic stressors.

The results of the present study suggest that tidal exposure can cause biochemical changes in the mussel *Mytilus galloprovincialis*. After 14 days, individuals under tidal exposure demonstrated an increase of metabolic capacity by increasing RR and ETS activity. However, the increased metabolic capacity was not converted into the activation of antioxidants enzymes, leading to oxidative damage. The inactivation of antioxidant defenses may be correlated to natural tolerance of these intertidal organisms to these factors. Nonetheless, tidal exposure demonstrated to cause oxidative damage in comparison with organisms always submersed which may be correlated with the high generation of ROS produced during the re-immersion periods, associated with higher RR and ETS.

This study evidenced that increased temperature caused oxidative stress in the mussels as well. In fact, when exposed to increased temperature, submersed organisms demonstrated a decrease of energy reserves associated with an increase of antioxidant defenses. However, under the combination of tidal exposure and increased temperature, mussels not only mobilized LIP content, but also increased antioxidant defenses (SOD and GPx) and GSSG, probably due to a burst in the production of ROS. Nonetheless, organisms couldn't prevent cellular damage evidenced by the increase of LPO levels. Therefore, air exposure showed to enhance the effects of increased temperature by inducing higher oxidative stress in mussels exposed to both stress conditions.

Furthermore, the present study demonstrated that contamination by MWCNTs may also affect *M. galloprovincialis* physiological and biochemical performance. Nevertheless, the present findings also revealed that mussels seemed to be able to tolerate oxidative stress caused by the ROS production induced by the contaminant since the stress induced was not enough to activate mussels' antioxidant defenses which resulted in oxidative damage.

However, contaminated organisms under air exposure were able to increase their metabolism, to activate their defense mechanisms and, therefore, prevent cellular damages due to high production of ROS. Nevertheless, longer exposure periods should be tested in the future, as possibility cellular damage may occur.

As proved by the present study, *M. galloprovincialis* may be able to tolerate tidal exposure, increased temperature and MWCNTs exposure if these stressors are acting alone. However, when under tidal exposure the effects of increased temperature or MWCNTs exposure seemed to be greatly increased inducing higher oxidative stress. Furthermore, the combination of air exposure and temperature demonstrated to be more stressful than the combination of air exposure and MWCNTs exposure, since it induced not only oxidative stress as the last condition, but also oxidative damage. Thus, organisms may be more sensitive to the air and water temperature variations than exposure of MWCNTs during a tidal regime. However, more studies would be needed to confirm this hypothesis since effects observed could be related to the low concentration of MWCNTs used (0.01mg/L) and short experimental period (14 days).

When evaluating the ability of organisms to respond to these different stressors under laboratory conditions, it should be considered the fact that the exposure to tides can act as a confounding factor to this stressor in the environment. This work as therefore demonstrated its importance in the evaluation of different abiotic factors and intertidal organisms for future studies.

5.2. Future considerations

In future works I would like to perform assays for a longer experimental periods to obtain more results. The study of the effects of other emergent pollutants and temperature differences, by itself and in combination, may be an asset to understand the importance of these stressors in a pollution and climatic altered panorama. Furthermore, since tidal regime can act as a cofactor of other stressors in intertidal organisms, mores studies should be conducted considering this parameter and different pollutants or climate change related factors. More physiological parameters, such as behavior and reproduction, should be assessed in the future since they are ecological relevant. Furthermore, the comparison between different mussels' species or different tidal regimes should be interesting to understand, especially since these differences are typical in estuaries.

Chapter 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. https://doi.org/10.1016/j.exger.2009.02.012.

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 6, 105–121. https://doi.org/10.1016/S0076-6879(84)05016-3.

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. https://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. https://doi.org/10.1016/j.jembe.2004.12.007.

Almeida, E.A., Bainy, A.C.D., Loureiro, A.P.M., Martinez, G.R., Miyamoto, S., Onuki, J., Barbosa, L.F., Garcia, C.C.M, Prado, F.M., Ronsein, G.F., Sigolo, C.A., Brochini, C.B., Martins, A.M.G., Medeiros, M.H.G., Mascio, P.D., 2007. Oxidative stress in *Perna perna* and other bivalves as indicators of environmental stress in the Brazilian marine environment: antioxidants, lipid peroxidation and DNA damage. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146(4), 688-600. https://doi.org/10.1016/j.cbpa.2006.02.040.

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. https://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. 25(9), 8903-8913. https://doi.org/10.1007/s11356-017-1123-7.

Andral, B., Stanisiere, J.Y., Sauzade, D., Damier, E., Thebault, H., Galgani, F., Boissery, P., 2004. Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. Mar. Pollut. Bull. 49, 704–712. https://doi.org/10.1016/j.marpolbul.2004.05.008.

Anestis, A., Lazou, A., Pörtner, H.-O., Michaelidis, B., 2007. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. AJP: Regul. Integr. Comp. Physiol. 293, 911 – 921. https://doi.org/10.1152/ajpregu.00124.2007.

Anisimova, A.A., Chaika, V.V., Kuznetsov, V.L., Golokhvast, 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.

Arndt, D.A., Moua, M., Chen, J., Klaper, R., 2013. Core structure and surface functionalization of carbon nanomaterials alter impacts to daphnid mortality, reproduction, and growth: acute assays do not predict chronic exposure impacts. Environ. Sci. Technol. 47 (16), 9444–9452. https://doi.org/10.1021/es4030595.

Attig, H., Kamel, N., Sforzini, S., Dagnino, A., Jamel, J., Boussetta, H., Viarengo, A., Banni, M., 2014. Effects of thermal stress and nickel exposure on biomarkers responses in *Mytilus galloprovincialis* (Lam). Mar. Environ. Res. 94, 65-71. https://doi.org/10.1016/j.marenvres.2013.12.006.

Azevedo, A., Sousa, A.I., Silva, J.D.L.E., Dias, J.M., Lillebø, A.I., 2013. Application of the generic DPSIR framework to seagrass communities of Ria de Aveiro: a better understanding of this coastal lagoon. J. Coast. Res. 1, 19-24. https://doi.org/10.2112/SI65-004.1.

Azpeitia, K., Ferrer, L., Revilla, M., Pagaldai, J., Mendiola, D., 2016. Growth, biochemical profile, and fatty acid composition of mussel (*Mytilus galloprovincialis* Lmk.) cultured in the open ocean of the Bay of Biscay (northern Spain). Aquaculture 454, 95-108. https://doi.org/10.1016/j.aquaculture.2015.12.022.

Banni, M., Hajer, A., Sforzini, S., Oliveri, C., Boussetta, H., Viarengo, A., 2014. Transcriptional expression levels and biochemical markers of oxidative stress in *Mytilus galloprovincialis* exposed to nickel and heat stress. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 160, 23-29. https://doi.org/10.1016/j.cbpc.2013.11.005.

Bayne, B.L., Newell, R.C., 1983. Physiological energetics of marine molluscs. In-The mollusca. Volume 4: Physiology, Part 1. Academic Press. New York.

Baughman, R.H., Zakhidov, A.A., de Heer, W.A., 2002. Carbon nanotubes — the route toward applications. Science 297 (5582), 787–792. https://doi.org/10.1126/science.1060928.

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

Berridge, M.V., Herst, P.M., Tan, A.S., 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol. Annu. Rev. 11, 127-152. https://doi.org/10.1016/S1387-2656(05)11004-7.

Bielen, A., Bošnjak, I., Sepčić, K., Jaklič, M., Cvitanić, M., Lušić, J., Lajtner, J., Simčič, T., Hudina, S., 2016. Differences in tolerance to anthropogenic stress between invasive and native bivalves. Sci. Total Environ. 543, Part A, 449–459. https://doi.org/10.1016/j.scitotenv.2015.11.049.

Boukadida, K., Banni, M., Gourves, P.-Y., Cachot, J., 2016. High sensitivity of embryo-larval stage of the Mediterranean mussel, *Mytilus galloprovincialis* to metal pollution in combination with temperature increase. Mar. Environ. Res. 122, 59-66. https://doi.org/10.1016/j.marenvres.2016.09.007.

Boveris, A., Oshino, N., Chance, B., 1972. The cellular production of hydrogen peroxide. Biochem. J. 128, 617–630. https://doi.org/10.1042/bj1280617.

Branch, G.M., Steffani, N.C., 2004. Can we predict the effects of alien species? A case-history of the invasion of South Africa by *Mytilus galloprovincialis* (Lamarck). J. Exp. Mar. Biol. Ecol. 300, 189–215. https://doi.org/10.1016/j.jembe.2003.12.007.

Burnett, L.E., 1997. The Challenges of Living in Hypoxic and Hypercapnic Aquatic Environment. Integr. Comp. Biol. 37, 633–640. https://doi.org/10.1093/icb/37.6.633.

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., 2010a. Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (Nano carbon black, C60 fullerene, Nano-TiO_2, Nano-SiO_2). Aquat. Toxicol. 100, 168-177. https://doi.org/10.1016/j.aquatox.2010.04.009.

Canesi, L., Ciacci, C., Vallotto, D., Marcomini, A., Pojana, G., 2010b. In vitro effects of suspensions of selected nanoparticles (C60 fullerene, TiO_2, SiO_2) on *Mytilus hemocytes*. Aquat. Toxicol. 96 (2), 151-158. https://doi.org/10.1016/j.aquatox.2009.10.017.

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.

Castro, H., Ramalheira, F., Quintino, V., Rodrigues, A.M., 2006. Amphipod acute and chronic sediment toxicity assessment in estuarine environmental monitoring: an example from Ria de Aveiro, NW Portugal. Mar. Pollut. Bull. 53, 91-99. https://doi.org/10.1016/j.marpolbul.2005.09.029.

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, https://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: the case of Thermaikos gulf, Greece. 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.

Çelik, M.Y., Karayücel, S., Karayücel,I., Öztürk, R., Eyüboğlu, B., 2012. Meat yield, condition index, and biochemical composition of mussels (*Mytilus galloprovincialis* Lamarck, 1819) in

Sinop, South of the Black Sea. J. Aquat. Food Prod. Technol. 21, 198-205. https://doi.org/10.1080/10498850.2011.589099.

Chance B., Sies H., Boveris A., 1979. Hydroperoxide metabolism in mammlian organs. Physiol. Rev. 59, 527–605.

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.

Cheng, Y., Zheng, Y., VanderGheynst, J.S., 2011. Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. Lipids 46, 95–103. https://doi.org/10.1007/s11745-010-3494-0.

Coelho, J.P., Pato, P., Henriques, B., et al., 2014. Long-term monitoring of a mercury contaminated estuary (Ria de Aveiro, Portugal): the effect of weather events and management in mercury transport. Hydrol. Process. 28, 352–360. https://doi.org/10.1002/hyp.9585.

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:1008228517955.

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.

Coppola, F., Almeida, Â., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E., Freitas, R., 2017. Biochemical impacts of Hg in *Mytilus galloprovincialis* under present and predicted warming scenarios. Sci. Total Environ. 601–602, 1129-1138, https://doi.org/10.1016/j.scitotenv.2017.05.201.

Coppola, F., Almeida, Â., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E., Freitas, R., 2018. Biochemical responses and accumulation patterns of *Mytilus galloprovincialis*

exposed to thermal stress and Arsenic contamination, Ecotoxicol. Environ. Saf. 147, 954-962. https://doi.org/10.1016/j.scitotenv.2017.05.201.

Correia, B., Freitas, R., Figueira, E., Soares, A.M.V.M, Nunes, B., 2016. Oxidative effects of the pharmaceutical drug paracetamol on the clam *Ruditapes philippinarum* under different salinities. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 179, 116-124. https://doi.org/10.1016/j.cbpc.2015.09.006.

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.

Davenport, J., Macalister, H., 1996. Environmental conditions and physiological tolerances of intertidal fauna in relation to shore zonation at Husvik, South Georgia. J. Exp. Mar. Biol. Ecol. 76, 985–1002. https://doi.org/10.1017/S0025315400040923.

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.

Dias, J.M., Lopes, J.F., 2006. Implementation and assessment of hydrodynamic, salt and heat transport models: the case of Ria de Aveiro Lagoon (Portugal). Environ. Model. Softw. 21, 1-15. https://doi.org/10.1016/j.envsoft.2004.09.002.

Dias, J.M., Lopes, J.F., Dekeyser, I., 1999. Hydrological characterisation of Ria de Aveiro, Portugal, in early summer. Oceanol. Acta 22, 473-485. https://doi.org/10.1016/S0399-1784(00)87681-1.

Dias, J.M., Lopes, J.F., Dekeyser, I., 2000. Tidal propagation in the Aveiro lagoon, Portugal. Phys. Chem. Earth Part B 25, 369-374. https://doi.org/10.1016/S1464-1909(00)00028-9.

Dias, J.M., Lopes, J.F., Dekeyser, I., 2003. A numerical system to study the transport properties in the Ria de Aveiro lagoon. Ocean Dyn. 53, 220–231. https://doi.org/10.1007/s10236-003-0048-5.

Dias, J.M., Picado, A., 2011. Impact of morphologic anthropogenic and natural changes in estuarine tidal dynamics. J. Coast. Res. 64, 1490-1494.

Dickinson, G.H., Ivanina, A.V., Matoo, O.B., Pörtner, H.O., Lannig, G., Bock, C., Beniash, E., Sokolova, I.M., 2012. Interactive effects of salinity and elevated CO2 levels on juvenile eastern oysters, *Crassostrea virginica*. J. Exp. Biol. 215, 29–43. https://doi.org/10.1242/jeb.061481.

Donaldson, K., Li, X.Y., MacNee, W., 1998. Ultrafine (nanometre) particle mediated lung injury. J. Aerosol Sci. 29 (5–6), 553–560. https://doi.org/10.1016/S0021-8502(97)00464-3.

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. https://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. https://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.

Dyer, K.R., 1997. Estuaries. A Physical Introduction. John Wiley & Sons, Chichester, 195 pp.

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.

EC, European Commission, 2011. Definition of a nanomaterial. http://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm

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.

Fanslow, D.L., Nalepa, T.F., Johengen, T.H., 2001. Seasonal changes in the respiratory electron transport system (ETS) and respiration of the zebra mussel, *Dreissena polymorpha* in Saginaw Bay, Lake Huron. Hydrobiologia, 448, 61-70. https://doi.org/10.1023/A:1017582119098.

FAO, Food and Agriculture Organization of the United Nations, 1998. Integrated coastal area management and agriculture, forestry and fisheries. FAO guidelines. Rome.

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.

Famme, P., Kofoed, L.H., 1980. The ventilatory current and ctenidial function related to oxygen in declining oxygen tension by the mussel *Mytilus edulis* L. Comp. Biochem. Physiol. 66, 161-171. https://doi.org/10.1016/0300-9629(80)90147-4.

Folch, J., M. Lees, M., Sloane Stanley G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497-509.

Foster, B.A., 1971. Desiccation as a factor in intertidal zonation of barnacles. Mar. Biol. 8, 12–29. https://doi.org/10.1007/bf00349341.

Filgueira, R., Guyondet, T., Comeau, L.A., Tremblay, R., 2016. Bivalve aquaculture-environment interactions in the context of climate change. Glob. Chang. Biol. 22, 3901-3913. https://doi.org/10.1111/gcb.13346.

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. https://doi.org/10.1002/9781444345988.ch3.

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. https://doi.org/10.1016/j.scitotenv.2017.04.005

Freitas, R., Almeida, A., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016a. The impacts of pharmaceutical drugs under ocean acidification: New data on single and combined long-term effects of carbamazepine on *Scrobicularia plana*. Sci. Tot. Environ. 541, 977-985. https://doi.org/10.1016/j.scitotenv.2015.09.138.

Freitas, R., Pires, A., Velez, C., Almeida, Â., Moreira, A., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2016b. 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., 2016c. Multiple stressors in estuarine waters: Effects of arsenic and salinity on *Ruditapes philippinarum*. Sci. Total Environ. 541, 1106-1114. https://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. https://doi.org/10.1016/S0045-6535(03)00477-6.

Gagné, F., Eullafroy, P. Blaise, C., 2006. Effects of selected pharmaceutical products on phagocytic activity in *Elliptio complanata* mussels. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143 (2), 179-86. https://doi.org/10.1016/j.cbpc.2006.01.008.

García-Martín, E.E., McNeill, S., Serret, P., Leakey, R.J.G., 2014. Plankton metabolism and bacterial growth efficiency in offshore waters along a latitudinal transect between the UK and Svalbard. Deep Sea Res. Part 1 Oceanogr. Res. Pap. 92, 141-151. https://doi.org/10.1016/j.dsr.2014.06.004.

Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J., O'Connor, W.A., Martin, S., Pörtner, H., Ross, P.M., 2013. Impacts of ocean acidification on marine shelled molluscs. Mar. Biol. 160, 2207–2245. https://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.

Gosling, E.M., 2003. Bivalve Molluscs: Biology, Ecology, and Culture. Oxford, Fishing News Books, Malden, MA.

Gosling, E. 2015. Marine Bivalve Molluscs. 2nd Edition. Wiley-Blackwell, Hoboken, USA.

Gray, J.S., Wu, R.S.S., Or, Y.Y., 2002. Effects of hypoxia and organic enrichment on the coastal marine environment. Mar. Ecol. Progr. Ser. 238, 249–279. http://dx.doi.org/10.3354/meps238249.

Griffiths, C.L., Griffiths, R.J., 1987. Bivalvia. In-Animal energetics. Volume 2: Bivalvia through Reptilia. Eds. Pandian, T. J. and Vernberg, F. J. Academic Press. New York.

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.

Hamza-Chaffai, A., 2014. Usefulness of Bioindicators and Biomarkers in Pollution Biomonitoring. Int. J. Biotechnol. Welness Ind. 3, 19-26. https://doi.org/0.6000/1927-3037.2014.03.01.4.

Helmuth, B.S., Hofmann, G.E., 2001. Microhabitats, thermal heterogeneity, and patterns of physiological stress in the rocky intertidal zone. Biol. Bull. 201, 374-384. https://doi.org/10.2307/1543615.

Helmuth, B., Denny, M.W., 2003. Predicting wave exposure in the rocky intertidal zone: Do bigger waves always lead to larger forces? Limnol. Oceanogr. 48, 1338–1345. https://doi.org/10.4319/lo.2003.48.3.1338.

Hockey, P.A.R., van Erkom Schurink, C., 1992. The invasive biology of the mussel *Mytilus galloprovincialis* on the southern African coast. Trans. R. Soc. S. Afr. 48, 123-139. https://doi.org/10.1080/00359199209520258.

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

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.

Hu, 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.

Hutchins, L.W., 1947. The Bases for Temperature Zonation in Geographical Distribution. Ecol. Monogr. 17 (3), 325–335. https://doi.org/10.2307/1948663.

IPCC, 2001. The scientific basis, summary for policy makers—contribution of working group I to the third assessment report of the Intergovermental Panel on Climate Change. Cambridge University Press, Cambridge, UK.

IPCC, 2007. Climate change 2007: the physical science basis. In: Contribution of Work Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate. Cambridge University Press, Cambridge, UK.

IPCC, 2014. Climate change 2014: Synthesis report. In: Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva, Switzerland.

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. https://doi.org/10.1007/s10872-006-0013-2.

Ivanina, A.V., Froelich, B., Williams, T., Solokov, 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.

Ivanina, A.V., Sokolova, I.M., 2016. Effects of intermittent hypoxia on oxidative stress and protein degradation in molluscan mitochondria. J. Exp. Biol. 219, 3794-3802. https://doi.org/10.1242/jeb.146209.

Jackson, P., Jacobsen, N.R., Baun, A., Birkedal, R., Kühnel, D., Jensen, K.A., et al., 2013. Bioaccumulation and ecotoxicity of carbon nanotubes. Chem. Cent. J. 7 (1), 154–165. https://doi.org/10.1186/1752-153X-7-154.

Jansen, J.M., Hummel, H., Bonga, S.W., 2009. The respiratory capacity of marine mussels (*Mytilus galloprovincialis*) in relation to the high temperature threshold. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 153, 399-40. https://doi.org/10.1016/j.cbpa.2009.03.013.

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. https://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.

Jones, K.M.M., Boulding, E.G., 1999. State-dependent habitat selection by an intertidal snail: the costs of selecting a physically stressful microhabitat J. Exp. Mar. Biol. Ecol. 242,149-177. https://doi.org/10.1016/S0022-0981(99)00090-8.

Kádár, E., Lowe, D.M., Solé, M., Fisher, A.S., Jha, A.N., Readman, J.W., Hutchinson, T.H., 2010. Uptake and biological responses to nano-Fe versus soluble $FeCl_3$ in excised mussel gills. Anal. Bioanal. Chem. 396, 657-666. https://doi.org/10.1007/s00216-009-3191-0.

Kamel, N., Attig, H., Dagnino, A., Boussetta, H., Banni, M., 2012. Increased temperatures affect oxidative stress markers and detoxification response to benzo[a]pyrene exposure in mussel *Mytilus galloprovincialis*. Arch. Environ. Contam. Toxicol. 63, 534–543. https://doi.org/10.1007/s00244-012-9790-3.

Kataoka, C., Nakahara, K., Shimizu, K., Kowase, S., Nagasaka, S., Ifuku, S., et al., 2016. Salinity-dependent toxicity of water-dispersible, single-walled carbon nanotubes to Japanese medaka embryos. J. Appl. Toxicol. 37 (4), 408–416. https://doi.org/10.1002/jat.3373.

Kefaloyianni, E., Gourgou, E., Ferle, V., Kotsakis, E., Gaitanaki, C., Beis, I., 2005. Acute thermal stress and various heavy metals induce tissue-specific pro- or anti-apoptotic avents via the p38-MAPK signal transduction pathway in *Mytilus galloprovincialis* (Lam.). J. Exp. Biol. 208, 4427-4436. https://doi.org/10.1242/jeb.01924.

Keller, A.A., McFerran, S., Lazareva, A., Suh, S., 2013. Global life cycle releases of engineered nanomaterials. J. Nanopart. Res. 15, 1692. https://doi.org/10.1007/s11051-013-1692-4.

Kiibus, M., Kautsky, N., 1996. Respiration, nutrient excretion and filtration rate of tropical freshwater mussels and their contribution to production and energy flow in Lake Kariba, Zimbabwe. Hydrobiologia 331, 25-32. https://doi.org/10.1007/BF00025404.

King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnol. Oceanogr. 20, 849–854. https://doi.org/10.4319/lo.1975.20.5.0849.

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.

Kristan, U., Kanduč, T., Osterc, A., Šlejkovec, Z., Ramšak, A., Stibilj, V., 2014. Assessment of pollution level using as a bioindicator species: The case of the Gulf of Trieste. Mar. Pollut. Bull. 89, 455-463. https://doi.org/10.1016/j.marpolbul.2014.09.046.

Letcher, T.M., 2009. Climate change: Observed impacts on planet earth. Elsevier, Amsterdam, the Netherlands.

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., Hanquet, 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.

Li, R., Brawley, S.H., 2004. Improved survival under heat stress in intertidal embryos (Fucus spp.) simultaneously exposed to hypersalinity and the effect of parental thermal history. Mar. Biol. 144, 205–213. https://doi.org/10.1007/s00227-003-1190-9.

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.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666. https://doi.org/10.1016/s0025-326x(01)00060-1.

Lopes, C.B., Lillebo, A.I., Pereira, E., Vale, C., Duarte, A.C., 2007. Nutrient dynamics and seasonal succession of phytoplankton assemblages in a Southern European Estuary: Ria de Aveiro, Portugal. Estuar. Coast. Shelf Sci. 71, 480-490. https://doi.org/10.1016/j.ecss.2006.09.015.

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.

Loschen G., Flohe L., Chance B., 1971. Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. FEBS Letters 18, 261– 264. https://doi.org/10.1016/0014-5793(71)80459-3.

Lucas, A., Beninger, P.G., 1985. The use of physiological condition indices in marine bivalve aquaculture. Aquaculture 44, 187–200, https:// doi.org/10.1016/0044-8486(85)90243-1.

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. https://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/s11356-016-6690-5.

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.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

McEneff, G., Barron, L., Kelleher, B., Paull, B., Quinn, B., 2013. The determination of pharmaceutical residues in cooked and uncooked marine bivalves using pressurised liquid extraction, solid-phase extraction and liquid chromatography–tandem mass spectrometry. Anal. Bioanal. Chem. 405 (29), 9509-9521. https://doi.org/10.1007/s00216-013-7371-6.

McEneff, G., Barron, L., Kelleher, B., Paull, B., Quinn, B., 2014. A year-long study of the spatial occurrence andrelative distribution of pharmaceutical residues in sewage effluent, receiving marine waters and marine bivalves. Sci.Total Environ. 476-477, 317-326. https://doi.org/10.1016/j.scitotenv.2013.12.123.

McLusky, D.S., Elliott, M., 2004. The Estuarine Ecosystem. Ecology, Threats and Management. Oxford University Press, Oxford.

Menconi, M., Cinelle, F., 1999. Spatial and temporal variability in the distribution of algae and invertebrates on rocky shores in the northwest Mediterranean. J. Exp. Mar. Biol. Ecol. 233, 1-23. https://doi.org/10.1016/S0022-0981(98)00123-3.

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. https://doi.org/10.1016/S0166-445X(97)00083-0.

Mitrano, D.M., Motellier, S., Clavaguera, S., Nowack, B., 2015. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. Environ. Int. 77,132-147. https://doi.org/10.1016/j.envint.2015.01.013.

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

Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ. Int. 32, 967-976. https://doi.org/10.1016/j.envint.2006.06.014.

Moore, M.N., Readman, J.A.J., Readman, J.W., Lowe, D.M., Frickers, P.E., Beesley, A., 2009. Lysosomal cytotoxicity of carbon nanoparticles in cells of the molluscan immune system: an in vitro study. Nanotoxicology 3, 40-45. https://doi.org/10.1080/17435390802593057.

Moreira, M.H., Queiroga, H., Machado, M.M., Cunha, M.R., 1993. Environmental gradients in a southern estuarine system: Ria de Aveiro, Portugal, implication for a soft bottom macrofauna colonization. Neth. J. Aquatic Ecol. 27, 465-482. https://doi.org/10.1007/BF02334807.

Mottier, A., Mouchet, F., Pinelli, É., Gauthier, L., Flahaut, E., 2017. Environmental impact of engineered carbon nanoparticles: from releases to effects on the aquatic biota. Curr. Opin. Biotechnol. 46, 1-6. https://doi.org/10.1016/j.copbio.2016.11.024.

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. https://doi.org/10.1021/es7029637.

Nardi, A., Mincarelli, L.F., Benedetti, M., Fattorini, D., d'Errico, G., Regoli, F., 2017. Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*. Chemosphere 169, 493-502. https://doi.org/10.1016/j.chemosphere.2016.11.093.

Newell, R.C. 1979. Biology of intertidal organisms. Marine Ecological Surveys, Faversham, UK.

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. https://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. https://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.

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.

Paital, B., Chainy, G.B.N., 2013. Seasonal variability of antioxidant biomarkers in mud crabs (*Scylla serrata*). Ecotoxicol. Environ. Saf., 87, 33-41. https://doi.org/10.1016/j.ecoenv.2012.10.006.

Parker, L.M., Ross, P.M., O'Connor, W.A., Pörtner, H.O., Scanes, E., Wright, J.M., 2013. Predicting the response of molluscs to the impact of ocean acidification. Biology 2, 651-692. https://doi.org/10.3390/biology2020651.

Petersen, E.J., Zhang, L., Mattison, N.T., O'Carroll, D.M., Whelton, A.J., Uddin, N., Nguyen, T., Huang, Q., Henry, T.B., Holbrook, R.D., Chen, K.L., 2011. Potential release pathways, environmental fate, and ecological risks of carbon nanotubes. Environ. Sci. Technol. 45, 9837-9856. https://doi.org/10.1021/es201579y.

Pereira, E., Rodrigues, S.M., Otero, M., Válega, M., Lopes, C.B., Pato, P., Coelho, J.P., Lillebø, A.I., Pardal, M.A., Rocha, R., Duarte, A.C., 2008. Evaluation of an interlaboratory proficiency-testing exercise for total mercury in environmental samples of soils, sediments and fish tissue. Trends Anal. Chem. 27, 959-970. https://doi.org/10.1016/j.trac.2008.09.001.

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.

Prandle, D., 2009. Estuaries: Dynamics, Mixing. Sedimentation and Morphology, Cambridge University Press.

Pörtner, H.-O., Langenbuch, M., Michaelidis, B., 2005. Synergistic effects of temperature extremes, hypoxia, and increases in CO_2 on marine animals: from earth history to global change. J. Geophys. Res. 110, 2156-2202. https://doi.org/10.1029/2004JC002561.

Pörtner, H.-O., 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J. Exp. Biol. 213, 881-893. https://doi.org/10.1242/jeb.037523.

Pörtner, H.-O., Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science, 315, 95-97. https://doi.org/10.1126/science.1135471.

Poulain, C., Lorrain, A., Flye-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, https://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.

Rawlings, T.A., 1999. Adaptations to physical stresses in the intertidal zone: The egg capsules of neogastropod molluscs. Integr. Comp. Biol. 39, 230-243. https://doi.org/10.1093/icb/39.2.230.

Reddy, K.R., DeLaune, R.D., 2008. Biogeochemistry of wetlands: Science and applications. Crc Press, Boca Raton.

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. https://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. https://doi.org/10.1007/s11051-006-9092-7.

Resgalla Jr., C., Brasil, E.S., Salomao, L.C., 2007. The effect of temperature and salinity on the physiological rates of the mussel *Perna perna* (Linnaeus 1758). Braz. Arch. Biol. Technol. 50 (3), 543-556. https://doi.org/10.1590/S1516-89132007000300019.

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. https://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. https://doi.org/10.1016/j.marenvres.2013.09.007.

Rodrigues A.M., Quintino V., Sampaio L., Freitas R., Neves R., 2011. Benthic biodiversity patterns in Ria de Aveiro, Western Portugal: Environmental-biological relationships. Estuar. Coast. Shelf Sci. 95, 338-348. https://doi.org/10.1016/j.ecss.2011.05.019.

Rodrigues, L.C., Bergh, J.C.J.M.V.D., Massa, F., Theodorou, J.A., Ziveri, P., Gazeau, F., 2015. Sensitivity of Mediterranean bivalve mollusc aquaculture to climate change, ocean acidification, and other environmental pressures: findings from a producer survey. J. Shellfish Res. 34, 1161-1176. https://doi.org/10.2983/035.034.0341.

Salam, M.A., Burk, R., 2017. Synthesis and characterization of multi-walled carbon nanotubes modified with octadecylamine and polyethylene glycol. Arab. J. Chem. 10 (1), S921-S927. https://doi.org/10.1016/j.arabjc.2012.12.028.

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.

Santos, A.L., Mendes, C., Gomes, N.C.M., Henriques, I., Correia, A., Almeida, A., Cunha, Â., 2009. Short-term variability of abundance, diversity and activity of estuarine bacterioneuston and bacterioplankton. J. Plankton Res. 31, 1545–1555. https://doi.org/10.1093/plankt/fbp083.

Santos, S., Luttikhuizen, P.C., Campos, J., Heip, C.H.R., van der Veer, H.W., 2011. Spatial distribution patterns of the peppery furrow shell *Scrobicularia plana* (da Costa, 1778) along the European coast: A review. J. Sea Res. 66, 238–247. https://doi.org/10.1016/j.seares.2011.07.001.

Schiedek, D., Broeg, K., Barsiene, J., Lehtonen, K.K., Gercken, J., 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces*

viviparus) from the southwestern Baltic Sea. Mar. Pollut. Bull. 53, 387–405. https://doi.org/10.1016/j.marpolbul.2005.11.013.

Schmidt, W., Power, E., Quinn, B., 2013. Seasonal variations of biomarker responses in the marine blue mussel (*Mytilus* spp.). Mar. Pollut. Bull. 74, 50–55. https://doi.org/10.1016/j.marpolbul.2013.07.033.

Scott-Fordsmand, J.J., Weeks, J.M., 2000. Biomarkers in earthworms. Rev. Environ. Contam. Toxicol. 165, 117–159. https://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.

Shahnawaz, S., Sohrabi, B., Najafi, M., 2017. the investigation of functionalization role in multi-walled carbon nanotubes dispersion by surfactants. Int. Electr. Conf. Synthetic Org. Chem. 18, 1-30. https://dor.org/10.3390/ecsoc-18-f002.

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

Shumway, S.E., Davis, C., Downey, R., Karney, R., Kraeuter, J., Parsons, j., Rheault, R and Wikfors, G., 2003. Shellfish aquaculture — In praise of sustainable economies and environments. World Aquacult. 34 (4), 8-10.

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. https://doi.org/10.1016/j.ecoenv.2004.12.008.

Silva, J., Santos, R., Calleja, M.L., Duarte, C.M., 2005b. Submerged versus air-exposed intertidal macrophyte productivity: from physiological to community-level assessments, J. Exp. Mar. Biol. Ecol. 317, 87-95. https://doi.org/10.1016/j.jembe.2004.11.010.

Sokolova, I.M., Granovitch, A.I., Berger, V.Ja., Johannesson. K., 2000. Intraspecific physiological variability of the gastropod *Littorina saxatilis* related to the vertical shore gradient in the White and North Seas. Mar. Biol. 137, 297-380. https://doi.org/10.1007/s002270000343.

Sokolova, I.M., Pörtner, H.O., 2003. Metabolic plasticity and critical temperatures for aerobic scope in a eurythermal marine invertebrate (*Littorina saxatilis*, Gastropoda: Litorinidae) from different latitudes. J. Exp. Mar. Biol. Ecol. 206, 195-207. https://doi.org/10.1242/jeb.00054.

Solarskaciuk, K., Gajewska, A., Glińska, S., Michlewska, S., Balcerzak, Ł., Jamrozik, A., Skolimowski, J., Burda, K., G. 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.

Sousa, A.I., Calado, R., Cleary, D.F.R., Nunes, C., Coimbra, M.A., Serôdio, J., Lillebø, A.I., 2017b. Effect of spatio-temporal shifts in salinity combined with other environmental variables on the ecological processes provided by *Zostera noltei* meadows. Sci. Rep. 7, 1336. https://doi.org/10.1038/s41598-017-01359-2.

Sousa, A.I., Santos, D.B., Ferreira da Silva, E., Sousa, L.P., Cleary, D.F.R., Soares, A.M.V.M., Lillebø, A.I., 2017a. Blue carbon' and nutrient stocks of Salt marshes at a temperate coastal lagoon (Ria De Aveiro, Portugal). Sci. Rep. 7, 41225. https://doi.org/10.1038/srep41225.

Sultan, C.S., Saackel, 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, Y., Fu, K., Lin, Y.I., 2002. Functionalized carbon nanotubes: properties and applications. Acc. Chem. Res. 35 (12), 1096–1104. https://doi.org/10.1021/ar010160v.

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.

Tett, P., Gowen, R., Painting, S., Elliott, M., Forster, R., Mills, D., Bresnan, E., Capuzzo, E., Fernandes, T., Foden, J., Geider, R., Gilpin, L., Huxham, M., McQuatters-Gollop, A., Malcolm, S., Saux-Picart, S., Platt, T., Racault, M.-F., Sathyendranath, S., van der Molen, J., Wilkinson, M., 2013. Framework for understanding marine ecosystem health. Mar. Ecol. Prog. Ser. 494, 1–27. https://doi.org/10.3354/meps10539.

Tomanek, L., Somero, G.N., 2000. Time course and magnitude of synthesis of heat-shock proteins in congeneric marine snails (*genus Tegula*) from different tidal heights. Physiol. Biochem. Zool. 73, 249-256. https://doi.org/10.1086/316740.

Tsuchiya, M., 1983. Mass mortality in a population of the mussel *Mytilus edulis* L. caused by high temperature on rocky shores. J. Exp. Mar. Biol. Ecol., 66, 101-111. https://doi.org/10.1016/0022-0981(83)90032-1.

Underwood, G.J.C., Kromkamp, J., 1999. Primary Production by Phytoplankton and Microphytobenthos in Estuaries, in: Advances in Ecological Research: Estuaries. 29, 93-139. https://doi.org/10.1016/S0065-2504(08)60192-0.

Vaz, N., Dias, J.M., Leitão, P.C., 2009. Cont. Shelf Res. 29, 29-41. Three-dimensional modelling of a tidal channel: the Espinheiro Channel (Portugal). https://doi.org/10.1016/j.csr.2007.12.005.

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, https://doi.org/10.1016/j.cbpa.2016.06.029.

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2015. Spatial distribution and bioaccumulation patterns in three clam populations from a low contaminated ecosystem. Estuar. Coast. Shelf Sci. 155. 114-125. https://doi.org/10.1016/j.ecss.2015.01.004.

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. https://doi.org/10.1016/j.aquatox.2016.04.016.

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2017. Effects of seawater temperature increase on economically relevant native and introduced clam species. Mar. Environ. Res. 123, 62-70. https://doi.org/10.1016/j.marenvres.2016.11.010.

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. https://doi.org/10.1016/j.scitotenv.2015.01.092.

Wethey, D.S., 1983. Geographic limits and local zonation: the barnacles *Semibalanus* (*Balanus*) and *Chthamalus* in New England. Biol Bull. 165, 330-341. https://doi.org/10.2307/1541373.

Widdows, J., Bayne, B.L., Livingstone, D.R., Newell, R.I.E., Donkin, P., 1979. Physiological and biochemical responses of bivalve molluscs to exposure in air. Comp. Biochem. Physiol. 62A, 301-308. https://doi.org/10.1016/0300-9629(79)90060-4.

Wolanski, E., Elliot, M., 2015. Estuarine Ecohydrology – An Introduction (Second Edition). Elsevier, Boston. https://doi.org/10.1016/B978-0-444-63398-9.00001-5.

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.

Zandee, D.I., Holwerda, D.A., Kluytmans, J.H., de Zwaan, A., 1986. Metabolic adaptations to environmental anoxia in the intertidal bivalve mollusc *Mytilus edulis* L. Neth. J. Zool. 36, 322-343. https://doi.org/10.1163/002829686X00117.

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 TiO_2 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.