Universidade de Aveiro Departamento de Biologia
Ano 2019

LUCIA DE MARCHI

RESPOSTAS DE DUAS ESPÉCIES INVERTEBRADAS AQUÁTICAS ÀS NANOPARTÍCULAS BASEADAS EM CARBONO SOB O CENÁRIO DE MUDANÇAS CLIMÁTICAS

RESPONSES OF TWO AQUATIC INVERTEBRATE SPECIES TO CARBON-BASED NANOPARTICLES UNDER A CLIMATE CHANGE SCENARIO

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Doutora Rosa Freitas, Investigadora do Departamento de Biologia da Universidade de Aveiro, Prof. Doutora Etelvina Figueira, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e Doutor Victor Fernando Santos Neto, Professor Auxiliar Departamento de Engenharia Mecânica da Universidade de Aveiro

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"*C'é una forza motrice più forte del vapore, dell'elettricità e dell'energia atomica: la volontà*" Albert Einstein.

Palavras-chave

Nanotubos de carbono não funcionalizados; nanotubos de carbono de paredes múltiplas carboxiladas; alterações de salinidade; variações de pH; *Ruditapes philippinarum*; *Hediste diversicolor*; *Diopatra neapolitana*; capacidade regenerativa; reservas energéticas; capacidade metabólica; estado oxidativo; neurotoxicidade.

Resumo

De acordo com publicações recentes, nos próximos 100 anos prevê-se um aumento na acidificação da água do mar e alterações na sua salinidade. Nas águas superficiais, o aumento do CO_2 atmosférico já causou uma diminuição do pH em mais de 0,1 unidades comparando com 8,1, a média referente à época pré-industrial. Está previsto que até ao final deste século, esta redução do pH possa atingir valores na ordem das 0,13 e 0,43 unidades. As alterações climáticas podem também resultar em alterações na salinidade da água do mar. A salinidade é mais alta quando as temperaturas são mais altas e os períodos de chuva são reduzidos enquanto que, eventos de chuva intensa diminuem a salinidade da água do mar. Em qualquer dos cenários, estas alterações irão promover respostas por parte das espécies. Portanto, é imperativo identificar os efeitos das alterações climáticas nos ecossistemas aquáticos de modo a conservar a sua biodiversidade. Para além das alterações climáticas, há uma preocupação crescente com o grande número de poluentes emergentes que têm sido descartados no meio ambiente sem serem devidamente regulamentados. Entre estes poluentes emergentes estão as nanopartículas artificiais (Engineered nanoparticles - ENPs). Um dos tipos de ENPs mais usados nos últimos anos são os Nanotubos de Carbono (Carbon nanotubes - CNTs). Devido às suas propriedades químicas e nano-toxicológicas únicas, é expectável que os CNTs entrem nos ambientes aquáticos e se acumulem na fauna que lá vive. De facto, a toxicologia dos CNTs em sistemas aquáticos é complexa. Numa primeira análise, o tamanho, a forma, a estrutura química e os agentes de revestimento desempenharão um papel no que diz respeito à estabilidade e, portanto, à biodisponibilidade da partícula. No entanto, a toxicidade dos nano-materiais tem sido atribuída não só à sua estrutura central e modificação/funcionalização da sua superfície, mas também aos parâmetros físico-químicos do meio em que os nanotubos se apresentam e que podem alterar a sua dispersão e consequentemente a sua deteção: agregação/desagregação, adsorção/dessorção, sedimentação/ressuspensão e dissolução. O impacto dos nanotubos no meio aquático já foi descrito por vários autores; no entanto, ainda não se sabe de que forma as alterações climáticas podem alterar a toxicidade dos CNTs e subsequentemente os efeitos sobre os organismos marinhos. As espécies bentónicas são um bom modelo para avaliar os impactos das Alterações Climáticas e ENPs, uma vez que são sensíveis às mudanças ambientais. Principalmente devido às características do seu ciclo de vida, bem como à sua resposta relativamente rápida à poluição, há vários estudos que usam espécies bentónicas como bioindicadores para fatores de stress antropogénicos e naturais. Sendo assim, é urgente avaliar os impactos dos CNTs, sob alterações de salinidade e redução do pH em organismos aquáticos. *Ruditapes philippinarum* (bivalve), *Hediste diversicolor* e *Diopatra neapolitana* (poliquetas) são invertebrados marinhos que respondem rapidamente a perturbações ambientais e são caracterizados por uma ampla distribuição espacial e relevância económica, nomeadamente em Portugal. Pelo que, esta tese pretendeu avaliar os efeitos tóxicos de desvios de salinidade, variação de pH e presença de CNTs (atuando isolados ou em combinação) ao nível da resposta bioquímica (reservas de energia e atividade metabólica, estado oxidativo e neurotoxicidade) e da resposta fisiológica (capacidade regenerativa) das espécies acima citadas. Neste estudo, os dois materiais de CNT selecionados foram os nanotubos de carbono de parede múltipla, não funcionalizados (pristine multi walled carbon nanotubes - Nf-MWCNTs) e os MWCNTs quimicamente funcionalizados através da introdução de grupos polares como grupos carboxilo (-COOH), que aumentam sua estabilidade e capacidade de dispersão no meio aquoso. Como ponto de partida, para cada concentração de exposição, avaliamos os possíveis efeitos da carboxilação/funcionalização da superfície dos MWCNTs nos organismos. Em todas as espécies de invertebrados foi possível observar uma relação positiva entre o aumento da dose e a toxicidade, principalmente no que diz respeito ao estado oxidativo, o que está de acordo com a informação disponível na literatura.

Além disso, comparando os efeitos tóxicos de ambos os CNTs, em todas as espécies de invertebrados, verificaram-se maior dano celular induzido pela forma carboxilada da MWCNT em comparação com a forma não funcionalizada. Posteriormente, selecionamos as duas concentrações de Nf-MWCNTs e f-MWCNTs mais perniciosas, e expusemos as três espécies de invertebrados à combinação destes materiais CNT com variações de salinidade e variações de pH, avaliando desta forma se estes fatores relacionados com as alterações climáticas modificavam a toxicidade de ambos os materiais MWCNT bem como a sensibilidade das espécies expostas a esses contaminantes. Os resultados obtidos salientam que Nf-MWCNTs e f-MWCNTs sob salinidade controlo e pH baixo, geraram grandes impactos tóxicos nos organismos em comparação com indivíduos mantidos em condições de salinidade baixa e pH controlo. Confirmou-se desta forma que, alterações de salinidade e variações de pH podem alterar o comportamento químico de ambos os MWCNTs e consequentemente o efeito em indivíduos expostos. Além disso, observamos que a sensibilidade ao contaminante é dependente da espécie o que confirma que a maior suscetibilidade observada em algumas espécies não é apenas um resultado das diferentes características dos compostos usados, mas também da fisiologia dessas espécies em particular.

Para uma melhor proteção do meio ambiente, a Avaliação de Risco Ecológico dos fatores de stress mencionados, deve incluir objetivos e cenários de exposição ecologicamente relevantes para impulsionar medidas de segurança corretas e adaptadas com respeito à conservação da biodiversidade.

Keywords

Pristine multi-walled carbon nanotubes; carboxylated multi-walled carbon nanotubes; salinity shifts; pH variations; *Ruditapes philippinarum*; *Hediste diversicolor*; *Diopatra neapolitana*; regenerative capacity; energy reserves and metabolic capacity; oxidative status; neuro status

Abstract

According to recent reports, increases in water acidification and changes in seawater salinity are predicted to occur in the next 100 years. The increase of atmospheric CO_2 already caused a pH reduction in surface waters by more than 0.1 units below the pre-industrial average of 8.1, and it is expected to decrease between -0.13 and -0.43 units by the end of this century. Climate change can also occur through alterations in seawater salinity. Warmer temperatures and reduced rainfall increase seawater salinity, while extreme rainy events decrease seawater salinity. Both situations will promote species responses. Therefore, identifying the effects of predicted climate change in aquatic ecosystems must be a priority in order to maintain their biodiversity. Aside from climate change, there is an increasing concern about the large number of emerging pollutants that have been released into the environment without yet being regulated. Among these emerging pollutants are Engineered nanoparticles (ENPs). One of the types of ENPs that are most commonly used in recent years are Carbon Nanotubes (CNTs). Due to their unique chemical and nanotoxicological properties, it is expected that CNTs enter aquatic environments and accumulate in aquatic biota. As a matter of fact, CNTs toxicology in aquatic systems is complex. In the first instance particle size, shape, chemistry and capping agents will all play a role regarding the stability, and thus bioavailability. However, nanomaterial toxicity not only has been attributed to core structure and surface modification/functionalization, but also by the physico-chemical parameters of the media where the CNTs are presented, altering their dispersion and consequently their detection: aggregation/disaggregation, adsorption/desorption, sedimentation/resuspension and dissolution. Several works have described their impacts in the aquatic environment; however, no information is known on how predicted Climate Change could alter the CNT's toxicity and their effects on marine organisms. Benthic species are a good model to evaluate the impacts of Climate Change and ENPs as they are sensitive to several environmental constrains. Essentially due to their life-history characteristics, as well as their relatively rapid response to pollution, several studies have been using benthic species as bioindicators for anthropogenic and natural stresses. Thus, the evaluation of the impacts of CNTs, under salinity changes and pH reduction on aquatic organisms is an urgent issue needing attention. Particularly, *Ruditapes philippinarum* (bivalve), *Hediste diversicolor* and *Diopatra neapolitana* (polychaetes) have been identified by several authors as a group of marine invertebrates that respond quickly to environmental disturbances, with a wide spatial distribution and economic relevance, namely in Portugal. Thus, the present proposal evaluated the toxic effects in terms of biochemical (energy reserves and metabolic activity, oxidative and neuro status) and physiological responses (regenerative capacity) in the cited species of salinity shifts and pH variation and the presence of CNTs acting along and in combinations. The two CNT materials selected in the present study were the pristine multi walled carbon nanotubes (Nf-MWCNTs) and the chemically functionalized MWCNTs, by introducing polar groups such as carboxyl groups (-COOH) increasing their stability and dispersibility in the water media. As a starting point we evaluated the possible effects of the carboxylation/functionalization of the surface of MWCNTs in organisms for each exposure concentration. In all invertebrate species it was possible to observe a dose-dependent increased of the toxicity, especially in terms of oxidative status, which is in line with the information provided by the literature. Moreover, comparing the toxic effects of both CNTs, in all invertebrate species major cellular damage was induced by carboxylated forms of MWCNTs in comparison to the pristine one. Subsequently we selected the two most deleterious concentrations of Nf-MWCNTs and f-MWCNTs, and we exposed the three invertebrate species to the combination of CNT materials with salinity shifts and pH variations assessing if both climate change factors altered the toxicity of both MWCNT materials as well as the sensitivity of all these species exposed to these contaminates.

The present findings underlined that Nf-MWCNTs and f-MWCNTs under control salinity and low pH generated major toxic impacts in the organisms compared to individuals maintained under low salinity and control pH, confirming that salinity shifts and pH variations may alter the chemical behaviour of both MWCNTs and consequent fate in exposed individuals. Moreover, we observed species-dependent sensitivity to contaminants confirming that the higher susceptibility observed in some species would however be expected not only to depend on the characteristics of the compounds, but also on the physiology of that particular species.

For a better environment protection, the Ecological Risk Assessment of the mentioned stressors must include ecologically relevant endpoints and exposure scenarios to drive accurate safety levels towards biodiversity conservation.

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CHAPTER 1. INTRODUCTION

1.1. Engineered nanoparticles

Nanotechnology is the science responsible for the study, fabrication and manipulation of structures, devices, materials or particles in the size range from 1 to 100 nanometers (or contain at least one component in this range); this size range is known as the nanoscale. Nanoparticles (NPs) exist since the very beginning of the Earth's history (Verma et al., 2002). The formation of NPs can have both natural and anthropogenic sources. Volcanic eruptions and forests fires both contribute for the formations of atmospheric NPs, as a natural source (Farré et al., 2009). Aquatic colloids, the fine fraction of desert sand, oil fumes, and certain atmospheric dust also represent NPs produced naturally (Ostiguy et al., 2006). Even some plants have the ability to synthesize NPs which are used to reduce the uptake of metals in polluted soils (Bernhardt et al., 2010). Engineered nanoparticles (ENPs) or anthropogenic NPs are intentionally created (Farré et al., 2009). They can be divided into two general classes: carbon-based (e.g., carbon nanotubes and fullerenes) and metal-containing (e.g., Ag, TiO_2, CeO_2, Fe) (Fadeel and Garcia-Bennett, 2010). Carbon-based NPs are allotropes of carbon with at least one dimension within the range of 1 to 100 nm. The main classes can be divided as buckyballs (spherical fullerenes), carbon nanotubes (CNTs) (cylindrical fullerenes) and carbon black (amorphous carbon) (Freixa et al., 2018). Regarding metals and metal oxides NPs, these particles can be formed of two or more metals (Au, Ag, Cu, Pt, Pd, Zn, etc.) which are combined with each other or bonded to metalloids (Irzhak, 2016). Particle size, surface chemistry and charge, crystallinity, phase purity, solubility and shape are essential characteristics to explain the homogeneity, stability, reactivity, bioavailability and application potential of ENPs in different media (Kahru and Dubourguier, 2010). As a consequence of their unique characteristics, the use of ENPs in consumer, industrial, and agricultural products, as well as in environmental technology is rapidly increasing, and Global production of ENPs are projected to grow to half a million tons with the number of ENPs-containing consumer products reaching 3400 by 2020 (www.nanoproject.org). Tubes and wires considered one-dimensional materials with different type of applications of nanotechnology. Nanowires are ultrafine wires or linear arrays of dots that are formed by self-assembly. Semiconductor nanowires are produced of silicon. Their dimensional nanomaterial applications include NPs like dendrimers and fullerenes. Dendrimers are spherical polymeric molecules that are formed through a nanoscale self-assembly process and fullerenes are carbon molecules arranged into a spherical shape resembling a geodesic dome characterized by a multiple spherical configuration which depend on the number of carbon atoms (Guo and Tan, 2009).

This fast expansion and use of ENPs have inevitably resulted in their release into the environment, either as the original or manufactured NPs. Of particular interest is the aquatic environment, which tends to be the ultimate sink for particulate contaminants (Selck et al., 2016). The releases of ENPs into the aquatic environment can be direct (sewage, effluents, river influx) or indirect (aerial deposition, dumping and run-off) (Rocha et al., 2015) reaching different types of

ecosystem compartments (water column and sediments). When into the aquatic system, ENPs behaviour and fate is dependent on their properties as well as the characteristics of the media where they are dispersed. Therefore, the toxic impacts of ENPs towards aquatic organisms will depend on the behaviour of the NPs as a consequence of their chemical-physical characteristics and as well as aquatic systems characteristics, which may change considering predicted climate changes.

There are also concerns about which aquatic organisms will be most at risk in the presence of ENPs. Currently, knowledge of biological effects in the aquatic environment is skewed toward studies on as-manufactured NPs in aqueous acute tests using pelagic organisms (Selck et al., 2016). However, because of the settling behaviour of particulates, benthic organisms are more likely to be exposed (Selck et al., 2016). Also, a review of Minetto et al. (2016) pointed to an important asymmetry: almost 76% of published paper employed freshwater animal species and only 24% were saline water or marine species.

1.1.1. Carbon Nanotubes (CNTs)

1.1.1.1. Characteristics

Nanotubes are members of the fullerene (carbon molecules arranged into a spherical shape) structural family, which includes buckyballs and carbon nanotubes (CNTs). While buckyballs are spherical in shape, CNTs are cylindrical, with at least one end typically capped with a hemisphere of the buckyball structure (McEnaney, 1999). They can be single-walled (SWCNT) with a diameter of less than 1 nanometer (nm) (Figure 1A) or multi-walled (MWCNT), consisting of several concentrically interlinked nanotubes, with diameters reaching more than 100 nm (Figure 1B). Their length can reach several micrometers or even millimeters. CNTs are chemically bonded with sp^2 bonds, an extremely strong form of molecular interaction (Baughman et al., 2002).

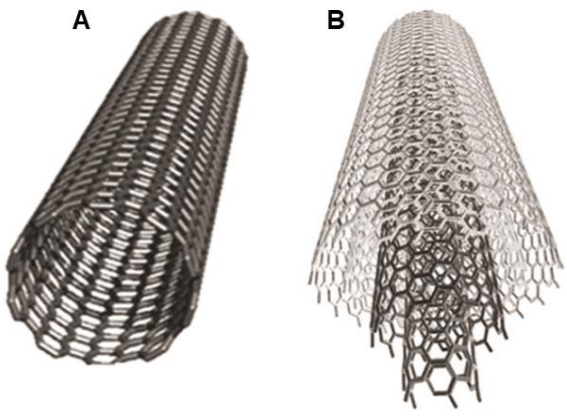

Figure 1. Schematic representation of a single-walled nanotube (SWCNT) (**A**) and a multiwalled nanotube (MWCNT) (**B**) (fuelcellstore.com).

Looking on their properties, CNTs have high thermal conductivity, high electrical conductivity, high aspect ratio, very high elasticity, high tensile strength, highly flexible — can be bent considerably

without damage, low thermal expansion coefficient and are considered good electron field emitters (Ajayan and Zhou, 2001).

1.1.1.2. Applications

Commercial applications are incorporating CNT materials, which are now entering the growth phase of their product life cycle. The most promising present and future commercial applications of CNTs include: field emission (field-emission flat-panel and in general types of low-voltage cold-cathode lighting sources, electron microscope sources, and lightning arrestors are other applications utilizing the field-emission characteristics of CNTs); conductive plastics (electrostatic dissipation (ESD); coatings for gaskets, enclosures, and other uses; radar-absorbing materials for low-observable ("stealth") applications; and antistatic materials and (even transparent) conductive coatings); energy storage (electrodes in capacitors and batteries; supercapacitor electrodes and fuel cell components); conductive adhesives and connectors (electromagnetic shielding, electronics packaging, including coaxial cables, potting compounds, adhesives and other types of connectors); molecular electronics (electronic circuit); structural composites; fibers and fabrics (woven fabrics and textiles, transmission line cables, and body and vehicle armor); catalyst supports; biomedical applications (anti-fouling coatings for ships and coatings for prosthetics, neuron growth and regeneration, and vascular stents); air and water filtration (water and air filtration devices able to block the tiniest particles as well as destroy most bacteria); ceramic applications. There are several other potential applications for CNTs, including solar collection, nanoporous filters, catalyst supports, and all kinds of coatings. There are almost certainly several surprising applications for this excellent material that will be revealed in the future, and which may prove to be the most significant and valuable ones of all. A number of researchers have been studying the conductive and/or waterproof paper produced using CNTs. CNTs have also been demonstrated to absorb infrared light and may hold applications in optics industry (De Volder et al., 2013).

1.1.1.3. Environmental concentrations

CNTs may enter the environment directly during unintentional release, during use and consumption of CNT containing goods or as waste from sewage treatment plants, waste incineration plants and landfills (Petersen et al., 2011). Looking on the most recent literature, the environmentally relevant concentrations (ERCs) of CNTs in water, based on a stochastic/probabilistic material-flow computer model, are in the µg/L or ng/L range (Sun et al., 2016) while the predicted environmental concentrations (PECs) were projected to approximately 0.001-1000 µg/L (Noura et al., 2013; Zhang et al., 2017).

1.1.1.4. Environmental behaviour

The environmental behaviour of CNTs in aquatic systems is related to their ability to interact and aggregate, creating clusters that exhibit colloidal behaviour. Despite the virtual water insolubility of individual CNT molecules, the formed aggregates are stable under certain environmental conditions. The properties of the aggregates (size, ζ-potential, shape, surface functionalization, sedimentation rate, critical flocculation concentration, etc.) are dependent on the alteration of their surface properties (Freixa et al., 2018). In the review proposed by Jackson et al. (2013), the authors reported that, because CNTs are difficult to disperse in water and polar matrices, many commercially available CNTs are therefore functionalized before final use preventing agglomeration in the composite matrices. Dispersants can be added to the test media to reduce CNT agglomeration (Kim et al., 2011; Najeeb et al., 2012). For example, organic matter will increase the pristine CNT dispersibility in aquatic solutions by covering the hydrophobic surface causing longer residence time in the water column and increases CNT mobility which in turn, intensifies risk of exposure and toxicity (Hyung et al., 2007; Ferguson et al., 2008; Kennedy et al., 2008; 2009; Edgington et al., 2010; Zhang et al., 2011). Functionalization is achieved also through chemical modification such as amidation and esterification of the nanotube-bound carboxylic acids (Sun et al., 2002). The functionalization breaks the nanotube bundles, which is essential for the solubility and the presence of functional groups on the nanotubes surface, increasing nanotubes dispersibility (Shahnawaz et al., 2010). Specifically, to disperse CNTs in aqueous media, the chemical functionalization of CNTs by introducing polar groups such as carboxyl groups (-COOH) is one of the most common approaches in order to achieve better dispersibility in water (Shahnawaz et al., 2010). Furthermore, the large specific surface area may facilitate pollutant adhesion and thus influence CNT toxicity in itself and/or toxicity of co-pollutants and influence the bioaccumulation of environmental contaminants (Ferguson et al., 2008). Their behaviour depends also on the surrounding conditions including salinity, pH, temperature, ionic strength, composition and concentration of natural organic matter which affect their aggregation/agglomeration or stabilisation (Freixa et al., 2018). Salinity and pH are among the main factors influencing CNTs behaviour (Chinnapongse et al., 2011). Changes in the salinity of the aqueous environment can influence the nanoparticles' stability, which might change their toxicity into the organisms (Jastrzębska et al., 2012). It has been already demonstrated that CNTs transferred from fresh water to seawater decreased their zeta potential (because of the higher ionic strength of seawater due to salinity), thus causing aggregation and precipitation (Wong et al., 2013). Looking on the pH, the decrease can facilitate the dissolution of CNTs in an aquatic medium, which may increase the uptake and biodistribution into the organisms generating synergistic and more toxic interactive effects of pH and NPs (Xia et al., 2018).

1.1.1.5. Toxicity

Regarding their toxicity, available data already demonstrated that CNTs can cross organism's membrane barriers inducing harmful effects (e.g., inflammatory and fibrotic reactions). Cell and CNT interactions include cellular uptake and processing of CNTs by different routes, effects on cell signaling, membrane perturbations, production of cytokines, chemokines and reactive oxygen species (ROS), overt toxic reactivity, cell apoptosis (Zhao et al., 2012). In detail, CNTs were reported to accumulate in various subcellular compartments, such as the cell cytosol (Al-Jamal, et al., 2011), endosomes (Antonelli et al., 2010; Wang et al., 2010), the perinuclear region (Lacerda et al., 2007), mitochondria (Zhou et al., 2010; Neves et al., 2012), or the nucleus (Shi Kam et al., 2004) according to their physicochemical properties and functionalisation. Also, indirect non-specific toxic effects of CNTs, which include physical irritation and occlusion of surface tissues (e.g., gills), have been found in some studies with aquatic organisms (Oberdörster et al., 2006). Growth inhibition and genotoxicity are other expressions of ecotoxicity (Mouchet et al., 2008).

1.2. Climate change

As a consequence of human activity, the carbon dioxide (CO_2) emissions increased at the beginning of the industrial age and are now enhancing the atmospheric concentration of CO_2 by 1–2 parts per million by volume (ppmv) annually (Davis, 2017), resulting in global warming and ocean acidification, actually recognized as important drivers of biological systems (Fabry et al., 2008) (Figure 2). Observations of the increase in global average air and ocean temperatures along with widespread melting of ice and rising sea level provide unequivocal evidence of climate warming (IPCC, 2014). Due to continued greenhouse gas emissions at or above present rates, climate models predict further warming in the global climate system during the 21st century. Risk of climate-related impacts results from the interaction of climate-related hazards (including hazardous events and trends) with the vulnerability and exposure of human and natural systems, including their ability to adapt. Several associated parameters of climate warming, such as increasing sea surface temperature, ocean acidification, changes in vertical mixing, upwelling, precipitation and evaporation patterns, may in turn impact resilience of many ecosystems (Moore et al., 2008). The overall risks of future climate change impacts can be reduced by limiting the rate and magnitude of climate change, including ocean acidification. The precise levels of climate change sufficient to trigger abrupt and irreversible change remain uncertain, but the risk associated with crossing such thresholds increases with rising temperature (IPCC, 2014). Considering coastal and aquatic ecosystems, there are expected damaging consequences due to increasing temperature, sea level rise, and decrease of oceanic pH (Harley et al., 2006). While the ocean plays an important role in moderating the build-up of atmospheric CO_2, especially in a climate change context (Caldeira and Wickett, 2003, Sabine et al., 2004), the acidification of seawater resulting from oceanic absorption of CO_2 will impact negatively on calcifying organisms (Fabry et al., 2008, Guinote and Fabry, 2008). The temperature increase will amplify hypoxia conditions worldwide, while it enhances the respiratory demand of the organisms, reduces oxygen solubility, and reduces the ventilation of coastal waters by affecting stratification patterns (Vaquer-Sunyer and Duarte, 2008). Increasing coastal flooding events are linked to sea level rise but were probably accelerated by historical losses of floodplains and erosion control provided by coastal wetlands, reefs, and submerged vegetation (Danielsen et al., 2005). Also, changing coastal currents may affect the distribution and recruitment of populations, and altered patterns of precipitation and runoff may affect estuarine and coastal management and strategies to control nonpoint sources of nutrients and other pollutants (Boesch, 1999). All these impacts lead, ultimately, to an overall biodiversity decline and, presently, there are already many registers of populations, key species and even entire functional groups being lost in estuaries, coral reefs and many other coastal systems around the world (Worm et al., 2006). By affecting ecosystems properties, biodiversity loss impairs at least three critical ecosystem services: number of viable fisheries; provision of nursery habitats; and filtering and detoxification services

provided by suspension feeders, submerged vegetation, and wetlands (Worm et al., 2006). Loss of filtering services contributes eventually to declining water quality and the increasing occurrence of harmful algal blooms, fish kills, shellfish and beach closures, and oxygen depletion (Dame et al., 2002). An increased number of species invasions over time coincided also with the loss of native biodiversity (Worm et al., 2006).

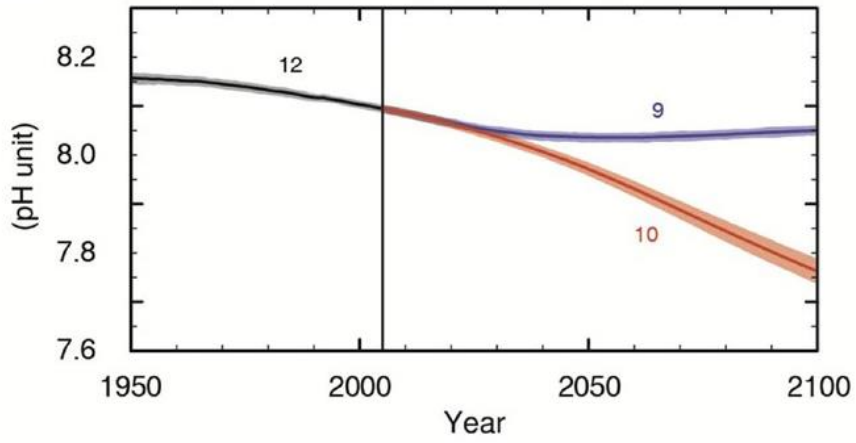

Figure 2. Multi-model simulated time series from 1950 to 2100 for global mean ocean surface pH. Time series of projections and a measure of uncertainty (shading) are shown for scenarios RCP2.6 (blue) (best case scenario) and RCP8.5 (red) (worst case scenario). Black (grey shading) is the modeled historical evolution using historical reconstructed forcings (IPCC, 2014).

1.2.1. pH variations

Absorption of anthropogenic CO_2, reduced pH, and lower calcium carbonate ($CaCO_3$) saturation in surface waters, where the bulk of oceanic production occurs, are well verified from models, hydrographic surveys, and time series data. In detail, seawater carbonate chemistry is governed by a series of chemical reactions:

$$CO_2\,(atmos) \rightarrow\leftarrow CO_2\,(aq) + H_2O \rightarrow\leftarrow H_2CO_3 \rightarrow\leftarrow H^+ + HCO^-{}_3 \rightarrow\leftarrow 2H^+ + CO_2{}^-{}_3$$

Air-sea gas exchange equilibrates surface water CO_2 to atmospheric levels with a timescale of approximately one year. Once dissolved in seawater, CO_2 gas reacts with water to form carbonic acid (H_2CO_3), which can then dissociate by losing hydrogen ions to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. The seawater reactions are reversible and near equilibrium (Millero et al., 2002); for surface seawater with pH of ~8.1, approximately 90% of the inorganic carbon is bicarbonate ion, 9% is carbonate ion, and only 1% is dissolved CO_2. Adding CO_2 to seawater increases aqueous CO_2, bicarbonate, and hydrogen ion concentrations. The projected 0.3–0.4 pH drops for the 21st century is equivalent to approximately a 150% increase in H^+ and a 50% decrease in CO_3^{2-} concentrations (Orr et al., 2005).

Considering that one-third of the anthropogenic CO_2 produced in the past 200 years has been taken up by the oceans (Sabine et al., 2004), the direct effect of CO_2 on ocean chemistry may affect marine biota. In this regard, most of the studies are focused on marine calcifying organisms, such as corals, mollusks, echinoderms and crustaceans, as a consequence of the reduction of carbonate ions that are necessary to produce $CaCO_3$ for the construction of their shells and skeletons (Feely et al., 2004). However, other physiological and biochemical indices appear to be correlated with the capacity for acid-base tolerance, including survival, growth, development, metabolism, and pH balance of marine non-calcifying organisms under elevated pCO_2.

1.2.2. Extreme weather events: salinity shifts

Changes in many extreme weather and climate events have been observed since about 1950. Some of these changes have been linked to human influences, including a decrease in cold temperature extremes, an increase in warm temperature extremes, an increase in extreme high sea levels and an increase in the number of heavy precipitation events in a number of regions (IPCC, 2014). Specifically, the normal range of weather patterns will be influenced in two ways. First, there will be gradual changes in average weather patterns. Incremental changes in precipitation patterns will result in either floods or droughts. Second, the increased variability of extreme weather events associated with changes in surface temperature and precipitation (Mirza et al., 2003). All these extreme weather events are affecting the ocean salinity, one of the determining factors for water density, column stability and circulation patterns in the ocean. Changes in sea surface salinity (SSS), particularly in coastal areas of the world, are largely determined by local meteorological processes, such as precipitation, evaporation and mixing (Henderson-Sellers and McGuffie, 2012). Precipitation data, in general, show considerable spatial and temporal variability. In the last century, precipitation has mostly increased over land, particularly in high northern latitudes and Asian regions, while decreases have dominated in tropics since the 1970s (IPCC, 2014). The frequency of extreme precipitation, however, has increased and different climate models agree with consistent increase in precipitation for the 21st century (Bates et al., 2008) causing, in turn, significant impact on biological and ecological aspects, from species to community levels (Walther et al., 2002), including biodiversity, population size, species community composition, geographical distribution, phenology and productivity. A study conducted by Durack et al. (2012) reported changing patterns of salinity in the global ocean during the past 50 years, marking a clear symptom of climate change (Figure 3).

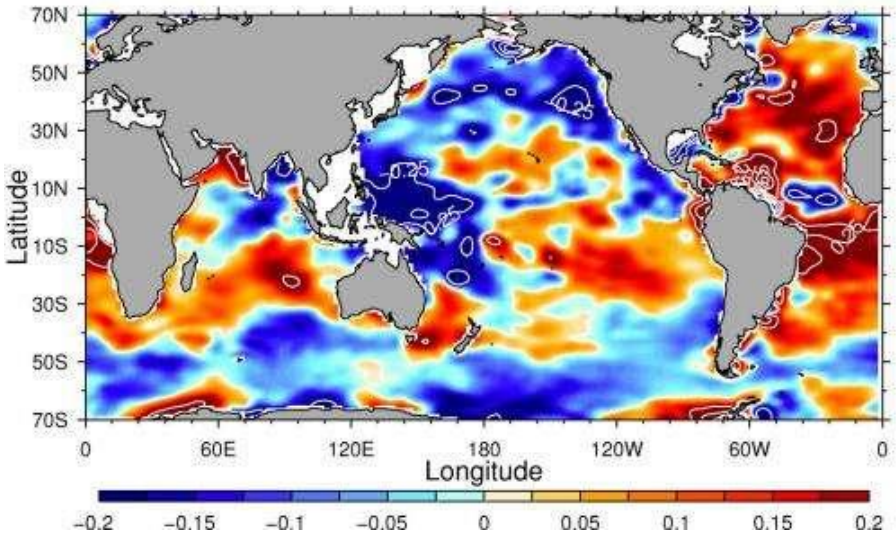

Figure 3. Surface salinity changes from 1950 to 2000. Red indicates regions becoming saltier, and blue regions becoming fresher (retrieved from Durack et al., 2012).

In the study the authors observed global surface salinity changes combined with changes from global climate models, presenting robust evidence of an intensified global water cycle at a rate of 8-5% per degree of surface warming. This rate is double the response projected by current-generation climate models and suggested that a substantial (16 to 24%) intensification of the global water cycle will occur in a future 2° to 3° warmer World.

1.3. Benthic invertebrates as bioindicators

The expression 'Bioindicator' is used as an aggregate term referring to all sources of biotic and abiotic reactions to ecological changes. Instead of simply working as gauges of natural change, taxa are utilized to show the impacts of natural surrounding changes, or environmental change. They are used to detect changes in natural surroundings as well as to indicate negative or positive impacts (Parmar et al., 2016). They can also detect changes in the environment due to the presence of pollutants which can affect the biodiversity of the environment, as well as species present in it and the condition of the environment is effectively monitored by the use of bioindicator species due to their resistance to ecological variability (Parmar et al., 2016). Particularly benthic invertebrates, due became a valuable fraction of the ecosystems and are frequently used as bioindicators in ecological assessments (McLusky and Elliott, 2004; Pinto et al., 2009). Using benthic communities has certain advantages because they are disturbance indicators with a real effect on the biota at the species-community level, and they are global pollution/disturbance indicators with easily worked elements. Several other characteristics make benthic organisms useful and suitable indicators, such as: (i) most benthos are relatively sedentary and reflect the quality of their immediate environment (Weisberg et al., 2008); (ii) many benthic species have relatively long life spans and their responses

integrate water and sediment quality changes over time (Reiss and Kröncke, 2005); (iii) they include diverse species with a variety of life features and tolerances to stress, which allow their inclusion into different functional response groups (Pearson and Rosenberg, 1978); (iv) they have a fundamental role providing links to the higher trophic levels and some species are, or are prey of, commercially important species (McLusky and Elliott, 2004, Reiss and Kröncke, 2005); (v) they affect fluxes of chemicals between sediment and water columns through bioturbation and suspension feeding activities, as well as playing a vital role in nutrient cycling (Reiss and Kröncke, 2005). For all these reasons, indices based on benthic invertebrates have proved to be effective measurements of coastal and estuarine conditions and are commonly used to assess the biological quality of the environment worldwide.

1.3.1. *Ruditapes philippinarum* (Adams & Reeve, 1850)

1.3.1.1. Distribution and habitat

The Manila clam *Ruditapes philippinarum* (Adams & Reeve, 1850) is a bivalve mollusc of the family Veneridae native to the Indo-Pacific region (https://www.cabi.org/isc/datasheet/61697). The Manila clam is found in the mid to upper portion of the intertidal and subtertidal areas (Carter, 2003).

Figure 4. Global distribution of *Ruditapes philippinarum* (https://www.marlin.ac.uk/species/detail/1426).

1.3.1.2. Description

Figure 5. *Ruditapes philippinarum.*

The shell is roundly triangular in shape strong and heavy, with radiating ridges and varies in colour from greyish white through yellow to buff brown. The shell also has distinctive black and white markings (Carter, 2003). The interior surface of the shell is smooth with a deep purple. It can grow up to 6 cm in length. The tip of the siphon is split. The body tissue of live specimens, especially the foot, are orange (Carter, 2003). band. The Manila clam has a wide feeding spectrum ranging across bacteria, algae and rotifers (Breber, 2002).

1.3.1.3. Potential Uses

Due to their biological features, these organisms are considered one of the major aquaculture species in the World as well as good sentinel organisms for monitoring the health status of marine ecosystems (Milan et al., 2011). Looking to this species as an economic resource, *R. philippinarum* introduction in several aquatic systems already provided growth in coastal communities through the increased direct sources from fishing, aquaculture and wholesaling (Cordero et al., 2017). The Manila clam culture represented 25% of global mollusk production in 2014 (Cordero et al., 2017) coming from the western coasts of the Pacific Ocean with China as the first worldwide producer (98.9%) (Cordero et al., 2017). In Europe, the wild reproduction favoured their geographical expansion, particularly in Italy, France, Spain, Portugal and Ireland where *R. philippinarum* proved to be more resistant and faster growing than the endemic clam, *Ruditapes decussatus*, and now represents the most important commercial species in Europe (Milan et al., 2011).

Accompanying *R. philippinarum* wide spatial distribution and associated economic importance, scientific studies and integrated management plans have raised. Recent experimental studies have investigated *R. philippinarum* biochemical and genotoxic responses to different environmental conditions, including the presence of pollutants as metals (Antunes et al., 2013; Wu et al., 2013; Ji et al., 2015; Velez et al., 2016; Matozzo et al., 2016) and pharmaceuticals (Antunes et al., 2013; Freitas et al., 2015a; Freitas et al., 2016a; Correia et al., 2016; Matozzo et al., 2016)

and, in the last decades, this species has been considered a suitable model for investigating the effects and mechanisms of action of NPs underlying the potential toxicity of these emerging contaminants in marine invertebrates (Marisa et al., 2015; Volland et al., 2015; Marisa et al., 2016; De Marchi et al., 2017a; b; c; De Marchi et al., 2018a).

1.3.2. *Hediste diversicolor* (O.F. Müller, 1776)

1.3.2.1. Distribution and habitat

Hediste diversicolor (O.F. Müller, 1776), commonly known as a ragworm, is a polychaete worm in the family Nereidae. This species is native to the north-east Atlantic. Its range extends from the Baltic Sea and the North Sea southwards to the Azores and the Mediterranean Sea. It has been introduced to the north-west Atlantic in the areas of Cobscook Bay, the Gulf of Maine and the Gulf of St Lawrence (Einfeldt et al., 2014) (Figure 6). It Inhabits muddy substrate in a more-or-less permanent U or J-shaped burrow that may be up to 20 cm in depth. Also occurs under stones on the mud where the burrow is adjacent to the stone (Budd, 2008).

Figure 6. Global distribution of *Hediste diversicolor* (https://www.marlin.ac.uk/species/detail/1426).

1.3.2.2. Description

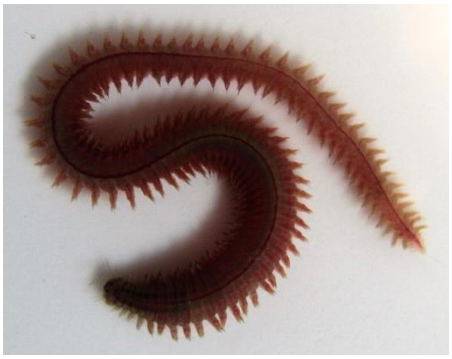

Figure 7. *Hediste diversicolor*.

Hediste diversicolor has an elongate, cylindrical body, somewhat inflated anteriorly and flattened posteriorly, which is divided into up to 120 chaetigers. The head is roughly triangular, but with concave sides and rounded corners. The head bears four eyes, two frontal antennae, and pair of stout conical palps (Budd, 2008). The antennae are much shorter than the palps. The head is flanked by four pairs of tentacular cirri. The posterior pair of tentacular cirri is longest and can reach back to chaetigers 5-7. Ventrally and anteriorly, the muscular, extendable, proboscis consists of two rings, terminating in a pair of toothed, amber-colored jaws, with 5-8 teeth. *H. diversicolor* can reach 200 mm, but rarely exceeds 120 mm. As its name suggests, this worm's colour is highly variable, including greenish, yellowish, orange-red, and reddish-brown, often with longitudinal brown stripes (Budd, 2008). *H. diversicolor* is omnivorous and exhibits a diversity of feeding modes; carnivory, scavenging, filter feeding on suspended particles and deposit-feeding on materials in and on the surface layers of the sediment (Barnes, 1994).

1.3.2.3. Potential Uses

H. diversicolor is a species of commercial and applied interest because of its use as bait in recreational fishing and as food in aquaculture. Individuals of this species are dug for sale as bait from intertidal mudflats of Europe (Bellan, 1964; Ansoloni et al., 1986). Worms are collected by professional bait diggers for ad hoc commercial sale and distributed live. In order to alleviate environmental pressure caused by the excess of demand over the optimal sustainable yield of bait fisheries, intensive aquaculture has been proposed (Scaps, 1992; Marty, 1997). At present, in the laboratory, it is possible to accelerate the maturity of individuals to induce their reproduction and to produce in a few months' individuals of commercial size (Scaps, 2002). In consequence, it is already imaginable to put in service a pilot of aquaculture in order to produce individuals in great quantities. Moreover, these omnivorous sediment-dwelling organisms are considered key species due to the remobilization of contaminants and nutrients linked to their burrowing and dietary behaviour (Amiard et al., 2007; Durou et al., 2007; Gillet et al., 2008). This confers to *H. diversicolor* the capacity to act as bioindicator species of estuarine environmental quality. In particular, this species has been commonly used to assess pollution impacts due to metals (Moreira et al., 2006; Burlinson et al., 2007; Pook et al., 2009; Bouraoui et al., 2010; Bouraoui et al., 2016; Freitas et al., 2017), polycyclic aromatic hydrocarbons (Sun et al., 2008; Catalano et al., 2012), pharmaceuticals (Maranho et al., 2014; Pires et al., 2016a) and more recently to ENPs (Cong et al., 2011; Matranga et al., 2012; Buffet et al., 2014a; b; Cong et al., 2014; Moschino et al., 2014; Thit et al., 2015; Bour et al., 2015; De Marchi et al., 2017d; De Marchi et al., 2018b).

1.3.3. *Diopatra neapolitana* (Delle Chiaje, 1841)

1.3.3.1. Distribution and habitat

Diopatra neapolitana (Delle Chiaje, 1841), is a genus of polychaete worms in the family Onuphidae native to Indo-Pacific, Northeast Atlantic and the Mediterranean (Figure 8), inhabiting the intertidal mudflats of estuaries and shallow water bodies.

Figure 8. Global distribution of *Diopatra neapolitana* (https://www.marlin.ac.uk/species/detail/1426: modified by Lucia De Marchi).

1.3.3.2. Description

Figure 9. *Diopatra neapolitana.*

D. neapolitana is with an approximate 30-35 cm length, which presents green coloration and 15-16 rings in the ceratophores. The species inhabits a tube, has a preference for sediments with mud or a mixture of mud and sand, and grows to about 60 cm (Pires et al., 2012a, b).The tube consists of an inner lining secreted by its inhabitant, and an outer layer of foreign particles like sand grains, fragments of hard parts from other animals, or plants (Cunha et al., 2005). The worm's tube is a food-catching tool that creates a small micro-reef where small invertebrate prey resides. *Diopatra* sp. dart partially out of the tube and grasp the prey with their maxillae and mandibles. Their large anterior parapodia help them to immobilize the prey (Wehe and Fiege, 2002). Moreover, among polychaetes, some *Diopatra* sp. are capable to regenerate anterior segments and prostomial

structures (including *D. sugokai*, *D. tuberculantennata*, *D. cuprea*, and *D. micrura*), posterior ends (*D. aciculata*) and the anterior and posterior regions (*D. dexiognatha*, *D. neapolitana*, *D. marocensis*) (Freitas et al., 2015c).

1.3.3.3. Potential Uses

This species plays important ecological roles: i) by constituting a food source for populations of marine biota such as some species of birds and fishes (Rangel and Santos, 2009); ii) as an ecosystem engineer by stabilising the sediment with its tubes, and thus increasing the structural complexity and biodiversity of its habitat (Bailey-Brock, 1984; Thomsen et al., 2011); iii) by providing refugia from disturbance and predation (Bailey-Brock, 1984) and iv) facilitating the settlement and the attachment of some algal species (Thomsen and McGlathery, 2005). Moreover, *D. neapolitana* is commonly used as fresh bait by sport and professional fishers to catch several important demersal fishes. Only the anterior part of the body (approximately 10 cm) is collected and utilised as bait. Digging activity to collect bait for recreational or professional purposes is widespread and has attained commercial significance in many parts of the world (Cunha et al., 2005). *D. neapolitana* is also a good bioindicator of metal contamination (Freitas et al., 2012; Coppola et al., 2016; Pires et al., 2017) organic matter enrichment (Carregosa et al., 2014), pharmaceuticals (Pires et al., 2016b; Freitas et al., 2015b), salinity shifts and pH decrease (Pires et al., 2015; Freitas et al., 2016b) and recently ENPs (De Marchi et al., 2017d, e).

1.4. Biomarkers

The term ‘biomarker’ is generally used in a broad sense to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO, 1993). A pollutant stress situation normally triggers a cascade of biological responses, each of which may, in theory, serve as a biomarker (McCarthy et al., 1991). Above a certain threshold (in pollutant dose or exposure time) the pollutant-responsive biomarker signals deviate from the normal range in an unstressed situation, finally leading to the manifestation of a multiple effect situation at higher hierarchical levels of biological organisation. The sequential order of responses to pollutant stress within a biological system, from the molecular to the ecosystem level (Bayne et al., 1985), has triggered the research to establish early-warning signals reflecting the adverse biological responses towards anthropogenic environmental toxins (Bucheli and Fent, 1995). When a contaminant first infects an organism, the primary effects are felt at the biochemical and biomolecular level (enzymes, DNA), only later can be noticed at higher levels of organisation. Biomarker usually is a cellular, tissue, body fluid, physiological or biochemical change that normally can be quantified (van der Oost et al., 2003). They have been classified as biomarkers of exposure to a toxicant, biomarkers of effects of exposure, or biomarkers of susceptibility to the effects of exposure (Peakall and Shugart, 1993). This definition has been challenged by several authors (Adams, 1990; McCarty and Munkittrick, 1996; Engel and Vaughan, 1996) and the term biomarker is now more commonly used in a more restrictive sense, namely biochemical sublethal changes resulting from individual exposure to xenobiotics (Hyne et al., 2003). Biomarkers used in environmental monitoring can be divided into categories according to the response at the level of biological organization (Fossi, 1998):

- DNA damage
- Changes in protein
- Changes in metabolic products
- Immune system disorders
- Histopathological abnormalities

Some contaminants have the ability to damage DNA, causing a series of alterations which cascade into changing the genetic material (double helix breaking, fragmentation of chromosomes) until a mutation occurs which can change the gene functionality (Shugart, 1995). The contact with a pollutant can, also, originate induction or inhibition of protein activity in an organism. The mechanisms involved can be protective, in order to proceed in the xenobiotic detoxification, some of the defence against heavy metals (metallothioneins), and others of inhibition (McCarthy et al., 1991). Some classes of pollutants can interfere with the normal metabolism of endogenous compounds and cause accumulation of intermediate products. Contaminants can also affect the immune system. Since it has the ability to neutralize foreign materials and to defend the body from

pathogens it is a good indicator of organism health. Phagocytosis activity and cytotoxic activity are examples of the biomarkers used. At last, histopathological abnormalities can occur, since the final stage of the toxic effect of many pollutant compounds can involve some target organs. It assesses the response to acute and chronic effects induced by contaminants (McCarthy et al., 1991).

1.4.1. Biomarkers and ENPs

Dissolution and release of ions from the particles, oxidative stress and cell injury in proteins, membrane and DNA damage are the major modes of action of ENPs in invertebrates. Moreover, alteration in terms of energy reserves and metabolism as well as neurotoxicity was also demonstrated as mechanisms of action by ENPs (see Rocha et al., 2015).

1.4.1.1. Energy reserves and metabolism

Energy metabolism plays a fundamental role in organisms' survival and function, as well as in stress adaptation and tolerance (Sokolova et al., 2012) and the controlled use of energy is an essential prerequisite of all life. Energy must be extracted from fuel molecules and preserved in a form that is readily available for mechanical and chemical work. Fuel molecules are highly reduced compounds that release energy when oxidized, and the purpose of energy metabolism is to provide ATP, the universal currency of energy. In addition, reduced electron carriers are provided for the biosynthesis of complex, reduced molecules (Haunerland et al., 2003). Therefore, all animals feed on various nutrients that are then digested and oxidized. This catabolism occurs in three stages. Initially, complex polymeric precursors are broken down to monomeric fuel molecules. These are transformed in the next stage into central metabolic intermediates, which in turn are oxidized in the mitochondria. Biological oxidations require the electron acceptors nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD). The reduced coenzymes NADH and $FADH_2$ are converted back to NAD^+ and $FADH_2$ when their electrons are transferred, via the electron transport chain or electron transport system (ETS), on to molecular oxygen. These redox processes lead to the establishment of an electrochemical gradient across the inner mitochondrial membrane, which provides the energy to phosphorylate adenosine diphosphate (ADP) and to produce adenosine triphosphate (ATP) (Haunerland et al., 2003). Carbohydrates (such as glycogen (GLY)), lipids (LIP), and proteins (PROT) are the major nutrients that fuel energy metabolism. The different properties and fates of these nutrients give them distinct roles in energy metabolism. However, metabolic rates, i.e. the rates by which ATP is produced and used, vary widely among and within invertebrate species and if they are subjected to long periods of anaerobiosis (Haunerland et al., 2003). Figures 10 and 11 illustrate aerobic and anaerobic energy metabolism.

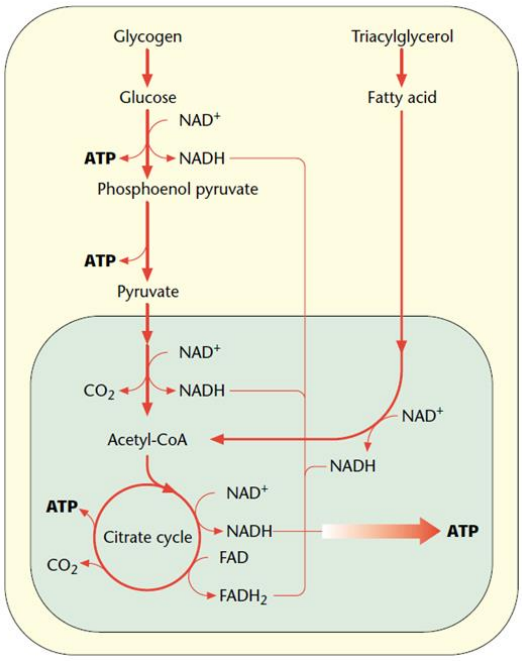

Figure 10. Aerobic energy metabolism. Glycogen and triacyglycerol are broken down in the cytosol to glucose and free fatty acid. In the glycolytic pathway, glucose is oxidized to pyruvate. In the mitochondria, pyruvate is oxidized to acetyl coenzyme A (acetyl-CoA), also the end product of b-oxidation of fatty acids. Acetyl-CoA is completely oxidized in the citrate cycle. The energy released during the transport of electrons from NADH or $FADH_2$ along the electron transport chain (bold arrow) is used for the synthesis of ATP (retrieved from Haunerland et al., 2003).

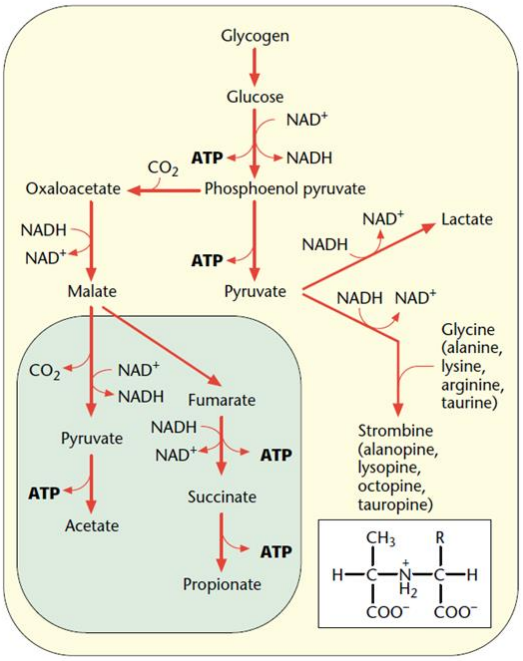

Figure 11. Anaerobic metabolism in invertebrates. In the absence of oxygen, pyruvate is reduced to lactate or to an opine, which is formed in a reaction of amino acid and pyruvate (see boxed structure). Alternatively, phosphoenolpyruvate reacts with carbon dioxide to oxaloacetate, which in turn is reduced to malate. Malate is

both oxidized to pyruvate, and reduced to fumarate. Further reactions produce additional ATP and lead to various end products (retrieved from Haunerland et al., 2003).

1.4.1.2. Oxidative stress and cellular damage

The best-developed paradigm to explain most of the cytotoxic effects exerted by ENPs is directly or indirectly mediated by reactive oxygen species (ROS) and free radicals' production (Rocha et al., 2015). ROS are naturally produced during several cellular pathways of aerobic metabolism including oxidative phosphorylation, electron transport chains in mitochondria and microsomes, the activity of oxido-reductase enzymes producing ROS as intermediates or final products, or even immunological reactions such as active phagocytosis (Halliwell and Gutteridge 2007). The main ROS generated by cellular metabolism are the singlet oxygen O_2, the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO•); these compounds can rapidly react to form other molecules like peroxynitrite (HOONO), hypochloric acid (HOCl), peroxyl radicals (ROO•) and alkoxyl radicals (RO•), to cite a few (Regoli and Giuliani, 2014). Under basal conditions, the adverse effects of oxyradicals are prevented by the antioxidant system, consisting of a wide array of low molecular weight scavengers and antioxidant enzymes which interact in a network with both direct and indirect effects. Scavengers neutralize ROS by direct reaction with them, thus being temporarily oxidized before being reconverted by specific reductases to the active form. Scavengers can act as antioxidants in the cytoplasm or are intended to arrest the propagation of lipid peroxidation (LPO) reactions on the membranes. The most abundant cytosolic scavenger is reduced glutathione (GSH), a tripeptide (γ- glutamil-cysteinyl glycine), which directly neutralizes several reactive species through its oxidation to oxidized glutathione (GSSG); in addition, GSH acts as a cofactor of several antioxidant glutathione-dependent enzymes. Compared to scavengers, which interact with more than one type of ROS, enzymatic antioxidants catalyze highly specific reactions with specific substrates. Superoxide dismutase (SOD) represents a source of H_2O_2, being thus necessary that its activity is coordinated with that of H_2O_2 reducing enzymes, like catalase (CAT) or glutathione peroxidases (GPx) (Figure 12) (Regoli and Giuliani, 2014). Mostly present within peroxisomes, CAT is an extremely active catalyst for reduction of H_2O_2 to H_2O (Halliwell and Gutteridge, 2007). H_2O_2 is substrate also for GPx, using GSH as electron donor to catalyze the reduction of H_2O_2 to H_2O (Figure 12). Moreover, GPx and some isoforms of glutathione S-transferases (GSTs), reduce lipid hydroperoxides to alcohol, with the concomitant oxidation of GSH to GSSG (Regoli and Giuliani, 2014). Oxidized glutathione is then reconverted to GSH by glutathione reductase (GR) which, despite not a real antioxidant enzyme, is nonetheless essential to maintain the correct GSH/GSSG ratio and the intracellular redox status in marine organisms (Regoli and Giuliani, 2014) (Figure 12).

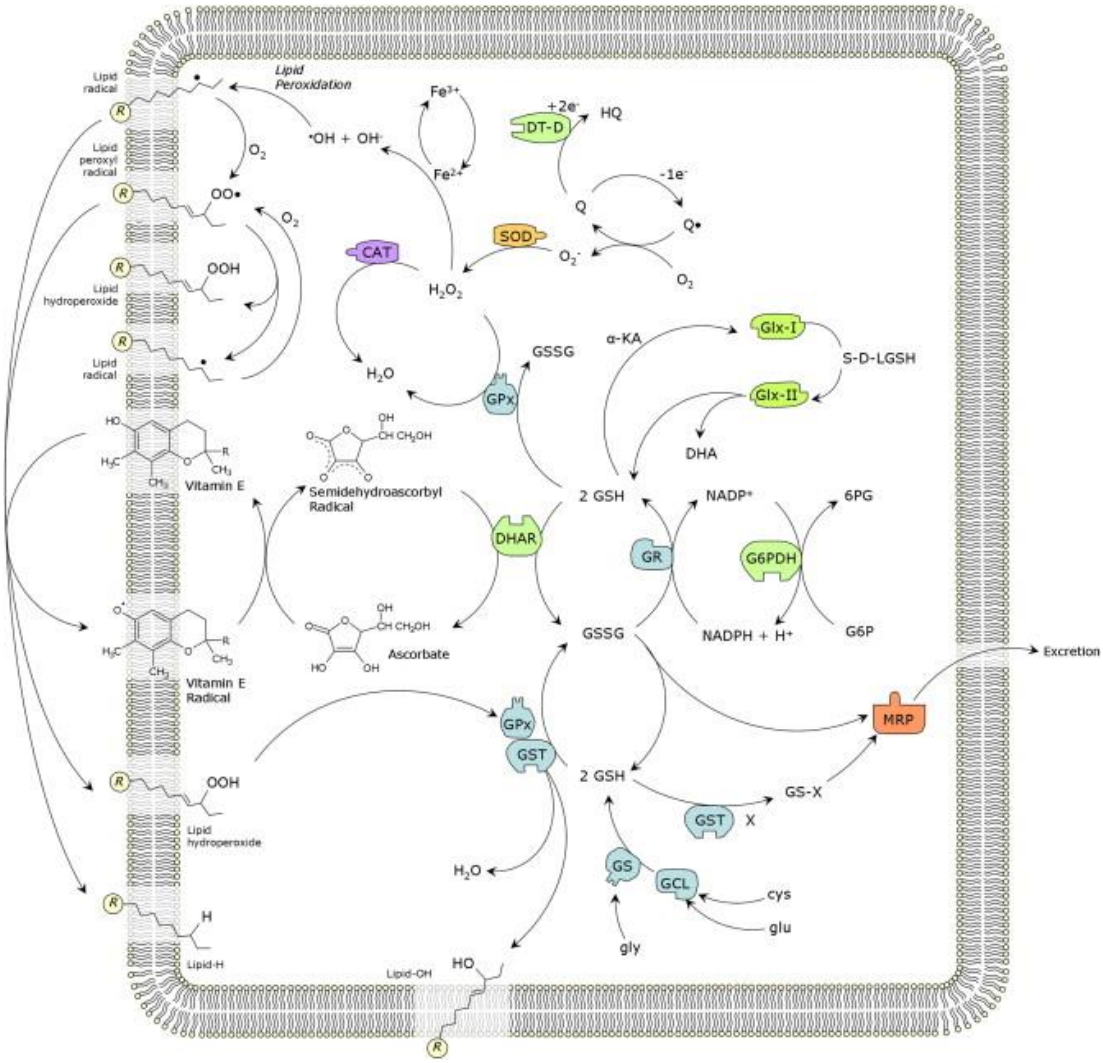

Figure 12. Main cellular antioxidant defences and antioxidant pathways (arranged in alphabetical order): 6PG: 6-phospogluconate; CAT: catalase; cys: cysteine; DHA: d-hydroxy acid; DHAR: dehydroascorbate reductase; DT-D: DT-diaphorase; G6P: glucose 6-phosphate; G6PDH: glucose 6-phosphate dehydrogenase; GCL: γ-glutamylcysteine synthetase; Glx-I: glyoxalase I; Glx-II: glyoxalase II; GPx: glutathione peroxidases; GR: glutathione reductase; gly: glycine; glu: glutamia acid; GS: glutathione synthetase; GSH: reduced glutathione; GSSG: oxidised glutathione; GST: glutathione S-transferases; GS-X: GSH conjugated xenobiotic; HQ: hydroquinone; αKA: α-keto aldehydes; MRP: multidrug resistance-related protein; Q: quinone; Q·: semiquinone radica; S-D904 LGSH: S-D-Lactoylglutathione; SOD: superoxide dismutase; X: xenobiotic (retrieved from Regoli and Giuliani, 2014).

1.4.1.3. Neurotoxicity

Among the biomarkers evaluated to date, there is a lot of interest in cholinesterase (ChE) activity as an indicator of the biological effects of exposure to neurotoxic compounds in aquatic organisms (Bocquené and Galgani, 1991). ChEs are a family of enzymes that includes acetylcholinesterase (AChE) or true cholinesterase and pseudocholinesterases (PsChE). AChE plays an important role in neurotransmission in both vertebrates and invertebrates, being responsible

for the hydrolysis of acetylcholine into choline and acetic acid at the cholinergic synapses and neuromuscular junctions (Figure 13). PsChE seems to have no specific natural substrates and has been proposed as a scavenging enzyme for certain classes of toxic compound (Massoulié et al., 2008). Focusing on AChE, the inhibitors or anti-cholinesterases inhibit the cholinesterase enzyme from breaking down ACh, increasing both the level and duration of the neurotransmitter action. According to the mode of action, AChE inhibitors can be divided into two groups: irreversible and reversible. Reversible inhibitors, competitive or non-competitive, mostly have therapeutic applications, while toxic effects are associated with irreversible AChE activity modulators (Colovic et al., 2013).

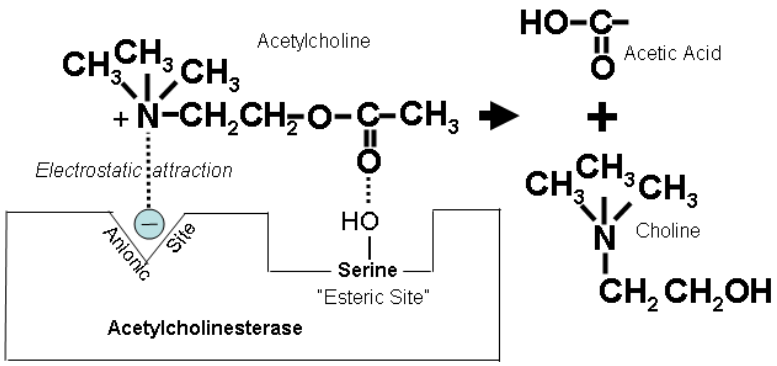

Figure 13. Breakdown of acetylcholine (retrieved from Agency for Toxic Substances and Disease Registry).

1.5. Thesis objectives

The main goal of the present thesis was to better understand how the presence of two different carbon nanotubes (CNTs) materials affect three different invertebrate species (the bivalve *Ruditapes philippinarum* and two polychaetes species *Hediste diversicolor* and *Diopatra neapolitana*) under different climate change scenarios (salinity and pH variations). To this end, this proposal was focused on the following main objectives:

1. Understand if the modification of CNTs surface caused toxic effects in all invertebrate species in terms of physiological (regenerative capacity) and biochemical (energy reserves and metabolisms, oxidative and neuro status) responses. For this purpose, the organisms were exposed for 28 days to environmental relevant concentrations of pristine multi-walled carbon nanotubes (MWCNTs) and chemically functionalized MWCNTs, by introducing carboxyl groups (-COOH) (f-MWCNTs) evaluating:
    I) the effects of exposure concentrations of both MWCNTs
    II) the effects of the carboxylation of the surface of the MWCNTs for each exposure concentration.
2. Understand if the combination of both MWCNTs and different salinity levels caused toxic effects in all invertebrate species both in terms of physiological al biochemical responses. For this purpose, the two most deleterious concentrations of both MWCNTs detected in the previous section were used in combination with two different salinity levels assessing:
    I) the effects of exposure concentrations of both MWCNTs maintained under both salinities
    II) the effects of different salinity levels in organisms exposed to both materials in each exposure concentration
    III) the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both salinities for each exposure concentration.
3. Understand if the combination of both CNTs and pH variations caused toxic effects in all invertebrate species both in terms of physiological al biochemical responses. For this purpose, the two most deleterious concentrations of both MWCNTs detected in the previous section were used in combination with two different pH levels assessing:
    I) the effects of exposure concentrations of both MWCNTs maintained under both pH levels
    II) the effects of pH variations in organisms exposed to both materials in each exposure concentration
    III) the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both pH levels for each exposure concentration.

CHAPTER 2. MATERIAL AND METHODS

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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. *Aquatic Toxicology*, 199, 199–211.

De Marchi, L., Neto, V., Pretti, C., Chiellini, F., Morelli, A., Soares, A.M.V.M., Figueira, E. & Freitas, R. (2018b). Does the exposure to salinity variations and water dispersible carbon nanotubes induce oxidative stress in *Hediste diversicolor*? *Marine Environmental Research*, 141, 186-195.

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2.1. Contaminants

2.1.1. Contaminants description

The Carbon Nanotubes (CNTs) used in the present thesis corresponded to two types of Multi Walled Carbon Nanotubes (MWCNTs): pristine MWCNTs (Nf-MWCNTs) (MWCNTs: NC7000 series, http://www.nanocyl.com) and chemically functionalized MWCNTs, by introducing polar groups such as carboxyl groups (-COOH) (f-MWCNTs) (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com). All the technical data of both materials are specified in Table 1.

Table 1. Characterization of the powder form of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs).

	Diameter (nm)	**Length (um)**	**Carbon Purity (%)**	**Surface Area (m^2/g)**	**Amorphous Carbon (mol%)**	**-COOH (wt%)**
Nf-MWCNTs	9.5	1.5	90	250-300	*	-
f-MWCNTs	2-5	10-30	98	400	8-10	3.86

* Pyrolytically deposited carbon on the surface of MWCNTs

The selection of these two CNTs was based on:

a. Wide industrial applicability

Three main properties of MWCNTs are specifically interesting for the industry: the electrical conductivity (as conductive as copper), their mechanical strength (up to 15 to 20 times stronger than steel and 5 times lighter) and their thermal conductivity (same as that of diamond and more than five times that of copper). A combination of these impressive properties enables a whole new variety of useful and beneficial applications (Li et al., 2011). In detail, f-MWCNTs are used as additives in polymers, catalysts electron field emitters for cathode ray lighting elements, flat panel display, gas-discharge tubes in telecom networks, electromagnetic-wave absorption and shielding, energy conversion, lithium-battery anodes, nanotube composites, nanoprobes, nanolithography, nanoelectrodes, drug delivery sensors reinforcements in composites and supercapacitor (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com). The Nf-MWCNTs are used in a different markets such as transportation (Automotive, Aeronautic, Boats), electronics (Electronic packaging, EMI-shielding, sensors), energy (Lithium-ion), industrial applications (Oil&Gas, dynamic rubber parts, coatings, heating elements) and sports goods (http://www.nanocyl.com/product/nc7000/).

b. Different physical and chemical properties and different behaviour on the water media

Carboxylated CNTs (f-MWCNTs) are more stable in salt water media in comparison to pristine CNTs as a consequence of their oxidation process which introduces oxygen-containing groups on the CNTs surface. These groups ionize in water charging the oxygen atoms negatively in the aqueous phase and the electrostatic repulsive forces between negative surface charges of the oxygen-containing groups can lead to stability of oxidized CNTs in the water column increasing the availability of these materials for the organisms (Peng et al., 2009).

c. Safety testing and risk assessment

The Organization for Economic Co-operation and Development (OECD)'s Working Party on Manufactured Nanomaterials (WPMN) launched a Sponsorship Programme for the Testing of Manufactured Nanomaterials (OECD, 2010) which promotes international co-operation on the human health and environmental safety of manufactured nanomaterials and involves the safety testing and risk assessment of ENPs. The OECD WPMN has published a list of ENPs selected considering their commercial use, the production volume of the materials, availability of such materials for testing and the existing information that would readily be available on the materials and this list comprised CNTs.

The exposure concentrations of both MWCNT were selected considering the PECs (0.001-1000 µg/L) of CNTs in aquatic systems (Zhang et al., 2017).

2.2. Study area

The Ria de Aveiro is a coastal lagoon (Northwest of Portugal) with four main channels which radiate from the ocean mouth with several branches, islands and mudflats (Figure 14). Tides are semi-diurnal and constitute the main forcing water circulation agent. The minimum and maximum tidal height ranges are about 0.6 m and 3.2 m at neap and spring tides, respectively (Dias et al., 2000). The most important freshwater input (the Vouga River) of the Ria de Aveiro flows through the Espinheiro channel, that is about 17 km long and is characterized by a strong horizontal gradient of salinity and water temperature which migrates back and forth with the spring/neap cycle (Vaz et al., 2005; Lillebø et al., 2015). The other freshwater sources are smaller, namely the Boco, Mira (sampling area) and Cáster rivers, the latter flowing through the 29 km long São Jacinto – Ovar channel (Figure 14).

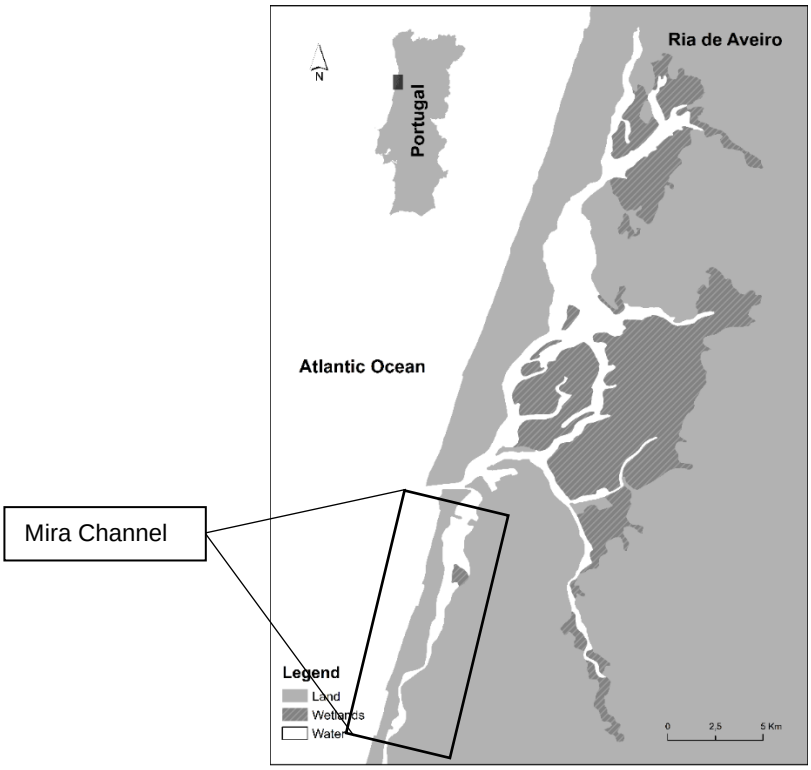

Figure 14. The Ria de Aveiro coastal lagoon (Portugal) indicating the sampling area (Mira Channel) in the northwest Atlantic coast of Portugal (40º38' N, 8º45' W).

2.3. Sampling and laboratory conditions

2.3.1. *Ruditapes philippinarum* (Adams & Reeve, 1850)

Ruditapes philippinarum specimens were collected in Mira Channel (Figure 14). Bivalves with similar size (mean length and weight of clams were 32.3 ± 0.19 mm and 19.2 ± 2 g, respectively) were used to prevent differences on organisms' CNTs accumulation and biochemical responses. For 7 days, the collected clams were placed in different aquaria (20 L each) for depuration and acclimation to laboratory conditions. Artificial seawater (salinity 28) was made by the addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water, one day prior to utilization. The temperature was kept at 18 ± 1 ºC, with a photoperiod of 12 h light: 12 h dark, and pH 8.0 ± 1 with constant aeration. Every two-three days' specimens were fed with AlgaMac Protein Plus, Aquafauna Bio-Marine, Inc (150,000 cells/animal).

2.3.2. *Hediste diversicolor* (O.F. Müller, 1776)

Hediste diversicolor was collected in Mira Channel (Figure 14). Specimens with similar weight (0.53 ± 0.2 g) were used to prevent differences in biochemical responses. Upon arrival, organisms were allowed to acclimate progressively (2 weeks) in different aquaria (20 L each; 50 specimens *per* aquarium). Each aquarium was filled with a mixture of fine and medium sediment from the sampling area. The sediment grain size was analysed by wet and dry sieving, following the procedure described in Quintino et al. (1989). The silt and clay fraction (fine particles, with diameter below 0.063 mm) and the gravel fraction (particles with diameter above 2.000 mm), were expressed as a percentage of the total sediment (sediment median value 1.59; percentage (%) of fine 6.75±0.79; percentage (%) of organic matter content 3.24±0.44). Artificial seawater (salinity 28) was made by the addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water, one day prior to utilization. The temperature was kept at 18 ± 1 ºC, with a photoperiod of 12 h light: 12 h dark, and pH 8.0 ± 1 with constant aeration. During this period, specimens were fed *ad libitum* with commercial fish food every two-three days (48.6% protein and 7.7% fat).

2.3.3. *Diopatra neapolitana* (Delle Chiaje, 1841)

Diopatra neapolitana were collected in Mira Channel (Figure 14). In the laboratory, organisms were pushed out from their tubes and placed in different aquaria (20 L each) for the acclimation period (20 days). The aquaria were filled with a mixture of fine and medium sediment from the sampling area (see sediment details in section 2.3.2.). Artificial seawater with the salinity 28 was used by the addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water, one day prior

to utilization. Temperature was kept to 18 ± 1 ºC, photoperiod of 12 h light: 12 h dark, pH 8.0 ± 1 and constant aeration. During this period every two-three days the specimens were fed ad libitum with small fragments of frozen cockles or mussels (Pires et al., 2012a). To assess the impact of different CNT materials on the regenerative capacity of *D. neapolitana,* immediately before the exposure assay individuals were removed from their new tubes, anaesthetized with a 4% $MgCl_2.6H_2O$ solution, and amputated at the 60^{th} chaetiger under a stereomicroscope (Pires et al., 2012a).

2.4. CNTs characterization analyses

2.4.1. Water media

In all the experiment conducted, immediately before the water renewal, water samples (50 mL) *per* replicate were collected to measure the average size distribution by dynamic light scattering (DLS) and the polydispersity index (PDI) of both MWCNT materials suspended in artificial seawater at different exposure conditions and exposure times (T0: time zero, immediately after the dispersion of the materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure). DLS measurements were carried out to obtain data regarding the tendency to aggregate and the settling behaviour of suspended CNT materials in aqueous media. Measurements were performed on 1000 µL of suspension in four samples *per* replicate (three replicates *per* condition), and five analyses per sample performed by DLS using a DelsaTM NanoC Particle Size Analyzer (Beckman Coulter). Each analysis was carried out by performing 120 acquisitions. Due to the inherent heterogeneity and colloidal instability of the analysed samples, DLS analyses were repeated several times to ensure reproducible results. Size distributions were obtained by analysing the autocorrelation functions through the Contin algorithm which is particularly appropriate for polydisperse and multimodal systems (Varenne et al., 2016). The cumulant method was used to obtain information on the particle's average hydrodynamic radii and on the PDI (Tardani and Mesa, 2015).

2.4.2. Sediment matrix

Thermogravimetric analysis (TGA) has been used as an innovative method to assess the presence of pristine MWCNTs (Nf-MWCNTs) aggregates in the sediments exposed to CNTs dispersions. To the best of our knowledge, no reports describing the use of TGA for such purpose are reported in the literature. TGA may represent an effective method to detect the presence of CNTs in the sediments since it records the weight loss of materials upon heating and can distinguish the contribution given by each component of a mixture by applying the derivative operation to the thermogravimetric curve (DTGA analysis). To this aim, the degradation behaviour of Nf-MWCNTs is unique and might be easily distinguished from that of both inorganic and organic background (Lehman et al., 2011). The detection of Nf-MWCNTs in sediments has been carried out on 50 mg samples by thermogravimetric analysis (TGA) by using a Mettler Toledo Star-system TGA/SDTA 851e apparatus, with air as the purge gas (60 mL/min) at a scan rate of 10°C/min. Samples were conditioned for 90 min. at 150° C prior to analysis in order to remove water. TGA analysis was not performed for sediment samples contaminated with f-MWCNTs due to the need to develop and

optimize *ad hoc* protocols for its use in this type of investigation, with particular reference to the selection and preparation of the samples / matrix that has to be analysed.

2.5. Exposure experiments

2.5.1. Single stressor experiments: CNTs exposure

Ruditapes philippinarum: 15 organisms for each condition (3 aquaria/replicates *per* condition, with 5 organisms *per* aquarium/replica). Each aquarium (10 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water).

Hediste diversicolor: 30 organisms for each condition (3 aquaria/replicates *per* condition, with 10 organisms *per* aquarium/replica). Each aquarium (20 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water) and sediment from the sampling area approximately 1/3 of the height of the aquarium (sediment median value 1.59 g; percentage (%) of fine 6.75±0.79; percentage (%) of organic matter content 3.24±0.44).

Diopatra neapolitana: 27 organisms for each condition (3 aquaria/replicates *per* condition, with 9 organisms *per* aquarium/replica). Each aquarium (20 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water) and sediment from the sampling area approximately 1/3 of the height of the aquarium (sediment median value 1.59 g; percentage (%) of fine 6.75±0.79; percentage (%) of organic matter content 3.24±0.44).

After the acclimation or depuration period, organisms were exposed during 28 days to two types of CNTs: pristine multi-walled carbon nanotubes (MWCNTs) (Nf-MWCNTs) and carboxylated MWCNTs (f-MWCNTs), both at the concentrations of 0.001; 0.01 and 0.10 mg/L. Food, Salinity, pH, temperature and aeration conditions in each aquarium were set up as in the acclimation period (see section 2.3.). Both materials were sonicated using a Hz ultrasound bath (IKA Labortechnik IKASONIC U50). The difference was only the time of sonication to promote the dispersion of the materials (1 h for Nf-MWCNTs, while the f-MWCNTs, due to the presence of carboxyl groups (Shahnawaz et al., 2010), was sonicated for few minutes). Both CNTs were weighed (stock solution of 50 mg/L), suspended in seawater and re-established weekly after complete water renewals to ensure the same exposure concentrations during the experiment. The added MWCNTs (f and Nf) were homogenously dispersed in the seawater using one submersible circulation pump *per* aquarium, increasing both CNTs mass suspended in the water column (Vonk et al., 2009).

2.5.2. Combination of stressors experiments: CNTs and salinity shifts

Ruditapes philippinarum: 15 organisms for each condition (3 aquaria/replicates *per* condition, with 5 organisms *per* aquarium/replica). Each aquarium (10 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water).

Hediste diversicolor: 30 organisms for each condition (3 aquaria/replicates *per* condition, with 10 organisms *per* aquarium/replica). Each aquarium (20 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water) and sediment from the sampling area approximately 1/3 of the height of the aquarium (sediment median value 1.59; percentage (%) of fine 6.75±0.79; percentage (%) of organic matter content 3.24±0.44).

Diopatra neapolitana: 27 organisms for each condition (3 aquaria/replicates *per* condition, with 9 organisms *per* aquarium/replica). Each aquarium (20 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water) and sediment from the sampling area and sediment from the sampling area approximately 1/3 of the height of the aquarium (sediment median value 1.59; percentage (%) of fine 6.75±0.79; percentage (%) of organic matter content 3.24±0.44).

After the acclimation period, organisms were exposed for 28 days to two different salinities (21-low and 28-control), each one combined with two different concentrations (0.01 and 0.10 mg/L) of both MWCNT materials (f-MWCNTs and Nf-MWCNTs). Prior to experiment initiation, the salinity was progressively decreased (2 units) every 2 days until the testing value was reached (salinity 21) while the other parameters (pH, temperature and aeration conditions) in each aquarium were set up as in the acclimation period (see section 2.3.). The two salinity levels were chosen to take in consideration i) the seasonal mean of salinity of the sampling area (control-salinity 28) (Santos et al., 2007; http://www.ipma.pt/pt/index.html); ii) extreme weather events such as the increases in fresh water runoff induced by global warming (IPCC, 2014), which caused negative salinity anomalies (i.e. a surface salinity that is less than salinity measured at depth of a few meters) at the ocean surface (Asher et al., 2014) (low-salinity 21).

2.5.3. Combination of stressors experiments: CNTs and pH variation

Ruditapes philippinarum: 15 organisms for each condition (3 aquaria/replicates *per* condition, with 5 organisms *per* aquarium/replica). Each aquarium (10 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water).

Hediste diversicolor: 30 organisms for each condition (3 aquaria/replicates *per* condition, with 10 organisms *per* aquarium/replica). Each aquarium (20 L) was filled with artificial seawater (addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water) and sediment from the sampling area approximately 1/3 of the height of the aquarium (sediment median value 1.59 g; percentage (%) of fine 6.75±0.79; percentage (%) of organic matter content 3.24±0.44).

Diopatra neapolitana: 27 organisms for each condition (3 aquaria/replicates *per* condition, with 9 organisms *per* aquarium/replica). Each aquarium (20 L) was filled with artificial seawater

(addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water) and sediment from the sampling area approximately 1/3 of the height of the aquarium (sediment median value 1.59 g; percentage (%) of fine 6.75±0.79; percentage (%) of organic matter content 3.24±0.44).

After the acclimation period, organisms were exposed for 28 days to two different pH levels (7.6-acidify and 8.0-control) each one combined with two different concentrations (0.01 and 0.10 mg/L) of both MWCNT materials (f-MWCNTs and Nf-MWCNTs). Lowered pH was set to 7.6 to give a 0.4 pH units' reduction relative to control (pH 8.0) and it was selected considering the predicted scenario of climate change for 2100 (IPCC, 2014). Low pH exposure was obtained by directly and automatically diffusing CO_2 into aquaria. Individual aquarium pH levels were continuously monitored and controlled using a pH-Stat system (Aquamedic). Immediately before the starting of the experiment, the acidified pH was progressively decreased to avoid additional osmotic stress to the organisms. During the exposure period, every week water was renewed and pH conditions re-established, with acidified water previously prepared for the low pH conditions. Immediately after and before water renewal water samples (50 mL) were collected for the physicochemical parameters of the used water (*R. philippinarum*: Table 2; *H. diversicolor*: Table 3; *D. neapolitana*: Table 4).

Table 2. *Ruditapes philippinarum*: carbonate system physicochemical parameters for pH experiments (mean±SD; n=3). Measured pH and determined total alkalinity (TA) from weekly water sampling of pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH 8.0 and acidify pH 7.6. Partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-), carbonate ion concentrations (CO_3^{2-}), saturation states of calcite (ΩCal) and aragonite (ΩAg), calculated with CO2SYS software.

CNTs (mg/L)	pH level		pH	TA (µmol/kg)	pCO_2 (µatm)	HCO^{3-} (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	ΩCal	ΩAra
0.00	8.0	Nf	7.99±0.02	3032.29±51.74	913.32±73.25	2710.64±54.13	137.88±8.22	3.41±0.20	2.17±0.13
		f	8.11±0.06	2053.47±217.79	453.93±101.93	1753.97±205.77	120.42±12.06	2.99±0.31	1.90±0.20
	7.6	Nf	7.60±0.01	3107.63±84.93	2384.18±132.82	2967.90±86.36	60.02±1.01	1.49±0.02	0.94±0.02
		f	6.62±0.01	2055.97±99.28	1542.64±50.85	1947.86±91.15	43.45±3.87	1.08±0.09	0.69±0.06
0.01	8.0	Nf	7.97±0.03	2946.88±37.26	902.96±52.41	2649.41±34.35	126.88±7.18	3.14±0.18	1.99±0.11
		f	8.10±0.07	1780.17±108.17	399.12±77.31	1517.59±105.17	102.50±11.52	2.54±0.29	1.62±0.18
	7.6	Nf	7.61±0.02	3177.50±17.89	2356.49±123.92	3033.10±111.55	62.14±4.68	1.54±0.12	0.97±0.07
		f	7.62±0.02	2236.50±129.48	1660.19±151.52	2118.97±123.71	47.97±2.84	1.19±0.06	0.76±0.04
0.10	8.0	Nf	7.98±0.02	2925.01±69.83	917.52±88.66	2629.00±75.04	127.53±6.80	3.25±0.23	2.05±0.13
		f	8.08±0.06	1955.68±338.72	466.04±153.30	1683.07±326.60	108.28±8.84	2.68±0.21	1.71±0.13
	7.6	Nf	7.60±0.02	3221.92±72.33	2444.67±132.18	3079.19±68.96	61.44±3.70	1.52±0.09	0.96±0.06
		f	7.62±0.01	2096.09±53.70	1542.33±29.16	1983.75±44.46	49.05±5.64	1.12±0.10	0.72±0.06

Table 3. *Hediste diversicolor:* Carbonate system physicochemical parameters for pH experiments (mean±SD; n=3). Measured pH and determined total alkalinity (TA) from weekly water sampling of pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH 8.0 and acidify pH 7.6. Partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-), carbonate ion concentrations (CO_3^{2-}), saturation states of calcite (ΩCal) and aragonite (ΩAg), calculated with CO2SYS software.

CNTs (mg/L)	pH level		pH	TA (µmol/kg)	pCO_2 (µatm)	HCO^{3-} (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	ΩCal	ΩAra
0.00	**8.0**	Nf	8.09±0.03	2022.54±20.88	442.53±46.82	1728.88±12.47	116.41±4.43	2.85±0.11	1.82±0.08
		f	8.09±0.03	2022.54±20.88	442.53±46.82	1728.88±12.47	116.41±4.43	2.85±0.11	1.82±0.08
	7.6	Nf	7.61±0.04	1821.96±55.24	1318.91±128.62	1721.08±60.99	39.08±6.69	0.05±0.16	0.61±0.11
		f	7.61±0.04	1821.96±55.24	1318.91±128.62	1721.08±60.99	39.08±6.69	0.05±0.16	0.61±0.11
0.01	**8.0**	Nf	8.08±0.04	2066.00±72.32	471.19±42.54	1779.09±79.73	114.52±11.96	2.81±0.28	1.79±0.19
		f	8.10±0.08	1888.82±57.90	441.08±78.91	1610.90±38.74	108.81±9.63	2.67±0.24	1.70±0.14
	7.6	Nf	7.65±0.02	1608.01±322.39	1225.99±271.34	1521.51±30.1	32.71±9.69	0.80±0.24	0.51±0.16
		f	7.63±0.03	2427.05±53.90	1701.73±16.70	2296.54±40.59	53.62±7.30	1.31±0.18	0.84±0.12
0.10	**8.0**	Nf	8.11±0.01	2045.95±26.53	429.37±47.62	1744.18±28.43	120.36±8.65	2.96±0.22	1.89±0.14
		f	8.12±0.05	2249.87±32.24	463.81±66.34	1914.82±43.44	135.94±11.51	3.33±0.29	2.13±0.19
	7.6	Nf	7.62±0.01	1440.85±32.24	1099.39±60.28	1361.04±24.18	29.19±4.85	0.71±0.12	0.46±0.08
		f	7.63±0.03	2102.78±57.90	1478.93±134.04	1987.70±60.53	46.02±4.86	1.13±0.12	0.72±0.08

Table 4. *Diopatra neapolitana*: Carbonate system physicochemical parameters for pH experiments (mean±SD; n=3). Measured pH and determined total alkalinity (TA) from weekly water sampling of pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH 8.0 and acidify pH 7.6. Partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-), carbonate ion concentrations (CO_3^{2-}), saturation states of calcite (ΩCal) and aragonite (ΩAg), calculated with CO2SYS software.

CNTs (mg/L)	pH level		pH	TA (µmol/kg)	pCO_2 (µatm)	HCO^{3-} (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	ΩCal	ΩAra
0.00	8.0	Nf	8.01±0.01	2011.51±19.81	544.51±36.75	1621.81±22.57	121.49±5.99	2.81±0.11	1.89±0.18
		f	8.01±0.01	2011.51±19.81	544.51±36.75	1621.81±22.57	121.49±5.99	2.81±0.11	1.89±0.18
	7.6	Nf	7.65±0.02	1724.91±35.21	1518.51±98.62	1691.10±40.96	38.12±7.59	0.51±0.10	0.21±0.11
		f	7.59±0.04	2087.21±61.02	1633.43±110.01	1970.43±56.32	46.42±3.69	0.70±0.05	1.11±0.08
0.01	8.0	Nf	8.04±0.02	2061.01±52.30	402.39±54.54	1579.21±72.43	151.42±8.91	2.86±0.21	1.74±0.11
		f	8.03±0.02	2012.50±23.18	542.21±26.48	1699.82±19.40	126.41±4.12	2.99±0.15	1.91±0.11
	7.6	Nf	7.61±0.03	1901.01±121.50	1741.99±102.14	1722.43±101.1	39.77±10.61	0.72±0.23	0.49±0.13
		f	7.59±0.02	2129.32±44.99	1734.32±99.10	2015.75±42.21	46.45±2.21	0.69±0.03	1.10±0.05
0.10	8.0	Nf	8.09±0.02	2051.65±46.51	472.17±57.00	1777.98±36.61	131.20±5.15	2.98±0.12	1.90±0.15
		f	7.99±0.02	2001±58	586.27±21.92	1743.32±49.33	102.81±4.75	2.54±0.12	1.68±0.07
	7.6	Nf	7.63±0.02	1640.81±34.34	1798.50±71.28	1358.12±54.21	31.29±5.81	0.77±0.09	0.49±0.18
		f	7.58±0.02	2027.45±62.99	1654.32±62.54	1920.21±55.43	43.80±3.26	0.74±0.05	1.14±0.08

2.6. Biological analyses

2.6.1. Physiological parameter: regenerative capacity

Only the polychaetes *D. neapolitana* were used to performed physiological analyses under different conditions. Nine *D. neapolitana* specimens *per* condition (3 *per* aquarium) were analysed every week during the experimental period (28 days). During the experiment, organisms for regenerative capacity analysis were inspected at day 11^{th}, 18^{th}, and 28^{th} after amputation. The width of the regenerated body part was measured, and the number of new segments counted. Percentage of regenerated body width was calculated by comparing the width of the new segments with the width of the old segments (Pires et al., 2012a). New segments were identified by the lighter colour and/or the narrower width compared to the old body segments (Pires et al., 2012a).

2.6.2. Biochemical parameters: energy reserves content and metabolic capacity, oxidative status, activity of antioxidant and biotransformation enzymes and neuro status

The individually whole body of frozen organisms (3 *per* aquarium) (*R. philippinarum* and *D. neapolitana)* or three pools of organisms, each corresponding to three whole body individuals (9 individuals in total *per* aquarium, 27 *per* condition) (*H. diversicolor*) were pulverized with liquid nitrogen and divided into 0.5 g fresh weight (FW) aliquots and used for biochemical analyses. Extractions were performed with specific buffers to determine: energy reserves content (protein (PROT) content, glycogen (GLY) content), metabolic capacity (electron transport system (ETS) activity), oxidative status (lipid peroxidation (LPO) levels, reduced (GSH) and oxidized (GSSG) glutathione content), activity of antioxidant (superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx)) and biotransformation (glutathione S-transferases (GSTs) enzymes) and neuro status (Acetylcholinesterase (AChE) activity). Biochemical analyses were performed twice for each sample and parameter. For ETS activity quantification, supernatants were extracted in homogenizing buffer (0.1 M Tris-HCl pH 8.5 with 15% (w/v) PVP, 153 µM magnesium sulphate ($MgSO_4$) and 0.2% (v/v) Triton X-100) in a 1:2 proportion using TissueLyser II set at frequency 20 1/s, during 1.30 s. and then centrifugated at 3000 g at 4ºC, for 20 min. For LPO determination supernatants were extracted using 20% (v/v) trichloroacetic acid (TCA) in a 1:2 proportion using TissueLyser II set at frequency 20 1/s., during 1.30 s. and then centrifugated at 10000 g at 4ºC, for 20 min. GSH and GSSG concentrations were determined in supernatants extracted with 0.6% sulfosalicylic acid in potassium phosphate buffer (0.1 M dipotassium phosphate; 0.1 M potassium dihydrogen phosphate; 5 mM EDTA; 0.1% (v/v) Triton X-100; pH 7.5) in a 1:2 proportion using

TissueLyser II set at frequency 20 1/s, during 1.30 sec. and then centrifugated at 10000 g at 4ºC, for 20 min. For CAT, SOD, GPx, GSTs and AChE activities, GLY and PROT contents, extraction was performed with potassium phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM dipotassium phosphate; 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (w/v) polyvinylpyrroli- done (PVP); 1 mM dithiothreitol (DTT); pH 7.0) in a 1:2 proportion using TissueLyser II set at frequency 20 1/s, during 1.30 sec. and then centrifugated at 10000 g at 4ºC, during 20 min. All supernatants were then reserved and stored at - 80 °C or used immediately.

2.6.2.1. Energy reserves content and metabolic capacity

The PROT content was determined according to Robinson and Hogden (1940), following the Biuret method that uses Bovine serum albumin (BSA) as standard (0–40 mg/mL). After 10 min incubation at 30 °C, the absorbance was determined spectrophotometrically and measured at a wavelength of 540 nm. The results were expressed in mg *per* g of FW.

The quantification of GLY content was done according to the sulphuric acid method (Dubois et al., 1956), using glucose standards (0-2 mg/mL). The absorbance was measured at of 492 nm. Concentrations of GLY were expressed in mg *per* g of FW.

The activity of ETS was determined by the amount of formazan formed after adding p-IodoNitroTetrazolium following King and Packard (1975) and modifications performed by De Coen and Janssen (1997). The absorbance was read spectrophotometrically at 490 nm for 10 min in 25 s. intervals. The amount of formazan formed was calculated using the extinction coefficient (ε) = 15900 M^{-1} cm^{-1} and the results were expressed nmol of formazan formed *per* min *per* g FW.

2.6.2.2. Oxidative status

The levels of LPO were measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to Ohkawa et al. (1979) protocol. This methodology is based on the reaction of LPO by-products, namely malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) forming TBARS. The amount of MDA was quantified spectrophotometrically and measured at 532 nm using ε = 1.56x105 M^{-1} cm^{-1}. Results were expressed as nmol of MDA equivalents *per* g FW.

GSH and GSSG contents were calculated following Rahman et al. (2014) protocols. The spectrophotometric reader assay method for GSH involves oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. The spectrophotometric reader assay method for

of GSSG in cell extracts is based on the measurement of NADPH consumption by GR, a process that reduces GSSG present in the sample. The cell extracts are treated with 2-vinylpyridine, which covalently reacts with GSH (but not GSSG). The excess 2-vinylpyridine is neutralized with triethanolamine. The absorbance was measurable at 412 nm. Reduced to oxidised glutathione ratio (GSH/GSSG) was calculated dividing GSH content by 2x the amount of GSSG.

The activity of SOD was measured using the method described by Beauchamp and Fridovich (1971). The standard curve was determined with SOD standards (0.25–60 U/mL). After 20 min in an orbital incubator set at room temperature, the enzyme activity was measured at 560 nm and expressed in unit of enzyme (U) *per* g FW. One U corresponds to a reduction of 50 % of Nitro blue tetrazolium (NBT).

The activity of CAT was measured by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson and Borg, 1988). The standard curve was determined using formaldehyde standards (0–150 µM). After 20 min in an orbital incubator at room temperature, the formaldehyde formation in the presence of Purpald was measured at 540 nm. The enzymatic activity was expressed in U *per* g FW. One U is defined as the amount of enzyme that generated the formation of 1.0 µmol formaldehyde *per* min.

The activity of GPx was quantified following Paglia and Valentine (1967). Enzyme activity catalyses the reduction of cumene hydroperoxide (an organic peroxide), oxidizing reduced glutathione (GSH) to form disulphide glutathione (GSSG). This oxidized glutathione is then reduced by glutathione reductase (GR) and NADPH forming NADP+ and recycling the GSH. This reaction results in decreased absorbance at 340 nm which is directly proportional to the GPx concentration. The activity was determined using $\varepsilon = 0.00622\ \mu M^{-1}\ cm^{-1}$ and the results were expressed in U *per* g FW where U represents the quantity of enzymes which catalyse the conversion of 1 µmol nicotinamide adenine dinucleotide phosphate (NADPH) *per* min.

The activity of GSTs activity was determined according to Habig et al. (1976). GSTs catalyse the conjugation of the substrate 1-chloro-2, 4-dinitrobenzene (CDNB) with glutathione, forming a thioether. Absorbance was measured at 340 nm and the activity of GSTs determined using $\varepsilon = 9.6\ mM\ cm^{-1}$ for CDNB. Results were expressed in U *per* g of FW where U is defined as the amount of enzymes that catalysis the formation of 1 µmol of dinitrophenyl thioether *per* min.

2.6.2.3. Neuro status

The activity of AChE activity was quantified according to Ellman et al. (1961) and modified by Mennillo et al. (2017). The activity was corrected for the spontaneous hydrolysis of the substrate (Acetylthiocholine iodide (ATChI, 5 mM)) and recorded spectrophotometrically for 5 min at 412 nm.

The results expressed in nmol *per* min *per* g FW, using $\varepsilon = 13.6 \times 10^3\ M^{-1}cm^{-1}$ (the yellow dianion of 5-thio-2-nitrobenzoic acid, TNB).

2.7. Data analyses

2.7.1. Single stressor experiments: CNTs exposure

Physiological (regenerative capacity) and biochemical (energy reserve contents and metabolic activity, oxidative status and neuro status) analyses were submitted to hypothesis testing using the PERMANOVA (permutational multivariate analysis of variance) + add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistically significant differences ($p \leq 0.05$), pairwise comparisons were performed. The *t*-statistic in the pair-wise comparisons was evaluated in terms of significance. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested for all the species were: I) for each biomarker and for each MWCNT, no significant differences existed among exposure concentrations (0.00, 0.001, 0.010, 0.10 mg/L); II) for each biomarker and for each exposure concentration, no significant differences existed between MWCNTs.

Looking on physiological analysis, significant differences ($p \leq 0.05$) among exposure concentrations (0.00; 0.001; 0.01 and 0.10 mg/L) for each MWCNT (f-MWCNTs and Nf-MWCNTs) were represented with different letters: uppercase and regular letters for Nf-MWCNTs and lower and regular letters for f-MWCNTs; Significant differences ($p \leq 0.05$) between f-MWCNTs and Nf-MWCNTs at each exposure concentration were represented with bold hashes (#). Regarding the biochemical analyses, significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) among the two MWCNTs at each exposure concentration were represented with asterisks (*).

2.7.2. Combination of stressors experiments: CNTs and salinity shifts

Physiological (regenerative capacity) and biochemical (energy reserve contents and metabolic activity, oxidative status and neuro status) analyses were submitted to hypothesis testing using the PERMANOVA (permutational multivariate analysis of variance) + add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistically significant differences ($p \leq 0.05$), pairwise comparisons were performed. The *t*-statistic in the pair-wise comparisons was evaluated in terms of significance. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested for all the species were: i) no significant impacts on organisms due to MWCNT concentrations, regardless the salinity shifts; ii) no effects of salinity levels on the toxicity of MWCNTs; iii) no effects of salinity levels on the sensitivity of organisms to MWCNTs. For this it

was verified if: I) for each biomarker and for each salinity level, no significant differences existed between both MWCNT exposure concentrations (0.01 and 0.10 mg/L); II) for each biomarker and for each MWCNT and exposure concentration, no significant differences exist between salinities; III) for each biomarker and for salinity level and exposure concentration, no significant differences exist between MWCNTs (Nf and f-MWCNTs).

Looking on physiological analysis, significant differences ($p \leq 0.05$) among exposure concentrations (0.00; 0.01 and 0.10 mg/L) for each MWCNT (f-MWCNTs and Nf-MWCNTs) and salinity (control-salinity 28 and low-salinity 21) were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and bold letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with bold asterisks (*****). Significant differences ($p \leq 0.05$) among f-MWCNTs and Nf-MWCNTs within each salinity at each exposure concentration were represented with bold hashes (#). Regarding the biochemical analyses, significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*). Significant differences ($p \leq 0.05$) among f-MWCNTs and Nf-MWCNTs within each salinity at each exposure concentration were represented with asterisks (*).

2.7.3. Combination of stressors experiments: CNTs and pH variation

Physiological (regenerative capacity) and biochemical (energy reserve contents and metabolic activity, oxidative status and neuro status) analyses were submitted to hypothesis testing using the PERMANOVA (permutational multivariate analysis of variance) + add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistically significant differences ($p \leq 0.05$), pairwise comparisons were performed. The *t*-statistic in the pair-wise comparisons was evaluated in terms of significance. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested for all the species were: i) no significant impacts on organisms due to MWCNT material concentrations, regardless the pH tested levels; ii) no effects of pH levels on the toxicity of MWCNT materials; iii) no effects of pH variations on the sensitivity of organisms to MWCNT materials. For this we verified if: I) for each biomarker and for each pH variation, no significant differences existed between both MWCNT exposure concentrations (0.01 and 0.10 mg/L); II) for each biomarker and for each MWCNT and exposure concentration, no significant differences exist

between pH; III) for each biomarker and for pH variation and exposure concentration, no significant differences exist between MWCNTs (Nf and f-MWCNTs).

Looking on physiological analysis, significant differences ($p \leq 0.05$) among exposure concentrations (0.00; 0.01 and 0.10 mg/L) for each MWCNT (f-MWCNTs and Nf-MWCNTs) and pH level (pH control- 8.0 and pH acidified-7.6) were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; lowercase and regular letters for Nf-MWCNTs at pH 7.6; uppercase and bold letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) among the two pH levels for each MWCNT and exposure concentration were represented with bold asterisks (*****). Significant differences ($p \leq 0.05$) among f-MWCNTs and Nf-MWCNTs within each pH level at each exposure concentration were represented with bold hashes (#). Considering biochemical analyses, significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) among the pH levels for each MWCNT and exposure concentration were represented with asterisks (*). Significant differences ($p \leq 0.05$) among f-MWCNTs and Nf-MWCNTs within each pH level at each exposure concentration were represented with asterisks (*).

CHAPTER 3. RESULTS AND DISCUSSION

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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. *Aquatic Toxicology*, 199, 199–211.

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3.1. Single stressor experiments: CNTs exposure

3.1.1. Results

3.1.1.1. *Ruditapes philippinarum* (Adams & Reeve, 1850)

3.1.1.1.1. Characterization analysis water media

Table 5 reportes the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.001; 0.01 and 0.10 mg/L) under control conditions (salinity 28; pH 8.0).

Table 5. *Ruditapes philippinarum*: average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed in each exposure concentration (0.001; 0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

	Size (nm)	PDI	Size (nm)	PDI
CNT concentration (mg/L)	**Nf-MWCNTs**		**f-MWCNTs**	
	T0		T0	
0.001	1987.2	0.21	987.6	n.d.
0.01	2018.3	0.76	l.d.	n.d.
0.10	2407.1	0.98	2545.1	1.13
	T7		T7	
0.001	1943.3	0.32	654.3	n.d.
0.01	1998.1	0.87	856.1	0.12
0.10	3 l.d.	n.d.	1888.1	0.31
	T14		T14	
0.001	*	*	-	-
0.01	*	*	1111.8	0.11
0.10	*	*	1975.1	0.03
	T21		T21	
0.001	*	*	-	-
0.01	*	*	553.6	0.22
0.10	*	*	l.d.	-
	T28		T28	
0.001	1985.2	1.43	432.1	0.09
0.01	2010.1	0.87	655.3	0.26
0.10	4542.7	1.81	2711.6	0.11

I.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions)
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
*: Not supplied sample

DLS and PDI analysis of samples exposed to different concentrations of Nf-MWCNTs did not allow for the detection of measurable macro/micro/nanosize particle aggregates observed among collection periods T14 and T21 reported in the table as “not supplied samples”, however at T0, T7 and T28 was evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 5). Furthermore, it was also possible to observe a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples. DLS and PDI analyses of experimental samples exposed to different concentrations of f-MWCNTs among collection periods (T0, T7, T14; T21 and T28) were characterized by the presence of micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 5). As for the Nf-MWCNTs, a time-dependent increase of the polydisperse sample in each condition due to the generation of large particles or aggregates in the investigated samples was observed. The time evolution of the mean values of the dimension of both suspended CNTs aggregates was similar among the different exposure periods.

Comparing the two CNTs materials, the mean recorded hydrodynamic diameter of f-MWCNTs aggregates were smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating higher dispersion of f-MWCNTs in aqueous media (Table 5).

3.1.1.1.2. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering two main topics: I) understand the effects of exposure concentrations of both CNT materials and II) understand the effects of the carboxylation of the surface of the CNTs for each exposure concentration.

Energy reserves content and metabolic capacity

I) *R. philippinarum* exposed to Nf-MWCNTs presented a decrease tendency in PROT content with the increase of exposure concentrations, however significantly lower content was only observed at 0.10 mg/L in comparison to all the remaining concentrations. Clams exposed to f-MWCNTs presented significantly lower PROT content under 0.01 and 0.10 mg/L in comparison to non-contaminated organisms and organisms exposed to the lowest f-MWCNTs concentration (Figure 15 A).

II) No significant differences were detected between the two MWCNTs at each of the tested concentrations (Figure 15 A).

I) Along with the increasing Nf-MWCNTs exposure concentrations, the clams maintained at 0.01 and 0.10 mg/L decreased significantly their GLY content in comparison to the other treatments, while the GLY content decreased only at 0.10 mg/L in clams under f-MWCNTs, with significantly lower content at the highest exposure concentration (Figure 15 B).

II) Between MWCNT materials, significant differences in GLY content were observed only at 0.01 mg/L, with the highest values in clams exposed to f-MWCNTs in comparison to Nf-MWCNTs (Figure 15 B).

I) The ETS activity in *R. philippinarum* submitted to Nf-MWCNTs significantly increased with the increasing exposure concentrations, while in clams exposed to f-MWCNTs, the activity of ETS was significantly higher only at 0.10 mg/L relative to the other treatments (Figure 15 C).

II) Between MWCNTs, significant differences in ETS activity were recorded at the two highest concentrations (0.10 and 1.00 mg/L) with higher activity in organisms exposed to Nf-MWCNTs in both cases (Figure 15 C).

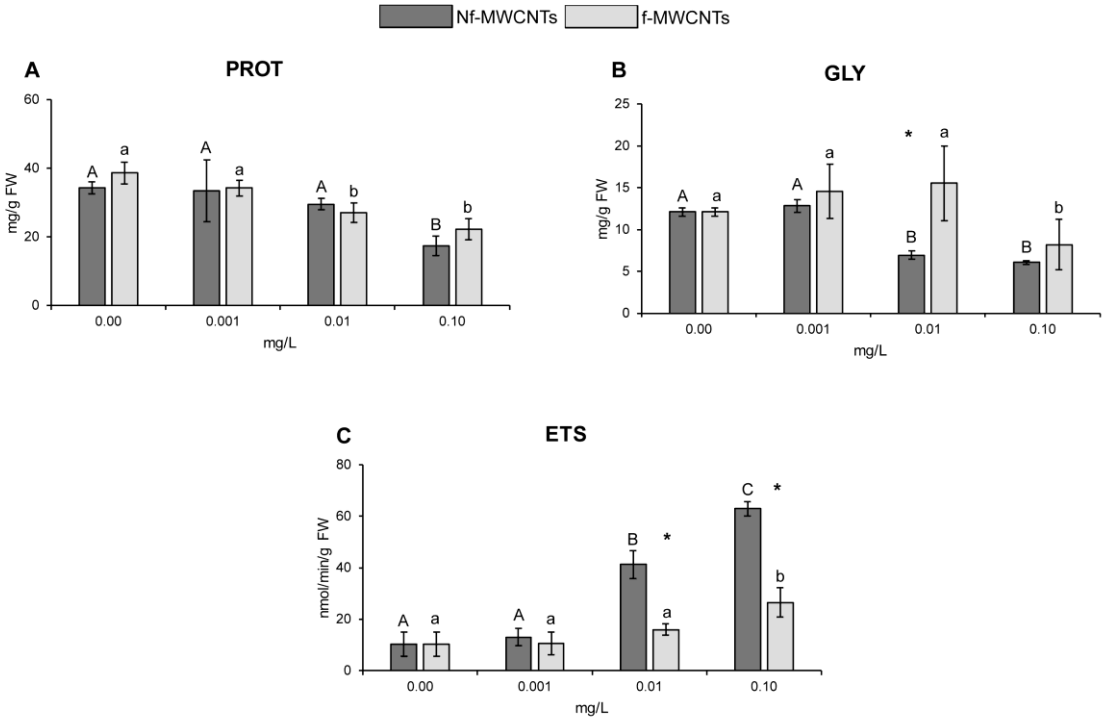

Figure 15. A: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron Transport System (ETS) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

Oxidative status

I) The level of LPO in clams exposed to Nf-MWCNTs increased with the increasing of exposure concentrations with significant differences between the two highest exposure concentrations and the remaining ones. In organisms exposed with f-MWCNTs, the LPO at 0.01 and 0.10 mg/L was significantly higher than values observed in control clams as well as in clams exposed to 0.001 mg/L, and no significant differences were observed between individuals exposed to these two concentrations (Figure 16 A).

II) Between MWCNTs, significant differences were observed at 0.01 and 0.10 mg/L, with the highest LPO levels in *R. philippinarum* under f-MWCNTs compared to Nf-MWCNTs (Figure 16 A).

I) At both tested MWCNTs, GSH/GSSG was significantly lower only in clams exposed to 0.10 mg/L in comparison to clams exposed to all the other concentrations. Clams exposed to control, 0.001 and 0.01 mg/L showed no significant differences in GSH/GSSG values (Figure 16 B).

II) Comparing GSH/GSSG values in organisms exposed to different MWCNTs at each of the tested concentrations, no significant differences were noticed between organisms exposed to different CNT materials (Figure 16 B).

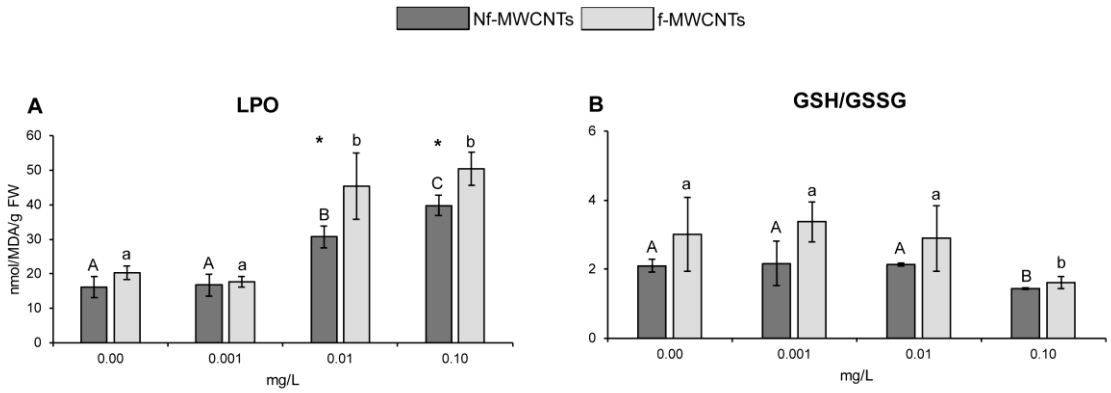

Figure 16. A: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

I) No significant differences in terms of SOD activity were observed between clams submitted to Nf-MWCNTs, while in organisms exposed to f-MWCNTs, SOD activity was only significantly higher at 0.10 mg/L in comparison to organisms exposed to the remaining treatments (Figure 17 A).

II) Significant differences between *R. philippinarum* submitted to different MWCNTs for each of the tested concentrations were observed at 0.10 mg/L, with higher SOD activity in organisms exposed to f-MWCNTs (Figure 17 A).

I) In terms of CAT activity, at both tested MWNCTs, no significant differences were observed between all concentrations (Figure 17 B).

II) No significant differences were observed in CAT activity between organisms exposed to different MWCNT materials (Figure 17 B).

I) The activity of GPx in clams exposed to Nf-MWCNTs significantly increased at 0.01 and 0.10 mg/L in comparison to the values of the other, with the highest activity recorded at 0.10 mg/L. Different GPx activity was observed in clams under f-MWCNTs, where significantly higher activity was found only when the clams were contaminated with 0.10 mg/L (Figure 17 C).

II) Between MWCNTs, significant differences in GPx activity were observed at 0.01 and 0.10 mg/L, with higher values in clams exposed to Nf-MWCNTs in comparison to f-MWCNTs in both cases (Figure 17 C).

I) No significant differences in terms of GSTs activity was recorded between clams submitted to Nf-MWCNTs, while *R. philippinarum* exposed to f-MWCNTs decreased the GSTs activity at 0.01 and 0.10 mg/L, with significant differences observed between clams exposed to these two concentrations and those exposed to the remaining ones (Figure 17 D).

II) Comparing GSTs activity in *R. philippinarum* exposed to different MWCNT materials, significant differences were only recorded at the highest exposure concentration, with organisms under Nf-MWCNTs presenting higher enzymatic activities (Figure 17 D).

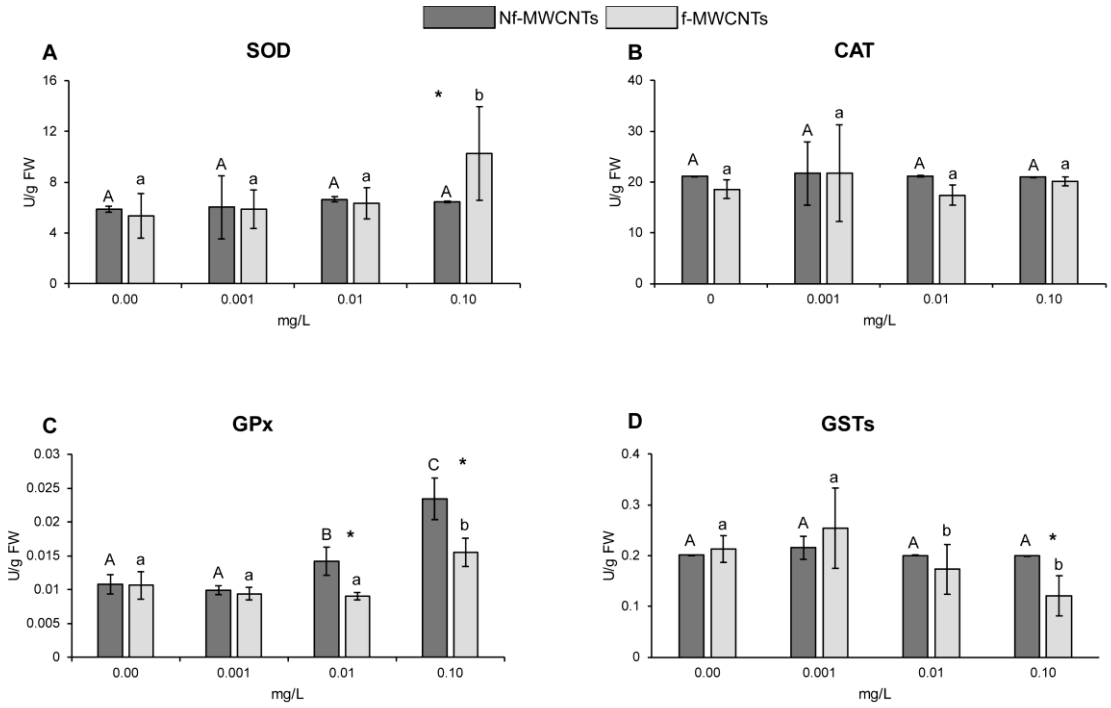

Figure 17. A: Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

Neuro status

I) Under both MWCNTs, AChE activity presented significantly lower values in contaminated organisms exposed to 0.01 and 0.10 mg/L in comparison to the other treatments, but no significant differences were observed between organisms exposed to these two concentrations for both Nf and f-MWCNTs (Figure 18).

II) Comparing AChE activity in clams exposed to different MWCNTs at each of the tested concentrations, no significant differences were noticed between organisms exposed to each of the tested concentration (Figure 18).

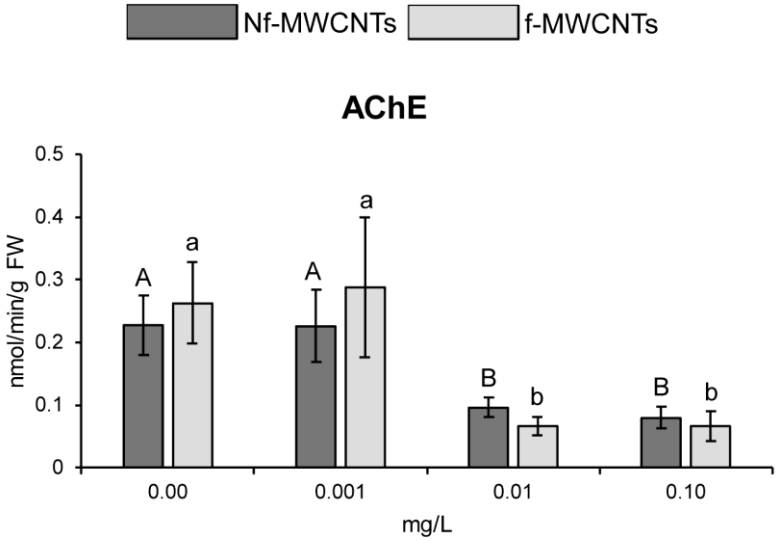

Figure 18. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

3.1.1.2. *Hediste diversicolor* (O.F. Müller, 1776)

3.1.1.2.1. Characterization analysis of water media

Table 6 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.001; 0.01 and 0.10 mg/L) under control conditions (salinity 28; pH 8.0).

Table 6. *Hediste diversicolor*: average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed in each exposure concentration (0.001; 0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

	Size (nm)	PDI	Size (nm)	PDI
CNT concentration (mg/L)	**Nf-MWCNTs**		**f-MWCNTs**	
	T0		T0	
0.001	1232.1	0.98	876.2	0.03
0.01	1863.1	1.26	5 I.d.	n.d.
0.10	5428.0	2.23	2545.1	1.13
	T7		T7	
0.001	1235.3	0.93	1234.2	0.76
0.01	5 I.d.	n.d.	5501.2	1.18
0.10	3217.4[a]	1.39	5 I.d.	n.d.
	T14		T14	
0.001	3 I.d.	n.d.	5 I.d.	n.d.
0.01	8603.7[a]	4.87	2344.9[a]	1.38
0.10	1381.0	1.25	5 I.d.	n.d.
	T21		T21	
0.001	3 I.d.	n.d.	5 I.d.	n.d.
0.01	5 I.d.	n.d.	5 I.d.	n.d.
0.10	5 I.d.	n.d.	1930.5[a]	0.88
	T28		T28	
0.001	1432.1	0.54	1001.2	0.21
0.01	5 I.d.	n.d.	5 I.d.	n.d.
0.10	5 I.d.	n.d.	5 I.d.	n.d.

I.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
a: Sample contaminated with sand grains and macroscopic blackish aggregates.

DLS and PDI analysis of samples exposed to different concentrations of Nf-MWCNTs did not allow for the detection of measurable macro/micro/nanosize particle aggregates observed among

collection periods T21 as a consequence of not detected colloidal material into the analyzed sample at the end of 120 acquisitions (I.d.). Same results were observed in some concentrations detected under T7 (0.01 mg/L); T14 (0.001 mg/L) and T28 (0.01 and 0.10 mg/L). However, at T0 (immediately after the dispersion of CNTs materials in a water medium) it was evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 6). Furthermore, a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples was observed under this time of exposure. Considering DLS and PDI analysis of experimental samples exposed to different concentrations of f-MWCNTs among collection periods, also, in this case, it was not possible to detect colloidal material into the analyzed sample at the end of 120 acquisitions in some concentrations of different exposure periods (Table 6). Nevertheless, the presence of micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples was observed (Table 6). As for the Nf-MWCNTs, a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples was observed. The time evolution of the mean values of the dimension of both suspended CNTs aggregates was similar between the different exposure periods.

Comparing the two CNTs materials, the mean recorded hydrodynamic diameter of f-MWCNTs aggregates were smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating higher dispersion of f-MWCNTs in aqueous media (Table 6).

3.1.1.2.2. Characterization analysis in sediment matrix

A preliminary study with TGA analysis showed the presence of aggregates in sediments exposed to Nf-MWCNTs dispersions stating the feasibility of experimental samples sediment only at the highest exposure concentration (Figure 19 A). DTGA analysis evidenced the presence of a negative peak at 600° C relevant to the maximum rate of degradation of Nf-MWCNTs. The thermal characterization was carried out on samples exposed to higher concentration of Nf-MWCNTs dispersions and compared to that of the sediment used as control (Figure 19 B). The weight loss (%) of all the analysed samples were negligible at 900° C due to the inorganic nature of the sediments. DTGA analysis of the obtained TGA curves was carried out in order to better understand the steps of degradation (Figure 19 C). The preliminary DTGA investigation did not clearly highlight the presence of negative peaks in correspondence with that of native MWCNTs (600° C, cf. Figure 19 A). This could be most probably due to the experimental conditions adopted to detect trace amount of MWCNTs particulate. In fact, first attempts at analysing Nf-MWCNTs samples with TGA gave us the opportunity to discuss the first results, indicating that A) TGA analysis could represent a promising tool for the detection of CNTs in inorganic sediments due to the different degradation behaviour of the materials from the background and could represent a valuable technique for their

quantification after proper optimization of the method but B) it is necessary to develop and optimize *ad hoc* protocols experimental condition in order to detect even traces of different CNT materials (both Nf-MWCNTs and f-MWCNTs) in this complex matrix.

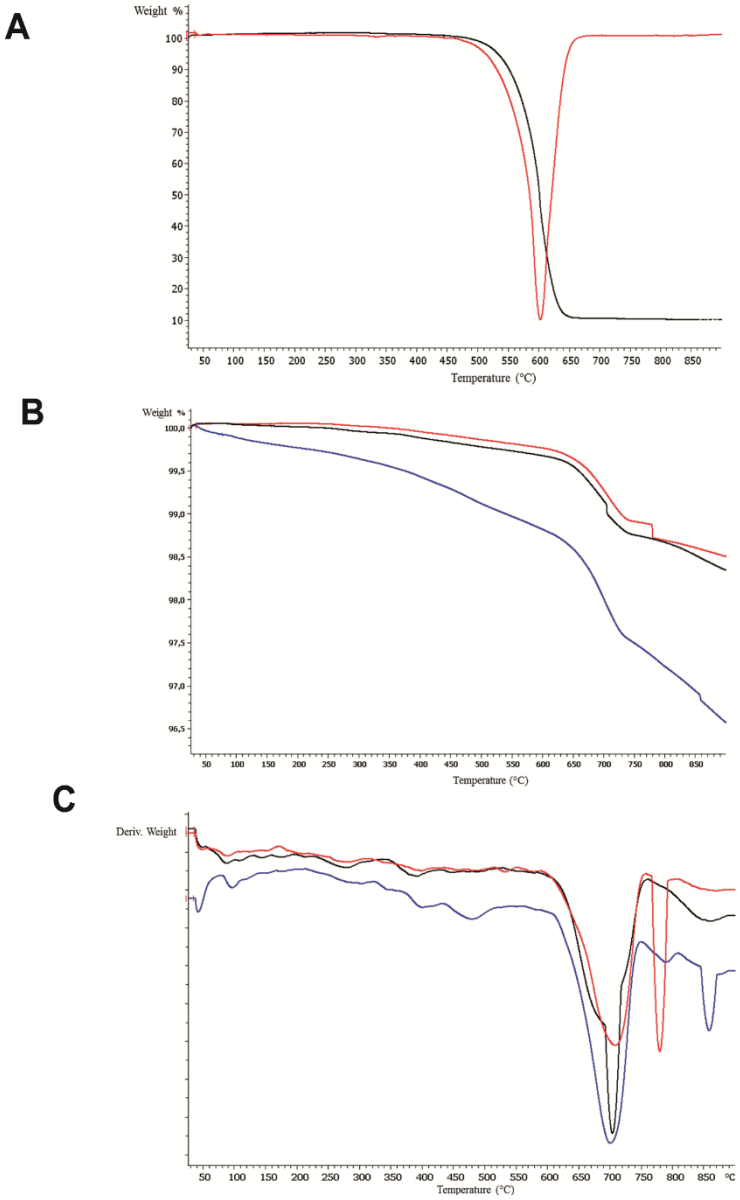

Figure 19. A: Thermogravimetric curve (black line) and derivative of the thermogravimetric curve (red line) of MWCNTs; **B:** Thermogravimetric curves of sediments: a) 0.00 mg/L (black line), b) T0 + 0.10 mg/L (red line), c) T7 + 0.10 mg/L (blue line); **C:** DTGA analysis of sediments: a) 0.00 mg/L (black line), b) T0 + 0.10 mg/L (red line), c) T7 + 0.10 mg/L (blue line).

3.1.1.2.3. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering two main topics: I) understand the effects of exposure concentrations of both CNTs materials and II) understand the effects of the carboxylation of the surface of the CNTs for each exposure concentration.

Energy reserves content and metabolic capacity

I) At both tested MWCNTs, no significant differences were observed among all concentrations in terms of PROT content (Figure 20 A).

II) No significant differences were detected in PROT content between organisms exposed to different MWCNT materials (Figure 20 A).

I) As for the PROT content, GLY content showed no significant differences among exposure concentrations, both in polychaetes exposed to Nf-MWCNTs and f-MWCNTs (Figure 20 B).

II) No significant differences were detected in GLY content between organisms exposed to different MWCNT materials (Figure 20 B).

I) Considering *H. diversicolor* exposed to Nf-MWCNTs, no significant differences in terms of ETS activity were found among concentrations, while specimens submitted to f-MWCNTs showed significantly higher ETS activity only at the highest exposure concentration (0.10 mg/L) (Figure 20 C).

II) No significant differences were detected in ETS activity between organisms exposed to different MWCNT materials (Figure 20 C).

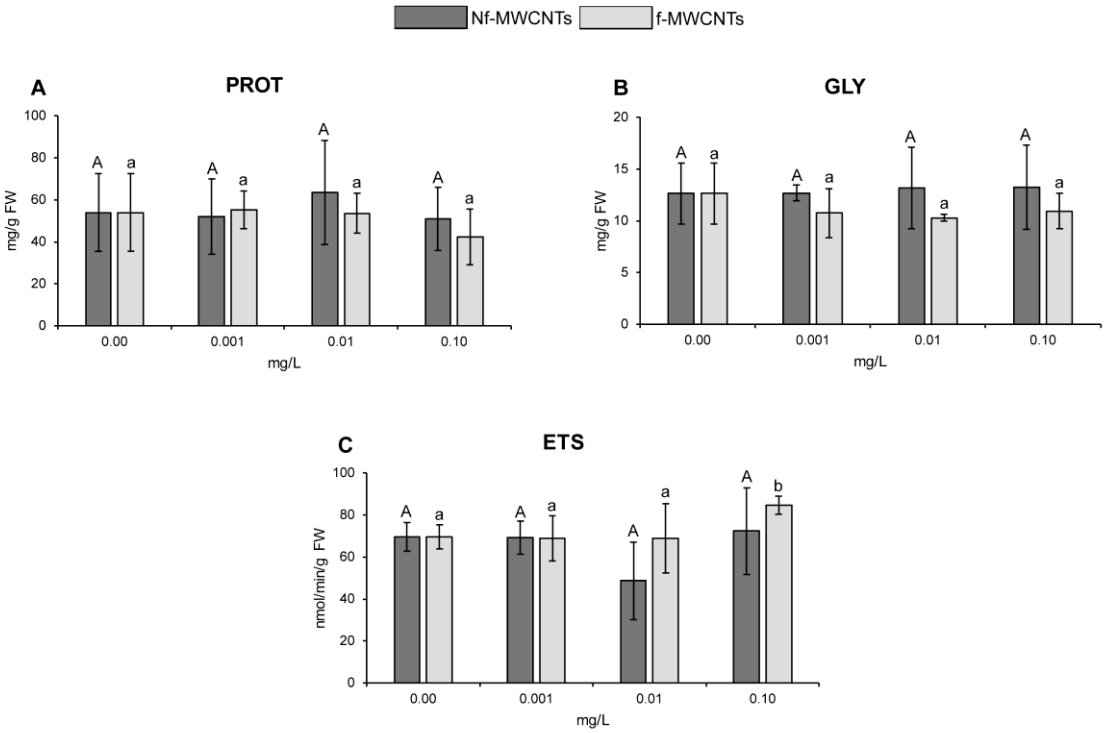

Figure 20. A: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron Transport System (ETS) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

Oxidative status

I) No significant differences in terms of LPO levels were recorded between polychaetes submitted to Nf-MWCNTs, while in *H. diversicolor* exposed to f-MWCNTs an increase of LPO was recorded at 0.01 and 0.10 mg/L, with significant differences observed between organisms exposed to the highest concentration (0.10 mg/L) and those exposed to 0.001 mg/L and control conditions (Figure 21 A).

II) Comparing LPO levels in *H. diversicolor* exposed to different MWCNTs, significant differences were only recorded at the highest concentration, with organisms under f-MWCNTs presenting higher cellular damage in comparison to polychaetes contaminated with Nf-MWCNTs (Figure 21 A).

I) No significant differences of GSH/GSSG were observed among polychaetes submitted to different Nf-MWCNT concentrations, while only a significantly decreased of GSH/GSSG was

detected in *H. diversicolor* exposed 0.10 mg/L f-MWCNTs in comparison to the remaining concentrations (Figure 21 B).

II) Significant differences between materials were observed between polychaetes exposed to 0.10 mg/L concentration, showing lower GSH/GSSG in *H. diversicolor* under f-MWCNTs in comparison to Nf-MWCNTs (Figure 21 B).

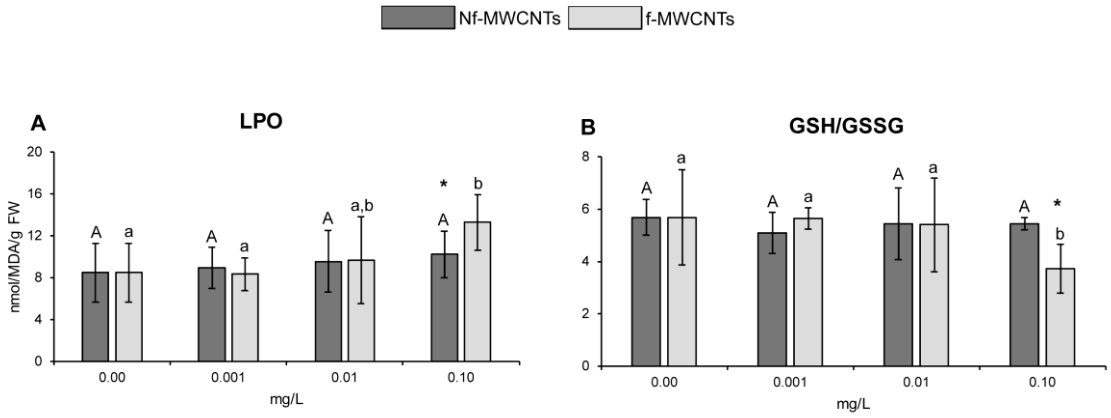

Figure 21. A: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

I) Significantly higher SOD activity was observed between polychaetes exposed to 0.01 and 0.10 mg/L Nf-MWCNTs and the remaining treatments, while in organisms exposed to f-MWCNTs SOD activity was only significantly higher at 0.10 mg/L in comparison to organisms exposed to the other concentrations (Figure 22 A).

II) Significant differences between *H. diversicolor* submitted to different MWCNTs for each of the tested concentrations were observed at 0.01 mg/L, with higher SOD activity in organisms exposed to Nf-MWCNTs in comparison to f-MWCNTs (Figure 22 A).

I) At both tested MWCNTs, no significant differences among all concentrations were observed in terms of CAT activity (Figure 22 B).

II) No significant differences were detected in CAT activity between organisms exposed to different MWCNT materials (Figure 22 B).

I) Under MWCNTs, significantly higher GPx activity was observed between polychaetes exposed to the two highest concentrations (0.01 and 0.10 mg/L) and the remaining ones (Figure 22 C).

II) Comparing GPx activity in *H. diversicolor* exposed to different MWCNTs, no significant differences were detected between materials (Figure 22 C).

I) In *H. diversicolor* exposed to Nf-MWCNTs the activity of GSTs was significantly lower only in polychaetes exposed to 0.10 mg/L relative to the remaining concentrations. Opposite behaviour was observed in polychaetes submitted to f-MWCNTs, observing a significantly higher activity at 0.01 and 0.10 mg/L concentrations in comparison to the other treatments (Figure 22 D).

II) Significant differences between materials were observed between polychaetes exposed to 0.01 and 0.10 mg/L concentrations, showing higher GSTs activity in *H. diversicolor* under f-MWCNTs in comparison to Nf-MWCNTs (Figure 22 D).

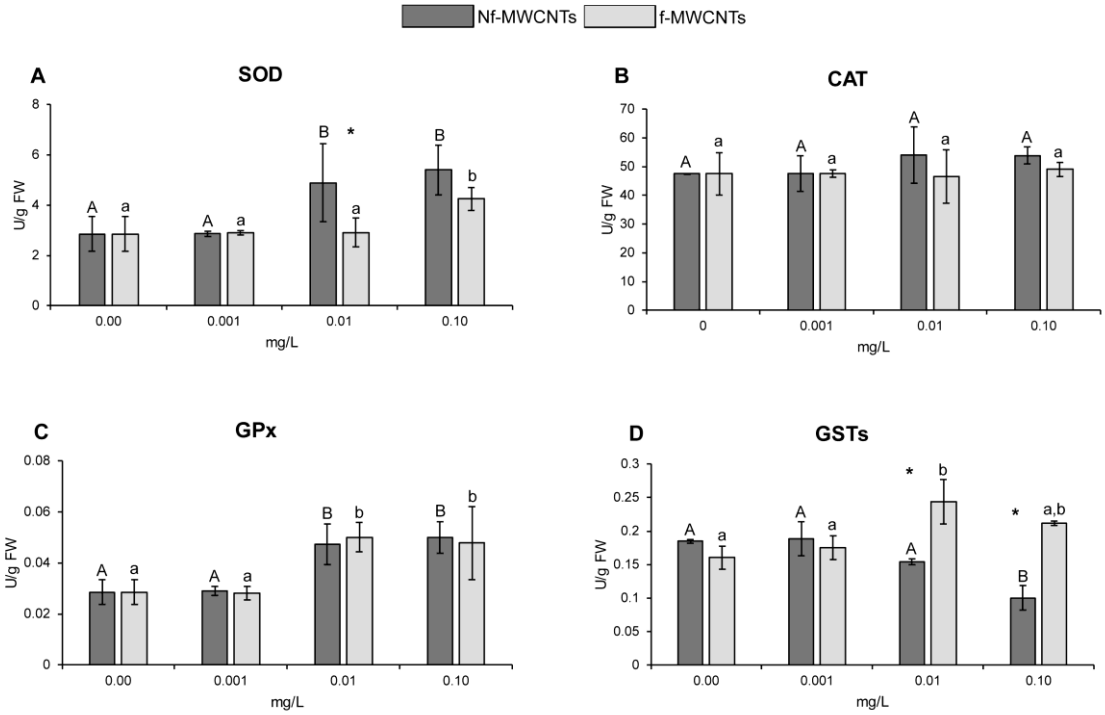

Figure 22. A: Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

Neuro status

I) *H. diversicolor* exposed to Nf-MWCNTs presented significant differences in terms of AChE activity among all the concentrations except between control and 0.001 mg/L, however significantly lower activity was observed at 0.01 mg/L in comparison to the other concentrations. Considering

polychaetes submitted to f-MWCNTs, significantly lower AChE value was detected only in organisms contaminated with 0.10 mg/L relative to the remaining concentrations (Figure 23).

II) Significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L, showing higher and lower AChE activities respectively in *H. diversicolor* under f-MWCNTs in comparison to Nf-MWCNTs (Figure 23).

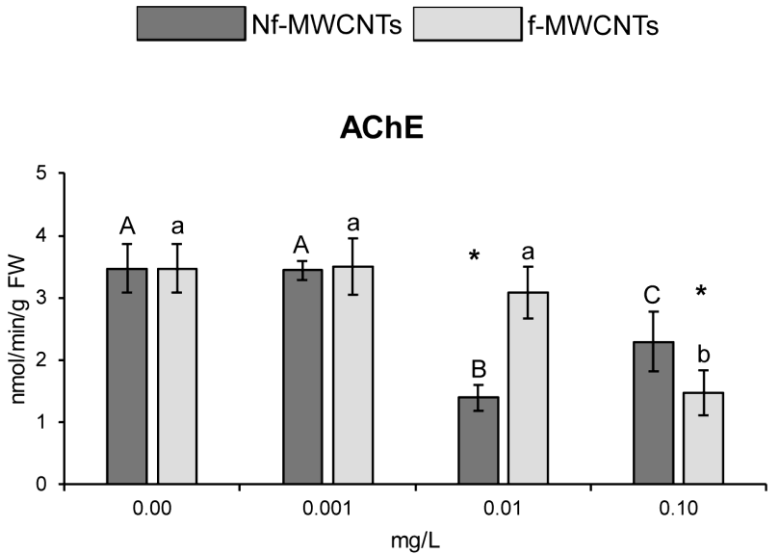

Figure 23. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

3.1.1.3. *Diopatra neapolitana (Delle Chiaje, 1841)*

3.1.1.3.1. Characterization analysis of water media

Table 7 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.001; 0.01 and 0.10 mg/L) under control conditions (salinity 28; pH 8.0).

Table 7. *Diopatra neapolitana*: average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed in each exposure concentration (0.001; 0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

	Size (nm)	PDI	Size (nm)	PDI
CNT concentration (mg/L)	**Nf-MWCNTs**		**f-MWCNTs**	
	T0		T0	
0.001	5 I.d.	n.d.	2211.4	1.02
0.01	2596.6	0.98	3634.9	1.50
0.10	4321.1	1.32	3987.2	1.45
	T7		T7	
0.001	5 I.d.	n.d.	5 I.d.	n.d.
0.01	5 I.d.	n.d.	5 I.d.	n.d.
0.10	3214.2	0.78	2098.7	1.72
	T14		T14	
0.001	5 I.d.	n.d.	5 I.d.	n.d.
0.01	5 I.d.	n.d.	1771.2	0.804
0.10	3998.8	1.24	3098.2	1.09
	T21		T21	
0.001	5 I.d. [a]	n.d.	5 I.d.	n.d.
0.01	3354.7	1.32	3354.7	1.50
0.10	3 I.d. [a]	n.d.	3987.2	1.89
	T28		T28	
0.001	5 I.d. [a]	n.d.	2121.3	0.90
0.01	5 I.d. [a]	n.d.	3 I.d.	n.d.
0.10	4098.2	1.98	5 I.d.	n.d.

I.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
a: Sample contaminated with sand grains and macroscopic blackish aggregates.

DLS and PDI analysis of samples exposed to different concentrations of Nf-MWCNTs did not allow for the detection of measurable macro/micro/nanosize particle aggregates observed among collection periods T7 (0.001 and 0.01 mg/L); T14 (0.001 and 0.01 mg/L); T21 (0.001 and 0.10 mg/L) and T28 (0.001 and 0.01 mg/L) as a consequence of not detected colloidal material in the analyzed sample at the end of 120 acquisitions (I.d.). Moreover, the reliability of the mean diameter values obtained at time 21 and 28 days was compromised by the presence of microaggregates of unknown origin as evidenced in Table 7. Similar results were observed in samples contaminated with f-MWCNTs: T7 (0.001 and 0.01 mg/L); T14 (0.001 mg/L); T21 (0.001 mg/L) and T28 (0.01 and 0.10 mg/L),. as a consequence of not detected colloidal material into the analyzed sample at the end of 120 acquisitions (I.d.). However, in this case, no contamination with sand grains was observed. Considering both materials (Nf-MWCNTs and f-MWCNTs) at T0 (immediately after the dispersion of CNTs materials in a water medium), it was evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 7). Furthermore, a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples was observed under this time of exposure.

Comparing the two CNTs materials, up to 14 days, it was possible to observe that Nf and f-MWCNTs displayed a different behavior: f-MWCNTs were found to agglomerate and remain dispersed in the medium while Nf-MWCNTs particles were not detectable by DLS analysis due to a possible settlement and/or uptake by marine organisms (Table 7).

3.1.1.3.2. Biological analysis: physiological parameter (regenerative capacity)

The mean values for the percentage (%) of regenerated body width and the number (#) of new chaetigers in *D. neapolitana* after 11th, 18th and 28th days of amputation are illustrated in Figure 24 and presented in Table 8. All the results were discussed considering: two main topics: I) understand the effects of exposure concentrations of both CNTs materials and II) understand the effects of the carboxylation of the surface of the CNTs for each exposure concentration.

11th day

After amputation all individuals were healing the cut region, however, no significant differences were observed in terms of percentage of regenerated body width as well as a number of new chaetigers between individuals non-exposed (0.00 mg/L) and exposed to both MWCNTs in all tested concentrations (0.001; 0.01 and 0.10 mg/L) (Table 8 and Figure 24).

18th day

I) The results of both percentages of regenerated body width and number of new chaetigers showed that for Nf-MWCNT significantly lower values were detected only in individuals exposed to 0.01 and 0.10 mg/L in comparison to remaining concentrations. Significantly lower values in terms of percentage of regenerated body width were observed only in individuals exposed to 0.10 mg/L f-MWCNTs related to the other treatments, while no significant differences between concentrations were found regarding the number of new chaetigers (Table 8).

II) Considering the effects of MWCNTs (Nf vs f), for each concentration (0.00; 0.001; 0.01 and 0.10 mg/L) significant differences were observed between organisms exposed to 0.10 mg/L, showing a lower percentage of regenerated body width in polychaetes contaminated with Nf-MWCNTs. Regarding the number of new chaetigers, significant differences between materials were detected only in polychaetes exposed to 0.01 and 0.10 mg/L presenting in both cases a lower number of chaetigers for individuals contaminated with Nf-MWCNTs (Table 8).

28th day

I) Considering the effects of exposure concentrations, *D. napolitana* exposed to Nf-MWCNTs showed significantly concentration-dependent decreased of both percentages of regenerated body width and number of new chaetigers, with the lowest values at the highest exposure concentration (0.10 mg/L). Significant differences in terms of percentage of regenerated body width were also observed in polychaetes exposed to f-MWCNTs. However, the lower value was noticed only at 0.10 mg/L in comparison to the remaining concentrations. No significant differences between concentrations were identified regarding the number of new chaetigers (Table 8 and Figure 24).

II) For each exposure concentration, differences between MWCNTs were only detected at 0.10 mg/L regarding the percentage of regenerated body width, showing significantly lower value in individuals exposed to Nf-MWCNT (Table 8 and Figure 24).

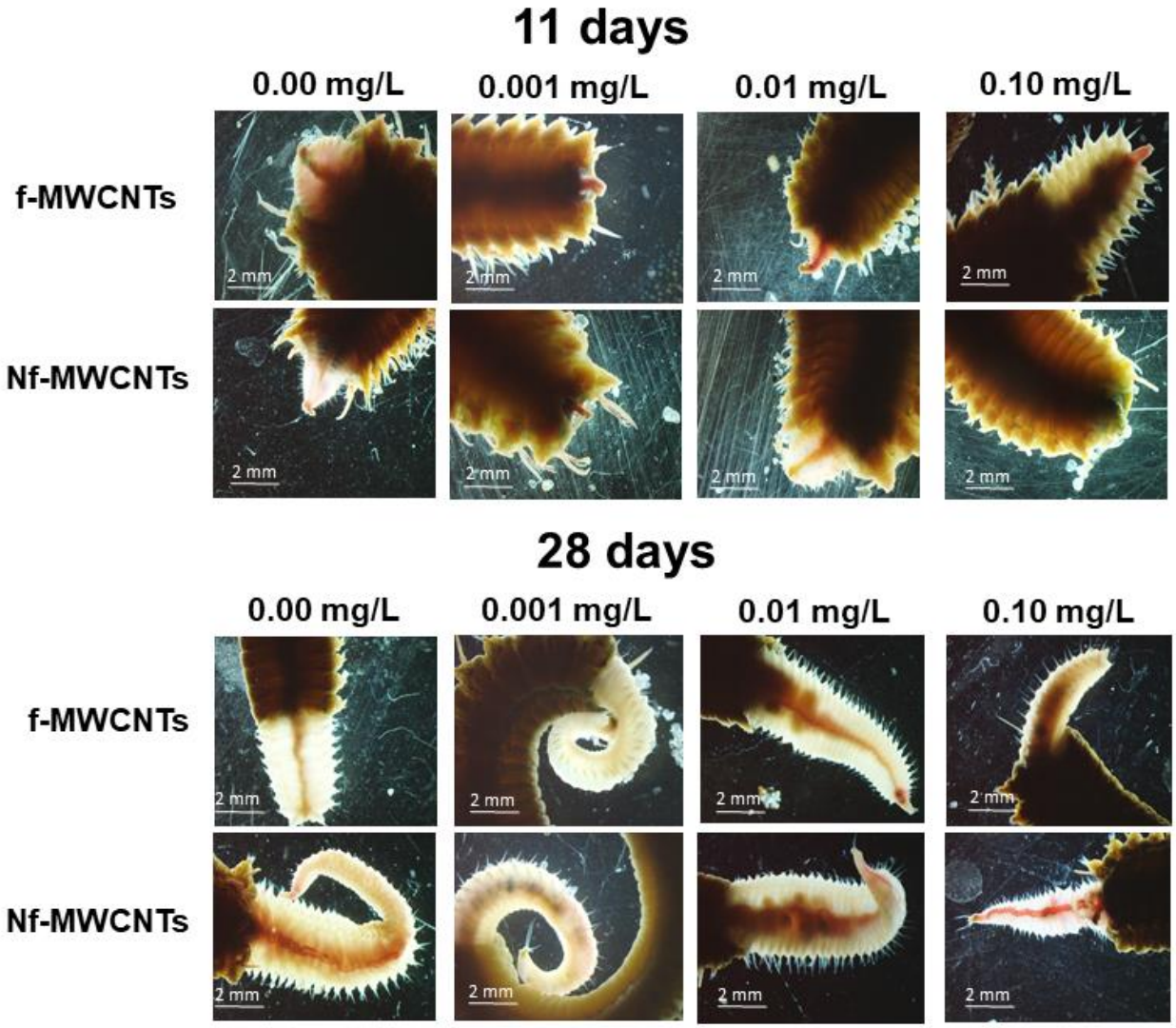

Figure 24. Regenerative capacity of *D. neapolitana* at 11th and 28th days after amputation, exposed to different MWCNTs (f and Nf) and concentrations (0.00; 0.001; 0.01 and 0.10 mg/L).

Table 8. Regeneration data (percentage (%) of body width and the number (#) of new chaetigers) for *D. neapolitana*, 11th, 18th and 28th days after amputation. Significant differences ($p \leq 0.05$) among exposure concentrations (0.00; 0.001; 0.01 and 0.10 mg/L) for each MWCNTs (f-MWCNTs and Nf-MWCNTs) were represented with different letters: uppercase and regular letters for Nf-MWCNTs and lower case and regular letters for f-MWCNTs; Significant differences ($p \leq 0.05$) between f-MWCNTs and Nf-MWCNTs at each exposure concentration were represented with bold hashes (#).

CNT concentrations (mg/L)		**11th days**		**18 th days**		**28 th days**	
		% body width	**# chaetigers**	**% body width**	**# chaetigers**	**% body width**	**# chaetigers**
0.00	f-MWCNTs	7.67±2.07 a	0.00±0.00 a	44.64±10.04 a	21.50±6.28 a	75.79±3.96 a	30.50±1.38 a
	Nf-MWCNTs	7.67±2.07 A	0.00±0.00 A	44.64±10.04 A	21.50±6.28 A	75.79±3.96 A	30.50±1.38 A
		11th days		**18th days**		**28th days**	
		% body width	**# chaetigers**	**% body width**	**# chaetigers**	**% body width**	**# chaetigers**
0.001	f-MWCNTs	7.83±4.62 a	0.00±0.00 a	43.13±6.42 a	18.83±1.72 a	72.61±7.05 a	28.17±2.14 a
	Nf-MWCNTs	8.33±2.73 A	0.00±0.00 A	43.75±11.39 A	17.83±3.92 A	73.12±7.74 A	29.67±1.63 A
		11th days		**18th days**		**28th days**	
		% body width	**# chaetigers**	**% body width**	**# chaetigers**	**% body width**	**# chaetigers**
0.01	f-MWCNTs	6.50±3.73 a	0.00±0.00 a	37.87±7.51 a	18.83±2.40 a #	70.09±12.21 a	28.67±1.51 a
	Nf-MWCNTs	8.83±4.53 A	0.00±0.00 A	19.12±4.83 B	11.17±5.95 B #	59.41±19.35 B	26.67±7.39 B
		11th days		**18th days**		**28th days**	
		% body width	**# chaetigers**	**% body width**	**# chaetigers**	**% body width**	**# chaetigers**
0.10	f-MWCNTs	5.60±3.90 a	0.00±0.00 a	29.06±7.45 b #	17.98±3.34 a #	59.12±10.14 b #	21.57±2.22 a
	Nf-MWCNTs	8.43±2.51 A	0.00±0.00 A	15.10±3.68 B #	9.50±3.94 B #	24.87±6.22 C #	11.33±2.58 C

3.1.1.3.3. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering two main topics: I) understand the effects of exposure concentrations of both CNTs materials and II) understand the effects of the carboxylation of the surface of the CNTs for each exposure concentration.

Energy reserves content and metabolic capacity

I) Considering the effects of exposure concentrations, PROT content significantly increased along the increasing exposure gradient in *D. neapolitana* contaminated with to Nf-MWCNTs, showing the highest value when exposed to 0.10 mg/L in comparison to the remaining concentrations. In individuals exposed to f-MWCNTs, no significant differences were revealed among exposure concentrations (Figure 25 A).

II) Significant differences between materials were observed between polychaetes exposed to 0.01 and 0.10 mg/L, with higher PROT content in *D. neapolitana* under Nf-MWCNTs in comparison to f-MWCNTs (Figure 25 A).

I) In polychaetes exposed to Nf-MWCNTs, the content of GLY was significantly higher only in specimens exposed to 0.10 mg/L in comparison to the remaining concentrations. Opposite behaviour was observed in *D. neapolitana* submitted to f-MWCNTs, showing a significantly lower content in all contaminated organisms compared to non-contaminated ones (Figure 25 B).

II) Significant differences between materials were detected in all contaminated organisms, with higher GLY content in organisms exposed to Nf-MWCNTs in comparison to f-MWCNTs, regardless the exposure concentrations (Figure 25 B).

I) Polychaetes contaminated with Nf-MWCNTs decreased significantly the activity of ETS when exposed to 0.01 mg/L Nf-MWCNTs, but at the highest exposure concentration (0.10 mg/L) the activity significantly increased to values higher than all the other concentrations. *D. neapolitana* exposed to f-MWCNTs showed significant ETS increased along the concentration gradient, with significantly higher value when exposed to the highest exposure concentration (0.10 mg/L) (Figure 25 C).

II) Significant differences between materials were observed between polychaetes exposed to 0.01 and 0.10 mg/L, showing lower and higher metabolic activity respectively in *D. neapolitana* under Nf-MWCNTs in comparison to f-MWCNTs (Figure 25 C).

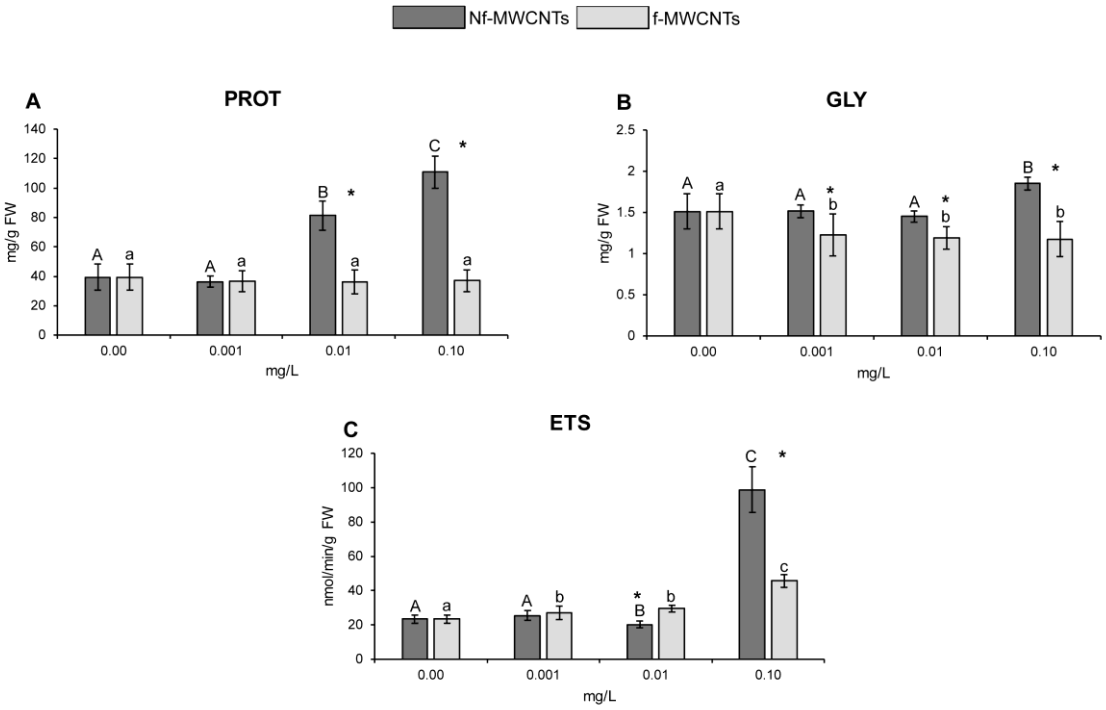

Figure 25. A: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron Transport System (ETS) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

Oxidative status

I) Significantly higher LPO levels were detected in polychaetes exposed to 0.01 and 0.10 mg/L Nf-MWCNTs compared to the remaining treatments. In organisms exposed to f-MWCNTs a significant dose-dependent increase of LPO levels was observed, showing higher values under the two highest exposure concentrations (0.01 and 0.10 mg/L) in comparison to the remaining ones (Figure 26 A).

II) Significant differences between materials were observed in specimens exposed to 0.01 and 0.10 mg/L, showing higher LPO levels in *D. neapolitana* exposed to Nf-MWCNTs compared f-MWCNTs (Figure 26 A).

I) In *D. neapolitana* exposed to Nf-MWCNTs, the GSH/GSSG was significantly lower in polychaetes exposed to 0.01 and 0.10 mg/L relative to the remaining concentrations. Significant dose-dependent decreased of GSH/GSSG was observed in individuals contaminated with f-MWCNTs, showing lower values at the two highest exposure concentrations (Figure 26 B).

II) Significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L, showing in both cases lower ratio in *D. neapolitana* contaminated with Nf-MWCNTs compared to f-MWCNTs (Figure 26 B).

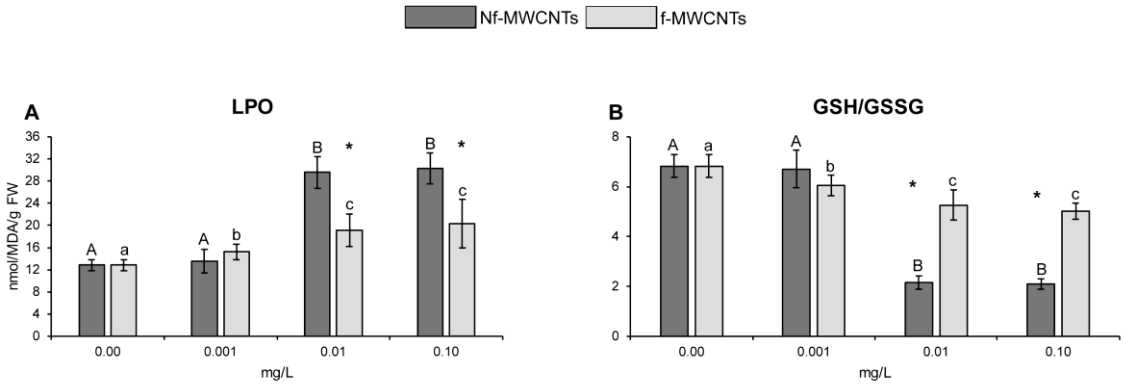

Figure 26. A: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

I) No significant differences between all concentrations were observed in terms of SOD activity in *D. neapolitana* submitted to Nf-MWCNTs, while polychaetes contaminated with f-MWCNTs, showed a significant dose-dependent increase of the antioxidant activity in comparison to control, with higher values at the highest exposure concentration (0.10 mg/L) (Figure 27A).

II) Significant differences between materials were detected in all contaminated organisms, showing for all concentrations higher SOD activity in organisms exposed to f-MWCNTs in comparison to Nf-MWCNTs (Figure 27 A).

I) At both tested MWCNTs, no significant differences between all concentrations were observed in terms of CAT activity (Figure 27 B).

II) No significant differences were detected in CAT activity between organisms exposed to different MWCNTs (Figure 27 B).

I) No significant differences between all concentrations were observed in terms of GPx activity in *D. neapolitana* submitted to Nf-MWCNTs, while significantly higher activity was observed in polychaetes exposed to 0.01 and 0.10 mg/L f-MWCNTs compared to the remaining treatments (Figure 27 C).

II) Comparing GPx activity in *D. neapolitana* exposed to different MWCNTs, no significant differences were detected between materials (Figure 27 C).

I) Polychaetes contaminated with Nf-MWCNTs decreased significantly the activity of GSTs when exposed to 0.001 mg/L Nf-MWCNTs, but at the two highest exposure concentrations (0.01 and 0.10 mg/L) the activity significantly increased to values higher than all the other concentrations. Opposite GSTs activity was detected in polychaetes exposed to f-MWCNTs, where the activity significantly decreased when the organisms were contaminated with the two highest concentrations (Figure 27 D).

II) Significant differences between materials were detected in all contaminated organisms, showing lower (0.001 mg/L) and higher (0.01 and 1.00 mg/L) GSTs activity in individuals exposed to Nf-MWCNTs in comparison to f-MWCNTs (Figure 27 D).

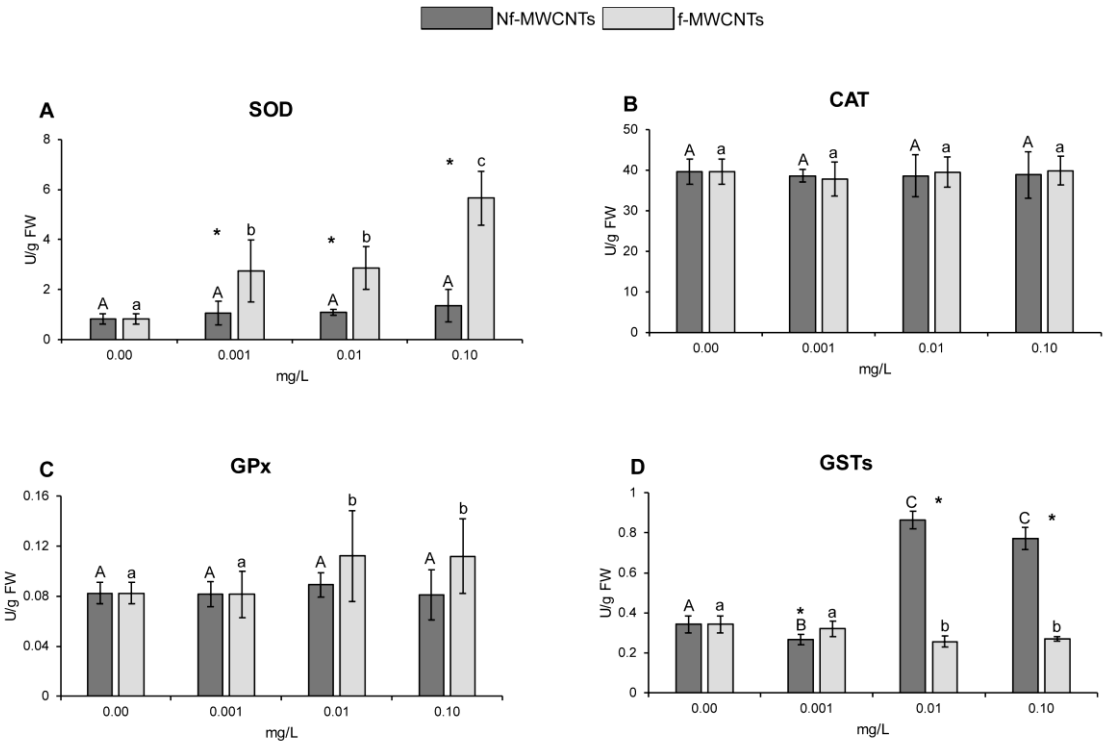

Figure 27. A: Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

Neuro status

I) Under Nf-MWCNTs *D. neapolitana* presented a significant increase of AChE activity only at 0.01 mg/L, while under f-MWCNTs significantly inhibition of the activity was detected only in polychaetes exposed to 0.10 mg/L in comparison to the remaining concentrations (Figure 28).

II) Significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L, showing in both cases lower AChE activity in *D. neapolitana* contaminated with f-MWCNTs compared to Nf-MWCNTs (Figure 28).

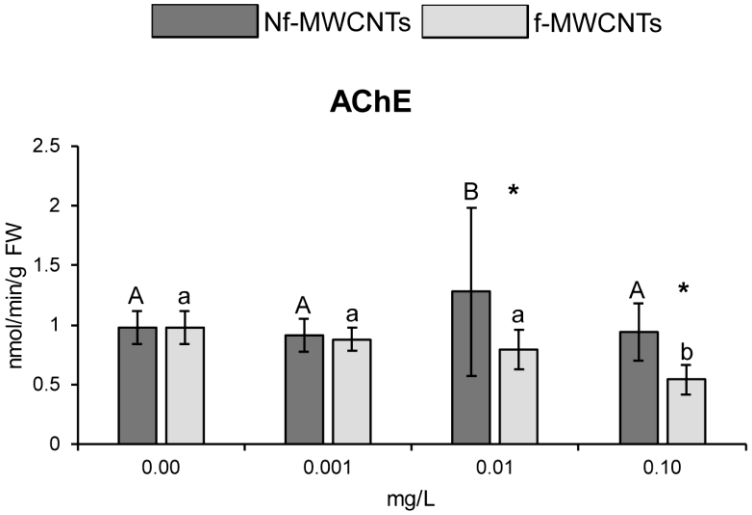

Figure 28. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.001; 0.01 and 0.10 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (uppercase letters for Nf-MWCNTs; lowercase letters for f-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNTs at each exposure concentration were represented with asterisks (*).

(*)

3.1.2. Discussion

3.1.2.1. Characterization analyses

In the present studies, it was demonstrated the presence of the CNT materials both in the water and sediment matrices. Considering the different species with different trophic behaviour such as the filter-feeder bivalve (which is able through their gills to capture particulate matter and particles greater than ca. 6 µm are captured with an efficiency >90% (Ward and Kach, 2009)), and the two omnivorous sediment dwelling polychaetes (which the ingestion of nano-contaminated sediment was directly related to their burrowing and dietary behaviour (Amiard et al., 2007; Durou et al., 2007; Gillet et al., 2008)), they may have come into contact also with the CNT materials present both in the water and sediment matrices. These could explain the alteration in terms of biological responses observed in the contaminated organisms. However, the behaviour and effects of CNTs are related to their ability to interact and aggregate, creating clusters that exhibit colloidal behaviour. Despite the virtual water insolubility of individual CNT molecules, the formed aggregates are stable under certain environmental conditions. The properties of the aggregates (size, ζ-potential, shape, surface functionalization, sedimentation rate, critical flocculation concentration, etc.) are dependent on the alteration of their surface (Jackson et al., 2013; Freixa et al., 2018). Jackson et al. (2013) reported that because CNTs are difficult to disperse in water and polar matrices, many commercially available CNTs are therefore functionalized before final use preventing agglomeration in the composite matrices (Kim et al., 2011; Najeeb et al., 2012). Overall, CNTs functionalization technology is currently being used for creation of more soluble forms of carbon NMs for various medical and industrial products such as multifunctional composites, chemical and biological sensors, molecular electronics, fuel cells, super capacitors, lithium batteries, solar cells, and drug and gene delivery systems (Klaper et al., 2010). Functionalization can be achieved through chemical modification such as amidation and esterification of the nanotube-bound carboxylic acids (Sun et al., 2002). The functionalization breaks the nanotube bundles, which is essential to solubility and the presence of functional groups on nanotubes surface therefore increases nanotubes dispersibility (Shahnawaz et al., 2010). Specifically, to disperse CNTs in aqueous media, the chemical functionalization of CNTs by introducing polar groups such as carboxyl groups (-COOH) is one of the most common approaches (Shahnawaz et al., 2010). This carboxylation process has been already demonstrated to have more amorphous carbon fragments as a result of increased oxidation of carbon, and these amorphous fragments can induce higher levels of toxicity to biological systems compared to non-functionalized CNTs (Arndt et al., 2013). Moreover, the large specific surface area may facilitate pollutant adhesion and thus influence CNT toxicity in itself and/or toxicity of co-pollutants and influence the bioaccumulation of environmental contaminants (Ferguson et al., 2008). These findings could justify the biological results observed in the present studies.

3.1.2.2. Biological analyses

3.1.2.2.1. Energy reserves content and metabolic capacity

Energy metabolism plays a fundamental role in organisms' survival and function, as well as in stress adaptation and tolerance (Sokolova et al., 2012). It has been already demonstrated that once the organisms are exposed to pollutants, they can increase their energy expenditure (GLY and PROT), considered a mechanism of cellular protection (Bielen et al., 2016). The balance between energy reserves and ETS activity is important to access if lower energetic availability can lead to impairment in organisms' reproduction and development (Smolders et al., 2004). The ETS activity can be used as a measure of metabolic capacity in different organisms (namely in invertebrates) in response to environmental disturbances (Cammen et al., 1990; Simčič et al., 2014; Aliko et al., 2015; Schmidlin et al., 2015; Bielen et al., 2016; Freitas et al., 2016c) due to the ability to release the energy stored within the reduced hydrogen carriers in order to synthesize ATP (Liu et al., 2002). However, ETS activity has also been recognized as one of the major cellular generators of reactive oxygen species (ROS), which include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl free radical (-OH) (Liu et al., 2002).

Ruditapes philippinarum (Adams & Reeve, 1850)

The present study demonstrated that *R. philippinarum* decreased the GLY and PROT content when exposed to both f-MWCNTs and Nf-MWCNTs, which may indicate that clams were using GLY and PROT to fuel their mechanisms of defense against CNTs toxicity. Moreover, after 28 days exposure to both Nf-MWCNTs and f-MWCNTs, the clams presented increased metabolic capacity (ETS) with the increase of exposure concentrations, especially at the highest exposure concentration, which can be related to the energy expenditure observed under these conditions. These results may be associated with detoxification and excretion processes (Holmstrup et al., 2011). In fact, the energy sources are mainly accumulated as GLY and consumed during reproduction or stress conditions. Similar results were observed by Gagné et al. (2016) in the mussels *Elliptio complanata* exposed to 1 and 10 µg/L zinc oxide NPs (nZnO) for 21 days,. revealing a decrease of energy reserves (GLY content) when the mussels were exposed to the contaminants.

Hediste diversicolor (O.F. Müller, 1776)

Looking to polychaetes energy reserves a similar behaviour was observed for individuals exposed to both Nf and f-MWCNT materials, demonstrating no differences in PROT and GLY contents between contaminated and non-contaminated polychaetes. Although it has been already demonstrated that the availability of energy reserves can be affected by chemical stressors (Scott-

Fordsmand and Weeks, 2000), the present results may indicate that the energy reserves were not used as a resource of energy to fuel polychaetes defence mechanisms when in the presence of both CNTs or most probably, there was no need for an extra expenditure of energy reserves under contaminated conditions suggesting that the concentration levels tested were not stressful enough to increase expenditure of energy reserves. Looking at the metabolic activity, different behaviour was observed between organisms exposed to both CNT materials. While in polychaetes submitted to Nf-MWCNTs no differences were observed between contaminated and non-contaminated organisms, suggesting that the used concentrations were not high enough to result in metabolic depression, the increase of ETS detected in polychaetes exposed to the highest f-MWCNTs concentration could be attributed to higher stress level that was also associated with membranes cellular damage (as demonstrated under this exposure condition) resulting from possible higher ROS production due to higher mitochondrial respiration. Similar results were also obtained by Bertrand et al. (2016) which exposed the bivalve *Scrobicularia plana* to silver (Ag) NPs observed an increase of ETS activity in clams that suffered from membranes cellular damage. In this study, the different observed behaviour of metabolic activity could be attributed to the surface functionalization of the CNTs which induced an increase of the metabolic activity only in polychaetes exposed to f-MWCNTs, while no differences were observed when the organisms were exposed to Nf-MWCNTs suggesting a possible higher uptake of the water-dispersible MWCNTs in comparison to the pristine one .

Diopatra neapolitana (Delle Chiaje, 1841)

The results of the present study revealed both CNTs have a negative effect on the regenerative capacity of *D. neapolitana* at the highest exposure concentration, showing a lower percentage of body width as well as the number of new chaetigers compared to the other conditions after 18th and 28th days exposure. Other studies also showed that CNTs can induce alterations in physiological functions in different invertebrate species (Mwangi et al., 2012; Moschino et al., 2014). For example, Moschino et al. (2014) demonstrated sub-lethal effects at the digestion level in the polychaete *H. diversicolor* exposed to three single-walled carbon nanohorns (SWCNHs) and Mwangi et al. (2012) showed that both MWCNTs and SWCNTs significantly reduced the survival and growth of an amphipod (*Hyalella azteca*), a midge (*Chironomus dilutus*), an oligochaete (*Lumbriculus variegatus*), and a mussel (*Villosa iris*). Moreover, in the present study, the ETS increased exponentially at the highest exposure concentration (0.10 mg/L) of Nf-MWCNTs, indicating that *D. neapolitana* may increase their metabolic activity under stressful conditions. The increase in ETS activity may explain the activation of defence or biotransformation mechanisms, such as the increase on GSTs activity, in contaminated organisms suggesting that *D. neapolitana* was capable to increase the metabolic potential to fuel up defence mechanisms, such as detoxification defences. However, although polychaetes metabolic capacity was enhanced under this condition, polychaetes were able to

increase their GLY and PROT contents. Such findings indicate that individuals may prevent energy expenditure in specific processes when under stress conditions (e.g. limiting their use for polychaetes regeneration) or were using other energy sources (such as lipids) to fuel up defence mechanisms. Similar results were also observed in previous studies that demonstrated that some polychaete species increase their energy reserves under stressful conditions (Carregosa et al., 2014; Maranho et al., 2014). For example, in *H. diversicolor* Maranho et al. (2014) showed an increase of energy reserves with the increase of antiepileptic drug carbamazepine (CBZ) concentrations. Carregosa et al. (2014) observed an increase of GLY content in *D. neapolitana* exposed to stressful organic matter enrichment conditions. These results were totally different in comparison to the results detected in *H. diversicolor* when exposed to Nf-MWCNTs (where no differences in energy reserves and metabolic activity were observed in the exposed individuals compared to control ones), revealing in this polychaetes species a possible major sensitiveness for this contaminant.

Considering the organisms exposed to f-MWCNTs, also in this case polychaetes were able to increase their metabolic capacity, however we observed a decrease of energy reserves (especially GLY content) and lower effect on the regenerative capacity caused by f-MWCNTs in comparison to Nf-MWCNTs, which could indicate that polychaetas under this condition were using their energy reserves to regenerate their body fighting against high CNTs concentration. Similar results were also obtained by Bertrand et al. (2016) which exposing *S. plana* to silver (Ag) NPs, observed an increase of ETS activity indicating impairment of metabolic activity in clams that suffered from the damage of their cellular membranes. As for *H. diversicolor*, the controversial behaviour of energy reserves observed in the present study could be attributed to the surface functionalization of the CNTs, showing also in this species, that f-MWCNTs induced higher levels of toxicity to biological systems in comparison to Nf-MWCNTs (Arndt et al., 2013).

3.1.2.2.2. Oxidative status

Interactions of CNTs with organisms can be external, as the attachment of the NPs onto the skin or exoskeleton, or internal, via food intake, or both (Mesarič et al., 2015). All of these interactions can cause different physiological disturbances, and the generation of oxidative stress, which leads to toxicity with direct damage of the lipid membranes, due to the high affinity of CNTs for lipid membranes (Mesarič et al., 2015). However, successful CNTs uptake in the exposed organisms are important prerequisites for bioaccumulation in the body and consequent cellular damage which are directly related to the characteristics of the CNTs such as heterogeneous purity, length, type of functionalization (Costa et al., 2016). The generation of LPO is known to be responsible for the activation/inactivation of antioxidant systems (scavengers and antioxidant enzymes), which play important roles in the total defence against oxidative damage (Ighodaro and Akinloye, 2017). Specifically, oxidative stress has often been associated with the reduced (GSH) and oxidized

(GSSG) glutathione ratio within the cell (Regoli and Giuliani, 2014). This ratio represents the major homeostatic regulator of redox equilibrium inside the cell and can be useful as a biomarker to detect protective or injurious cellular reactions by measuring the rate and level of ratio alterations (Mocan et al., 2010). Other components of the antioxidant systems are the antioxidant enzymes. SOD is essential for maintaining the normal function of mitochondria-rich organs (Gomes et al., 2012). Biochemically, SOD is the enzyme responsible for the removal of O_2^- with the formation of H_2O_2 that can be used by CAT or GPx enzymes (which uses GSH as an electron donor to catalyze the reduction of H_2O_2 to H_2O) (Regoli and Giuliani, 2014). However, under stressful conditions, ROS can be overproduced and may not be eliminated by defense mechanisms such as antioxidant enzymes leading to LPO (Regoli and Giuliani, 2014). Biochemically, ROS readily interact with polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids and the end-products of such LPO reactions are especially dangerous for the viability of cells, even tissues (Ayala et al., 2014). It has been proven that a major mechanism of toxicity for NPs is oxidative stress, associated with increases in reactive radicals that may affect the balance between antioxidants and oxidative damage, causing significant sub-lethal toxicity to organisms. Therefore, LPO has been used in invertebrates as an indicator of oxidative damage (Tedesco et al., 2010; Zhu et al., 2011; Mccarthy et al., 2013; Ayala et al., 2014; Buffet et al., 2014a; Gomes et al., 2014; Anisimova et al., 2015; Cid et al., 2015; Volland et al., 2015; De Marchi et al., 2017b; c; d; e). Moreover, as multicomponent enzymes involved in the detoxification of different xenobiotics, GSTs play important role in protecting tissues from oxidative stress (Fournier et al., 1992) and they have been already used as biomarkers of cellular damage as these enzymes exhibit many of the required characteristics, i.e. specific localization, high cytosolic concentration and relatively short half-life (Pérez et al., 2004).

Ruditapes philippinarum (Adams & Reeve, 1850)

The results obtained in the present study showed that clams exposed to Nf-MWCNTs showed a gradual increase of LPO levels with the increase of exposure concentrations, and clams exposed to f-MWCNTs presented an increase of LPO levels at the two highest exposure concentrations. Similarly, Anisimova et al. (2015) exposed the mussel *Crenomytilus grayanus* to 12-14 nm diameter MWCNTs (100 mg/L) for 48h and showed that CNTs were responsible for the increase of LPO levels. NPs also showed to affect the processes involved in the maintenance of tissue redox balance in invertebrates, expressed as the decrease of GSH/GSSG (Tedesco et al., 2010) and increase of GSH or GSSG content (Zhu et al., 2011; Anisimova et al., 2015; Falfushynska et al., 2015). In the present study GSH/GSSG values, under both MWCNTs, decreased at the highest exposure concentration, indicating that the stress induced by carbon NPs led to a decrease of GSH that was oxidized to GSSG. Using pristine MWCNTs, Anisimova et al. (2015) observed GSH increased in hemolymph of

C. grayanus on the second day of exposure with respect to control. As mentioned above, invertebrate species are known to increase the activity of SOD in response to the generated cellular oxidative stress (Buffet et al., 2011; Zhu et al., 2011; Gomes et al., 2012; Mccarthy et al., 2013; Buffet et al., 2014a; Gomes et al., 2014). Similar results were observed in the present study where, in clams exposed to f-MWCNTs, the activity of SOD increased indicating a possible enzymatic response to eliminate ROS and to prevent cellular damage (e.g. LPO) under this condition. However, although the activation of antioxidant enzyme such as SOD, a possible elevated concentrations of ROS cells resulted in oxidative stress and LPO still occurred, especially under the highest exposure condition. Differently, in organisms exposed to Nf-MWCNTs, SOD activity did not increase along the increasing exposure concentrations, suggesting a loss of compensatory mechanisms as a consequence of insufficient mechanism of the antioxidant activity (Fukai and Ushio-Fukai, 2011; Walters et al., 2016) and thus contribute to higher LPO levels recorded under these conditions. Although GPx activity is proportionally lower in invertebrate than in vertebrate species compared to the other key antioxidant enzymes (CAT and SOD) (Gamble et al., 1995), the activation of this enzyme in invertebrate species when exposed to NPs has been demonstrated (Gomes et al., 2012; 2014; Volland et al., 2015). Also, in the present study, GPx was activated in clams exposed to both CNT materials at the highest exposure concentration, suggesting that the H_2O_2 produced by SOD may possibly be converted by these antioxidant systems which contribute in the defence against oxidative stress. In the presence of NPs, invertebrates may also increase the activity of GSTs (Ciacci et al., 2012; Garaud et al., 2014; Minetto et al., 2014; Volker et al., 2014; Cid et al., 2015). On the contrary, in the present study, the activity of this biotransformation enzymes, did not increase among in increasing exposure concentrations (Nf-MWCNTs), or decrease at the highest exposure conditions (f-MWCNTs), suggesting that that this group of enzymes was not involved in the biotransformation of CNTs into less toxic excreted substance. In agreement with the present results, Anisimova et al. (2015) observed a decrease of GSTs activity in *C. grayanus* exposed to 12-14 nm diameter of MWCNTs (100 mg/L) after 48h.

Hediste diversicolor (O.F. Müller, 1776)

Considering the generation of the oxidative stress in *H. diversicolor*, the present results showed that while the LPO levels of polychaetes contaminated with Nf-MWCNTs did not present a dose-dependent increased probably due to low concentrations tested or low solubility and consequently low toxicity of non-functionalized MWCNTs, damage of the lipid membranes was observed under the two highest f-MWCNT concentrations, assuming that all these different responses were directly related to the availability of the CNT materials for the organisms or their different biological reactivity. Although the results present till now did not evidence possible severe toxic effects caused by Nf-MWCNTs in exposed individuals, an activation of antioxidant enzymes (such as SOD and GPx) was

observed when the polychaetes were exposed to the two highest concentrations. These results suggested an attempt by these enzymes to cope as compensatory response of cellular defence systems against cellular damage, which may have led to arrest the propagation of LPO reactions on the membranes. In fact, under basal conditions, the adverse effects of oxyradicals are prevented by the antioxidant system, which are able to neutralize ROS by direct or indirect reaction with them, thus being temporarily oxidized before being reconverted by specific reductases to the active form (Regoli and Giuliani, 2014). A similar increase of the antioxidant defences were also observed in polychaetes exposed to f-MWCNTs, however, in this case, the enzymatic responses were not enough to prevent cellular damage and LPO occurred under these conditions as a consequence of ROS overproduction. Again, these antioxidant and biotransformation defences could be associated with the type of NPs and consequent uptake by the exposed organisms as well as higher sensitivity of the species to f-MWCNTs compared to Nf-MWCNTs. Looking the activity of the biotransformation enzymes, under Nf-MWCNTs GSTs activity decreased along the increasing exposure gradient, indicating that under the presence of these NPs, GSTs may be inactivated. Opposite behaviour was observed in polychaetes exposed to f-MWCNTs, where dose-dependent increase of GSTs activity was observed. Other studies that exposed polychaetes to different quantum dots and metal-based NPs exposures, showed an increase in the activity of these biotransformation enzymes (Buffet et al., 2011; Marques et al., 2013; Díaz-Jaramillo et al. 2013; Buffet et al., 2014a; b; Mouneyrac et al., 2014). Therefore, we may hypothesize that the activity of this group of enzymes could be dependent on the NPs used, which was previously hypothesised by Canesi and Corsi (2015).

Diopatra neapolitana (Delle Chiaje, 1841)

Looking to the results of the present study in terms of oxidative status, while in the organisms exposed to Nf-MWCNTs the LPO increased only at the highest exposure concentrations, in polychaetes exposed to f-MWCNTs the damage of the lipid membranes was observed also at the lowest exposure concentration, assuming that these different responses were directly related to the availability of the CNT materials. As for *H. diversicolor*, the dose-dependent increased of the LPO in *D. neapolitana* under f-MWCNTs, led a consequence dose-dependent decrease of GSH/GSSG as well as the increase of SOD and GPx activities, but not from CAT activity, which means that this enzyme was not involved in the antioxidant defences. This result suggested a compensatory response of cellular defence systems against cellular damage; however, the enzymatic responses were not enough to prevent cellular damage and LPO occurred under these conditions. Considering *D. neapolitana* exposed to Nf-MWCNTs, the GSH/GSSG decreased at the highest exposure concentration but the SOD, GPx and CAT activities did not increase. In this case, the results were different in comparison to *H. diversicolor* exposed to the same condition. In fact, while in *H. diversicolor* the activation of the antioxidant systems may have generated the arrest the propagation

of LPO reactions on the membranes, in *D. neapolitana* the observed behaviour of the antioxidant systems may be due to an excessive ROS production, especially under the highest exposure concentration, which may have caused the oxidative damage and a loss of compensatory mechanisms as a consequence of insufficient antioxidant mechanisms (Fukai and Ushio-Fukai 2011; Walters et al., 2016) contributing to higher LPO levels recorded at this condition. In previously published studies GSTs showed different mechanisms of action when exposed to different NPs, assuming that GSTs activity may be either increased or decreased due to the production of lipid hydroperoxides (Kos et al., 2017) and also the type of NPs (Lehman et al., 2011). For example, Canesi et al. (2010) exposing the *M. galloprovincialis* to different CNPs (nano carbon black-nNCB, C60 fullerene), reported that all materials induced changes in GSTs activities, with contrasting trends, depending on NPs type and solubility. The results of the present study, as well as for *H. diversicolor*, are in line with such findings, showing a decreased GSTs activity when organisms were exposed to Nf-MWCNTs (insoluble) and increased activities in organisms exposed to f-MWCNTs (soluble).

3.1.2.2.3. Neuro status

The Cholinesterases class includes specific cholinesterase (acetylcholinesterase (AChE)) and non-specific cholinesterase (or pseudo cholinesterase). AChE hydrolyses the neurotransmitter acetylcholine, producing choline and an acetate group (Lionetto et al., 2011). Among the various types of biomarkers studied, the inhibition of AChE activity receives special attention in ecotoxicological studies. Recently the inhibition of cholinesterase in invertebrates has been used as a sensitive biomarker of exposure to various NPs (Gomes et al., 2011; Buffet et al., 2014a; Luis et al., 2016; Marisa et al., 2016), demonstrated high adsorption or directly interaction with AChE and the contaminants (Lionetto et al., 2011). So far, there is no clear explanation of how the NPs interact with these enzymes. One of the most plausible hypotheses is that NPs have the capacity to bind to ChEs due to the lipophilicity of the NPs and the hydrophobicity of the enzyme (Šinko et al., 2014) and recently studies already demonstrated CNTs high affinity for AChE adsorption and inhibition in different invertebrate species (Šinko et al., 2014; Calisi et al., 2016).

Ruditapes philippinarum (Adams & Reeve, 1850)

The present results revealed that both MWCNT materials impaired the hydrolytic activity of ChEs which resulted in a significant inhibition of AChE activity in *R. philippinarum* exposed to Nf-MWCNTs and f-MWCNTs compared to control and the lowest CNT concentrations. These results may have been caused due to the high affinity of MWCNT for AChE, and their ability to cause 76–88% AChE activity reductions (Wang et al., 2009), assuming that the perturbation of the structure influencing the function of enzyme subunits may be the common mode of ChE inhibition by NPs.

Hediste diversicolor (O.F. Müller, 1776)

The results of the present study revealed that when polychaetes were exposed to Nf-MWCNTs, the activity of the neurotransmitter AChE was inhibited especially 0.01 mg/L but then increase again when exposed to the highest concentration. Different behaviour was observed regarding f-MWCNTs, where the activity of the neurotransmitter was lower only at the highest exposure concentration. Looking on DLS analysis, the mean size of the f-MWCNTs was always lower in comparison to Nf-MWCNTs, which could help us to justify the higher availability of the carboxylated form of MWCNTs also at the highest concentration for the organisms, intensifying the risk of exposure and possible absorption of the NPs, leading to a much higher neuro status damage in comparison to the insoluble form of MWCNTs.

Diopatra neapolitana (Delle Chiaje, 1841)

Looking the neuro status of *D. neapolitana*, as for *H. diversicolor*, the results showed an inhibition of AchE activity when the organisms were exposed to the highest concentration of f-MWCNTs, while when polychaetes were contaminated with Nf-MWCNTs, showing no inhibition of AChE activity. Such result may be related again to the availability of the NPs as already described for *H. diversicolor* section.

3.1.3. Final considerations

The results presented in the previous section allowed a better understanding the effects in three different invertebrate species (clam: *R. philippinarum*, and polychaetes: *H. diversicolor* and *D. neapolitana*) due to the exposure concentrations of two different forms of CNTs: one pristine MWCNTs (Nf-MWCNTs) and the other one chemically functionalized MWCNTs, by introducing polar groups such as carboxyl groups (-COOH) (f-MWCNTs). This carboxylated forms of CNTs are more stable in salt water media in comparison to pristine CNTs as a consequence of their oxidation process which introduces oxygen-containing groups on the CNTs surface. For these reasons, the possible effects of the carboxylation/functionalization of the surface of MWCNTs in organisms for each exposure concentration were also evaluated.

The use of three different species with different trophic behaviour was necessary to fully understand the fate of the contaminants in all different natural matrices. The selection of these three invertebrate species was appropriate due to the presence of a filter-feeder bivalve (which is able through their gills to capture particulate matter and particles greater than ca. 6 µm are captured with an efficiency >90% (Ward and Kach, 2009)) and the two polychaetes species (which the ingestion of nano-contaminated sediment is crucial for uptake and cellular internalization of NPs by polychaetes (Magesky et al., 2018)).

Looking at the obtained findings, it was demonstrated that the contamination by both Nf-MWCNTs and f-MWCNTs may affect all species' biochemical performance. In detail, both CNT materials caused alteration of the energy reserve contents and metabolism in *R. philippinarum* and *D. neapolitana*, while in the *H. diversicolor* the used concentrations of Nf-MWCNTs were not high enough to result in metabolic depression or alteration of the energy contents. These results may be due to higher tolerance responses by the polychaetes to the pristine form of MWCNTs or the lower uptake of these materials by the organisms. This was also observed in terms of oxidative stress. In the presence of Nf-MWCNTs, *H. diversicolor* seemed to be able to tolerate oxidative stress caused by the high production of ROS being able to increase their defense mechanisms and, therefore, preventing cellular damages under these exposure concentrations. Differently, in the organisms contaminated with f-MWCNTs, the observed impairment of metabolic activity could be attributed to membranes cellular damage despite the activation of antioxidant enzymes. Regarding *R. philippinarum* and *D. neapolitana*, both CNTs generated toxic impacts in terms of oxidative status. In fact, under both CNT materials, although the activation of the antioxidant systems, it was possible to observe oxidative damage and a loss of compensatory mechanisms as a consequence of insufficient antioxidant mechanisms. Considering the neuro status, in the two polychaete species, it

was possible to observe an inhibition of the neurotransmitter only when exposed to the carboxylated MWCNT, while in *R. philippinarum* both MWCNTs were able to generate neurotoxicity.

Overall, when comparing the toxic effects of both CNTs, in all invertebrate species major cellular damage was induced by the carboxylated forms of MWCNTs in comparison to the pristine one. Water-dispersible MWCNTs, due to the presence of higher amorphous carbon fragments in comparison to pristine one, can induced higher levels of toxicity to biological systems, as also demonstrated by Arndt et al. (2013), causing higher cellular damage with the activation of antioxidant mechanisms (Freixa et al., 2018). In fact, the release of ions resulting from the dissolution of the NPs caused higher oxidative stress mediated by ROS generation at the NPs surface, a process already suggested to be a major responsible factor for NPs toxicological effects (Freixa et al., 2018).

In conclusion, looking at the obtained results, it was clearly demonstrated that nanomaterial toxicity can be attributed to the core structure and surface functionalization, which have been shown to alter the level of toxicity to biological systems. Considering the increase of the use of CNTs in different fields and industrial applications and consequent release into aquatic ecosystems, the presented studies provide valuable information regarding the potential risk of these materials in the aquatic environment and living organisms. However, there is still a lack of information regarding CNTs fate and toxicology in the aquatic environment. The study of the toxicity of these CNTs may lack of ecological relevance since in the environment different conditions may act in combination, changing the behavior and toxicity of NPs. Considering that the simultaneous exposure of marine organisms to CNTs and climate changes is likely an ecologically relevant scenario, in the next section the two most deleterious concentrations of Nf-MWCNTs and f-MWCNTs detected in the previous section were selected and all the three invertebrate species were exposed to the combination of CNT materials with salinity shifts and pH variations assessing if both climate change factors altered the toxicity of both MWCNT materials as well as the sensitivity of all these species exposed to these contaminants.

3.2. Combination of stressors experiments: CNTs and salinity shifts

3.2.1. Results

3.2.1.1. *Ruditapes philippinarum* (Adams & Reeve, 1850)

3.2.1.1.1 Characterization analysis of water media

Table 9 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.01 and 0.10 mg/L) under control salinity (28) and low salinity (21).

Results of DLS and PDI analysis of experimental samples exposed to different concentrations of Nf-MWCNTs (0.01 and 0.10 mg/L) among collection periods (T0, T7, T21 and T28) under salinity 28 showed the presence of unstable micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 9). Furthermore, it was also possible to observe a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples. At T14 the DLS analysis was not carried out as a consequence of not supplied samples collected under this exposure period reported in the table as “not supplied sample”. DLS analysis of samples exposed to Nf-MWCNTs at salinity 21 at different exposure periods evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 9). Moreover, the mean dimensions of the particle aggregates showed a general decrease in the hydrodynamic radius of the aggregates at both tested concentrations probably due to a fractional deposition of larger particles occurring during the period of exposure. The decrease of the PDI was directly correlated with the detected aggregates in the investigated samples.

DLS and PDI analyses of samples exposed to different concentrations of f-MWCNTs (0.01 and 0.10 mg/L) at salinity 28 did not allow for the detection of measurable macro/micro/nanosized particle aggregates observed among collection periods (T7, T21 and T28), however at T0 it was evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 9). The time evolution of the mean values of the dimension of the suspended f-MWCNTs aggregates exposed to salinity 21 was similar to that recorded for Nf-MWCNTs at the same experimental conditions.

The mean recorded hydrodynamic diameter of f-MWCNTs aggregates was smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating a higher dispersion of f-MWCNTs in aqueous media (Table 9). Comparing the aggregates of both MWCNT

materials under salinity 21 and 28, it was possible to observe larger mean diameters on both carbon NPs under salinity 21 compared the ones under control salinity 28. However, under salinity 28, it was identified through a visual observation the presence floated macro-particle with larger particle sizes compared to the ones at salinity 21, which the instrument was not able to record.

Table 9. *Ruditapes philippinarum*: average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed under control salinity (28) and low salinity (21) in each exposure concentration (0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

CNT concentration (mg/L)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
	Nf-MWCNTs				**f-MWCNTs**			
	28		**21**		**28**		**21**	
	T0				T0			
0.01	5330.4	1.79	2407.1	0.98	4551.8	1.86	3244.8	1.30
0.10	6714.4	1.75	7845.3	2.83	5714.4	1.45	6264.2	2.17
	T7				T7			
0.01	3 l.d.	n.d.	3938.3	1.23	3 l.d.	n.d.	5 l.d.	n.d.
0.10	3602.9	1.39	5543.2	1.83	5 l.d.	n.d.	5548.8	1.74
	T14				T14			
0.01	*	*	*	*	*	*	*	*
0.10	*	*	*	*	*	*	*	*
	T21				T21			
0.01	5 l.d.	n.d.	3841.9	1.09	3 l.d.	n.d.	1661.8	0.10
0.10	n.d.	n.d.	6230.6	2.29	5 l.d.	n.d.	2953.8	0.75
	T28				T28			
0.01	4542.7	1.81	3276.1	1.32.	5 l.d.	n.d.	5 l.d.	n.d.
0.10	3765.1	1.40	4432.9	1.40	5 l.d.	n.d.	5 l.d.	n.d.

l.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
*: Not supplied sample.

3.2.1.1.2. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

The results presented here were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both salinity levels (control-28 and low-21); II) understand the effects of salinity in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both salinity levels at each exposure concentration.

Energy reserves content and metabolic capacity

I) Considering the effects of exposure concentrations, results of PROT content in *R. philippinarum* contaminated with Nf-MWCNTs under salinity 28 showed significantly lower PROT content only in clams exposed to 0.10 mg/L in comparison to the remaining concentrations, while under salinity 21 no significant differences were detected among concentrations (Figure 29 A). In organisms exposed to f-MWCNTs under salinity control, significantly lower PROT content was observed in contaminated organisms in comparison to control organisms, while under salinity 21 significantly lower content was assessed only at the highest exposure concentration (0.10 mg/L) compared to the other treatments (Figure 29 A).

II) For each MWCNTs (f and Nf) at each exposure concentration, no significant differences were observed between salinities (28 and 21) (Figure 29 A).

III) When comparing organisms exposed to the same salinity and exposure concentration, no significant differences were observed in PROT content between organisms exposed to different MWCNTs (Table 10).

I) Along with the increasing Nf-MWCNTs exposure concentrations, all the clams maintained at control salinity decreased their GLY content, with significant differences between contaminated and non-contaminated treatments, while under salinity 21 no significant differences were observed among concentrations (Figure 29 B). *R. philippinarum* contaminated with f-MWCNTs under salinity 28 showed lower GLY content when exposed to the highest Nf-MWCNTs concentration, with significant differences compared to the other concentrations. Under salinity 21, no significant differences were detected between concentrations (Figure 29 B).

II) For each MWCNTs (f and Nf) at each exposure concentration, significant differences between salinities were observed in all tested concentrations for individuals exposed to Nf-MWCNTs, with lower content in organisms maintained to control salinity 28 compared to the ones under salinity 21 (Figure 29 B).

III) Comparing organisms under each salinity and each exposure concentration, significant differences between materials were observed in organisms exposed to 0.01 mg/L under control salinity, with lower values in clams contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 10).

I) A significant dose-dependent increase of ETS activity was observed in *R. philippinarum* maintained at salinity 28 and contaminated with Nf-MWCNTs, with the highest value detected at 0.10 mg/L. At salinity 21, the activity of ETS was significantly higher only in clams exposed to 0.10 mg/L relative to the remaining concentrations (Figure 29 C). Results of ETS in *R. philippinarum* contaminated with f-MWCNTs under both salinities showed significantly higher metabolic activity only in clams exposed to 0.10 mg/L in comparison to the remaining concentrations (Figure 29 C).

II) For each MWCNT at each exposure concentration, differences between salinities were observed in individuals exposed to 0.10 mg/L Nf-MWCNTs and control individuals, with higher and lower values respectively at salinity 21 in comparison to salinity 28 (Figure 29 C). Also, individuals exposed to f-MWCNTs showed significant differences between salinities in all conditions, with higher ETS activity at salinity 21 (Figure 29 C).

III) When comparing *R. philippinarum* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in clams exposed to 0.01 and 0.10 mg/L under salinity 28 showing an increase of the metabolic activity for individuals contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 10).

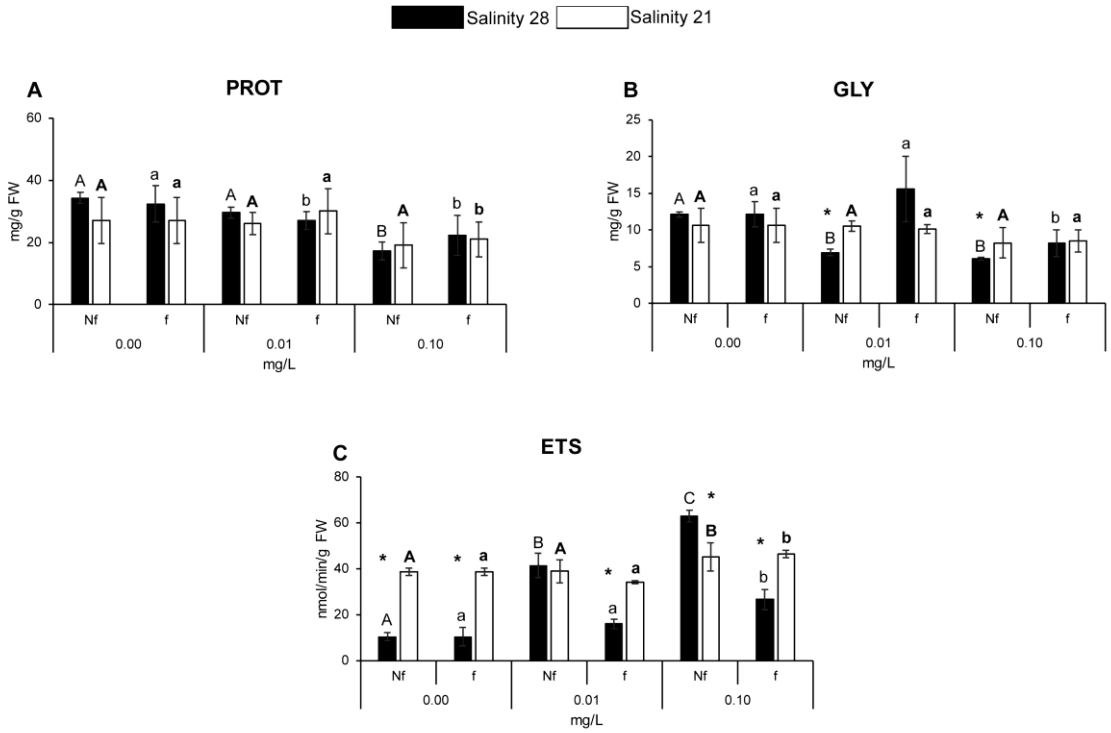

Figure 29. **A**: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron transport system (ETS) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

Table 10. Effect on oxidative stress biomarkers (Protein (PROT) content; Glycogen (GLY) content; Electron transport system (ETS) activity; Lipid peroxidation (LPO) levels; GSH/GSSG; Superoxide dismutase (SOD) activity; Catalase (CAT) activity; Glutathione peroxidase (GPx) activity; Glutathione S-Transferases (GSTs) activity; Acetylcholinesterase (AChE) activity) in *R. philippinarum* by pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control salinity (28) and low salinity (21). Significant differences ($p \leq 0.05$) between MWCNTs within each salinity at each exposure concentration were represented with asterisks (*).

CNT (mg/L)	Salinity		PROT	GLY	ETS	LPO	GSH/GSSG	SOD	CAT	GPx	GSTs	AChE
0.00	28	Nf	34.32±1.85	12.11±0.38	10.38±1.86	19.07±2.04	2.10±0.19	5.87±0.25	21.13±0.10	0.011±0.001	0.20±0.00	0.23±0.05
		f	32.39±5.86	12.11±1.73	10.38±4.04	20.27±2.01	2.01±1.00	5.34±1.77	20.57±1.84	0.012±0.005	0.21±0.02	0.26±0.07
	21	Nf	27.10±7.34	10.61±2.34	38.78±1.59	23.68±1.83	2.60±0.52	5.08±1.86	19.29±1.61	0.012±0.003	0.20±0.01	0.25±0.05
		f	27.10±7.34	10.61±2.34	38.78±1.59	23.68±1.83	2.60±0.52	5.08±1.86	19.29±1.61	0.012±0.003	0.20±0.01	0.25±0.05
0.01	28	Nf	29.58±1.71	6.94±0.50	41.31±5.36	30.69±3.21	2.14±0.05	6.67±0.21	21.16±0.10	0.014±0.002	0.20±0.00	0.10±0.02
		f	26.98±2.86	15.55±4.45*	16.01±2.14*	45.44±9.63*	2.90±0.95	6.34±1.23	17.38±1.97	0.009±0.001*	0.17±0.04	0.07±0.01
	21	Nf	26.20±3.56	10.55±0.72	38.89±5.00	29.25±3.27	2.83±0.43	5.49±1.88	21.68±11.77	0.013±0.002	0.22±0.17	0.05±0.01
		f	30.10±7.31	10.13±0.57	34.18±0.65	22.02±1.43	2.66±0.57	5.66±1.05	22.63±8.94	0.012±0.002	0.21±0.04	0.05±0.02
0.10	28	Nf	17.36±2.87	6.09±0.20	62.92±2.66	39.83±3.03	1.44±0.03	6.47±0.08	21.04±0.04	0.023±0.003	0.20±0.00	0.08±0.02
		f	22.29±6.33	8.22±1.82	26.57±4.45*	50.40±4.79*	1.63±0.18	10.28±3.68*	20.10±0.86	0.016±0.002*	0.12±0.04	0.07±0.02
	21	Nf	19.10±7.32	8.22±2.05	45.19±6.14	36.89±2.95	1.77±0.53	6.93±2.93	22.22±1.72	0.015±0.005	0.17±0.03	0.05±0.01
		f	21.01±5.58	8.52±1.52	46.44±1.68	45.77±1.83*	2.08±1.14	7.94±2.50*	21.88±1.81	0.015±0.003	0.19±0.01	0.05±0.02

Oxidative status

I) Under both salinities the level of LPO in clams exposed to Nf-MWCNTs increased with the increasing of exposure concentrations, with significant differences among all treatments (Figure 30 A). Increased LPO levels were also observed in clams under f-MWCNTs and salinity control, with significant differences among all exposed and non-exposed conditions. Under salinity 21, significantly higher levels were observed only in clams exposed to the highest exposure concentration compared to the remaining ones (Figure 27 A).

II) For each MWCNT at each exposure concentration, significant differences between salinities were only observed in contaminated clams with 0.01 mg/L f-MWCNTs, showing higher levels in individuals maintained at control salinity 28 compared to individuals under salinity 21 (Figure 30 A).

III) Comparing organisms under the same salinity and exposure concentration, significantly higher LPO levels in all tested concentrations were observed in clams exposed to f-MWCNTs compared to Nf-MWCNTs under salinity 28, as well as in clams exposed to 0.10 mg/L under salinity 21, showing also in this case higher LPO levels under f-MWCNTs (Table 10).

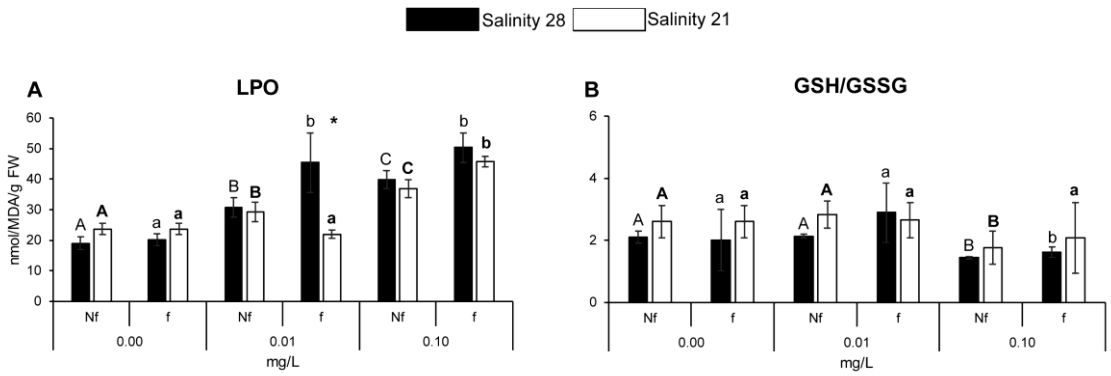

Figure 30. **A**: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

I) No significant differences in terms of SOD activity were observed in *R. philippinarum* contaminated with Nf-MWCNTs in comparison to control organisms maintained under both salinities (28 and 21). Regardless of the salinity levels, significantly higher antioxidant activity was detected

only at the highest exposure concentration of f-MWCNTs in comparison to all the other treatments (Figure 31 A).

II) For each MWCNT at each exposure concentration, significant differences between salinities were only observed in contaminated clams with 0.10 mg/L f-MWCNTs, showing higher SOD activity in individuals maintained at control salinity compared to individuals under salinity 21 (Figure 31 A).

III) When comparing *R. philippinarum* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in clams exposed to 0.10 mg/L under both salinities showing an increase of the antioxidant enzyme activity for individuals contaminated with f-MWCNTs (Table 10).

I) In all organisms submitted to both salinities and both MWCNTs, no significant differences in terms of CAT activity were observed among concentrations (Figure 31 B).

II) For each MWCNT at each exposure concentration, no significant differences between salinities were detected (Figure 31 B).

III) Comparing *R. philippinarum* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were assessed (Table 10).

I) A significantly dose-dependent increase of GPx activity was observed in clams exposed to Nf-MWCNTs under salinity 28, with higher values at the highest exposure concentration, while under low salinity the activity of GPx showed no significant differences among all exposure concentrations (Figure 31 C). Regarding organisms contaminated with f-MWCNTs, significantly higher activity was recorded only at 0.10 mg/L in comparison to the remaining treatments, while at salinity 21 no significant differences were detected between concentrations (Figure 31 C).

II) For each MWCNT at each exposure concentration, differences between salinities were observed at 0.01 mg/L f-MWCNTs, showing higher GPx activity in individuals maintained at salinity 21 in comparison to organisms under salinity 28, and at 0.10 mg/L Nf-MWCNTs, with higher activity recorded in clams under salinity 28 compared to salinity 21 (Figure 31 C).

III) When comparing *R. philippinarum* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in clams exposed to 0.01 and 0.10 mg/L under salinity 28 showing higher antioxidant enzyme activity for individuals contaminated with Nf-MWCNTs (Table 10).

I) Regardless of salinity levels, no significant differences in terms of GSTs activity were observed between concentrations in clams contaminated with Nf-MWCNTs (Figure 31 D). Significantly lower activity was assessed in specimens exposed to 0.10 mg/L f-MWCNTs under salinity 28 in comparison to control individuals. Under low salinity, no significant differences were observed among concentrations (Figure 30 D).

II) For each MWCNT at each exposure concentration, differences between salinities were observed in organisms exposed to 0.01 mg/L f-MWCNTs, with higher GSTs activity under salinity 21 compared to individuals under salinity 28. Significant differences between salinities were also recorded in individuals submitted to 0.10 mg/L Nf-MWCNTs and f-MWCNTs, with higher and lower enzyme activity respectively in individuals under salinity 28 in comparison to salinity 21 (Figure 31D).

III) Comparing *R. philippinarum* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were observed (Table 10).

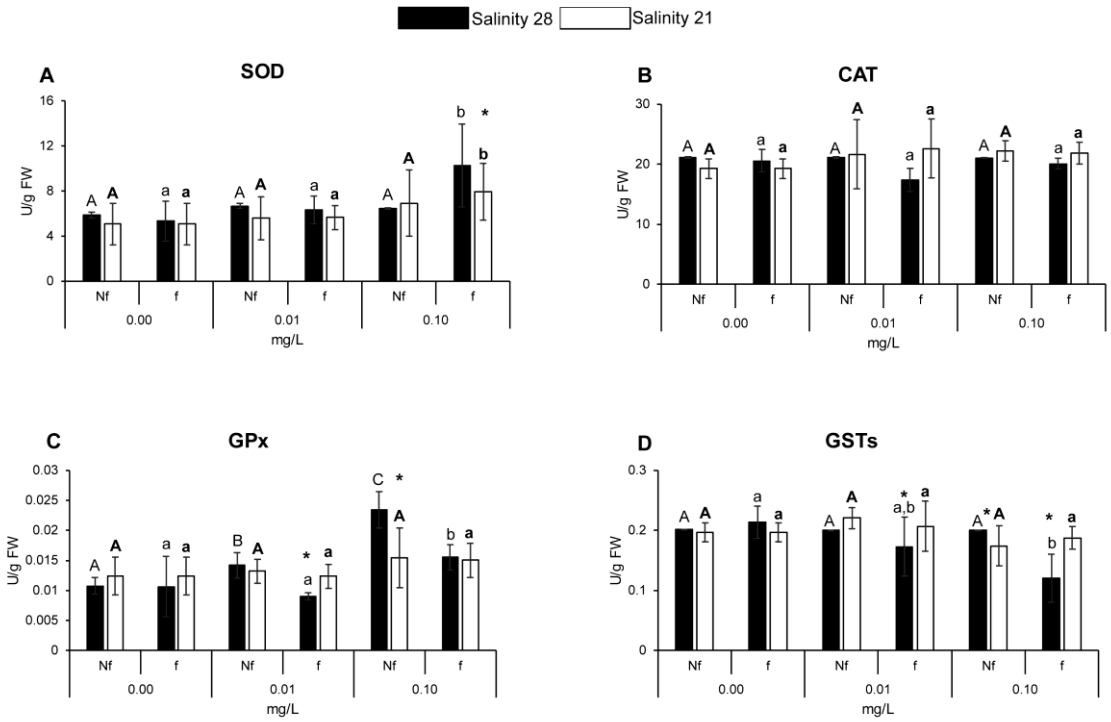

Figure 31. **A:** Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

Neuro status

I) In clams maintained under both salinities and contaminated with both MWCNTs, the AChE activity was significantly lower in exposed compared to non-exposed organisms (Figure 32).

II) For each MWCNT at each exposure concentration, no significant differences were observed between salinities (Figure 32).

III) When comparing organisms exposed to the same salinity and exposure concentration but different MWCNTs, no significant differences in terms of AChE activity were identified between both materials (Table 10).

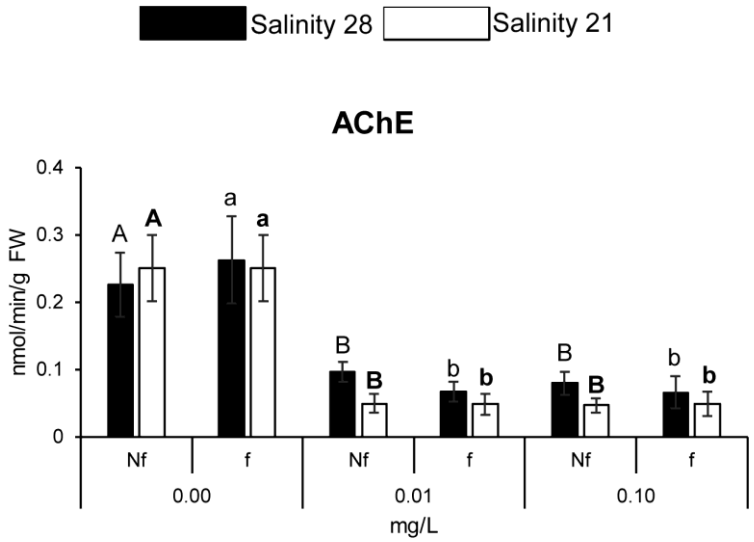

Figure 32. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

3.2.1.2. *Hediste diversicolor* (O.F. Müller, 1776)

3.2.1.2.1. Characterization analysis of water media

Table 11 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.01 and 0.10 mg/L) under control salinity (28) and low salinity (21).

Results of DLS and PDI analyses of experimental samples exposed to different concentrations of Nf-MWCNTs (0.01 and 0.10 mg/L) among collection periods (T0, T7 and T28) under salinity 28 showed the presence of unstable micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 11). Time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples was also observed. At T14 and T21 the DLS analyses were not carried out as a consequence of not supplied samples collected under this exposure period indicated in the table as “not supplied sample”. DLS analysis of samples exposed to Nf-MWCNTs at salinity 21 at T0, T7 and T21 evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 11). The mean dimensions of the particle aggregates recorded after different exposure periods showed similar hydrodynamic radius of the aggregates at both tested concentrations. Comparing the aggregates of Nf-MWCNT materials under salinity 28 and 21, it was possible to observe higher mean diameters on Nf-MWCNTs under salinity 28 compared to the ones under low salinity 21, confirming that higher salinity causes the formation of large-size aggregates, which will increase the chance of physical retention, such as gravitational sedimentation, interception and straining of NPs (Hu et al., 2017).

Regarding the results of DLS and PDI analyses of experimental samples exposed to different concentrations of f-MWCNTs (0.01 mg/L and 0.10 mg/L) among collection periods (T0, T7, T14, T21 and T28) under salinity 28, it was possible to observe also in this case unstable micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples as well as a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples (Table 11). DLS analysis of samples exposed to f-MWCNTs at salinity 21 among collection periods (T0, T7, T21 and T28) evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples. The increase of the PDI was directly correlated with the detected aggregates in the investigated samples (Table 11). Comparing the aggregates of f-MWCNT materials under salinity 28 and 21, it was possible to observe higher mean diameters on f-MWCNTs under salinity 28 compared to the ones under low salinity 21. Considering both salinities, the mean

recorded hydrodynamic diameter of f-MWCNTs aggregates were smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating higher dispersion of f-MWCNTs in aqueous media (Table 11).

Table 11. *Hediste diversicolor*: average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed under control salinity (28) and low salinity (21) in each exposure concentration (0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

CNT concentrations (mg/L)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
	Nf-MWCNTs				**f-MWCNTs**			
	28		**21**		**28**		**21**	
	T0				T0			
0.01	2098.3	1.32	1987.3	1.02	1987.2	0.99	1654.2	0.76
0.10	5707.5	1.91	2562.9	1.21	3244.8	1.30	3225.5	1.32
	T7				T7			
0.01	1999.2	1.21	1009.1	1.52	1543.2	0.54	1321.1	0.43
0.10	3 I.d.	n.d.	1776.2	0.51	5 I.d.	n.d..	5 I.d.	n.d.
	T14				T14			
0.01	*	*	*	*	5 I.d.	n.d.	3 I.d.	n.d.
0.10	*	*	*	*	4542.7	1.82	1654.5	0.55
	T21				T21			
0.01	*	*	*	*	n.d.	n.d.	n.d.	n.d.
0.10	*	*	*	*	4328.5	1.38	1661.8	0.10
	T28				T28			
0.01	1909.2	0.34	1876.2	0.65	1321.1	0.76	1009.1	0.21
0.10	3 I.d.	n.d.	2005.7	0.73	3276.5	1.32	5 I.d.	n.d.

I.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
* Not supplied sample.

3.2.1.2.2. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both salinity levels (control-28 and low-21); II) understand the effects of salinity in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both salinity levels at each exposure concentration.

Energy reserves content and metabolic capacity

I) Under both salinities and both MWCNTs, significantly lower PROT content was observed only when polychaetes were exposed to 0.10 mg/L in comparison to the other treatments (Figure 33 A).

II) For both MWCNTs (f and Nf) at each exposure concentration, no significant differences were observed between salinities in terms of PROT content (Figure 33 A).

III) Comparing *H. diversicolor* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were detected (Table 12).

I) Regardless of salinity levels, no significant differences in terms of GLY content were observed between concentrations in polychaetes contaminated with Nf-MWCNTs (Figure 33 B). Significantly lower content was detected in specimens exposed to 0.10 mg/L f-MWCNTs under both salinities in comparison to the remaining treatments (Figure 33 B).

II) For each MWCNT at each exposure concentration, no differences between salinities were observed (Figure 33 B)

III) Comparing polychaetes exposed to different MWCNTs at the same salinity and exposure concentration, significant differences were identified in specimens exposed to 0.10 mg/L under both salinities, showing lower GLY content in *H. diversicolor* contaminated with f-MWCNTs in comparison to Nf-MWCNTs (Table 12).

I) Under both salinities and both MWCNTs, significantly higher ETS activity was observed only when polychaetes were exposed to 0.10 mg/L in comparison to the other concentrations (Figure 33 C).

II) For each MWCNT at each exposure concentration, significant differences between salinities were detected only at 0.10 mg/L Nf-MWCNTs, showing higher ETS activity when the individuals were maintained under salinity control compared to low salinity (Figure 33 C).

III) Comparing *H. diversicolor* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were detected (Table 12).

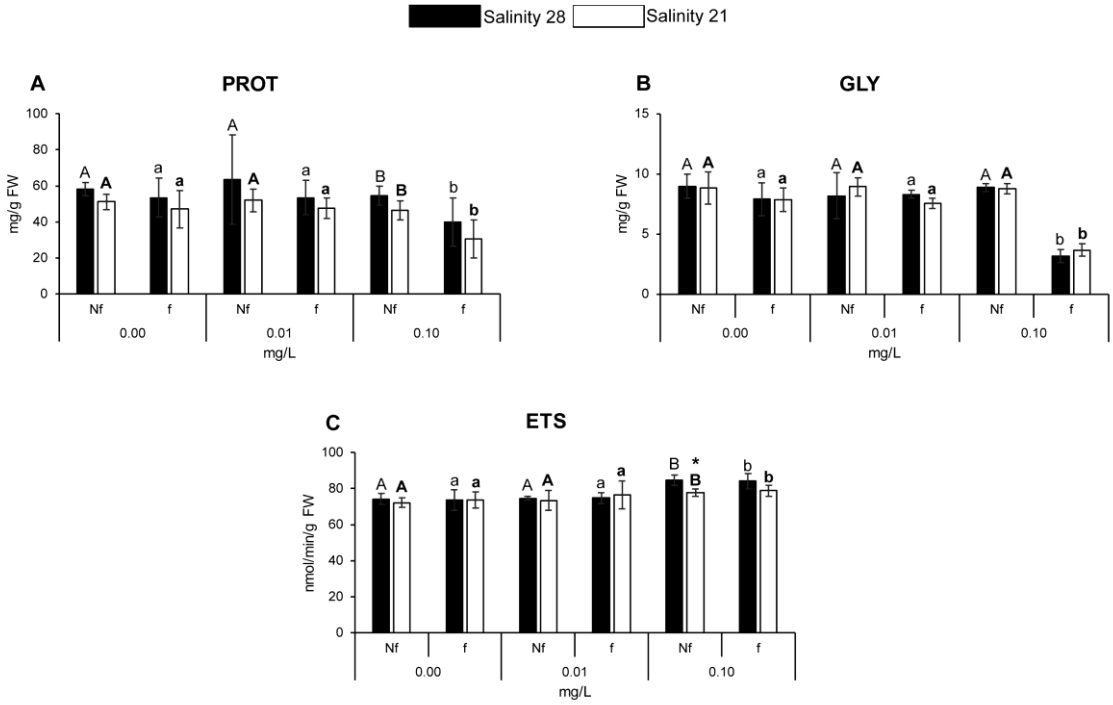

Figure 33. **A**: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron transport system (ETS) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

Table 12. Effect on oxidative stress biomarkers (Protein (PROT) content; Glycogen (GLY) content; Electron transport system (ETS) activity; Lipid peroxidation (LPO) levels; GSH/GSSG; Superoxide dismutase (SOD) activity; Catalase (CAT) activity; Glutathione peroxidase (GPx) activity; Glutathione S-Transferases (GSTs) activity; Acetylcholinesterase (AChE) activity) in *H. diversicolor* by pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control salinity (28) and low salinity (21). Significant differences ($p \leq 0.05$) between MWCNTs within each salinity at each exposure concentration were represented with asterisks (*).

CNT (mg/L)	Salinity		PROT	GLY	ETS	LPO	GSH/GSSG	SOD	CAT	GPx	GSTs	AChE
0.00	**28**	Nf	58.19±3.57	8.97±0.99	74.27±2.96	13.90±0.66	6.68±0.69	3.62±0.11	55.18±0.24*	0.016±0.002*	0.18±0.00	3.47±0.38*
		f	53.50±10.81	7.88±1.35	73.70±5.75	16.11±0.98	7.30±1.16	3.22±0.73	47.47±0.24	0.011±0.002	0.20±0.01	1.79±0.20
	21	Nf	51.28±4.26	8.84±1.34	72.25±2.78	14.22±0.58	5.41±0.57	3.51±0.25	55.15±0.23	0.019±0.002*	0.18±0.00	1.44±0.77
		f	47.07±10.31	7.83±0.97	73.85±4.48	17.16±1.22	7.77±1.55	3.79±1.24	51.72±5.72	0.011±0.001	0.18±0.01	1.70±0.26
0.01	**28**	Nf	63.52±24.61	8.17±1.92	74.65±1.03	13.21±4.32	6.44±0.32	4.89±1.54	54.09±9.75	0.025±0.002*	0.15±0.00*	1.40±0.21
		f	53.52±9.52	8.30±0.33	74.87±3.09	20.27±1.92*	6.31±0.54	3.98±0.99	46.64±9.29	0.017±0.006	0.19±0.00	1.54±0.21
	21	Nf	51.98±6.43	8.94±0.76	73.43±5.43	14.01±0.43	5.44±1.22	3.91±1.22	55.98±4.30	0.028±0.001*	0.18±0.08	1.43±0.26
		f	47.62±5.76	7.54±0.43	76.43±7.65	19.20±0.54*	7.87±0.87	3.98±1.01	52.01±2.10	0.018±0.007	0.19±0.05	1.67±0.34
0.10	**28**	Nf	54.40±5.28	8.86±0.32*	84.54±2.90	18.74±1.23*	2.60±0.23	6.20±0.10	55.42±0.12*	0.028±0.004	0.20±0.00*	2.29±0.48*
		f	39.89±13.44	3.17±0.55	84.08±4.22	28.17±2.41	4.02±2.09	6.60±0.86	49.06±2.93	0.021±0.004	0.10±0.03	1.36±0.33
	21	Nf	46.52±5.40	8.74±0.43*	77.85±2.06	17.88±1.46	2.25±0.36	6.26±0.09	55.59±0.20	0.031±0.003*	0.20±0.00*	1.56±0.65
		f	30.57±10.46	3.66±0.54	78.90±2.97	20.41±1.66	4.47±2.10	6.43±0.71	56.80±4.81	0.019±0.003	0.10±0.02	1.67±0.50

Oxidative status

I) Regardless of the salinity levels, significantly higher LPO levels were only observed when polychaetes were exposed to 0.10 mg/L Nf-MWCNTs in comparison to the other concentrations (Figure 34 A). Considering *H. diversicolor* contaminated with f-MWCNTs under salinity 28, significantly dose-dependent increase of LPO levels was detected, with the highest value at 0.10 mg/L, while under salinity 21, significantly higher LPO was observed only in polychaetes exposed at 0.10 mg/L compared to the other treatments (Figure 34 A).

II) For each MWCNT at each exposure concentration, significant differences between salinities were detected only at 0.10 mg/L f-MWCNTs, showing higher LPO levels when the organisms were maintained under salinity control compared to low salinity (Figure 34 A).

III) Comparing polychaetes exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 mg/L under both salinities and 0.10 mg/L under salinity control, showing in all cases higher LPO levels in *H. diversicolor* contaminated with f-MWCNTs in comparison to Nf-MWCNTs (Table 12).

I) At both salinities tested and both CNT materials, GSH/GSSG significantly decreased in polychaetes at the highest exposure concentration (0.10 mg/L) in comparison to the remaining concentrations (Figure 34 B).

II) For each MWCNT at each exposure concentration, no significant differences were observed between organisms exposed to different salinities (control-28 and low-21) (Figure 34 B).

III) Comparing *H. diversicolor* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were detected (Table 12).

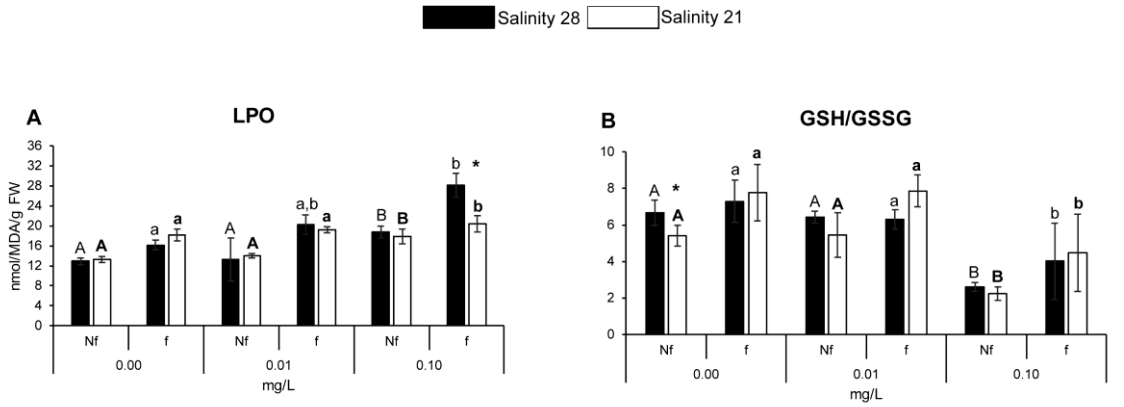

Figure 34. **A**: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

I) In polychaetes contaminated with Nf-MWCNTs under salinity 28, significantly higher SOD activity was observed in exposed individuals compared to non-exposed ones, while under salinity 21 the activity significantly increased only at the highest exposure concentration (Figure 35 A). Considering polychaetes exposed to f-MWCNTs, the antioxidant activity significantly increased in the contaminated specimens in comparison to control organisms maintained under both salinities (28 and 21) (Figure 35 A).

II) For each MWCNT (f and Nf) at each exposure concentration, no significant differences between salinities were detected (Figure 35 A).

III) When comparing *H. diversicolor* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were observed in terms of SOD activity (Table 12).

I) In all organisms submitted to both salinities and both MWCNTs, no significant differences in terms of CAT activity were observed among concentrations (Figure 35 B).

II) For each MWCNT at each exposure concentration, no significant differences between salinities were detected (Figure 35 B).

III) Comparing *H. diversicolor* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were assessed (Table 12).

I) The activity of GPx significantly increased in all contaminated polychaetes with both MWCNTs maintained under both salinities compared to non-contaminated organisms (Figure 35 C).

II) For each MWCNT at each exposure concentration, no significant differences between salinities were detected (Figure 35 C).

III) Comparing polychaetes exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in all conditions with the exception of organisms exposed to 0.10 mg/L under salinity 28, showing in all cases higher GPx activity in *H. diversicolor* contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 12).

I) Polychaetes contaminated with Nf-MWCNTs under salinity control exhibited significantly lower GSTs activity only when exposed to 0.10 mg/L compared to the other treatments, while under salinity 21 no significant differences among concentrations were detected (Figure 35 D). Considering *H. diversicolor* exposed to f-MWCNTs, significantly lower GSTs activity was observed under 0.10 mg/L compared to the remaining concentrations regardless of the salinity tested (Figure 35 D).

II) For both MWCNTs (f and Nf) at each exposure concentration, significant differences were assessed only when polychaetes were exposed to 0.10 mg/L Nf-MWCNTs, showing a decrease of the activity under salinity control compared to low salinity (Figure 35 D).

III) When comparing *H. diversicolor* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed at 0.01 mg/L under salinity 28, showing lower GSTs activity in organisms contaminated with Nf-MWCNTs. Significant differences between materials were also detected at 0.10 mg/L under both salinities, with lower activity under f-MWCNTs compared to Nf-MWCNTs in both cases (Table 12).

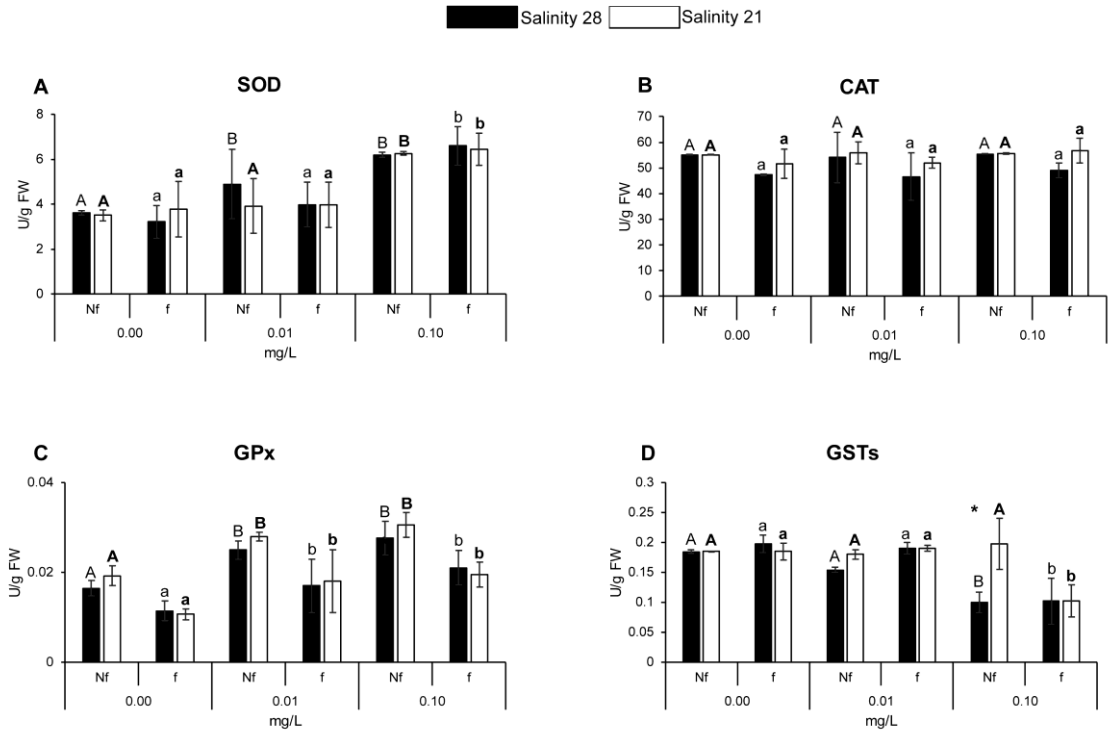

Figure 35. **A:** Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

Neuro status

I) *H. diversicolor* exposed to Nf-MWCNTs under salinity 28 presented significant differences in terms of AChE activity between all the concentrations, however significantly lower activity was observed at 0.01 mg/L in comparison to the other concentrations. No significant differences between concentrations were detected in polychaetes submitted to salinity 21 (Figure 36). Considering polychaetes exposed to f-MWCNTs, significantly lower AChE values were observed only in organisms contaminated with 0.10 mg/L relative to the remaining concentrations, while no significant differences between concentrations were assessed under salinity 21 (Figure 36).

II) For each MWCNT at each exposure concentration, no significant differences between salinities were found (Figure 36).

III) Comparing polychaetes exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in control and polychaetes exposed to 0.10 mg/L both under salinity 28, showing lower AChE activity when submitted to f-MWCNTs compared to Nf-MWCNTs (Table 12).

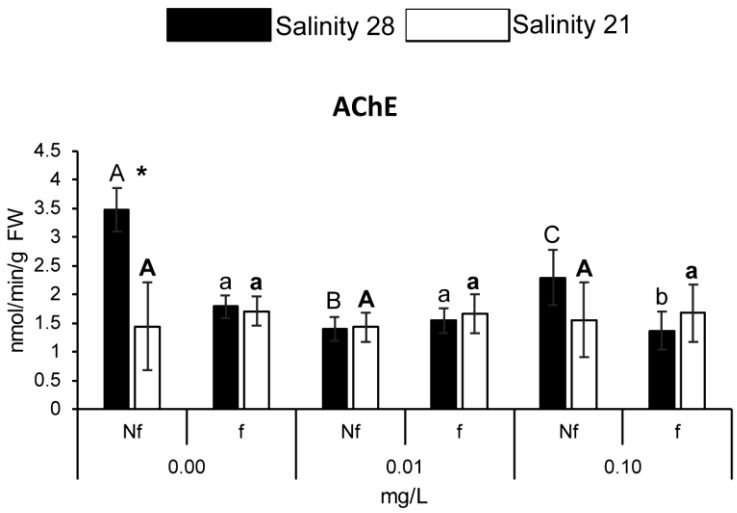

Figure 36. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

3.2.1.3 *Diopatra neapolitana* (Delle Chiaje, 1841)

3.2.1.3.1. Characterization analysis of water media

Table 13 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.01 and 0.10 mg/L) under control salinity (28) and low salinity (21).

Results of DLS and PDI analyses of experimental samples exposed to different concentrations of Nf-MWCNTs (0.01 and 0.10 mg/L) among collection periods (T0, T7 and T28) under salinity 28 showed the presence of unstable micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 13). Time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples was also observed. DLS analysis of samples exposed to Nf-MWCNTs under salinity 21 at T0, T7 and T21 evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 13). The mean dimensions of the particle aggregates recorded after different exposure periods showed the similar hydrodynamic radius of the aggregates at both tested concentrations. Comparing the aggregates of Nf-MWCNT materials under salinity 28 and 21, it was possible to observe higher mean diameters on Nf-MWCNTs under control salinity compared to the ones under low salinity, confirming that higher salinity causes the formation of large-size aggregates, which will increase the chance of physical retention, such as gravitational sedimentation, interception and straining of NPs (Hu et al., 2017).

Regarding the results of DLS and PDI analyses of experimental samples exposed to different concentrations of f-MWCNTs (0.01 and 0.10 mg/L) among collection periods (T0, T7, T14, T21 and T28) under salinity 28, it was possible to observe also in this case unstable micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples as well as a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples (Table 13). DLS analysis of samples exposed to f-MWCNTs at salinity 21 among collection periods (T0, T7, T21 and T28) evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples. The increase of the PDI was directly correlated with the detected aggregates in the investigated samples (Table 13).

Comparing the aggregates of f-MWCNT materials under salinity 28 and 21, it was possible to observe higher mean diameters on f-MWCNTs under salinity 28 compared to the ones under low salinity 21. Considering both salinities, the mean recorded hydrodynamic diameter of f-MWCNTs aggregates were smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating a higher dispersion of f-MWCNTs in aqueous media (Table 13).

Table 13. *Diopatra neaopolitana:* average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed under control salinity (28) and low salinity (21) in each exposure concentration (0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

CNT concentration (mg/L)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
	Nf-MWCNTs				**f-MWCNTs**			
	28		**21**		**28**		**21**	
	T0				T0			
0.01	2596.6	0.98	2236.0	1.04	3634.9	1.50	1963.6	0.80
0.10	4321.1	1.32	3090.1	1.21	3987.2	1.45	2098.2	0.98
	T7				T7			
0.01	5 l.d.	n.d.	3431.0	1.50	5 l.d.	n.d.	5 l.d.	n.d.
0.10	3214.2	0.78	3009.1	1.19	2098.7	1.72	2998.8	1.24
	T14				T14			
0.01	5 l.d.	n.d.	4191.8	1.91	1771.2	0.804	2796.6	1.42
0.10	3998.8	1.24	3211.1	1.65	3098.2	1.09	2987.4	1.50
	T21				T21			
0.01	3354.7	1.32	4548.1	1.87	3354.7	1.50	2912.8	1.87
0.10	3 l.d.	n.d.	5 l.d.	n.d.	3987.2	1.89	3 l.d.	n.d.
	T28				T28			
0.01	5 l.d.	n.d.	5588.7	2.12	3 l.d.	n.d.	7013.0	2.87
0.10	4098.2	1.98	3009.1	1.98	5 l.d.	n.d.	2009.1	1.98

l.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (l.d.) results in 3 out of 5 samples).

3.2.1.3.2. Biological analysis: physiological parameter (regenerative capacity)

The mean values for the percentage (%) of regenerated body width and the number (#) of new chaetigers in *D. neapolitana* after 11th, 18th and 28th days of amputation are illustrated in Figure 37 and presented in Table 14. All the results were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both salinity levels (control-28 and low-21); II) understand the effects of salinity in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both salinity levels at each exposure concentration.

11th day

After amputation all individuals were healing the cut region, however no significant differences were observed in terms of percentage of regenerated body width as well as number of new chaetigers between individuals non-exposed (0.00 mg/L) and polychaetes exposed to both MWCNT materials in all tested concentrations (0.01 and 0.10 mg/L) under both salinity levels (control-28 and low-21) (Figure 37; Table 14).

18th day

I) Looking on the effects of exposure concentrations for the same MWCNTs, the results of percentage of regenerated body width for f-MWCNT submitted to both salinities showed significantly lower values only in individuals exposed to 0.10 mg/L in comparison to the remaining concentrations, while no significant differences were observed between concentrations in terms of number of new chaetigers. Regarding polychaetes contaminated with Nf-MWCNTs, under salinity 28 significantly lower percentage of regenerated body width as well as a number of new chaetigers were detected in exposed individuals compared to control ones. Under salinity 21, significantly lower values in terms of percentage of regenerated body width was observed only in individuals exposed to 0.10 mg/L in comparison to remaining treatments, while no significant differences between concentrations were found considering the number of new chaetigers under this condition (Table 14).

II) Considering the effects of salinity, for each MWCNT at each exposure concentration, differences between salinities were only observed at 0.01 mg/L Nf-MWCNTs with lower percentage of regenerated body width in individuals maintained under salinity 28 in comparison to individuals maintained under salinity 21, while no significant differences between salinities were found in terms of number of new chaetigers for both MWCNT materials (Table 14).

III) Considering the effects of MWCNTs at each concentration and each salinity (28 or 21), no significant differences were observed between organisms exposed to different MWCNTs in terms of percentage of regenerated body width, while regarding the number of new chaetigers significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L under salinity 28 showing a lower number of chaetigers for individuals contaminated with Nf-MWCNTs compared to f-MWCNTs.

28th day

I) The results of percentage regenerated body width showed only significantly lower values in individuals exposed to 0.10 mg/L f-MWCNTs under both salinities in comparison to the remaining concentrations. Looking the results of the number of new chaetigers, individuals exposed to f-MWCNTs under salinity 28 showed no significant differences between concentrations, while under salinity 21 significantly lower number was detected in specimens contaminated with 0.10 mg/L compared to the remaining treatments. Considering the results observed in individuals exposed to Nf-MWCNTs, significantly dose-dependent decreased of the percentage regenerated body width was observed in polychaetes maintained under salinity control, while only at 0.10 mg/L under low salinity the percentage was significantly lower compared to the remaining concentrations. The results obtained for the number of new chaetigers in individuals contaminated with Nf-MWCNTs, showed a significantly lower value only when *D. neapolitana* was exposed to 0.10 mg/L under both salinities compared to the other treatments (Figure 37; Table 14).

II) For each MWCNT at each exposure concentration, differences between salinities were not observed both for a percentage of regenerated body width and number of new chaetigers (Figure 37; Table 14).

III) When comparing organisms exposed to the same salinity and exposure concentration, significant differences between materials in terms of both percentages of regenerated body and the number of new chaetigers were assessed in polychaetes exposed to 0.10 mg/L under salinity control, showing, in a both cases, significantly lower values when contaminated with Nf-MWCNTs compared to f-MWCNTs (Figure 37; Table 14).

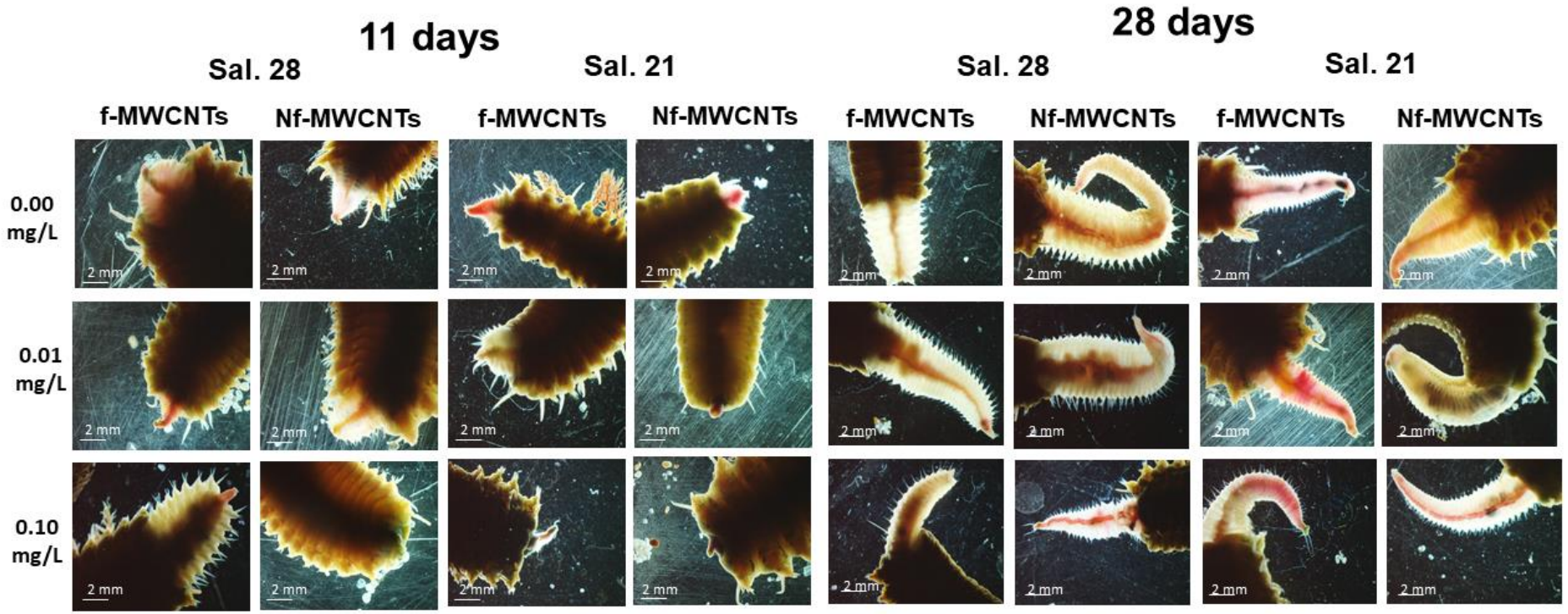

Figure 37. Regenerative capacity of *D. neapolitana* at 11th and 28th days after amputation, exposed to different MWCNTs (f and Nf) and concentrations (0.00; 0.01 and 0.10 mg/L) under two different salinities (control-28; low-21).

Table 14. Regeneration data (percentage (%) of body width and the number (#) of new chaetigers) for *D. neapolitana*, 11th, 18th and 28th days after amputation. Significant differences ($p \leq 0.05$) among exposure concentrations (0.00; 0.01 and 0.10 mg/L) for each MWCNT (f-MWCNTs and Nf-MWCNTs) and salinity (control 28 and low- 21) were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and bold letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with bold asterisks (*****). Significant differences ($p \leq 0.05$) between f-MWCNTs and Nf-MWCNTs within each salinity at each exposure concentration were represented with bold hashes (**#**).

CNT concentrations (mg/L)	Salinity		11 days		18 days		28 days	
			% body width	# chaetigers	% body width	# chaetigers	% body width	# chaetigers
0.00	28	f-MWCNTs	7.67±2.07 **A**	0.00±0.00 **A**	44.64±10.04 **A**	21.50±6.28 **A**	75.79±3.96 **A**	30.50±1.38 **A**
		Nf-MWCNTs	7.67±2.07 A	0.00±0.00 A	44.64±10.04 A	21.50±6.28 A	75.79±3.96 A	30.50±1.38 A
	21	f-MWCNTs	9.83±1.72 **a**	0.00±0.00 **a**	45.34±13.72 **a**	20.00±3.22 **a**	74.40±4.54 **a**	29.83±1.72 **a**
		Nf-MWCNTs	9.83±1.72 a	0.00±0.00 a	45.34±13.72 a	20.00±3.22 a	74.40±4.54 a	29.83±1.72 a
			11 days		**18 days**		**28 days**	
			% body width	**# chaetigers**	**% body width**	**# chaetigers**	**% body width**	**# chaetigers**
0.01	28	f-MWCNTs	6.50±3.73 **A**	0.00±0.00 **A**	37.87±7.51 **A**	18.83±2.40 **A #**	70.09±12.21 **A**	28.67±1.51 **A**
		Nf-MWCNTs	8.83±4.53 A	0.00±0.00 A	19.12±4.83 B *****	11.17±5.95 B **#**	59.41±19.35 B	26.67±7.39 A
	21	f-MWCNTs	8.00±2.28 **a**	0.00±0.00 **a**	39.92±6.28 **a**	18.67±2.16 **a**	71.63±9.89 **a**	27.67±1.37 **a**
		Nf-MWCNTs	7.67±3.83 a	0.00±0.00 a	39.50±5.59 a *****	17.17±4.26 a	70.68±5.60 a	28.83±2.14 a
			11 days		**18 days**		**28 days**	
			% body width	**# chaetigers**	**% body width**	**# chaetigers**	**% body width**	**# chaetigers**
0.10	28	f-MWCNTs	5.60±3.90 **A**	0.00±0.00 **A**	29.06±7.45 **B #**	17.98±3.34 **A #**	59.12±10.14 **B #**	25.57±2.22 **A #**
		Nf-MWCNTs	8.43±2.51 A	0.00±0.00 A	15.10±3.68 B #	9.50±3.94 B #	24.87±6.22 C #	11.33±2.58 B #
	21	f-MWCNTs	5.48±2.31 **a**	0.00±0.00 **a**	25.98±5.41 **b #**	16.92±2.10 **a**	54.43±5.80 **b**	19.61±2.41 **b**
		Nf-MWCNTs	7.99±2.81 a	0.00±0.00 a	14.40±5.23 b #	14.01±5.32 a	30.61±5.23 b	12.92±3.19 b

3.2.1.3.3. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both salinity levels (control-28 and low-21); II) understand the effects of salinity in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both salinity levels at each exposure concentration.

Energy reserves content and metabolic capacity

I) Considering the effects of exposure concentrations, results of PROT content in *D. neapolitana* exposed of Nf-MWCNT under salinity 28 showed a significant dose-dependent increase with higher values under the highest exposure concentration, while no significant differences among concentrations were observed when submitted to salinity 21. In individuals exposed to f-MWCNTs under both salinities, no significant differences were observed among exposure concentrations (Figure 38 A).

II) Significant differences between salinities (28 and 21) were observed when organisms were exposed to 0.01 and 0.10 mg/L of Nf-MWCNTs, showing in both cases higher PROT content in individuals maintained at salinity control in comparison to low salinity (Figure 38 A).

III) When comparing *D. neapolitana* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L under salinity 28 showing an increase of the content for individuals contaminated with Nf-MWCNTs (Table 15).

I) Under salinity 28, significant differences in terms of GLY content were detected only at 0.10 mg/L Nf-MWCNTs compared to the other treatments, while under salinity 21 along with the increasing Nf-MWCNTs exposure concentrations, the polychaetes decreased significantly their GLY content in comparison to non-exposed ones (Figure 38 B). In organisms submitted to f-MWCNTs, the GLY content significantly decreased in exposed individuals under salinity 28 in comparison to the control, while no significant differences among different concentrations and control were observed when *D. neapolitana* was submitted to low salinity 21 (Figure 38 B).

II) Significant differences between salinities were observed in GLY content when organisms were exposed to 0.10 mg/L of Nf-MWCNTs, showing higher content in individuals maintained at salinity control in comparison to low salinity (Figure 38 B).

III) When comparing *D. neapolitana* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L under salinity 28 showing higher GLY content in polychaetes contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 15).

I) At salinity 28 *D. neapolitana* presented a significant dose-dependent increase of ETS activity, with higher values at the highest exposure concentration of both MWCNTs. Under salinity 21 no significant differences were observed among concentrations of Nf-MWCNTs, while significantly higher ETS activity was observed in contaminated individuals with f-MWCNTs compared to control ones (Figure 38 C).

II) For each MWCNT and exposure concentration, significant differences between salinities were detected at 0.01 mg/L Nf-MWCNTs, with lower metabolic activity under salinity 28 compared to salinity 21. Opposite results were observed at 0.10 mg/L both f-MWCNTs and Nf-MWCNTs, with higher ETS activity in individuals maintained at control salinity in comparison to organisms under low salinity (Figure 38 C).

III) When comparing specimens exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were only observed in polychaetes exposed to 0.01 mg/L under salinity 28, showing lower activity for individuals contaminated with Nf-MWCNTs in comparison to individuals exposed to f-MWCNTs (Table 15).

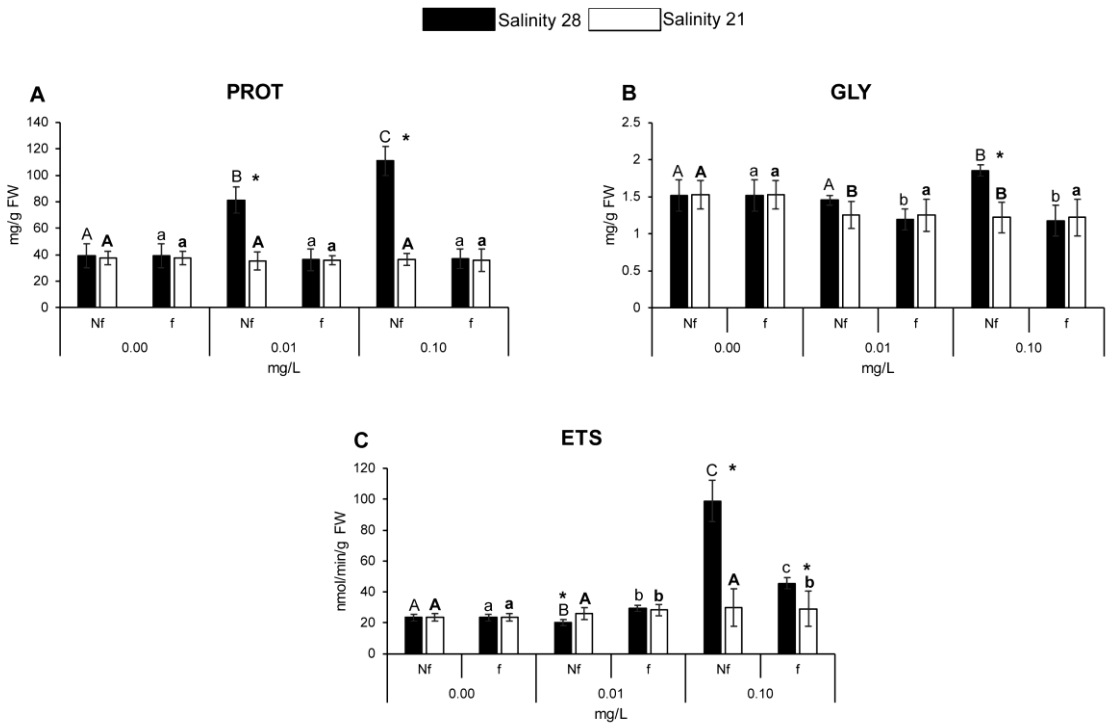

Figure 38. **A**: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron transport system (ETS) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs)

both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

Table 15. Effect on oxidative stress biomarkers (Protein (PROT) content; Glycogen (GLY) content; Electron transport system (ETS) activity; Lipid peroxidation (LPO) levels; GSH/GSSG; Superoxide dismutase (SOD) activity; Catalase (CAT) activity; Glutathione peroxidase (GPx) activity; Glutathione S-Transferases (GSTs) activity; Acetylcholinesterase (AChE) activity) in *D. neapolitana* by pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control salinity (28) and low salinity (21). Significant differences ($p \leq 0.05$) between MWCNTs within each salinity at each exposure concentration were represented with asterisks (*).

CNTs (mg/L)	Salinity		PROT	GLY	ETS	LPO	GSH/GSSG	SOD	CAT	GPx	GSTs	AChE
0.00	28	Nf	39.45±9.03	1.51±0.21	23.47±2.29	12.83±0.94	6.83±0.45	0.83±0.21	39.68±3.10	0.082±0.009	0.34±0.04	0.98±0.14
		f	39.45±9.03	1.51±0.21	23.47±2.29	12.83±0.94	6.83±0.45	0.83±0.21	39.68±3.10	0.082±0.009	0.34±0.04	0.98±0.14
	21	Nf	37.56±5.30	1.53±0.19	23.63±2.45	13.80±0.76	6.91±0.30	1.02±0.46	39.10±1.38	0.079±0.009	0.32±0.04	0.97±0.15
		f	37.56±5.30	1.53±0.19	23.63±2.45	13.80±0.76	6.91±0.30	1.02±0.46	39.10±1.38	0.079±0.009	0.32±0.04	0.97±0.15
0.01	28	Nf	81.33±10.09	1.45±0.07	20.29±1.83	29.59±2.88	2.16±0.27	1.09±0.11	38.63±5.15	0.089±0.010	0.86±0.04	1.28±0.71
		f	36.24±8.02*	1.19±0.14*	29.52±2.06*	19.11±2.97*	5.26±0.60*	2.86±0.85*	39.55±3.17	0.112±0.036	0.26±0.03*	0.79±0.16*
	21	Nf	35.31±35.91	1.26±0.18	25.96±3.83	16.15±2.37	6.88±0.69	1.10±0.51	38.99±1.38	0.081±0.006	0.30±0.03	0.88±0.13
		f	35.91±3.36	1.25±0.22	28.31±3.61	16.81±3.04	6.50±0.60	2.41±0.63*	38.98±1.28	0.093±0.010	0.27±0.03	0.88±0.14
0.10	28	Nf	110.86±10.91	1.85±0.08	98.88±13.99	30.27±2.75	2.10±0.20	1.36±0.64	38.90±5.76	0.081±0.020	0.77±0.05	0.94±0.24
		f	36.98±7.54*	1.18±0.21*	45.65±3.65*	20.32±4.32*	5.02±0.32*	5.65±1.09*	39.88±3.54*	0.112±0.030	0.27±0.01*	0.54±0.12*
	21	Nf	36.43±4.32	1.22±0.21	30.10±12.10	16.20±2.87	6.98±1.20	1.14±0.54	39.30±3.21	0.085±0.010	0.32±0.01	0.87±0.12
		f	35.87±8.43	1.22±0.25	29.10±11.30	18.20±3.98	6.90±1.11	2.43±1.11*	39.11±8.76	0.099±0.030	0.26±0.01	0.76±0.11*

Oxidative status

I) Under both salinities, the LPO levels significantly increased in the contaminated polychaetes compared to non-contaminated ones, regardless of the MWCNTs (Figure 39 A).

II) For each MWCNT and exposure concentration, differences between salinities were detected at 0.01 and 0.10 mg/L Nf-MWCNTs, showing in both cases significantly major cellular damage in *D. neapolitana* maintained under salinity control in comparison to salinity 21 (Figure 39 A).

III) When comparing specimens exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L under salinity 28, with higher LPO levels in individuals contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 15).

I) Regardless the type of material, significantly lower ratio of GSH and GSSG was observed in organisms contaminated with both MWCNTs under salinity 28 in comparison to non-contaminated specimens, while no significant differences were observed in individuals maintained at salinity 21 (Figure 39 B).

II) For each of the MWCNT and exposure concentration, differences between salinities were observed in all exposed polychaetes to MWCNTs (both f and Nf), with the lower GSH/GSSG values in individuals maintained at control salinity 28 compared to individuals under salinity 21 (Figure 39 B).

III) When comparing organisms exposed to the same salinity and exposure concentration, significant differences between MWCNT materials were observed only in *D. neapolitana* exposed to 0.01 mg/L at salinity 28, showing lower values in individuals contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 15).

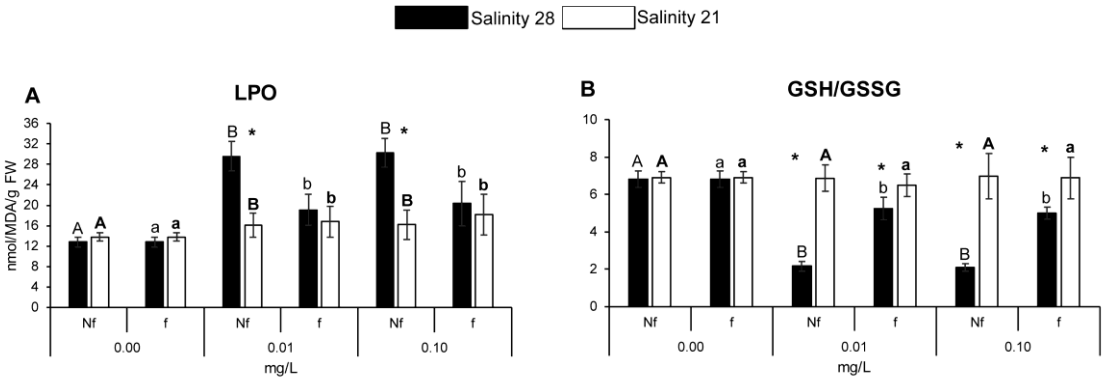

Figure 39. **A**: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure

concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

I) Considering the effects of exposure concentrations, results of SOD activity in *D. neapolitana* showed that for Nf-MWCNTs under both salinities (28 and 21), no significant differences were observed among all concentrations. In polychaetes exposed to f-MWCNTs, the SOD activity significantly increased with the increasing exposure concentrations under salinity 28, while under salinity 21 the activity of this enzyme significantly increased in the exposed individuals in comparison to control ones (Figure 40 A).

II) For each of the MWCNT and exposure concentration, differences between salinities were observed only at the highest exposure concentration for specimens under f-MWCNTs, with significantly higher SOD activity in organisms maintained to control salinity 28 in comparison to organisms under salinity 21 (Figure 40 A).

III) Comparing organisms under the same salinity and exposure concentration, significant differences between materials were observed in all exposed polychaetes under both salinities, showing in all cases higher SOD activity in polychaetes contaminated with f-MWCNTs comparted to Nf-MWCNTs (Table 15).

I) In all organisms submitted to both salinities (28 and 21) and both MWCNTs (f and Nf), no significant differences in terms of CAT activity were observed among concentrations (Figure 40 B).

II) For each MWCNT at each exposure concentration, no significant differences between salinities were detected (Figure 40 B).

III) Comparing *D. neapolitana* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were assessed (Table 15).

I) Regardless of the salinity levels, no significant differences in terms of GPx activity were observed among Nf-MWCNTs concentrations. Considering polychaetes contaminated with f-MWCNTs, significantly higher GPx activity was recorded in the exposed organisms compared to non-exposed ones under both salinities (Figure 40 C).

II) For each of the MWCNT and exposure concentration, differences between salinities were observed in polychaetes contaminated with 0.01 mg/L f-MWCNTs, showing higher antioxidant activity under salinity control compared to low salinity (Figure 40 C).

III) Comparing *D. neapolitana* exposed to different MWCNTs at the same salinity and exposure concentration, no significant differences between materials were assessed (Table 15).

I) At salinity 28 *D. neapolitana* presented a significant increase of GSTs activity in organisms exposed to Nf-MWCNTs in comparison to control individuals, while at salinity 21 no significant differences were observed among concentrations. An opposite behaviour was observed in organisms exposed to f-MWCNTs, where, regardless of the salinity levels, GSTs activity decreased significantly in contaminated individuals compared to non-contaminated ones (Figure 40 D).

II) For each of the MWCNT and exposure concentration, differences between salinities were detected at 0.01 and 0.10 mg/L Nf-MWCNTs, showing significantly higher GSTs activity under salinity 28 compared to salinity 21 (Figure 40 D).

III) When comparing organisms exposed to the same salinity and exposure concentration, significant differences between MWCNTs were observed in *D. neapolitana* exposed to 0.01 and 0.10 mg/L at salinity 28, showing in both cases major activity in individuals contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 15).

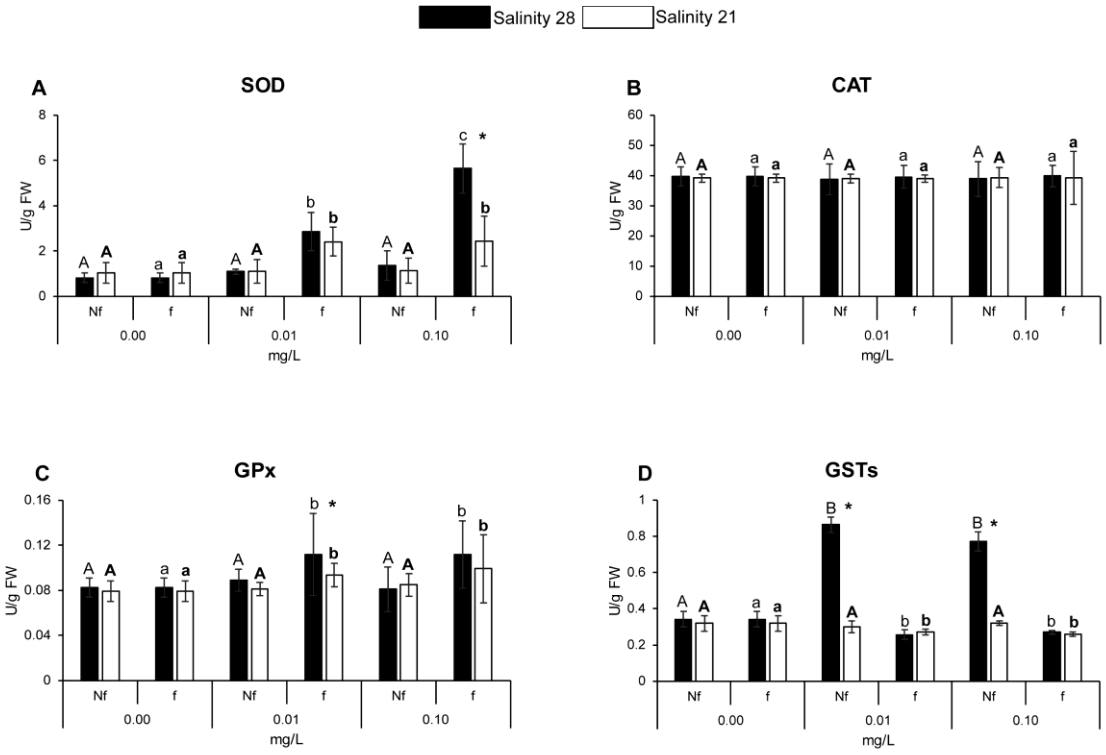

Figure 40. **A:** Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

Neuro status

I) *D. neapolitana* exposed to Nf-MWCNTs under salinity 28 presented significantly higher activity only at 0.01 mg/L in comparison to the remaining concentrations, while under salinity 21 no significant differences among concentrations were detected (Figure 41). Considering polychaetes exposed to f-MWCNTs, significantly lower AChE value was observed only in organisms contaminated with 0.10 mg/L under salinity control relative to the remaining concentrations, while no significant differences among concentrations were assessed under salinity 21 (Figure 41).

II) For each MWCNT at each exposure concentration, significant differences between salinities were observed at 0.01 mg/L Nf-MWCNTs, showing higher AChE activity under salinity 28 compared to salinity 21. Differences between salinities were also detected at 0.10 mg/L f-MWCNTs but in this case, significantly higher inhibition of the activity was recorded under salinity control (Figure 41).

III) Comparing polychaetes exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 mg/L under salinity 28, showing higher AChE activity when submitted to Nf-MWCNTs compared to f-MWCNTs. Significant differences were also detected in polychaetes exposed to 0.10 mg/L under both salinities, with lower values under f-MWCNTs compared to Nf-MWCNTs (Table 15).

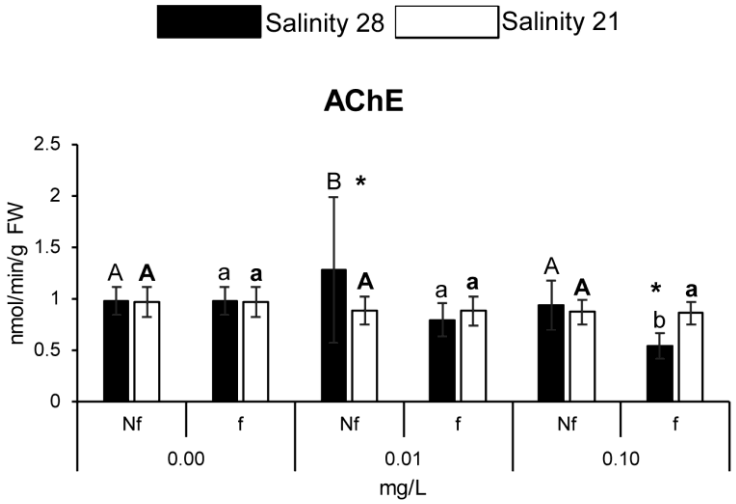

Figure 41. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different salinities range (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNTs at salinity 28; uppercase and bold letters for Nf-MWCNTs at salinity 21; lowercase and regular letters for f-MWCNTs at salinity 28; lowercase and bold letters for f-MWCNTs at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks (*).

3.2.2. Discussion

3.2.2.1. Characterization analyses

Looking to the DLS and PDI analyses of experimental samples of *R. philippinarum* exposed to different concentrations of Nf-MWCNTs and f-MWCNTs among collection periods under salinity 28 and 21, the results showed larger mean diameters of both CNTs under salinity 21 compared to ones under control salinity 28. However, under control salinity, it was noted, through visual observation, the presence floated macro-particle with larger particle sizes compared to the ones at low salinity, which the instrument was not able to record. Moreover, the confirmation of the presence of larger diameter of both CNTs under salinity 28 compared to salinity 21, was detected on the experimental samples of *H. diversicolor* and *D. neapolitana*, supporting the theory that the particle diameter and sedimentation rate increased at increasing salt concentrations, due to the effect of ionic strength (Rotini et al., 2017). It has been already demonstrated from the literature that the higher salinity causes the formation of large-size aggregates, which will increase the chance of physical retention, such as gravitational sedimentation, interception and straining of NPs (Hu et al., 2017). Aggregation of NPs can alter their biological effects by affecting ion release from the surface and their reactive surface area, affecting the mode of cellular uptake of NPs together with subsequent biological responses in the organisms (Hotze et al., 2010). Although in the literature it is reported that the increased particle size at high salinity determines a decrease of the total surface area implying a decrease of the superficial reactivity of NPs (because of agglomeration) which, in turn, produces a reduction of the toxic effects (Rotini et al., 2017), the results of the present study disagreed with the cited information, showing major toxic effects under high salinity in comparison to those detected under low salinity. The biological effects vary depending on whether or not a particle is aggregated, among other confounding factors (e.g., endotoxins, adsorption of biomacromolecules, etc.) (Hotze et al., 2010). These differences are presumably due to the fact that the protein corona generated by the aggregation state of CNTs may vary, and the receptors on which CNTs act differ depending on the complex conditions to ultimately affect cell responsiveness. In a study conducted by Kuroda et al. (2018), the authors observed different cytotoxicity and immune responses depending on the aggregation state of the CNTs. The aggregated CNT were taken up in the phagosomes, however highly dispersed CNTs was scattered and incorporated in the cells, but the amount of uptake was clearly less than that of aggregates. This result may be due to the fact that the surface charges and surface modifications of aggregated and highly dispersed CNTs were different. Thus, receptors mediating uptake by macrophages that recognize carbon NPs may differ, even for the same carbon nanomaterial, due to differences in particle shape and aggregation state (Kuroda et al., 2018). Besides, in a study conducted by Ward and Kach (2009), the authors revealed that the larger

aggregates can increase the uptake and bioavailability of NPs to suspension filter-feeding bivalves. The authors reported that the aggregates were likely broken down by the action of cilia on the gills and labial palps and the constituent particles ingested, due to the a longer gut retention time, has undergone more extensive extracellular digestion and, perhaps, been transported to the digestive glands for more complete intracellular digestion. This finding could explain the biological results obtained in the present study regarding *R. philippinarum*. Looking the results of *H. diversicolor* and *D. neapolitana*, it was observed that the toxic effects under high salinity was similar or higher respectively than those detected under low salinity. The occurring aggregation and sedimentation suggest that NPs may accumulate in sediments (Buffet et al., 2011) and therefore, benthic organisms are supposed to be the most exposed to NPs. Considering that these two polychaete species have predominant impacts on sedimentary processes due to its ecological characteristics (burrowing depth, bio-irrigation activities and sediment reworking) (Coelho et al., 2008) part of the NPs accumulation could be derived from sediment ingestion, which may explain the biological responses observed in the present studies.

Moreover, considering the aggregation behaviour between pristine and functionalized MWCNTs, the results of all the present studies showed that Nf-MWCNTs generated larger aggregates compared to f-MWCNTs under both salinity levels confirming that the carboxylated forms of CNTs are more stable in salt water media in comparison to pristine CNTs as a consequence of their oxidation process which introduces oxygen-containing groups on the CNTs surface.

Furthermore, higher toxic impacts were caused by f-MWCNTs compared to Nf-MWCNTs. In fact, while raw CNTs do not readily cross biological barriers, water dispersible MWCNTs due to the presence of higher amorphous carbon fragments in comparison to pristine MWCNTs, induced higher levels of toxicity to biological systems (Arndt et al., 2013) causing higher cellular damage with the activation of antioxidant mechanisms (Freixa et al., 2018).

3.2.2.2. Biological analyses

In the present studies, physiological (regenerative capacity) and biochemical responses (energy reserve contents and metabolic capacity, oxidative and neuro status) of two concentrations (0.10 and 1.00 mg/L) of Nf-MWCNTs and f-MWCNTs in the three invertebrate species maintained at two different salinity levels (control-28 and low-21) were investigated. For all the studies the three main hypotheses assessed were: **I)** both MWCNT materials generated toxic impact on the organisms after 28 days of exposure under different salinities; **II)** different salinity may alter the sensitivity of the individuals to the CNTs; **III)** the alteration induced by different salinity levels on the chemical behaviour of both materials changed the toxicity of the MWCNTs and consequent fate in exposed organisms.

3.2.2.2.1. Impacts of MWCNT concentrations under different salinity levels

Ruditapes philippinarum (Adams & Reeve, 1850)

I) For each MWCNT (f and Nf) and for each salinity (28 and 21), significant differences between exposure concentrations in organisms exposed to Nf-MWCNT and f-MWCNT were found. Specifically, despite the type of NPs, the present study demonstrated that *R. philippinarum* under salinity 28 presented a concentration-dependent decrease of energy reserves, especially the GLY content (which is already demonstrated to be considered one of their main energy reserves in bivalves (Beninger and Lucas, 1984)) suggesting the use of GLY consumption by organisms to fight against high CNTs concentration. Under salinity 21, no differences in energy content were observed. Moreover, the present results reported that the clams increased their ETS with the increasing exposure concentrations, both at nonfunctionalized and functionalized MWCNTs under both salinities, confirming that the increase of metabolic capacity in contaminated organisms is a common strategy in bivalves in response to different stressors (Bielen et al., 2016; De Marchi et al., 2017a).

Considering the oxidative status, in the present study, oxidative conditions upon exposure to both MWCNTs under both salinities were evidenced by an increase in LPO level with increasing of exposure concentrations and decrease GSH/GSSG. Various studies have already reported higher levels of LPO in bivalves with the increase of NPs concentrations (Kádár et al., 2010; Tedesco et al., 2010; Gomes et al., 2011; 2012; Gagné et al., 2013; Trevisan et al., 2014; Anisimova et al., 2015; Volland et al., 2015; Cid et al., 2015; De Marchi et al., 2017a) and a consequent decrease of GSH/GSSG (Tedesco et al., 2010; De Marchi et al., 2017a), confirming a concentration-dependent increase of lipids damage and lost of redox balance in organisms exposed to these contaminants.

When organisms are under stressful conditions, ROS are overproduced and bivalves may be able to increase the activity of antioxidant enzymes in response to the generated cellular oxidative stress. These antioxidant mechanisms are found to be associated with NPs exposure concentrations, showing increased activity of antioxidant enzymes in response to an increase of ROS production at the highest exposure concentration (Buffet et al., 2011; Gomes et al., 2012; Mccarthy et al., 2013; Gomes et al., 2014; Volland et al., 2015; De Marchi et al., 2017a). Similar results were observed in the present study in terms of antioxidant enzymes activity (SOD and GPx) considering the clams exposed to the highest concentration of f-MWCNTs under both salinities, suggesting a possible enzymatic response to eliminate ROS and to prevent cellular damage (e.g. lipid peroxidation) under this condition. However, although the activation of antioxidant enzyme, possible elevated concentrations of ROS cells resulted in oxidative stress and LPO still occurred. Differently, in organisms exposed to Nf-MWCNTs under both salinities, SOD activity did not increase along the increasing exposure concentrations, suggesting a loss of compensatory mechanisms as a consequence of insufficient mechanism of the antioxidant activity (Fukai and Ushio-Fukai, 2011; Walters et al., 2016) and this contribute to higher LPO levels recorded under these conditions. Nevertheless, activation of GPx activity in clams expose to Nf-MWCNTs at the highest exposure concentration was detected, suggesting that the H_2O_2 produced by SOD may possibly be converted by these antioxidant systems which contribute in the defence against oxidative stress. Considering the biotransformation enzymes, the results of the present study showed decrease of the activity in clams submitted to f-MWCNTs under salinity 28 confirming a concentration-dependent inhibition (decrease) of these enzymes under this condition, while in all other treatments, no differences were observed, indicating that these group of enzymes could be not involved in the biotransformation process under these conditions, suggesting that the behavior of this enzyme could be influenced by the type of materials as well as by different salinity levels.

Looking on the neuro status, it was observed a dose-response inhibition of the neurotransmitter AChE activity regardless the different salinities and CNT materials, confirming possible high affinity for AChE with the NPs as well as the high sensibility of the organisms to the contaminants.

Hediste diversicolor (O.F. Müller, 1776)

I) *H. diversicolor* showed increased ETS activity with the increase of exposure concentration of both MWCNTs at both salinity conditions, which might indicate that the increase of metabolic capacity of *H. diversicolor* was necessary to activate defence mechanisms to mitigate oxidative stress induced by MWCNTs, and this response could lead to the consumption of energy reserves. The present findings are in agreement with this hypothesis, showing a decrease of energy reserves

(measured by GLY and PROT content) when *H. diversicolor* specimens were exposed to both CNT materials, a response that was similar under control (28) and low (21) salinity conditions, indicating that the impacts induced by MWCNTs were neither altered by salinity shifts or by the different form of CNTs. In agreement with the present results, also Bertrand et al. (2016) demonstrated that in the clams *S. plana,* exposed for 7 days to 10 µg/L silver (Ag) NPs, the ETS activity increased and higher activity was observed at salinity control (30) in comparison to low salinity 15. These results confirmed that the energy expenditure in invertebrates can be enhanced when exposed to different pollutants, restricting the amount of energy reserves necessary for survival, homeostasis and reproduction.

Looking the impacts of NPs in terms of oxidative stress, the present results showed a concentration-dependent increase of cellular damage (LPO) when polychaetes where exposed to both MWCNTs and salinity levels, confirming that these NPs are able to produce ROS inside and outside of the cell caused by absorption of toxic substance to their surface, which is recognised one of the main factors that caused oxidative stress (Fu et al., 2014; Simin et al., 2014). In the present study, the generation of oxidative stress induced the activation/inactivation of the antioxidant defence system. In detail, the activity of SOD increased in organisms exposed to both MWCNTs under both salinities, especially at the highest concentration (0.10 mg/L), showing a possible adaptive response to increase ROS production due to dysfunction of the mitochondrial respiratory chain. On the other hand, CAT tended to maintain the activity regardless of the CNTs exposure conditions and salinity levels. These results may be explained by the fact that the excess of H_2O_2 produced by SOD could be eliminated by another antioxidant enzyme with similar capacity and, in the present study, this hypothesis was confirmed by an increase of GPx activity in polychaetes contaminated with 0.10 mg/L Nf-MWCNTs and f-MWCNTs under both salinities compared to non-contaminated. Similar results were reported by Zhu et al. (2008) which, exposed the fish *Carassius auratus* to fullerene C_{60} (nominal concentrations: 0.4–1.0 mg/L) for 32 days, and observed lower levels of LPO in brain and gills of the organisms due to the activation of antioxidant enzymes. However, regardless the activation of the antioxidant enzymes, in all conditions (both MWCNTs and both salinity levels) of the present study, the LPO increased with the increase of exposure concentrations showing that antioxidant mechanisms were not enough to eliminate the excess of ROS as a consequence of the excess of stressful conditions. Moreover, the GSH/GSSG values decreased with the increase of exposure concentrations, at both salinities, indicating that regardless of different salinity conditions and CNTs materials, MWCNTs were inducing oxidative stress in *H. diversicolor*. Also, Anisimova et al. (2015) exposed the bivalve *M. modiolus* to 12-14 nm diameter MWCNTs (100 mg/L) for 48h and demonstrated that CNTs were responsible for the increase of LPO levels and increase of reduced glutathione (GSH). Regarding the biotransformation enzymes, in the present study GSTs activity was one of the few enzymes that modified their activity behaviour between salinities. In detail, polychaetes exposed to f-MWCNTs under both salinities and in Nf-MWCNTs under salinity 28

revealed GSTs decreased activity along the increasing exposure gradient, indicating that under these conditions the activity may be inactivated due to the excess of cellular damage. Considering the effects in polychaetes under salinity 21 and Nf-MWCNTs, the GSTs activity did not change along the increasing exposure gradient, suggesting that this group of enzymes could be not involved in the biotransformation of the NPs into a less toxic excreted substance.

About the activity of the neurotransmitter AChE, the results of the present study revealed a neuro state inhibition in organisms exposed to both f-MWCNT concentrations under both salinity conditions, assuming that the perturbation of the structure influences the function of enzyme subunits may be the common mode of ChE inhibition by CNTs, independently of the salinity. Different results were observed for Nf-MWCNTs. In this case, the results revealed inhibition of the neurotransmitter activity at 0.01 mg/L but then increase again when exposed to the highest concentration. Looking on DLS analysis, the mean size of the f-MWCNTs was always lower in comparison to Nf-MWCNTs, which could explain the higher availability of the carboxylated form of MWCNTs also at the highest concentration for the organisms, intensifying the risk of exposure and possible absorption of the NPs, leading to a much higher neuro status damage in comparison to the insoluble form of MWCNTs.

Diopatra neapolitana (Delle Chiaje, 1841)

I) The results of the present study demonstrated that both MWCNTs under both salinities have a negative effect on the regenerative capacity of *D. neapolitana* at the highest exposure concentration showing a lower percentage of body width as well as the number of new chaetigers compared to the other conditions after 18^{th} and 28^{th} days exposure. As already cited in a previous section, these results represent the confirmation that both CNT materials have toxic effects on the regenerative capacity of *D. neapolitana*. Moreover, these results were observed under both salinities, suggesting a possible successful CNT uptake and bioaccumulation in the body of the exposed organisms regardless of the two different salinity levels. However, looking to the present results of energy reserves and metabolic activity in organisms exposed to Nf-MWCNTs under salinity control, the ETS activity increased between non-exposed (control) and exposed (MWCNTs) individuals, indicating that *D. neapolitana* may increase their metabolic activity under stressful conditions, while under low salinity no differences were detected between exposed and non-exposed organisms. The increase in ETS activity may highlight that this species was capable to increase the metabolic potential to fuel up defence mechanisms, such as detoxification defences under this condition. The present findings further revealed that although polychaetes metabolic capacity was enhanced in Nf-MWCNTs contaminated organisms, they were able to increase or at least maintain their GLY and PROT concentrations similar to control levels under both salinity levels. Such findings indicated that individuals may prevent energy expenditure in specific processes when under stress conditions (e.g.

limiting their use for polychaetes regeneration) or were using other energy sources to fuel up defence mechanisms. The present results are in agreement with previous studies that demonstrated that some polychaete species increase their energy reserves under stressful conditions (Maranho et al., 2014; Carregosa et al., 2014), while opposite results were obtained if compared with those observed for *H. diversicolor* exposed to the same condition, concluding that the different behavior could be related to the different sensitivity of the species to the contaminants (Nf-MWCNTs). Considering the results obtained for *D. neapolitana* exposed to f-MWCNTs under both salinities, although a loss of regenerative capacity in the exposed individuals, a decrease of energy reserves (especially GLY content) and an increase of metabolic capacity (ETS activity) were observed under these conditions. These results could indicate that polychaetas under this condition were using their energy reserves to regenerate their body fighting against high CNTs concentration. Moreover, the increase of ETS could be due to the activation of defense mechanisms, such as the increase on SOD activity, as demonstrated under this exposure condition in contaminated organisms with f-MWCNTs. Similar results were also observed in *H. diversicolor* exposed to the same conditions, concluding that or both invertebrate organisms had similar sensitivity to f-MWCNTs or the modes of toxic action of the contaminant was similar for both species.

Regarding the oxidative status, the results of the present study in the organisms exposed to both MWCNTs and submitted to both salinities, the LPO levels were observed in all materials concentrations, as for *H. diversicolor* exposed to the same conditions, confirming the high affinity of CNTs for lipid membranes (Mesarič et al., 2015). In detail, the polychaetes exposed to f-MWCNTs under both salinities showed a dose-dependent increase of the LPO with a consequence activation of antioxidant systems, suggesting a compensatory response of cellular defence systems against cellular damage, while in organisms exposed to Nf-MWCNTs under both salinities, no variations in terms of the antioxidant system were assessed. Although the different behaviours of the antioxidant systems, in both cases the possible excessive ROS production, especially under the highest exposure concentration, led to oxidative damage and contributing to higher LPO levels recorded at these conditions. Regarding the biotransformation enzymes, it has been already demonstrated that GSTs showed different mechanisms of action when exposed to different NPs, assuming that GSTs activity may be either increased or decreased due to production of lipid hydroperoxides (Kos et al., 2017) and also the type of NPs (Canesi et al., 2010; Lehman et al., 2011). The results of the present study are in line with such findings, showing an increase of GSTs activity when organisms were exposed to Nf-MWCNTs (insoluble) and a decreased activity in organisms exposed to f-MWCNTs (soluble), both under control salinity. Looking at the results of polychaetes exposed to salinity 21, also in this case differences in terms of GSTs activity were observed between materials, assuming that the low salinity may modify the behaviour of the NPs or the sensitivity of the organisms to the contaminants.

Looking the neuro status, controversial results were observed in the present study, showing no inhibition of AChE activity in exposed organisms under both materials and both salinities with the exception of f-MWCNTs at the highest exposure concentration under salinity control. Such result may be related to the fact that organisms try to reduce neurotransmitter excess in the synaptic clefts, which was already showed in the bivalve *Perna indica* exposed to arsenic (As) (Rajkumar, 2013).

3.2.2.2.2. Impacts of salinity on the sensitivity of the organisms to MWCNTs

Ruditapes philippinarum (Adams & Reeve, 1850)

II) The present results showed that, for each exposure concentration and for each MWCNT different salinity levels altered the toxicity of both CNTs materials as well as the sensitivity of *R. philippinarum* exposed to these contaminants in terms of clams' energy reserves and oxidative status. Despite estuarine bivalves are often exposed to short-term (tidal) and long-term (rain periods) that cause changes in salinity (Verdelhos et al., 2015), different studies revealed that bivalves exhibited physiological and morphological abnormalities with ensuing mortalities when exposed to low salinity (Sarà et al., 2008; Coughlan et al., 2009; Munari et al., 2011). However, in the present study, both Nf-MWCNTs and f-MWCNTs under salinity 28 generated greater alterations of energy reserve and metabolic activity, oxidative stress responses and antioxidant enzymes activities compared to individuals maintained under salinity 21, demonstrating that the alteration induced by salinity on the chemical behavior of both MWCNTs and consequent fate in exposed clams caused major toxicity in comparison to the sensitivity of the clams to low salinity. These results may be explained by relationships among physicochemical characterization, salinity and toxicity. It has been already demonstrated from the literature that higher salinity causes the formation of large-size aggregates, which will increase the chance of physical retention, such as gravitational sedimentation, interception and straining of NPs (Hu et al., 2017). Aggregation of NPs can alter their biological effects by affecting ion release from the surface and their reactive surface area, affecting the mode of cellular uptake of NPs together with subsequent biological responses in the organisms (Hotze et al., 2010). Ward and Kach (2009) revealed that the bigger aggregates can considerably increase the uptake and bioavailability of NPs to suspension filter-feeding bivalves. These authors, exposing mussels *Mytilus edulis* and oysters *Crassostrea virginica* to polystyrene NPs at a concentration of ca.1.3 x 10^4 particles/mL which were either dispersed or embedded within aggregates, showed that both of these species more efficiently captured and ingested NPs that were incorporated into aggregates than those freely suspended. Also Gagné et al. (2008) mentioned that cadmium-telluride quantum dots tended to aggregate at medium (4 mg/L) and high (8 mg/L) concentrations. If so, then the aggregated quantum dots probably were ingested by mussels at a higher rate than those not

aggregated (i.e., at 1.6 mg/L^{-1}). This idea is in agreement with the present results, showing major toxic impacts in organisms exposed to the higher salinity 28.

Hediste diversicolor (O.F. Müller, 1776)

II) The results obtained regarding energy reserves and metabolic biomarkers showed a similar trend at each tested concentration and for both salinities, deducing that salinity may not alter the response of organisms. In agreement with the present results, Durou et al. (2007) observed no influence of salinity (ranging between 15 and 25 g/L) on energy reserves and metabolism, confirming that the salinity did not alter the sensitivity of *H. diversicolor* from two contrasting areas: a non-contaminated site (Authie estuary) and a contaminated area (Seine estuary). Moreover, generally, no differences were observed between salinities in terms of oxidative status with the exception of GSTs activity, while differences in terms of neuro status between individuals exposed to salinity 28 and 21 were detected. These differences could be the result of the impacts of salinity on the toxicity of the CNTs. Surface properties, particle aggregation status and dissolution attributes of NPs are determined by the characteristics of the medium in which they are suspended. Therefore, the toxicity of NPs towards aquatic organisms can be expected to depend on the exposure medium characteristics, including salinity (Fu et al., 2014). A study conducted by Kataoka et al. (2015) demonstrated salinity-dependent toxicity of NPs in fish. These authors used different concentrations of ERM (embryo-rearing medium) (1×, 5×, 10×, 15×, 20× and 30×). The results showed that from freshwater (1× ERM) to seawater (30× ERM), the salinity increased the toxicity of silver nanocolloids (SNC) to medaka embryos. Also, Bertrand et al. (2016) demonstrated that under normal salinity conditions (30) clams were stressed by oxidative mechanisms inducing LPO of cellular membranes after Ag NPs (10 µg Ag/L) exposure. The results presented here are in part in agreement with the cited studies, showing that at the highest CNTs concentration under salinity 28 (which presented the higher aggregation forms demonstrated with DLS analysis) the response of antioxidant (namely GSTs activity) and neurotransmitter enzymes were modified in comparison to individuals maintained under salinity 21. These results pointed out that the modification induced by salinity on the chemical behaviour of CNTs and consequent fate in exposed polychaetes could cause major toxicity compared to the possible sensitivity changes of the polychaetes exposed to low salinity.

Diopatra neapolitana (Delle Chiaje, 1841)

II) Alteration induced by low salinity modifying the sensitivity of the polychaetes and the toxicity of the CNTs was also observed in the present study. It was already demonstrated that organisms exposed to salinity stress must increase their energy expenditure to successfully acclimate to the stressor and ensure cellular protection (Rivera-ingraham, 2017). When organisms are exposed to low salinity it is initiated a series of mechanisms (energetically costly) that allow them

to hyper-regulate (i.e. to maintain their extracellular fluids at a higher osmolality than that of their surrounding medium) and this osmoregulation is considered to be an energetically costly process (Rivera-ingraham, 2017). This hypothesis supported the obtained results, showing that when *D. neapolitana* were exposed to salinity 21 especially under f-MWCNTs, there was an increase of the energy expenditure (showed by a decrease of the GLY) and an increase of metabolic activity (expressed by an increase of ETS activity). Considering that the same result was also observed in *H. diversoclor* under the same conditions, it is possible to conclude that the alteration induced by a decrease of salinity can modify the sensitivity of the polychaetes to this contaminant. Mitochondria, as the main energy producers in eukaryotic cells, play a central role in acclimation processes. However, they also represent the main source of reactive oxygen and nitrogen species (ROS/RNS), although the relationship between mitochondrial respiration and ROS/RNS formation is not fully understood. ROS/RNS can potentially lead to the LPO (such as those composing cellular membranes), as well as damaging other cellular molecules; ROS/RNS potentially have negative consequences for acclimation to hyper- and hypo-osmotic conditions. However, the lipid electrophiles resulting from such processes can have, along with ROS/RNS themselves, a role in the activation of cellular defences (Rivera-ingraham, 2017; Sokolova, 2018). In the present study, organisms exposed to both f-MWCNTs and Nf-MWCNTs under low salinity presented an increase of LPO, however only under f-MWCNTs the exposed polychaetes presented an activation of antioxidant enzymes in terms of increase of SOD activity as well as decrease of GSH/GSSG and decrease of GSTs especially at the highest exposure concentration, demonstrating possible alteration induced by salinity decrease on the sensitivity of the polychaetes as well as the variation of the chemical behaviour of both MWCNTs under this condition. In fact, despite estuarine invertebrates are often exposed to short-term (tidal) and long-term (rain periods) changes in salinity, the increased stress may lead to physiological and morphological abnormalities when exposed to low salinity (Verdelhos et al., 2015).

3.2.2.2.3. Impacts of salinity on the toxicity of MWCNTs

Ruditapes philippinarum (Adams & Reeve, 1850)

III) For each salinity and for each exposure concentration, the obtained results demonstrated clearly that nanomaterial toxicity has been attributed also to the surface functionalization showing greater toxic impacts in clams exposed to f-MWCNTs compared to Nf-MWCNTs. This was particularly evident by a greater antioxidant enzymes activity such as SOD and GPx in organisms exposed to f-MWCNTs compared to Nf-MWCNTs, demonstrating that these enzymes could act as indicators of compensatory cellular response to this NPs exposure. Different studies already demonstrated the behavior of the antioxidant enzymes is dependent also on the type of NPs (Canesi

and Corsi, 2015). As a consequence, the bioavailability, as well as biodistribution and consequent biological responses, are dependent on the interactions of NPs inside the body of the organism. This hypothesis may explain the different responses of the antioxidant enzymes in clams exposed to two different CNTs. As mentioned above, controversial behavior of GSTs enzyme was also observed, demonstrating that the behavior of the antioxidant enzymes not only depend on the exposure concentrations and salinity but also on the type of NPs (Lehman et al., 2011). In agreement with the present results, Canesi et al. (2010) exposed *M. galloprovincialis* to different carbon-based NMs (nano carbon black-nNCB, C60 fullerene) (0.05, 0.2, 1, 5 mg/L) for short-term exposure (24 h), showing that to both induced changes in GSTs activities, with increases and decreases of the activity respectively, depending on NP type and concentration. Although the activation of antioxidant enzymes activities in *R. philippinarum* exposed to both MWCNTs under both salinities, the present results showed that these mechanisms were not enough to eliminate the excess of ROS and LPO increased with the increasing of both NPs exposure concentrations under both salinities, with major lipid membrane destruction in clams exposed to f-MWCNTs. These results confirmed the hypothesis that the presence of amorphous carbon fragments in the carboxylated form of MWCNTs, as a result of increased oxidation of carbon, can induce higher levels of toxicity (expressed as cellular damage) to the biological systems (Arndt et al., 2013).

Hediste diversicolor (O.F. Müller, 1776)

III) As for *R. philippinarum*, the results of the present study showed, in general, higher toxic impact in organisms exposed to f-MWCNTs in comparison to Nf-MWCNTs. Such results were particularly evident in terms of cellular damage (LPO), confirming higher toxicity in polychaetas exposed to the functionalized form of MWCNTs in comparison to the pristine one, that could be attributed to the release of surface ions resulting from dissolution of the NPs which caused oxidative stress, mediated by ROS generation at the NPs surface, a process already assessed to be a major responsible factor for NPs toxicological effects.

Diopatra neapolitana (Delle Chiaje, 1841)

III) In general, in the present study, both Nf-MWCNTs and f-MWCNTs under salinity 28 generated greater alterations on energy reserves and metabolic activity, oxidative stress biomarker responses and antioxidant enzymes activities compared to individuals maintained under salinity 21, assuming that exposed polychaetes tend to be more sensitive to the alteration induced by salinity variations on the chemical behaviour of both MWCNTs in comparison to salt stress. Moreover, the controversial behavior of energy reserves and metabolic activity as well as oxidative and neuro status observed in the present study could be attributed also to the surface functionalization of the CNTs, showing higher toxic effects caused by the f-MWCNTs compared to the Nf-MWCNTs. These

results were also observed in the two invertebrate species (*R. philippinarum* and *H. diversicolor*) previously discussed in the thesis confirming again that the presence of amorphous carbon fragments in the carboxylated form of MWCNTs as a result of increased oxidation of carbon can induce higher levels of toxicity (expressed as cellular damage) to the biological systems (Arndt et al., 2013) independently on the sensitiveness of the species to the contaminants.

3.2.3. Final considerations

In the presented studies, physiological and biochemical responses in the three invertebrate species *(R. philippinarum, H. diversicolor* and *D. neapolitana)* exposed of two concentrations of Nf-MWCNTs and f-MWCNTs maintained at two different salinity levels were investigated. For all the studies the three main hypotheses assessed were: **I)** both MWCNT materials generated toxic impact on the organisms after 28 days of exposure under different salinities; **II)** different salinity may alter the sensitivity of the individuals exposed to the CNTs; **III)** the alteration induced by different salinity levels on the chemical behaviour of both materials changed the toxicity of the MWCNTs and consequent fate in exposed organisms.

I) The present results demonstrated alterations in terms of physiological and biochemical responses in all three species caused by both MWCNTs, especially at the highest exposure concentration, concluding that both materials were able to generate toxic action effects in all exposed organisms proving that the three species were sensitive to both compounds. In general, in all invertebrate species a dose-dependent increase of the toxicity caused by both CNT materials under both salinities was detected, especially in terms of oxidative status, which is in line with the information provided by the literature confirming that among the oxidative damage induced by NPs, the breakdown of the antioxidant defence system, as well as LPO, are the common harmful effects caused by these materials in the exposed organisms (Rocha et al., 2015). These results can be justified by the successful CNT uptake, translocation and retention in the exposed organism. Different bioaccumulation studies provide evidence that CNTs are ingested by invertebrate organisms and are subsequently excreted (Jackson et al., 2013), confirming that organisms containing CNT may become a source of entry of CNTs into the food chain when ingested by larger animals, potentially leading to biomagnification.

II) The present findings demonstrated that Nf-MWCNTs and f-MWCNTs under salinity 28 generated greater toxic impacts in the organisms compared to individuals maintained under salinity 21, confirming that salinity shifts may alter the chemical behaviour of both MWCNTs and consequent fate in exposed individuals. It has been already demonstrated from the literature that higher salinity causes the formation of large-size aggregates (Hu et al., 2017) and although the increased particle size (aggregation) determines a decrease of the total surface area which in turn could decrease of the superficial reactivity of NPs (because of agglomeration) producing a reduction of the toxic effects (Rotini et al., 2017), different studies already demonstrated an increase in toxicity with increasing salinity, along with a greater tendency for the particles to form aggregates. For these reasons, it is important to consider that biological effects depend not only to the state of aggregations of the NPs, but also whether or not a particle is aggregated and among other confounding factors (e.g.,

endotoxins, adsorption of biomacromolecules, etc.) (Hotze et al., 2010). These differences are presumably due to the fact that the protein corona generated by the aggregation state of CNTs may vary, and the receptors on which CNTs act differ depending on the complex conditions to ultimately affect cell responsiveness justifying why under higher salinity level we detected major toxic impacts. Moreover, the results also showed a species-dependent sensitivity to contaminants under the two salinities tested. While *R. philippinarum* and *D. neapolitana* were more susceptible to the contaminant exposure under salinity 28, *H. diversicolor,* showed similar biochemical responses between the two salinities. These results suggested a interspecies differences in sensitivity to a chemical, confirming that the susceptibility observed in these invertebrate species would, however, be expected not only to depend on the characteristics of the compounds but also on the physiology of that particular species as well as by the changes in environmental conditions (e.g. alteration of salinity levels) that can affect the sensitivity of the species to the contaminants.

III) Comparing the toxic effects of both CNTs, in all invertebrate species major cellular damage was induced by carboxylated forms of MWCNTs in comparison to the pristine one. These results supported the theory that while raw CNTs do not readily cross biological barriers, water dispersible MWCNTs due to the presence of higher amorphous carbon fragments in comparison to pristine MWCNTs, induced higher levels of toxicity to biological systems (Arndt et al., 2013).

Based on the results here presented, it is possible to confirm that nanomaterials toxicity was not only attributed to the core structure and surface functionalization, but also to the physico-chemical parameters of the media which alter the behaviour of the CNTs and consequently the toxicity in the exposed organisms. Moreover, data obtained highlight the need to develop standard protocols for CNTs toxicological testing to characterize the behaviour and fate of these materials in different compartments of the aquatic environment, exposure conditions following environmental relevant concentrations and point out the importance of using a broad range of biomarkers to evaluate the possible toxic effects of these new emerging pollutants. This study improved the understanding of biological responses of polychaetes exposed to combined CNTs and predicted climate change scenarios. Considering that Ocean Acidification is one of the central problems that impacts the ocean ecosystem, in the next section, the two most deleterious concentrations of Nf-MWCNTs and f-MWCNTs were selected and all the three invertebrate species were exposed to the combination of CNT materials with pH variations assessing if this climate change factor may alter the toxicity of both MWCNTs as well as the sensitivity of all these species exposed to these contaminants.

3.3. Combination of stressors experiments: CNTs and pH variation

3.3.1. Results

3.3.1.1. *Ruditapes philippinarum* (Adams & Reeve, 1850)

3.3.1.1.1. Characterization analysis of water media

Table 16 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.01 and 0.10 mg/L) under pH control (8.0) and low pH (7.6).

At T0, T7 and T28 samples prepared with 0.01 and 1.10 mg/L of Nf-MWCNTs at both tested pH levels (8.0 and 7.6) were unstable and characterized by the presence of micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 16). For each condition, no differences were observed among collection periods under pH control. Considering the water samples under pH 7.6, the ones collected after 7 and 28 days at 0.10 mg/L were characterized by a mean diameter (nm) larger compared to those recorded to the time zero (Table 16). Regarding the PDI, it was possible to observe a time-dependent increased of the polydispersity index in each condition (pH 8.0 and 7.6) and exposure materials (Nf and f) due to the formation of large particles or aggregates in the analyzed samples. DLS and PDI analysis of samples exposed to different concentrations of Nf-MWCNTs at 14 and 21 days, did not allow for the detection of measurable macro/micro/nanosize particle aggregates reported in the table as “not supplied samples”. Similar dimension of Nf-MWCNTs among exposure concentrations were observed between pH 8.0 and 7.6.

Regarding samples contaminated with f-MWCNTs, DLS analysis evidenced the presence of suspended material at all the analysed conditions notwithstanding the pH of the sample medium and the time of exposure of the aquatic organisms to the carbon nanotubes. However, larger aggregates were observed under pH control in comparison to low pH. The temporal evolution of the mean diameter values of f-MWCNTs highlighted the aggregation behaviour of functionalized nanotubes detected as micrometric material during the course of the experiments.

Considering both pH levels, the mean recorded hydrodynamic diameter of f-MWCNTs aggregates were smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating a higher dispersion of f-MWCNTs in aqueous media (Table 16).

Table 16. *Ruditapes philippinarum*: average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed under control pH 8.0 and low pH 7.6 in each exposure concentration (0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

CNT concentration (mg/L)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
	Nf-MWCNTs				**f-MWCNTs**			
	8.0		**7.6**		**8.0**		**7.6**	
	T0				T0			
0.01	2018.3	0.76	1321.1	0.25	I.d.	-	I.d.	-
0.10	2407.1	0.98	1712.6	0.75	2545.1	1.13	2116.1	0.95
	T7				T7			
0.01	1998.1	0.87	1654.2	1.22	856.1	0.12	690.5	.0.05
0.10	3 I.d.	n.d.	4120.2	1.33	1888.1	0.31	464.9	0.15
	T14				T14			
0.01	*	*	*	*	1111.8	0.11	725.9	0.24
0.10	*	*	*	*	1975.1	0.03	767.4	0.38
	T21				T21			
0.01	*	*	*	*	.553.5	0.22	904.3	0.13
0.10	*	*	*	*	3 I.d.	n.d	825.6	0.26
	T28				T28			
0.01	2010.1	0.87	1543.1	1.43.	655.3	0.26	211.4	0.22
0.10	4542.7	1.81	4128.1	1.97	2711.6	0.11	914.3	0.10

I.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
*: Not supplied sample.

3.3.1.1.2. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both pH levels (control-8.0 and acidify-7.6); II) understand the effects of pH levels in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both pH levels at each exposure concentration.

Energy reserves content and metabolic capacity

I) Significant differences in terms of PROT content were detected in clams contaminated with 0.10 mg/L Nf-MWCNTs under pH control, showing lower content compared to the remaining treatments, while no significant differences in terms of PROT content were detected between concentrations in organisms exposed to acidified pH (Figure 42 A). Individuals exposed to f-MWCNTs, showed significantly lower PROT content only in specimens exposed to 0.10 mg/L f-MWCNTs under pH 8.0, while opposite behavior was observed in bivalves maintained under pH 7.6, showing significantly higher content at the highest exposure concentration compared to the remaining treatments (Figure 42 A).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were observed at 0.10 mg/L f-MWCNTs, showing higher PROT content when the organisms were exposed to low pH compared to control pH (Figure 42 A)

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were assessed in non-contaminated specimens and contaminated with 0.10 mg/L under pH 7.6, showing higher PROT content in *R. philippinarum* exposed to f-MWCNTs in comparison to Nf-MWCNTs (Table 17).

I) A Significant dose-dependent decrease of GLY content was observed in bivalves contaminated with Nf-MWCNTs under pH 8.0, with the lowest value at the highest exposure concentration. In individuals maintained under pH 7.6, significantly lower GLY content was observed in contaminated bivalves compared to non-contaminated ones (Figure 42 B). Opposite behavior was detected for *R. philippinarum* exposed to f-MWCNTs, showing significantly higher PROT content when exposed to 0.10 mg/L under pH control compared to the remaining concentrations, while under low pH significantly higher content was detected in the contaminated bivalves compared to non-contaminated ones (Figure 42 B).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were observed at 0.10 mg/L f-MWCNTs, with higher GLY content observed in bivalves maintained under pH 7.6 compared to pH 8.0 (Figure 42 B).

III) Comparing *R. philippinarum* exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were assessed at 0.01 mg/L under both pH levels and 0.10 mg/L at pH 7.6, showing in all cases higher GLY content in bivalves contaminated with f-MWCNTs compared to Nf-MWCNTs (Table 17).

I) A significant dose-dependent increase of ETS activity was observed in bivalves contaminated with Nf-MWCNTs under pH 8.0, with higher value at the highest exposure concentration. In individuals maintained under pH 7.6, significantly higher metabolic activity was observed in contaminated bivalves compared to non-contaminated ones (Figure 42 C). Significant differences were also detected in organisms exposed to f-MWCNTs under pH control, showing higher activity at 0.10 mg/L compared to the other concentrations, while no significant differences among concentrations were observed under low pH (Figure 42 C).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were observed at 0.10 mg/L both Nf and f-MWCNTs, showing higher ETS activity in bivalves under pH 8.0 compared to pH 7.6 (Figure 42 C).

III) Comparing *R. philippinarum* exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were assessed in all exposed bivalves under both pH levels, showing in all cases higher metabolic activity in organisms contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 17).

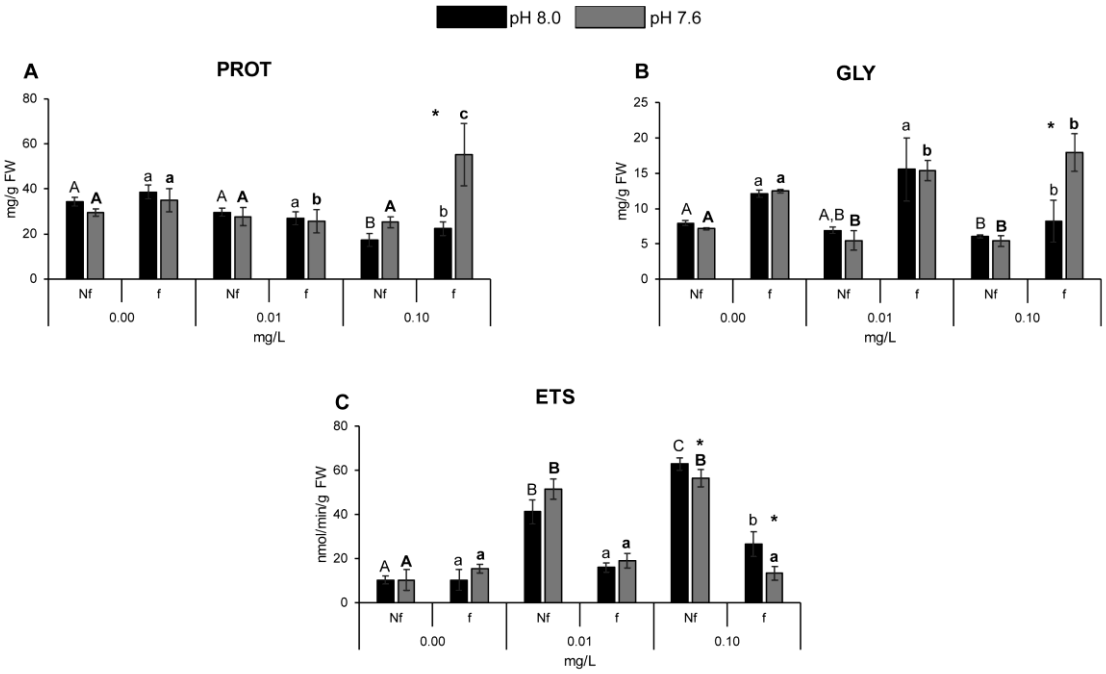

Figure 42. **A**: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron transport system (ETS) activity (mean ± standard deviation) in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

Table 17. Effect on oxidative stress biomarkers (Protein (PROT) content; Glycogen (GLY) content; Electron transport system (ETS) activity; Lipid peroxidation (LPO) levels; GSH/GSSG; Superoxide dismutase (SOD) activity; Catalase (CAT) activity; Glutathione peroxidase (GPx) activity; Glutathione S-Transferases (GSTs) activity; Acetylcholinesterase (AChE) activity) in *R. philippinarum* by pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH (8.0) and low pH (7.6). Significant differences ($p \leq 0.05$) between MWCNTs within each pH level at each exposure concentration were represented with asterisks (*).

CNT (mg/L)	pH		PROT	GLY	ETS	LPO	GSH/GSSG	SOD	CAT	GPx	GSTs	AChE
0.00	**8.0**	Nf	34.32±1.85	7.93±0.38	10.38±1.86	16.07±3.04	2.10±0.19	5.87±0.25	21.13±0.10	0.011±0.001	0.20±0.00	0.23±0.04
		f	38.61±3.12	12.11±0.48	10.38±4.77	25.70±2.69*	4.36±0.79*	5.54±1.45	28.97±7.22	0.011±0.002	0.50±0.63*	0.28±0.01
	7.6	Nf	29.57±1.63	7.17±0.16	10.32±4.81	29.09±1.50	2.10±0.19	6.60±0.17	21.12±0.12	0.012±0.004	0.20±0.00	0.21±0.11
		f	35.03±5.23*	12.52±0.25	15.44±1.88	24.46±7.58	4.07±0.35*	4.57±0.73*	28.10±7.65	0.013±0.001	0.45±0.15*	0.26±0.04
0.01	**8.0**	Nf	29.58±1.71	6.94±0.50	41.31±5.36	30.69±3.21	2.14±0.05	6.67±0.21	21.16±0.10	0.014±0.002	0.20±0.00	0.10±0.01
		f	26.98±2.86	15.55±4.45*	16.01±2.14*	30.48±2.36	2.77±0.54	7.52±0.71	29.56±7.86	0.012±0.003	0.37±0.11*	0.11±0.02
	7.6	Nf	27.73±4.15	5.49±1.42	51.55±4.45	31.53±4.29	2.35±0.66	6.47±1.94	23.35±3.31	0.011±0.003	0.23±0.15	0.04±0.01
		f	25.63±5.04	15.39±1.41*	19.15±3.35*	33.72±3.93	3.77±0.36*	2.46±0.80	41.62±7.37*	0.017±0.003*	0.43±0.06*	0.13±0.02*
0.10	**8.0**	Nf	17.36±2.87	6.09±0.20	62.92±2.66	39.83±3.03	1.44±0.03	6.47±0.08	21.04±0.04	0.023±0.003	0.20±0.00	0.08±0.01
		f	22.28±3.01	8.22±2.99	26.57±5.64*	29.38±5.02*	2.49±0.65*	9.28±2.35*	43.73±5.49*	0.017±0.002*	0.17±0.02	0.10±0.04
	7.6	Nf	25.17±2.48	5.43±0.75	56.48±3.90	38.12±0.85	1.63±0.11	6.52±0.03	21.13±0.05	0.022±0.002	0.20±0.00	0.03±0.02
		f	55.21±13.8*	17.95±2.69*	13.41±3.17*	35.55±4.70	3.37±0.87*	1.62±0.63*	21.55±4.49	0.012±0.002*	0.39±0.10*	0.12±0.05*

Oxidative status

I) Regardless the pH level, a significant dose-dependent increase of LPO levels was observed in *R. philippinarum* exposed to Nf-MWCNTs, showing higher value under 0.10 mg/L compared to the remaining concentrations (Figure 43 A). Considering the bivalves contaminated with f-MWCNTs, no significant differences were observed between concentrations in organisms under pH control, while at low pH, significantly higher LPO levels were detected in all exposed bivalves compared to control individuals (Figure 43 A).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were observed in control organisms, showing higher LPO levels under pH 7.6 compared to pH 8.0 (Figure 43 A).

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were assessed in control and exposed organisms to 0.10 mg/L maintained at pH control, showing higher and lower LPO levels respectively under f-MWCNTs compared to Nf-MWCNTs (Table 17).

I) Significantly lower GSH/GSSG was detected in bivalves exposed to 0.10 mg/L Nf-MWCNTs under both pH levels compared to the remaining concentrations (Figure 43 B). Considering individuals exposed to f-MWCNTs, significantly lower ratio was assessed in exposed *R. philippinarum* compared to controls under pH 8.0, while under pH 7.6 no significant differences were detected among concentrations (Figure 43 B).

II) For each MWCNT at each exposure concentration, no significant differences between pH levels were detected (Figure 43 B).

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were detected in control individuals under both pH levels, exposed organisms to 0.01 mg/L at pH 7.6 as well as in bivalves exposed to 0.10 mg/L under both pH levels, showing in all cases lower GSH/GSSG under Nf-MWCNTs compared to f-MWCNTs (Table 17).

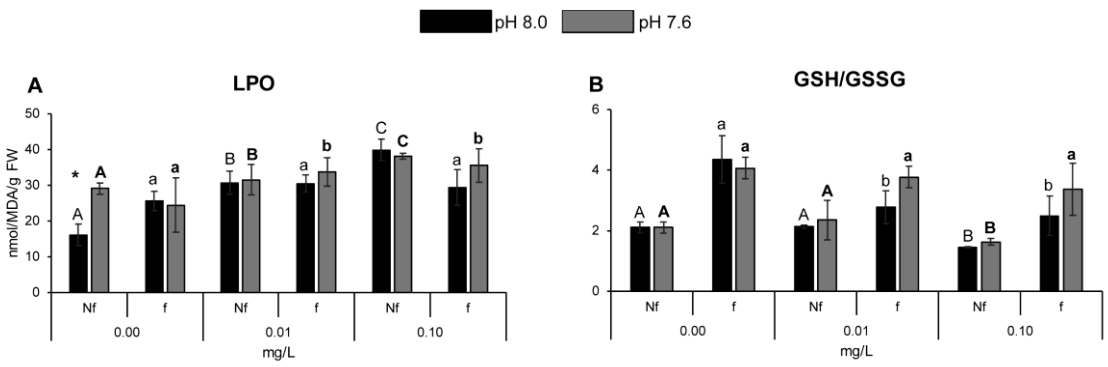

Figure 43. **A**: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

I) Regardless of the pH level, no significant differences between concentrations were detected in terms of SOD activity in bivalves exposed to Nf-MWCNTs (Figure 44 A). A Significant dose-dependent increase of SOD activity was observed in exposed organisms to f-WMCNTs under pH control, while opposite behavior was observed at low pH, with significantly lower values in contaminated bivalves compared to non-contaminated ones (Figure 44 A).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were detected in bivalves exposed to 0.01 and 0.10 mg/L f-MWCNTs, showing in both cases higher antioxidant activity under pH 8.0 compared to pH 7.6 (Figure 44 A).

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were assessed in control condition under pH 7.6 showing higher SOD activity under Nf-MWCNTs compared to f-MWCNTs. Significant differences were also assessed in exposed bivalves to 0.10 mg/L under both pH levels, showing lower (pH 8.0) and higher (pH 7.6) antioxidant activity in individuals contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 17).

I) Regardless of the pH level, no significant differences in terms of CAT activity were detected in bivalves exposed to Nf-MWCNTs (Figure 44 B). *R. philippinarum* exposed to f-MWCNTs showed significantly higher CAT activity in exposed specimens compared to non-exposed ones when submitted to pH control. Under low pH, significant differences were only detected when the organisms were exposed to 0.10 mg/L f-MWCNTs, showing the highest value under this concentration compared to the remaining ones (Figure 44 B).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were detected in bivalves exposed to 0.01 and 0.10 mg/L f-MWCNTs, showing lower and higher CAT activity respectively under pH control compared to pH 7.6 (Figure 44 B).

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were assessed under 0.01 mg/L pH 7.6 and 0.10 mg/L pH 8.0, showing in both cases higher CAT activity in bivalves contaminated with f-MWCNTs compared to Nf-MWCNTs (Table 17).

I) A significant dose-dependent increase of GPx activity was observed in *R. philippinarum* exposed to Nf-MWCNTs under pH 8.0, with higher values under the highest exposure concentration, while under pH 7.6 significant differences were observed only in individuals exposed to 0.10 mg/L, showing higher GPx activity under this concentration compared to the other ones (Figure 44 C). Bivalves contaminated with f-MWCNTs showed significantly higher GPx activity when exposed to 0.10 mg/L under pH control, while at pH 7.6, significantly higher activity was detected when organisms were exposed to 0.01 mg/L compared to the remaining concentrations (Figure 44 C).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were detected in bivalves exposed to 0.01 and 0.10 mg/L f-MWCNTs, showing lower and higher GPx activity respectively under pH control compared to low pH (Figure 41 C).

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed in exposed organisms to 0.01 mg/L under pH 7.6 with higher antioxidant activity in *R. philippinarum* contaminated with f-MWCNTs compared to Nf-MWCNTs. Significant differences between materials were also identified in bivalves exposed to 0.10 mg/L under both pH levels, showing in both cases, higher GPx activity under Nf-MWCNTs compared to f-MWCNTs (Table 17).

I) GSTs activity showed no significant differences between concentrations in bivalves exposed to Nf-MWCNTs under both pH levels (Figure 44 D). Considering *R. philippinarum* exposed to f-MWCNTs, a significant dose-dependent decrease of GSTs activity was observed under pH 8.0, with lower value at the highest exposure concentration, while under pH 7.6, no significant differences among concentrations were detected (Figure 44 D).

II) For each MWCNTs material at each exposure concentration, significant differences between pH levels were observed only in bivalves exposed to 0.10 mg/L f-MWCNTs, showing lower activity under pH control compared to pH 7.6 (Figure 44 D).

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were revealed in all conditions with the exception of 0.10 mg/L under pH 8.0, observing in all cases lower GSTs activity in specimens contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 17).

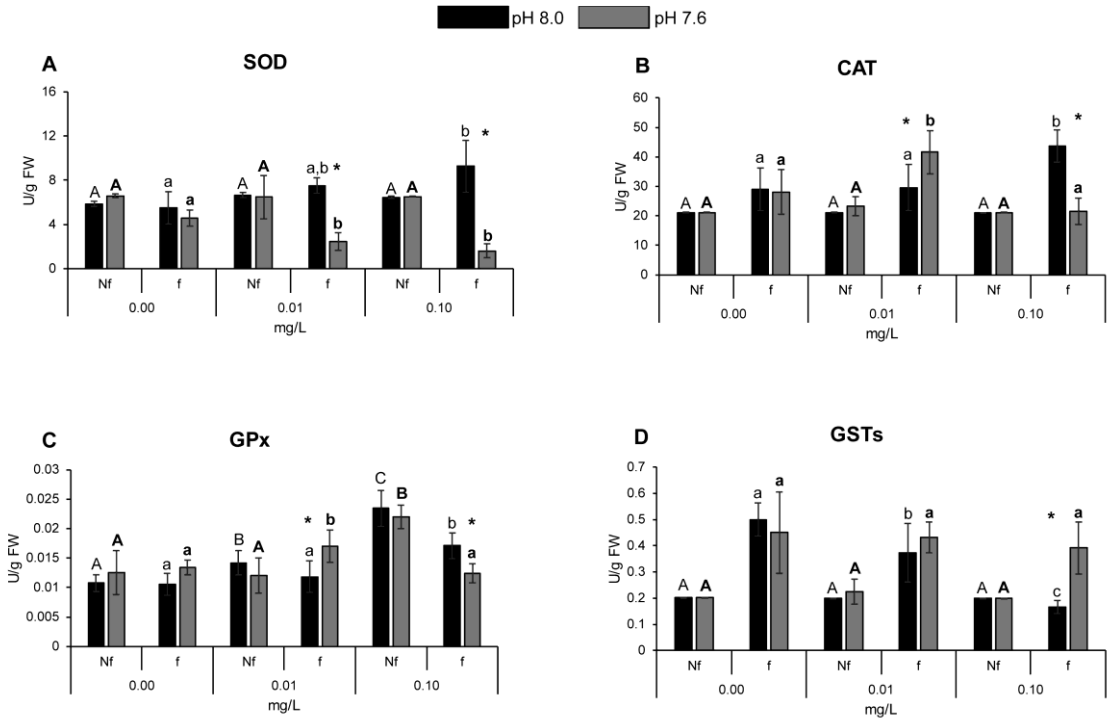

Figure 44. **A:** Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

Neuro status

I) Regardless of the pH variations as well as the different CNT materials, significantly lower AChE activity was observed in all contaminated bivalves compared to non-contaminated ones (Figure 45).

II) For each MWCNT at each exposure concentration, significant differences between pH levels were detected at 0.01 and 0.10 mg/L Nf-MWCNTs, with the lowest values observed in organisms under low pH compared to control pH (Figure 45).

III) Comparing bivalves exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were identified at 0.01 and 0.10 mg/L under pH 7.6, with lower neuroactivity in specimens contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 17).

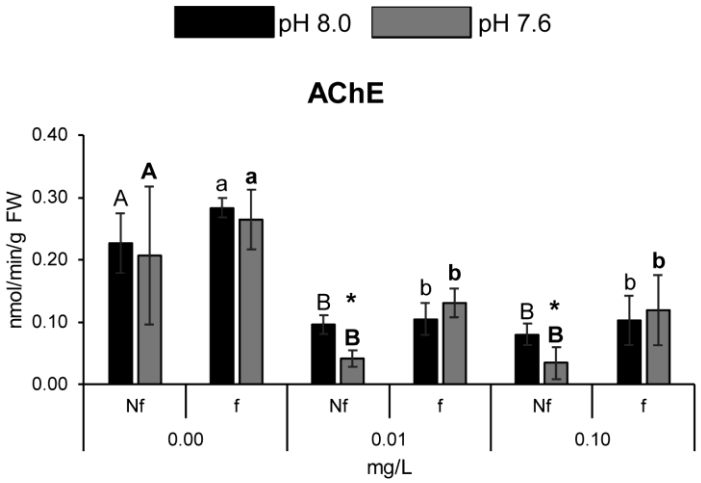

Figure 45. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *R. philippinarum* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

3.3.1.2. *Hediste diversicolor* (O.F. Müller, 1776)

3.3.1.2.1. Characterization analysis of water media

Table 18 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.01 and 0.10 mg/L) under pH control (8.0) and low pH (7.6).

At T0 samples prepared with 0.01 and 1.10 mg/L of Nf-MWCNTs at both tested pH levels (8.0 and 7.6) were unstable and characterized by the presence of micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 18). Regarding the PDI, it was possible to observe a time-dependent increase of the polydispersity index in each condition (pH 8.0 and 7.6) and exposure materials (Nf and f) due to the formation of large particles or aggregates in the analysed samples. DLS and PDI analyses of samples exposed to different concentrations of Nf-MWCNTs under acidifying pH at 7, 14 and 21 days, did not allow for the detection of measurable macro/micro/nanosize particle aggregates reported in the table as “not supplied samples” indicating the settlement and/or uptake of the material. The results obtained at T0 showed bigger aggregates under pH control compared to low pH especially at the highest exposure concentration (0.10 mg/L).

Regarding samples contaminated with f-MWCNTs, DLS analysis evidenced the presence of suspended material at all the analysed conditions whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples. Time-dependent increased of the polydispersity index in each condition (pH 8.0 and 7.6) and exposure materials (Nf and f) due to the formation of large particles or aggregates in the analysed samples was observed (Table 18). Moreover, f-MWCNTs suspended at 0.10 mg/L under pH 7.6 were found to agglomerate and remain dispersed in the medium until 28 days. Larger aggregates were observed under pH control in comparison to low pH.

Considering both pH levels, the mean recorded hydrodynamic diameter of f-MWCNTs aggregates were smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating a higher dispersion of f-MWCNTs in aqueous media (Table 18).

Table 18. *Hediste diversicolor*: average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed under control pH 8.0 and low pH 7.6 in each exposure concentration (0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

CNT concentration (mg/L)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
	Nf-MWCNTs				**f-MWCNTs**			
	8.0		**7.6**		**8.0**		**7.6**	
	T0				T0			
0.01	1863.1	1.26	1431.3	0.69	5 l.d.	n.d.	5 l.d.	n.d.
0.10	5428.0	2.23	3713.4	2.10	2545.1	1.13	2116.1	0.97
	T7				T7			
0.01	5 l.d.	n.d.	*	*	5501.2	1.18	5 l.d.	n.d.
0.10	3217.4[a]	1.39	5411.8	2.07	5 l.d.	n.d.	3116.0	1.23
	T14				T14			
0.01	8603.7[a]	4.87	*	*	2344.9[a]	1.38	5 l.d.	n.d.
0.10	1381.0	1.25	*	*	5 l.d.	n.d.	4353.9	1.61
	T21				T21			
0.01	5 l.d.	n.d.	*	*	5 l.d.	n.d.	2158.1[a]	1.23
0.10	5 l.d.	n.d.	*	*	1930.5[a]	0.88	2768.1	1.26
	T28				T28			
0.01	5 l.d.	n.d.	5 l.d.	n.d.	5 l.d.	n.d.	5 l.d.	n.d.
0.10	5 l.d.	n.d.	3893.8	1.26	5 l.d.	n.d.	2818.2	1.53

l.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
a: Sample contaminated with sand grains and macroscopic blackish aggregates.
*: Not supplied sample.

3.3.1.2.2. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both pH levels (control-8.0 and low-7.6); II) understand the effects of pH levels in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both pH levels at each exposure concentration.

Energy reserves content and metabolic capacity

I) Considering the effects of exposure concentrations on organisms PROT content, in individuals exposed to Nf-MWCNTs under both pH levels, no significant differences were observed among exposure concentrations (Figure 46 A). The same trend was observed in *H. diversicolor* exposed to f-MWCNTs under pH control, while a significantly lower content was recorded when organisms were submitted to low pH and exposed to 0.01 mg/L in comparison to control (Figure 46 A).

II) Significant differences between pH levels were observed in PROT content when organisms were maintained under control conditions, exposed to 0.01 mg/L of Nf-MWCNTs and 0.10 mg/L of both CNT materials, showing in all cases higher content in individuals maintained at pH 7.6 in comparison to pH 8.0 (Figure 46 A).

III) When comparing *H. diversicolor* exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed only in polychaetes exposed to 0.01 mg/L under pH 7.6, showing higher PROT content in individuals contaminated with Nf-MWCNTs (Table 19).

I) No significant differences in terms of GLY content were observed among Nf-MWCNT exposure concentrations in individuals maintained under pH control, while under pH 7.6 polychaetes exposed to 0.10 mg/L showed significantly higher content in comparison to the remaining concentrations (Figure 46 B). In individuals exposed to f-MWCNTs under both pH levels no significant differences were observed among all exposure concentrations (Figure 46 B).

II) Significant differences between pH levels were observed in GLY content when organisms were exposed to 0.01 mg/L of f-MWCNTs and at 0.10 mg/L of both MWCNTs, showing in all cases higher content in individuals under pH 7.6 in comparison to pH 8.0 (Figure 46 B).

III) When comparing organisms exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed only in polychaetes exposed

to 0.01 mg/L under pH 7.6, with higher GLY content in individuals contaminated with f-MWCNTs (Table 19).

I) Considering the effects of exposure concentrations, results of ETS activity in *H. diversicolor* showed that for Nf-MWCNTs under pH control no significant differences were observed among tested conditions, while at low pH organisms presented a significant decrease of their ETS activity at 0.10 mg/L Nf-MWCNTs in comparison to the remaining concentrations (Figure 46 C). For the individuals exposed to f-MWCNTs significant increase of the ETS activity was observed only at the highest concentration when maintained at pH control, while under low pH an opposite behaviour was observed, with significant inhibition of ETS activity at 0.10 mg/L in comparison to the remaining concentrations (Figure 46 C).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed at 0.10 mg/L of both MWCNTs, with lower ETS activity in individuals maintained at pH 7.6 in comparison to organisms under pH 8.0 (Figure 46 C).

III) When comparing specimens exposed to different MWCNTs at the same pH and exposure concentration, no significant differences between materials were observed (Table 19).

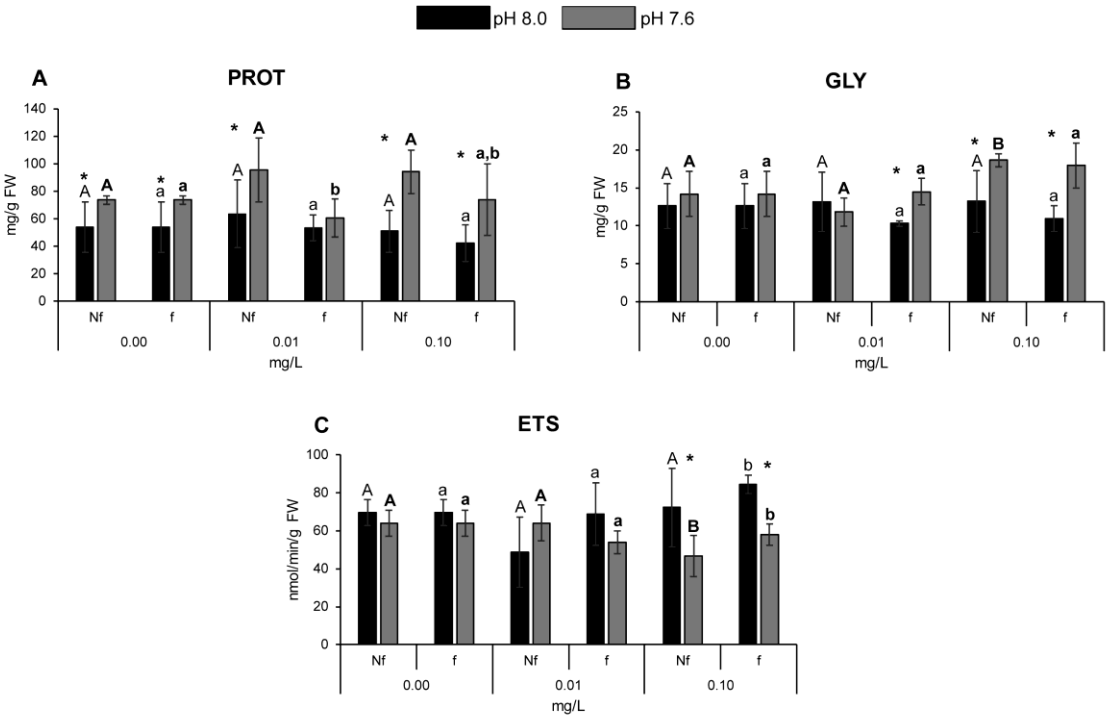

Figure 46. **A**: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron transport system (ETS) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold

letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

Table 19. Effect on oxidative stress biomarkers (Protein (PROT) content; Glycogen (GLY) content; Electron transport system (ETS) activity; Lipid peroxidation (LPO) levels; GSH/GSSG; Superoxide dismutase (SOD) activity; Catalase (CAT) activity; Glutathione peroxidase (GPx) activity; Glutathione S-Transferases (GSTs) activity; Acetylcholinesterase (AChE) activity) in *H. diversicolor* by pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH (8.0) and low pH (7.6). Significant differences ($p \leq 0.05$) between MWCNTs within each pH level at each exposure concentration were represented with asterisks (*).

CNT (mg/L)	pH		PROT	GLY	ETS	LPO	GSH/GSSG	SOD	CAT	GPx	GSTs	AChE
0.00	**8.0**	Nf	53.92±18.38	12.63±2.97	69.61±6.66	8.49±2.77	5.69±1.83	2.86±0.68	47.47±7.32	0.029±0.005	0.16±0.02	3.47±0.38
		f	53.92±18.38	12.63±2.97	69.61±6.66	8.49±2.77	5.69±1.83	2.86±0.68	47.47±7.32	0.029±0.005	0.16±0.02	3.47±0.38
	7.6	Nf	73.79±3.11	14.19±2.94	64.01±6.72	8.57±0.30	6.42±1.56	2.25±0.74	46.66±7.75	0.026±0.002	0.10±0.01	2.59±0.20
		f	73.79±3.11	14.19±2.94	64.01±6.72	8.57±0.30	6.42±1.56	2.25±0.74	46.66±7.75	0.026±0.002	0.10±0.01	2.59±0.20
0.01	**8.0**	Nf	63.52±24.61	13.17±3.92	48.70±18.38	9.56±2.97	5.81±1.37	4.89±1.54	54.09±9.75	0.047±0.008	0.15±0.00	1.40±0.21
		f	53.52±9.52	10.30±0.33	68.77±16.52	9.67±4.18	5.41±1.79	2.91±0.57*	46.64±9.29	0.050±0.006	0.24±0.03*	3.08±0.42*
	7.6	Nf	95.52±23.10	11.81±1.84	64.18±9.27	10.74±1.51	4.62±0.91	4.13±1.65	59.59±4.39	0.039±0.006	0.11±0.05	3.04±0.35
		f	60.59±13.67*	14.48±1.77*	54.00±6.11	11.32±2.01	5.78±1.32	3.70±1.46	52.19±6.57	0.036±0.007	0.20±0.05*	2.70±0.78
0.10	**8.0**	Nf	50.99±15.18	13.23±4.08	72.30±20.57	10.24±2.23	5.45±1.84	5.40±0.99	53.86±2.93	0.050±0.006	0.09±0.03	2.29±0.49
		f	42.32±13.29	10.93±1.71	84.60±4.79	13.29±2.64*	3.73±0.94*	4.25±0.46	49.06±2.50	0.048±0.014	0.21±0.00*	1.47±0.36*
	7.6	Nf	94.25±15.84	18.62±0.88	46.56±10.83	12.72±2.99	3.88±0.86	4.09±1.29	55.55±8.04	0.039±0.009	0.21±0.04	3.47±0.64
		f	73.85±26.02	17.93±2.98	57.98±5.70	17.70±4.48*	3.47±0.56	5.66±1.33	55.83±3.86	0.048±0.011	0.18±0.06	1.23±0.68*

Oxidative status

I) Results of LPO levels in *H. diversicolor* showed that for Nf-MWCNTs under pH control no significant differences were observed among all concentrations, while at pH 7.6 the levels significantly increased in contaminated organisms in comparison to non-contaminated ones (Figure 47 A). A similar trend was also observed in polychaetes exposed to f-MWCNTs under both pH levels, showing a concentration-dependent increase of LPO levels in organisms exposed to the CNTs compared to control individuals (Figure 47 A).

II) For each MWCNT and exposure concentration, no differences were observed between pH levels (Figure 47 A).

III) Significant differences between materials were observed in polychaetes exposed to 0.10 mg/L under both pH levels, with higher values in individuals contaminated with f-MWCNTs in comparison to organisms contaminated with Nf-MWCNTs (Table 19).

I) Results of GSH/GSSG in *H. diversicolor* showed that for Nf-MWCNTs under pH control no significant differences were observed between concentrations, while under pH 7.6 organisms presented a significant dose-dependent decrease of their GSH/GSSG compared to control individuals (Figure 47 B). Considering polychaetes exposed to f-MWCNTs, significantly lower ratio was detected when exposed to 0.10 mg/L under both pH levels compared to the remaining concentrations (Figure 47 B).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed at 0.10 mg/L of Nf-MWCNTs, with lower GSH/GSSG in individuals maintained at pH 7.6 in comparison to organisms under pH 8.0 (Figure 47 B).

III) When comparing organisms exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed only in polychaetes exposed to 0.10 mg/L under pH 8.0, with a lower values in specimens contaminated with f-MWCNTs (Table 19).

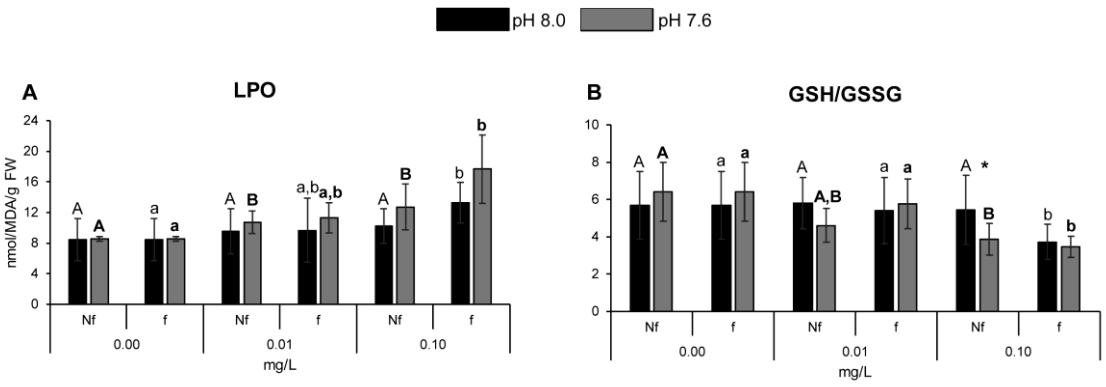

Figure 47. **A**: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

I) Regardless of the pH levels, polychaetes contaminated with Nf-MWCNTs showed significantly higher SOD activity in exposed organisms compared to non-exposed ones (Figure 48 A). When the organisms were contaminated with f-MWCNTs under pH 8.0, the SOD activity significantly increases only at the highest exposure concentration in comparison to the remaining treatments, while under pH 7.6, a significantly concentration-dependent increase of the antioxidant enzyme activity was detected in exposed organisms compared to control individuals (Figure 48 A).

II) For each MWCNT and exposure concentration, no significant differences were observed between pH levels (Figure 48 A).

III) When comparing individuals exposed to different MWCNTs at each pH and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 mg/L under pH control, with higher values in individuals contaminated with Nf-MWCNTs in comparison to organisms contaminated with f-MWCNTs (Table 19).

I) Considering the effects of exposure concentrations, the results of CAT activity in organisms exposed to Nf-MWCNTs at pH 8.0 showed no significant among concentrations, while a significant increase on the activity of this enzyme was observed in individuals exposed to 0.01 mg/L under pH 7.6 in comparison to uncontaminated individuals (Figure 48 B). Results of CAT activity in *H. diversicolor* exposed to f-MWCNTs under pH 8.0, showed no significant differences among all concentrations, while a significant concentration-dependent increase of the activity was recorded in organisms under pH 7.6 (Figure 48 B).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed in organisms exposed to 0.10 mg/L f-MWCNTs, showing higher CAT activity under pH 7.6 compared to individuals under pH 8.0 (Figure 48 B).

III) When comparing individuals exposed to different MWCNTs at the same pH and exposure concentration, no significant differences between materials were observed (Table 19).

I) Considering the effects of exposure concentrations, results of GPx activity in *H. diversicolor* showed that for both Nf-MWCNTs and f-MWCNTs under both pH levels, significantly higher activity was observed in exposed individuals compared to controls (Figure 48 C).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed in organisms exposed to 0.01 mg/L f-MWCNTs, showing higher GPx activity under pH 8.0 compared to individuals under pH 7.6 (Figure 48 C).

III) When comparing specimens exposed to different MWCNTs at the same pH and exposure concentration, no significant differences between materials were observed (Table 19).

I) Polychaetes contaminated with Nf-MWCNTs under pH control showed significantly lower GSTs activity when exposed to 0.10 mg/L compared to the remaining concentrations, while under low pH, opposite behavior was detected, showing a significantly increased of the activity only the highest exposure concentration (Figure 48 D). When exposed to f-MWCNTs under pH 8.0, polychaetes presented significantly higher GSTs activity at 0.01 mg/L compared to non-contaminated individuals, while under pH 7.6, significantly higher GSTs activity was recorded in all exposed individuals compared to non-exposed ones (Figure 48 D).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed in organisms exposed to 0.10 mg/L Nf-MWCNTs, showing higher GSTs activity under pH 7.6 compared to individuals under pH 8.0 (Figure 45 D).

III) When comparing individuals exposed to different MWCNTs at each pH and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 mg/L under both pH levels as well as to 0.10 mg/L pH control, showing in all cases higher values in individuals contaminated with f-MWCNTs in comparison to Nf-MWCNTs (Table 19).

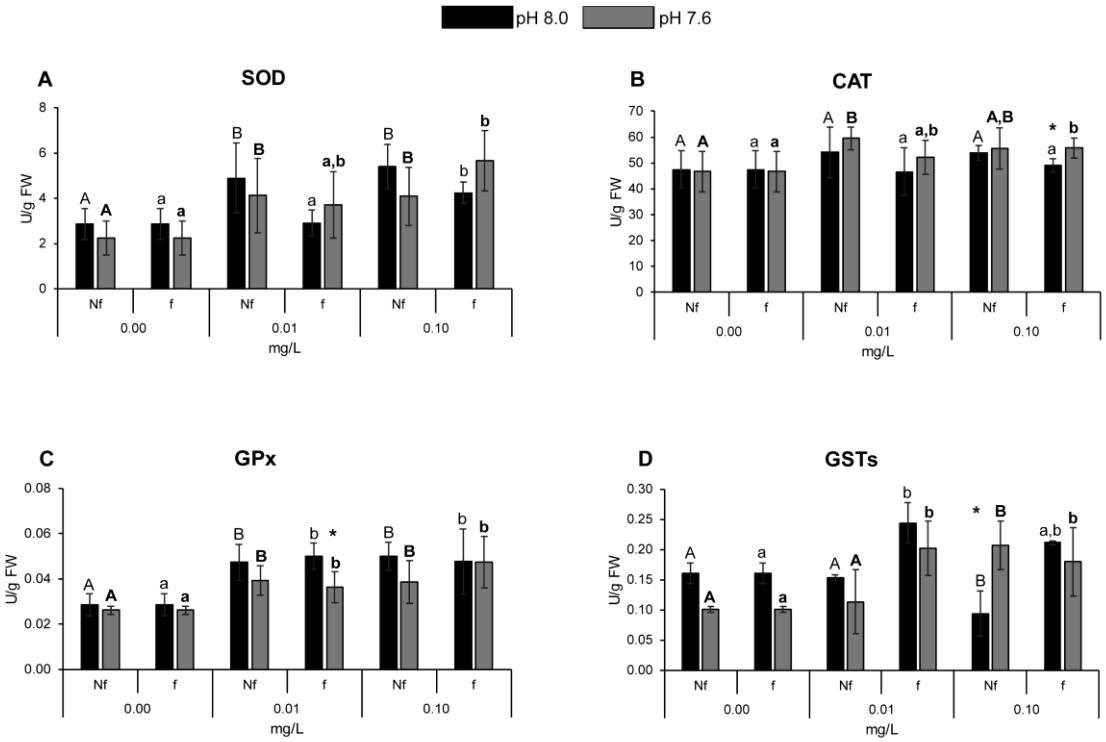

Figure 48. **A:** Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

Neuro status

I) Considering the effects of exposure concentrations, results of AChE activity showed significant differences among individuals exposed to different Nf-MWCNT concentrations under pH 8.0, with the lower value recorded under 0.01 mg/L in comparison to the other conditions. Under pH 7.6, the activity of the neuro-enzyme was significantly lower only at 0.01 mg/L Nf-MWCNTs in comparison to the remaining concentrations (Figure 49). Regarding the individuals contaminated with f-MWCNTs under both pH levels, significantly lower AChE activity was observed only in organisms exposed to 0.10 mg/L compared to all the remaining concentrations (Figure 49).

II) For each MWCNT at each exposure concentration, differences between pH levels were observed between control individuals, showing lower AChE activity in organisms under pH 7.6. Differences between pH levels were also recorded in *H. diversicolor* exposed to 0.01 and 0.10 mg/L

Nf-MWCNTs, with significantly lower enzyme activity in organisms maintained under pH control compared to pH 7.6 (Figure 46).

III) When comparing organisms exposed to the same pH and exposure concentration, significant differences between polychaetes exposed to different MWCNTs were observed at 0.01 mg/L under pH 8.0, with lower activity in *H. diversicolor* exposed to Nf-MWCNTs compared to individuals exposed to f-MWCNTs. Significant differences between materials were also observed in individuals exposed to 0.10 mg/L maintained under both pH levels, with lower activity in individuals contaminated with f-MWCNTs in comparison to Nf-MWCNTs (Table 19).

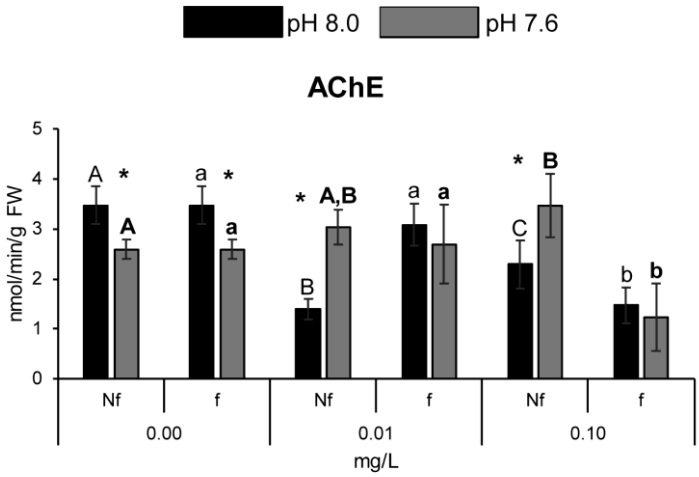

Figure 49. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *H. diversicolor* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

3.3.1.3. *Diopatra neapolitana* (Delle Chiaje, 1841)

3.3.1.3.1. Characterization analysis of water media

Table 20 reports the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media at different concentrations (0.01 and 0.10 mg/L) under pH control (8.0) and low pH (7.6).

At different times of exposure (T0, T7, T14, T21 and T 28) samples prepared with 0.01 and 0.10 mg/L of Nf-MWCNTs at both tested pH levels (8.0 and 7.6) were unstable and characterized by the presence of micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 20). It was possible to observe also a time-dependent increased of the polydispersity index in each condition (pH 8.0 and 7.6) and exposure materials (Nf and f) due to the formation of large particles or aggregates in the analysed samples. DLS and PDI analyses of samples exposed to different concentrations of Nf-MWCNTs under low pH at 21 days, did not allow for the detection of measurable macro/micro/nanosize particle aggregates reported in the table as “not supplied samples” indicating the settlement and/or uptake of the material. The results obtained at different exposure times showed bigger aggregates under pH control compared to pH 7.6 under both Nf-MWCNT exposure concentrations (0.01 mg/L and 0.10 mg/L).

Regarding samples contaminated with f-MWCNTs, DLS analysis evidenced the presence of suspended material at all the analysed conditions whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples. Time-dependent increased of the polydispersity index in each condition (pH 8.0 and 7.6) and exposure materials (Nf and f) due to the formation of large particles or aggregates in the analysed samples was detected (Table 20). Also in this condition, larger aggregates were observed under pH control in comparison to low pH.

Considering both pHs and both concentrations, the mean recorded hydrodynamic diameter of f-MWCNTs aggregates were smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating higher dispersion of f-MWCNTs in aqueous media (Table 20).

Table 20. *Diopatra neapolitana:* average size distribution (nm) and polydispersity index (PDI) of pristine MWCNTs (Nf-MWCNTs) and MWCNTs-COOH (f-MWCNTs) suspensions analysed under control pH 8.0 and low pH 7.6 in each exposure concentration (0.01 and 0.10 mg/L) at different exposure periods: T0: time zero, immediately after the dispersion of CNT materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure.

CNT concentrations (mg/L)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
	Nf-MWCNTs				**f-MWCNTs**			
	8.0		**7.6**		**8.0**		**7.6**	
	T0				T0			
0.01	2596.6	0.98	1431.3	0.69	3634.9	1.50	999.3	0.20
0.10	4321.1	1.32	3713.4	2.10	3987.2	1.45	1001.1	0.92
	T7				T7			
0.01	5 I.d.	n.d.	1321.2	0.54	5 I.d.	n.d.	5 I.d.	n.d.
0.10	3214.2	0.78	1411.8	1.07	2098.7	1.72	3.I.d.	n.d.
	T14				T14			
0.01	5 I.d.	n.d.	1234.1	0.40	1771.2	0.804	865.5	0.18
0.10	3998.8	1.24	1.321.2	0.67	3098.2	1.09	992.1	0.21
	T21				T21			
0.01	3354.7	1.32	*	*	3354.7	1.50	5 I.d.	n.d.
0.10	3 I.d.	n.d.	3 I.d.	n.d.	3987.2	1.89	992.1	0.26
	T28				T28			
0.01	5 I.d.	n.d.	3 I.d.	n.d.	3 I.d.	n.d.	5 I.d.	n.d.
0.10	4098.2	1.98	2893.8	1.06	5 I.d.	n.d.	3 I.d.	n.d.

I.d.: “Invalid data” (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).
n.d.: “No data” (Invalid data (I.d.) results in 3 out of 5 samples).
*: Not supplied sample.

3.3.1.3.2. Biological analysis: physiological parameter (regenerative capacity)

The mean values for the percentage (%) of regenerated body width and the number (#) of new chaetigers in *D. neapolitana* after 11th, 18 th and 28 th days of amputation are illustrated in Figure 50 and presented in Table 21. All the results were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both pH levels (pH 8.0 and pH 7.6); II) understand the effects of pH levels in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both pH levels at each exposure concentration.

11th day

After amputation all individuals were healing the cut region, however no significant differences were observed in terms of percentage of regenerated body width as well as number of new chaetigers between individuals non-exposed (0.00 mg/L) and exposed polychaetes to both MWCNTs in all tested concentrations (0.01 and 0.10 mg/L) under both pH (Figure 50; Table 21).

18th day

I) Looking on the effects of exposure concentrations, the percentage of regenerated body width for f-MWCNT under control pH showed significantly lower value only in individuals exposed to 0.10 mg/L in comparison to the remaining concentrations, while no significant differences were observed between concentrations in terms of number of new chaetigers. Regarding polychaetes contaminated with Nf-MWCNTs under pH control, significantly lower percentage of regenerated body width as well as number of new chaetigers were detected in exposed individuals compared to control ones (Table 21). When polychaetes were submitted to low pH and contaminated with both MWCNTs, significantly lower values in terms of percentage of regenerated body width and number of new chaetigers were observed in exposed individuals in comparison to non-exposed ones (Table 21).

II) Considering the effects of different pH levels, for each MWCNT at each exposure concentration, differences between pH levels were observed at 0.01 mg/L f-MWCNTs with lower percentage of regenerated body width as well as number of new chaetigers in individuals maintained under pH 7.6 in comparison to individuals maintained under pH 8.0. Significant differences between pH levels were also detected at 0.10 mg/L f-MWCNTs in terms of number of new chaetigers, showing also in this case, lower value in *D. neapolitana* maintained under pH 7.6 compared to pH 8.0 (Table 21).

III) Considering the effects of MWCNTs at each concentration and each pH, significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L under pH control, showing a lower percentage of regenerated body width (0.10 mg/L) and lower number of chaetigers (both 0.01 and 0.10 mg/L) in organisms contaminated with Nf-MWCNTs compared to f-MWCNTs (Table 21).

28th day

I) The results of percentage of regenerated body width for f-MWCNT under control pH, showed significantly lower value only in organisms exposed to 0.10 mg/L in comparison to the remaining concentrations, while no significant differences were observed among concentrations in terms of number of new chaetigers. Looking to polychaetes contaminated with Nf-MWCNTs under pH control, the results showed a significantly dose-dependent decreased of percentage of regenerated body width, with the lower value at the highest exposure concentration, while significantly lower number of new chaetigers were only observed in polychaetes contaminated with 0.10 mg/L compared to the remaining concentrations (Figure 50; Table 21). Considering the results observed in individuals exposed to low pH, significantly dose-dependent decreased of the percentage regenerated body width was observed in *D. neapolitana* contaminated with f-MWCNTs, while only at 0.10 mg/L the number of new chaetigers were significantly lower compared to the remaining concentrations. The results obtained for individuals contaminated with Nf-MWCNTs under pH 7.6, showed significantly lower percentage regenerated body width in exposed individuals compared to control ones, while significantly lower number of new chaetigers were identified when *D. neapolitana* was exposed to the highest concentration compared to the other treatments (Figure 50; Table 21).

II) For each MWCNT at each exposure concentration, differences between pH levels were observed only for the percentage of regenerated body width when the organisms were exposed to 0.01 and 0.10 mg/L f-MWCNTs, showing in both concentrations lower values when the polychaetes were exposed to pH 7.6 compared to pH 8.0 (Figure 50; Table 21).

III) Comparing organisms exposed to the same pH and exposure concentration, significant differences between materials were observed only in terms of percentage of regenerated body width when specimens were exposed to 0.10 mg/L under both pH 8.0 and 7.6, showing significantly higher and lower values respectively when contaminated with f-MWCNTs compared to Nf-MWCNTs (Figure 50; Table 21).

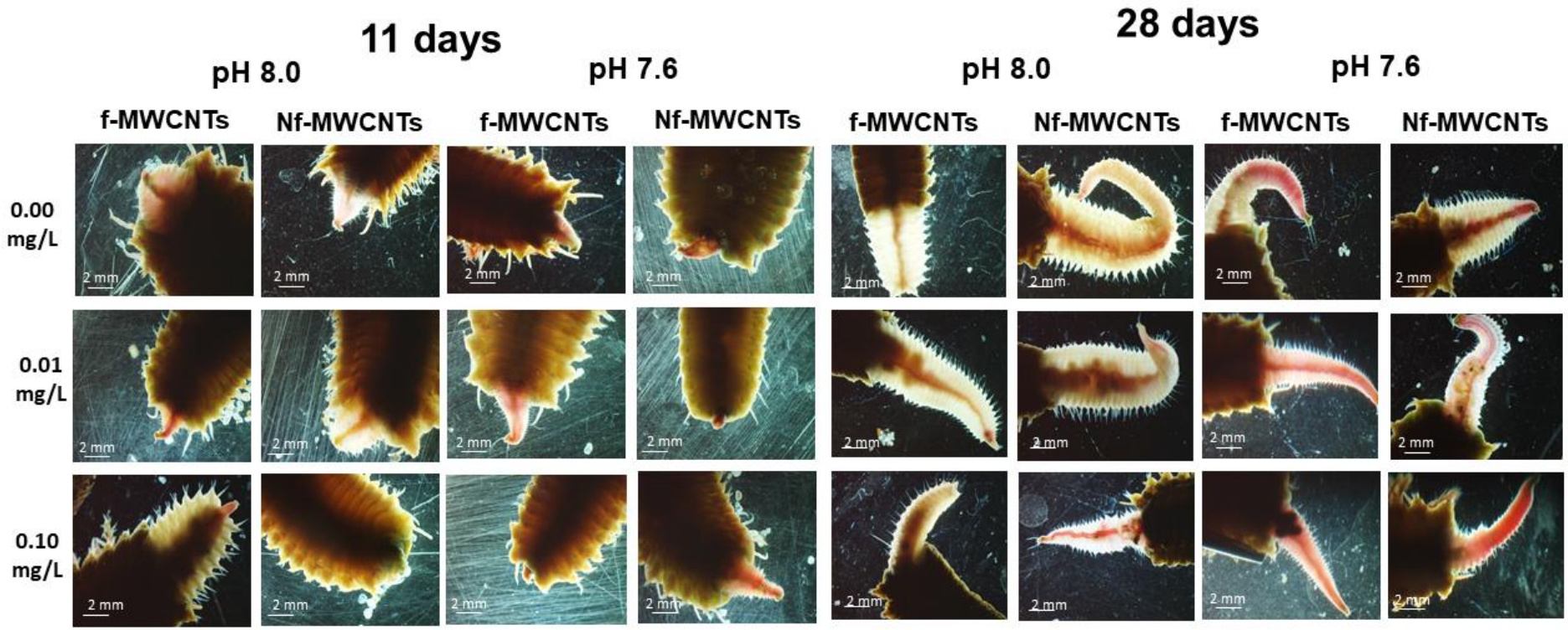

Figure 50. Regenerative capacity of *D. neapolitana* at 11th and 28th days after amputation, exposed to different MWCNTs (f and Nf) and concentrations (0.00; 0.01 and 0.10 mg/L) under two pH levels (control pH-8.0; low pH-7.6).

Table 21. Regeneration data (percentage (%) of body width and the number (#) of new chaetigers) for *D. neapolitana*, 11th, 18th and 28th days after amputation. Significant differences ($p \leq 0.05$) among exposure concentrations (0.00; 0.01 and 0.10 mg/L) for each MWCNT (f-MWCNTs and Nf-MWCNTs) and pH level (control pH-8.0 and low pH- 7.6) were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; lowercase and regular letters for Nf-MWCNTs at pH 7.6; uppercase and bold letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the two pH levels for each MWCNT and exposure concentration were represented with bold asterisks (*****). Significant differences ($p \leq 0.05$) between f-MWCNTs and Nf-MWCNTs within each pH at each exposure concentration were represented with bold hashes (**#**).

CNT concentrations (mg/L)	**pH**		**11 days**		**18 days**		**28 days**	
			%body width	**# chaetigers**	**%body width**	**# chaetigers**	**%body width**	**# chaetigers**
0.00 mg/L	8.0	f-MWCNTs	7.67±2.07 **A**	0.00±0.00 **A**	44.64±10.04 **A**	21.50±6.28 **A**	75.79±3.96 **A**	30.50±1.38 **A**
		Nf-MWCNTs	7.67±2.07 A	0.00±0.00 A	44.64±10.04 A	21.50±6.28 A	75.79±3.96 A	30.50±1.38 A
	7.6	f-MWCNTs	7.85±1.92 **a**	0.00±0.00 **a**	39.31±10.52 **a**	21.03±1.20 **a**	59.41±5.51 **a**	25.84±4.71 **a**
		Nf-MWCNTs	8.01±1.22 a	0.00±0.00 a	36.94±8.22 a	21.10±5.28 a	61.28±6.53 a	29.21±4.23 a
			11 days		**18 days**		**28 days**	
			%body width	**# chaetigers**	**%body width**	**# chaetigers**	**%body width**	**# chaetigers**
0.01 mg/L	8.0	f-MWCNTs	6.50±3.73 **A**	0.00±0.00 **A**	37.87±7.51 **A** *****	18.83±2.40 **A** **#** *****	70.09±12.21 **A***	28.67±1.51 **A**
		Nf-MWCNTs	8.83±4.53 A	0.00±0.00 A	19.12±4.83 B	11.17±5.95 B #	59.41±19.35 B	26.67±7.39 A
	7.6	f-MWCNTs	7.76±1.45 **a**	0.00±0.00 **a**	19.87±3.21 **b** *****	10.55±5.26 **b** *****	41.97±10.01 **b***	23.22±4.35 **a**
		Nf-MWCNTs	7.97±4.81 a	0.00±0.00 a	16.80±8.19 b	11.67±2.45 b	41.99±10.20 b	21.33±8.24 a
			11 days		**18 days**		**28 days**	
			%body width	**# chaetigers**	**%body width**	**# chaetigers**	**%body width**	**# chaetigers**
0.10 mg/L	8.0	f-MWCNTs	5.60±3.90 **A**	0.00±0.00 **A**	29.06±7.45 **B #**	17.98±3.34 **A # ***	59.12±10.14 **B # ***	21.57±2.22 **A**
		Nf-MWCNTs	8.43±2.51 A	0.00±0.00 A	15.10±3.68 B #	9.50±3.94 B #	34.87±4.22 C #	11.33±2.58 B
	7.6	f-MWCNTs	7.48±3.21 **a**	0.00±0.00 **a**	19.99±10.89 **b**	8.99±3.50 **b** *****	27.76±2.80 **c # ***	15.27±5.44 **b**
		Nf-MWCNTs	7.96±1.82 a	0.00±0.00 a	15.76±7.20 b	9.22±8.30 b	39.99±3.45 b #	11.45±7.29 b

3.3.1.3.3. Biological analyses: biochemical parameters (energy reserves content and metabolic capacity, oxidative and neuro status)

All the results were discussed considering three main topics: I) understand the effects of exposure concentrations of both MWCNTs maintained under both pH levels (control-8.0 and low-7.6); II) understand the effects of pH levels in organisms exposed to both MWCNTs in each exposure concentration; III) understand the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both pH levels at each exposure concentration.

Energy reserves content and metabolic capacity

I) Considering the effects of exposure concentrations in individuals exposed to Nf-MWCNTs under pH 8.0, significant dose-dependent increase was detected in terms of PROT content, with the higher values at the highest exposure concentration (Figure 51 A). Looking the results under pH 7.6, significantly higher PROT content was detected in exposed polychaetes compared to non- exposed ones (Figure 51 A). Same trend was observed in *D. neapolitana* exposed to f-MWCNTs under low pH, while no significant differences were recorded when organisms were submitted to control pH (Figure 51 A).

II) Significant differences between pH levels were observed in PROT content when organisms were exposed to 0.01 and 0.10 mg/L of Nf-MWCNTs, showing higher content in individuals maintained at pH 8.0 in comparison to pH 7.6 (Figure 51 A).

III) When comparing *H. diversicolor* exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L under pH 8.0, showing higher PROT content in individuals contaminated with Nf-MWCNTs (Table 22).

I) Under pH 8.0 polychaetes exposed to 0.10 mg/L Nf-MWCNTs showed significantly higher GLY content in comparison to the remaining concentrations, while no significant differences were observed among exposure concentrations in individuals maintained under pH 7.6 (Figure 51 B). The same trend was also detected in individuals exposed to f-MWCNTs under pH 7.6, while significantly lower GLY content was observed in contaminated individuals compared to non-contaminated ones when maintained under pH control (Figure 51 B).

II) Significant differences between pH levels were observed in all conditions, showing in all cases significantly higher GLY content when organisms were exposed to low pH compared to control pH (Figure 46 B).

III) When comparing organisms exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 and 0.10 mg/L under pH 8.0, with higher GLY content in individuals contaminated with Nf-MWCNTs (Table 22).

I) Considering the effects of exposure concentrations, results of ETS activity in *D. neapolitana* showed that for both MWCNTs under pH control a significant dose-dependent increase of the activity was observed, with the higher value at 0.10 mg/L. Considering the organisms exposed to Nf-MWCNTs under low pH, they presented a significant increase of their ETS activity only at 0.10 mg/L Nf-MWCNTs in comparison to the remaining concentrations, while when exposed to f-MWCNTs, the metabolic activity significantly increased in all contaminated polychaetes compared to control ones (Figure 51 C).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed at 0.01 mg/L f-MWCNTs, with higher ETS activity in individuals maintained at pH 7.6 in comparison to organisms under pH 8.0 (Figure 46 C). Significant differences between pH levels were also detected in organisms exposed to 0.10 mg/L Nf-MWCNTs, showing lower activity under pH 7.6 (Figure 51 C).

III) When comparing specimens exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed at 0.01 mg/L under both pH levels as well as at 0.10 mg/L under pH 8.0, showing in all cases higher metabolic activity when the organisms were contaminated with f-MWCNTs compared to Nf-MWCNTs (Table 22).

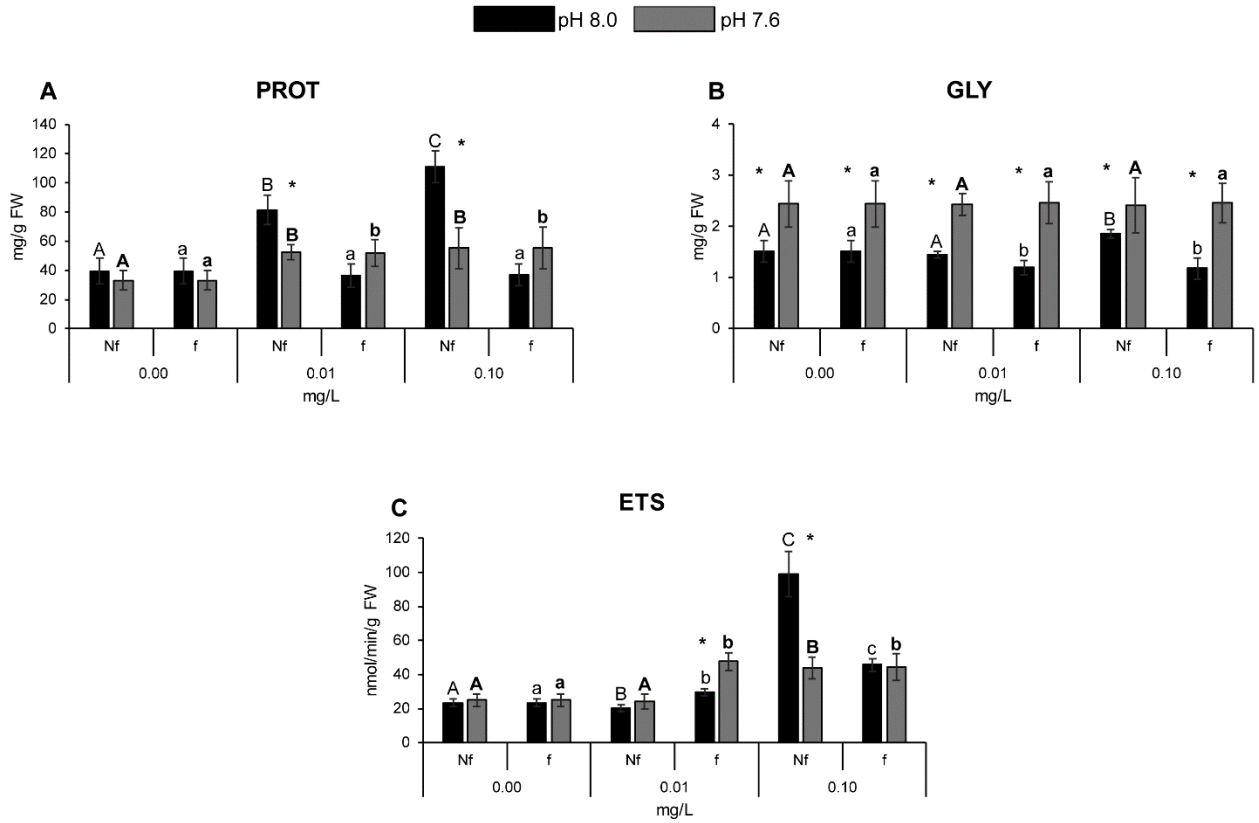

Figure 51. **A**: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** Electron transport system (ETS) activity (mean ± standard deviation), in *D.neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

Table 22. Effect on oxidative stress biomarkers (Protein (PROT) content; Glycogen (GLY) content; Electron transport system (ETS) activity; Lipid peroxidation (LPO) levels; GSH/GSSG; Superoxide dismutase (SOD) activity; Catalase (CAT) activity; Glutathione peroxidase (GPx) activity; Glutathione S-Transferases (GSTs) activity; Acetylcholinesterase (AChE) activity) in *D. neapolitana* by pristine MWCNTs (Nf) and MWCNTs-COOH (f) at each of the tested concentrations (control-0.00, 0.01, 0.10 mg/L) under control pH (8.0) and low pH (7.6). Significant differences ($p \leq 0.05$) between MWCNTs within each pH level at each exposure concentration were represented with asterisks (*).

CNT (mg/L)	pH		PROT	GLY	ETS	LPO	GSH/GSSG	SOD	CAT	GPx	GSTs	AChE
0.00	8.0	Nf	39.45±9.03	1.51±0.21	23.47±2.29	12.83±0.94	6.83±0.45	0.83±0.21	39.68±3.10	0.082±0.009	0.34±0.04	0.98±0.14
		f	39.45±9.03	1.51±0.21	23.47±2.29	12.83±0.94	6.83±0.45	0.83±0.21	39.68±3.10	0.082±0.009	0.34±0.04	0.98±0.14
	7.6	Nf	33.13±6.72	2.44±0.45	25.00±3.63	12.85±1.64	6.32±1.20	0.89±0.32	40.12±9.32	0.079±0.009	0.40±0.05	1.29±0.48
		f	33.13±6.72	2.44±0.45	25.99±3.63	12.85±1.64	6.32±1.20	0.89±0.32	40.12±9.32	0.079±0.009	0.40±0.05	1.29±0.48
0.01	8.0	Nf	81.33±10.09	1.45±0.07	20.29±1.83	29.59±2.88	2.16±0.27	1.09±0.11	38.63±5.15	0.098±0.010	0.86±0.04	1.28±0.71
		f	36.24±8.02*	1.19±0.14*	29.52±2.06*	19.11±2.97*	5.26±0.60*	2.86±0.85*	39.55±3.17	0.112±0.036	0.26±0.03*	0.79±0.16*
	7.6	Nf	52.43±5.33	2.43±0.21	24.20±4.30	30.21±4.32	4.21±0.54	0.99±0.16	39.19±3.21	0.080±0.015	0.48±0.08	1.34±0.21
		f	51.87±9.00	2.46±0.41	47.64±5.07*	13.91±5.43*	6.14±0.65*	2.99±0.99*	37.19±4.21*	0.120±0.019*	0.25±0.03*	1.27±0.20
0.10	8.0	Nf	110.86±10.91	1.85±0.08	98.88±13.99	30.27±2.75	2.10±0.20	4.36±1.64	38.90±5.76	0.099±0.020	0.77±0.05	0.94±0.24
		f	36.98±7.54*	1.18±0.21*	45.65±3.65*	20.32±4.32*	5.02±0.32*	5.65±1.09*	39.88±3.54*	0.112±0.030	0.27±0.01*	0.54±0.12*
	7.6	Nf	55.21±13.99	2.41±0.54	43.85±6.25	70.26±5.98	4.32±0.32	2.41±0.32	39.22±4.29	0.144±0.010	0.44±0.08	1.39±0.40
		f	55.56±14.20	2.46±0.39	44.28±7.80	34.02±7.54*	4.01±0.22	3.99±0.65*	37.43±5.87*	0.124±0.013	0.21±0.06*	0.58±0.28*

Oxidative status

I) Results of LPO levels in *D. neapolitana* showed that for Nf-MWCNTs under pH 8.0 significantly higher levels were observed in the exposed organisms compared to non-exposed ones. Under pH 7.6 were detected significant dose-dependent increase of the levels, with higher values under the highest Nf-MWCNTs concentration (Figure 52 A). When the polychaetes were contaminated with f-MWCNTs under pH control, the levels of LPO significantly increased in the exposed individuals compared to control ones, while under low pH, the levels increased only at 0.10 mg/L compared to the remaining concentrations (Figure 52 A).

II) For each MWCNT and exposure concentration differences between pH levels were observed at 0.10 mg/L under both MWCNTs, showing higher LPO levels when the organisms were submitted to pH 7.6 compared to pH 8.0 (Figure 52 A).

III) Significant differences between materials were observed in all contaminated polychaetes with both MWCNTs and under both pH levels, showing in all cases higher values in individuals contaminated with Nf-MWCNTs in comparison to organisms contaminated with f-MWCNTs (Table 22).

I) Results of GSH/GSSG showed that for Nf-MWCNTs under both pH levels significant differences were observed between contaminated and non-contaminated polychaetes, with lower values in the exposed individuals compared to control ones (Figure 52 B). Similar trend was also observed in the organisms submitted to f-MWCNTs under pH 8.0, while under pH 7.6, significantly lower values were detected only when *D. neapolitana* was exposed to 0.10 mg/L compared to the remaining concentrations (Figure 52 B).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed at 0.01 mg/L of Nf-MWCNTs, with lower GSH/GSSG in individuals maintained at pH control in comparison to organisms at low pH (Figure 52 B). Significant differences between pH levels were also detected at 0.10 mg/L of both MWCNTs, showing lower (Nf-MWCNTs) and higher (f-MWCNTs) values in organisms exposed to pH 8.0 compared to pH 7.6 (Figure 52 B).

III) When comparing organisms exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 mg/L under both pH levels as well as at 0.10 mg/L under pH 8.0, with lower GSH/GSSG in specimens contaminated with Nf-MWCNTs (Table 22).

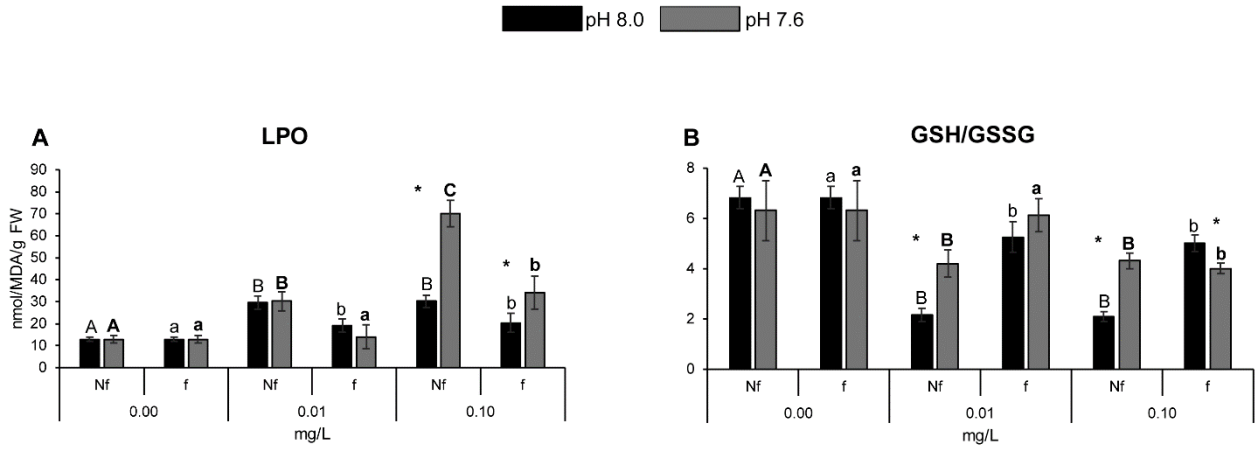

Figure 52. **A**: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

I) Polychaetes contaminated with Nf-MWCNTs at pH 8.0 did not show significant differences among concentrations in terms of SOD activity, while in organisms submitted to pH 7.6 the activity of the antioxidant enzyme significantly increase only at 0.10 mg/L compared to the remaining concentrations (Figure 53 A). When the organisms were contaminated with f-MWCNTs under pH 8.0, significant dose-dependent increase of SOD activity was observed, with the highest values at the higher exposure concentration, while the exposed specimens under low pH presented a significantly increase of activity compared to control individuals (Figure 53 A).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed at 0.10 mg/L both Nf and f-MWCNTs, with higher and lower SOD activity respectively under pH 7.6 compared to pH 8.0 (Figure 53 A).

III) When comparing individuals exposed to different MWCNTs at each pH and exposure concentration, significant differences between materials were observed in all contaminated polychaetes under both pH levels, showing in all cases higher values in individuals contaminated with f-MWCNTs in comparison to Nf-MWCNTs (Table 22).

I) Considering the effects of exposure concentrations, the results of CAT activity in *D. neapolitana* exposed to both MWCNTs under both pH levels showed no significant differences among concentrations (Figure 53 B).

II) For each MWCNT and exposure concentration, no significant differences were observed between pH levels (Figure 53 B).

III) When comparing individuals exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were detected at 0.01 mg/L at low pH with higher CAT activity when contaminated with Nf-MWCNTs compared to f-MWCNTs. Significant differences between materials were also detected at 0.10 mg/L under both pH levels, showing higher (pH 8.0) and lower (pH 7.6) enzyme activity when organisms were contaminated with f-MWCNTs in comparison to Nf-MWCNTs (Table 22).

I) Considering the effects of exposure concentrations, results of GPx activity in *D. neapolitana* showed that for Nf-MWCNTs under pH control, no significant differences were observed among concentrations, while at low pH, the activity of GPx significantly increased only at 0.10 mg/L compared to the other concentrations (Figure 53 C). Polychaetes contaminated with f-MWCNTs at both pH levels showed significantly higher GPx only in exposed individuals compared to control ones (Figure 53 C).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed in organisms exposed to 0.10 mg/L Nf-MWCNTs, with higher GPx activity under pH 7.6 compared to individuals under pH 8.0 (Figure 53 C).

III) When comparing specimens exposed to different MWCNTs at the same pH and exposure concentration, significant differences between materials were observed at 0.01 mg/L at pH 7.6 showing higher antioxidant activity in polychaetes exposed to f-MWCNTs compared to Nf-MWCNTs (Table 22).

I) When polychaetes were contaminated with Nf-MWCNTs under pH control, the GSTs activity significantly increased compared to control condition, while at low pH no significant differences were observed among concentrations (Figure 53 D). Considering *D. neapolitana* exposed to f-MWCNTs at both pH levels, the activity of the biotransformation enzymes significantly decreased in exposed polychaetes compared to non exposed ones (Figure 53 D).

II) For each MWCNT and exposure concentration, significant differences between pH levels were observed in organisms contaminated with 0.01 and 0.10 mg/L Nf-MWCNTs, showing higher GSTs activity under pH 8.0 compared to individuals under pH 7.6 (Figure 53 D).

III) When comparing individuals exposed to different MWCNTs at each pH and concentration, significant differences between materials were observed in all contaminated polychaetes under both pH levels, showing in all cases higher values in individuals contaminated with Nf-MWCNTs in comparison to f-MWCNTs (Table 22).

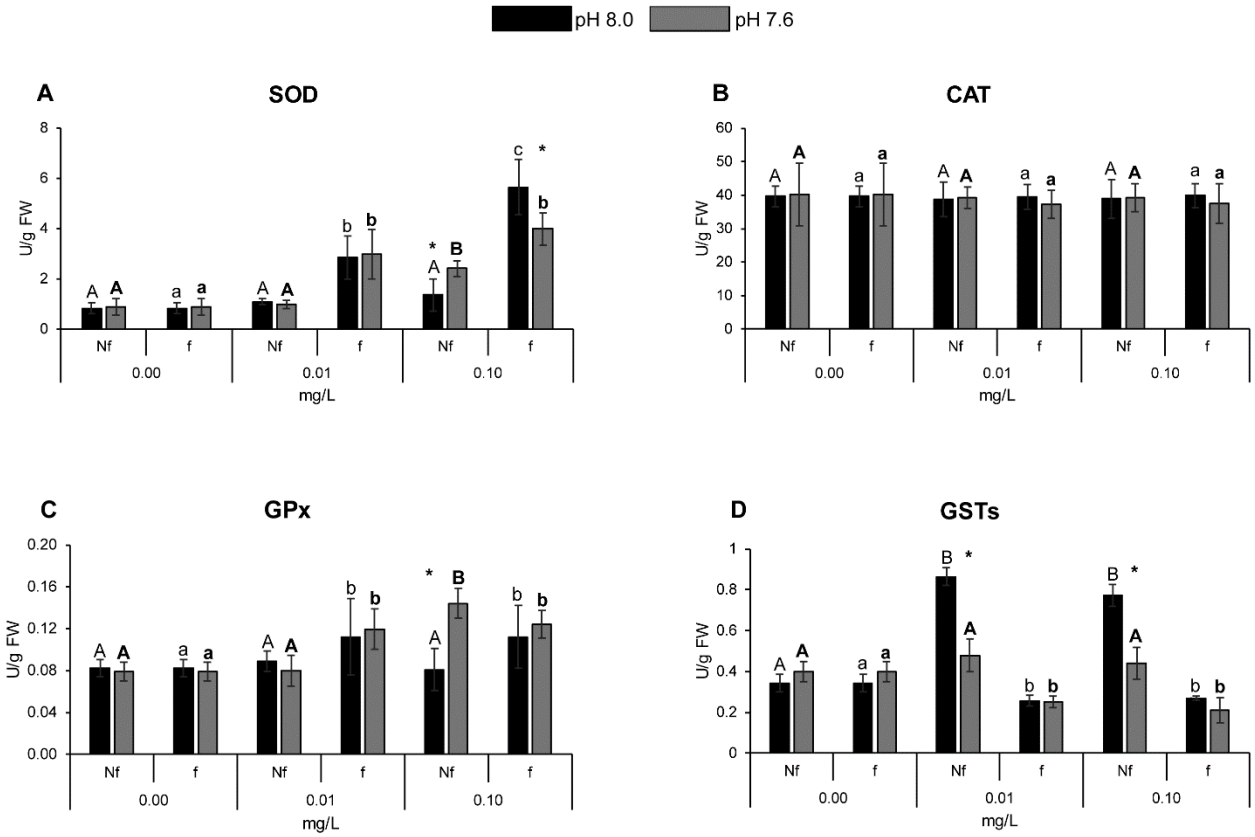

Figure 53. **A:** Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

Neuro status

I) Considering the effects of exposure concentrations, results of AChE activity showed significantly higher values only in polychaetes exposed to 0.01 mg/L Nf-MWCNTs under pH 8.0 compared to the other conditions, while under pH 7.6, no significant differences were observed among concentrations (Figure 54). Regarding the individuals contaminated with f-MWCNTs under both pH levels, significantly lower AChE activity was observed only in organisms exposed to 0.10 mg/L compared to the remaining concentrations (Figure 54).

II) For each MWCNT at each exposure concentration, differences between pH levels were observed between organisms exposed to 0.01 mg/L f-MWCNTs, with significantly lower enzyme activity in organisms maintained under pH control compared to pH 7.6 (Figure 54).

III) When comparing organisms exposed to the same pH and exposure concentration, significant differences between materials were observed in polychaetes exposed to 0.01 mg/L under pH 8.0 and and 0.10 mg/L at both pH levels, with lower activity in *D. neapolitana* contaminated with f-MWCNTs compared to Nf-MWCNTs (Table 22).

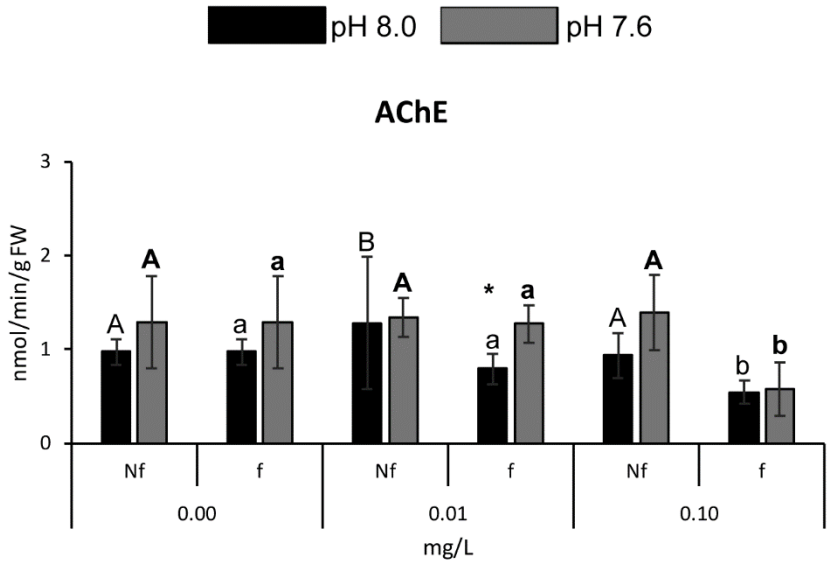

Figure 54. Acetylcholinesterase (AChE) activity (mean ± standard deviation), in *D. neapolitana* exposed to different MWCNTs (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01 and 0.10 mg/L) under different pH levels (control pH 8.0 and low pH 7.6). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and pH level were represented with different letters: uppercase and regular letters for Nf-MWCNTs at pH 8.0; uppercase and bold letters for Nf-MWCNTs at pH 7.6; lowercase and regular letters for f-MWCNTs at pH 8.0; lowercase and bold letters for f-MWCNTs at pH 7.6. Significant differences ($p \leq 0.05$) between the pH levels for each MWCNT and exposure concentration were represented with asterisks (*).

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3.3.2. Discussion

3.3.2.1. Characterization analyses

Looking the characterization results of the three invertebrate species, DLS and PDI analyses showed the presence of micro-sized aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples for both CNT materials under both pH levels. In detail, the results related to *R. philippinarum* showed similar dimension of Nf-MWCNTs among exposure concentrations between pH 8.0 and 7.6, while smaller aggregates were detected for f-MWCNTs under acidify pH compared to control ones. Considering the results of *H. diversicalor* and *D. neapolitana*, both MWCNTs had smaller aggregates under pH 7.6 compared to those detected under pH 8.0.

The stability of NPs in aquatic environment is closely related to different environmental factors (as already demonstrated in a previous section) including the pH level. A recent study by Xia et al. (2018) reported that the decrease of the pH can facilitate the dissolution of metal NPs in aquatic medium increasing their bioavailability in the water media. Considering carbon NPs, a study conducted by Nepal and Geckeler (2006), showed that preparing CNTs in an aqueous solution using a combination of ultrasonication and the product was a pH-sensitive dispersion, which remained in a highly dispersed state at pH<8 and pH>11 while in an aggregated state between pH 8 and 11. The rsults presented here are in line with the present finding, showing through DLS analysis less aggregate state of MWCNTs especially under pH 7.6 compared to pH 8.0. hypothesizing that Ocean Acidification could alleviate aggregation and agglomeration of the used materials in comparison to normal pH. Looking the biological responses, it was observed higher toxic impacts under pH 7.6 compared to control pH 8.0, suggesting that mechanism of enhanced toxicity in the exposed organisms should be attributed to slighter aggregation and more suspended MWCNTs in acidify seawater, which may increase the uptake and bioaccumulation into the organisms generating some synergistic and more toxic interactive effects of pH and NPs. Abiotic factors such as pH may influence the bioavailability of water dispersible NPs compared to pristine ones. Although studies in the literature reported that larger aggregates can generate more toxic effects of NPs to invertebrates in comparison to those freely suspended (Ward and Kach, 2009; Hotze et al., 2010), it has been also demonstrated that the increased particle size (aggregation) determines a decrease of the total surface area causing a decrease of the superficial reactivity of NPs (because of agglomeration) which, in turn, produces a reduction of the toxic effects (Rotini et al., 2017). This theory could justify the results obtained in the present studies.

Considering the aggregation behaviour between pristine and functionalized MWCNTs, the results of the present studies showed that Nf-MWCNTs generated larger aggregates compared to f-

MWCNTs under both pH levels confirming that the carboxylated forms of CNTs are more stable in seawater in comparison to pristine CNTs as a consequence of their oxidation process which introduces oxygen-containing groups on the CNTs surface. Furthermore, higher toxic impacts were caused by f-MWCNTs compared to Nf-MWCNTs due to the presence of higher amorphous carbon fragments in comparison to pristine MWCNTs, inducing higher levels of toxicity to biological systems (Arndt et al., 2013).

3.3.2.2. Biological analyses

In the present studies, physiological (regenerative capacity) and biochemical responses (energy reserve contents and metabolic capacity, oxidative and neuro status) of two concentrations (0.10 and 1.00 mg/L) of Nf-MWCNTs and f-MWCNTs in the three invertebrate species maintained at two different pH levels (control pH-8.0 and acidify pH-7.6) were investigated. For all the studies the three main hypotheses assessed were: **I)** both MWCNT materials generated toxic impact on the organisms after 28 days of exposure under different pH levels; **II)** different pH levels may alter the sensitivity of the individuals to the CNTs; **III)** the alteration induced by different pH levels on the chemical behaviour of both materials changed the toxicity of the MWCNTs and consequent fate in exposed organisms.

3.3.2.2.1. Impacts of MWCNT concentrations under different pH levels

Ruditapes philippinarum (Adams & Reeve, 1850)

I) Regarding energy reserves and metabolic capacity of the clams, the present study showed that when exposed to Nf-MWCNTs, *R. philippinarum* increased their metabolism, which resulted into the expenditure of their reserves. This response was similar under control and low pH conditions, indicating that the impacts induced by Nf-MWCNTs were not altered by acidified conditions. Similar results were also detected when the organisms were exposed to f-MWCNTs under pH control, showing an increase of the metabolism especially at 0.10 mg/L f-MWCNTs, assuming that sublethal stress had generated compensatory changes in the organism's energy metabolism leading to increased energy expenditure during basal metabolism to cope with stress induced by these NPs. In fact, also in this case as for the Nf-MWCNTs under both pH levels, a significant reduction of energy reserves was observed in *R. philippinarum* maintained under this condition. Similar results were observed in the previous section when *R. philippinarum* was exposed to both MWCNTs under different salinity levels, which confirmed that the toxic action of the materials in the exposed clams, suggesting a successful uptake of the NPs. However, when the organisms were submitted to pH 7.6 and f-MWCNTs an opposite behaviour was detected, observing a slightly decreased or maintained

of the ETS probably as a mechanism of defence to prevent accumulation of the NPs that were more available especially under the highest exposure concentration, and significant increase of the energy reserves. These different responses may be attributed to the acidified condition that could negatively affected the efficiency of organism's metabolism or most probably, the acidified pH may modify the availability of the f-MWCNTs, changing as a consequence the sensibility of the organisms to this contaminant.

Looking the results of the oxidative status, a dose-dependent increase of LPO levels in clams exposed to Nf-MWCNTs and both pH levels was detected. This behaviour could be traced to an inhibition of the antioxidant defences as a consequence of the persistent cellular damage, as demonstrated by an activation only of GPx activity but an inhibition of all the other defences such as SOD and CAT activities as well as by GSH/GSSG, which did not increase in the exposed individuals under these conditions, promoting a shift in the balance between oxidants and antioxidants in favour of oxidants, resulting in the enhancement of pollutants-induced oxidative effects (Chatziargyriou and Dailianis, 2010). The present findings further demonstrated that pH decrease did not change clams' responses towards Nf-MWCNTs. Similar results were reported by Canesi et al. (2010) using different NPs such as nano carbon black (NCB), nano fullerene (C60), silicon dioxide NPs (SiO_2) and titanium dioxide NPs ($nTiO_2$) at the concentrations of 0.05, 0.2, 1 and 5 mg/L for 24 h, showed that the activity of the antioxidant enzymes in *M. galloprovincialis* was inhibited at highest concentrations of NCB and $nSiO_2$. Similar results were also observed in the previous section when *R. philippinarum* was exposed to Nf-MWCNTs under both salinity levels (28 and 21), confirming again the sensibility of this species to the Nf-MWCNT contaminants independently of the different extreme weather events.

Considering bivalves exposed to f-MWCNTs under pH control, as for *R. philippinarum* under different salinities, the antioxidant system significantly increased their activities especially at the highest exposure concentration, indicating that the clams may try to eliminate the excess ROS produced, which was more noticeable at the highest exposure concentration. However, the activation of the antioxidant systems led to a compensatory response of cellular defence systems against cellular damage, and LPO did not occur under this condition. The present results further revealed that under pH acidified the antioxidant defences decreased significantly their activities, suggesting that under this condition the excessive ROS production may lead to oxidative damage and a loss of compensatory mechanisms as a consequence of insufficient mechanism of the antioxidant activity and this contribute to higher LPO levels recorded under low pH. An increase of antioxidant enzymes activities such as SOD and GPx was also observed by Buffet et al. (2011) which using copper NPs (CuNPs), showed that *S. plana* specimens were able to activate the antioxidant enzymes SOD and CAT to cope against cellular damage. Again, the possible explanation of these results is that under pH 7.6 the behaviour of f-MWCNTs was modified by the low pH, making them more available into the water media in comparison to control pH, facilitating the uptake by the organisms and,

consequently, more toxicity (Xia et al., 2018). In agreement with the present results, Huang et al. (2016) investigated the combined effects of low pH and nanoscale titanium dioxide (nano-TiO_2) in the mussel *Mytilus coruscus* showing that ROS increased with nano-TiO_2 concentrations under low pH conditions. Looking the biotransformation enzymes, the present findings demonstrated that the activity of GSTs did not change with the increasing exposure concentration of Nf-MWCNTs at both pH levels, indicating that this group of enzymes may be not involved in the biotransformation of Nf-MWCNTs into a less toxic and easily excreted substance. Considering the clams exposed to f-MWCNTs, opposite activity was observed between organisms maintained under pH control (dose-dependent decrease) and low pH (no differences between exposed and non-exposed individuals) confirming that the pH 7.6 modified the availability and the structure of the f-MWCNTs. The confirmation that the activity of this biotransformation enzymes could be modified depending on the type of materials was also demonstrated by Ciacci et al. (2012) that showed a stimulation of biotransformation enzymes activities when mussels *M. galloprovincialis* were exposed to different nano-oxides ($nSiO_2$, nZnO and nano-Cerium dioxide ($nCeO_2$)) while Barmo et al. (2013) exposing the same species to $nTiO_2$ suspensions demonstrating that $nTiO_2$ induced a decrease in GSTs activity.

Considering the neuro status of the clams, the present results showed a significant neuro state inhibition in organisms exposed to both CNTs under both pH levels assuming that the perturbation of the structure influencing the function of enzyme subunits may be the common mode of ChE inhibition by NPs, independently of the pH. In a study conducted by Šinko et al. (2014), where the authors aimed to investigate the effects of Ag NPs on AChE, the authors suggested that the inhibition effect of the NPs may be attributed to the larger surface area as a consequence of their smaller size and this hypothesis was also confirmed by Wang et al. (2009) which proposed that inhibition by NPs is primarily caused by adsorption or interaction with AChE protein. Overall, taking together evidence from the literature and our present findings, perturbation of the structure influencing the function of enzyme subunits may be the common mode of ChE inhibition by carbon NPs.

Hediste diversicolor (O.F. Müller, 1776)

I) Looking to polychaetes energy reserves and metabolism, an opposite behaviour was observed for the individuals exposed to low pH and control pH. Under pH 7.6 *H. diversicolor* exposed to both CNTs presented in general a dose-dependent decrease of ETS activity and an increase of GLY or PROT contents. These results may be related to an impairment of the function of ETS activity as a consequence of an over production of ROS which caused in turn LPO (Bielen et al., 2016). Considering *H. diversicolor* exposed to pH control and f-MWCNTs, as already demonstrated in a previous section, a slightly increase of the metabolic capacity and decrease of energy reserves

(especially PROT content) was observed under the highest concentration of f-MWCNTs, may be due to the activation of defence mechanisms, as observed under this exposure condition. Organisms submitted to Nf-MWCNTs under pH control, showed no differences among concentrations in terms metabolism as well as energy reserves, which may indicate that under this condition the used concentrations were not high enough to result in metabolic depression. These results showed how the variation of abiotic factors (such as pH as well as salinity levels) can modify the behaviour of the NP, especially the f-MWCNTs, as well the sensibility of this species to the different contaminates.

Looking the oxidative status, in the present study, in the organisms exposed to Nf-MWCNTs under pH control the LPO did not increase along with the increasing exposure concentrations while under acidify pH the level was higher in the contaminated organisms in comparison to non-contaminated ones, clearly indicating higher impact of pH in polychaetes in comparison to the impacts caused by CNTs (probably due to low concentrations tested, low solubility and consequently low toxicity of non-functionalized MWCNTs or no effect of pH on CNPs toxicity). Different results were observed when the organisms were exposed to f-MWCNTs, observing the damage of the lipid membranes under both pH conditions and assuming that all these different responses were directly related to the availability of the CNT materials as well as to the chemistry of the water media where the NPs were dispersed since higher damage was observed under low pH. These results indicated again higher toxicity of the CNTs at acidified conditions or, at the same time, higher sensitivity of polychaetes to CNTs under this condition. Considering the defence system against oxidative damage, in the present study, similar antioxidant defences and biotransformation activities under both CNTs were detected, however, major activation of defence activities were detected under acidified pH, especially at the highest exposure concentration of both materials. These results are in agreement with LPO levels described previously, suggesting an attempt by these enzymes to cope as compensatory response of cellular defence systems against cellular damage. Nevertheless, the excessive ROS production, especially under the highest exposure concentration, may lead to oxidative damage and a loss of compensatory mechanisms which may contribute to higher LPO levels recorded under this condition. In agreement with the present results, also Huang et al. (2018) showed higher activities of antioxidant enzymes such as SOD, CAT and GPx in gills and hemocytes of the mussels *M. coruscus* when the organisms were subject to low pH and high concentration of nanoparticulate zinc oxide (nano-ZnO) suggesting a major oxidative stress responses under the combination of the two stressors comparing the toxic effects caused by the NPs acting alone.

Regarding the neuro status, when organisms were exposed to Nf-MWCNTs under pH control the activity was inhibited especially under 0.01 mg/L while when polychaetes were submitted to acidify pH no neuro-inhibition was observed. Different behaviour was observed regarding f-MWCNTs, where under both pH levels, the activity of the neurotransmitter was lower only at the

highest exposure concentration. Looking on DLS analysis, the mean size of the f-MWCNTs was always lower in comparison to Nf-MWCNTs under both pH levels respectively, which could justify the higher availability of the carboxylated form of MWCNTs also at the highest concentration for the organisms, intensifying the risk of exposure and possible absorption of the NPs.

Diopatra neapolitana (Delle Chiaje, 1841)

I) The results of the present study demonstrated that both MWCNTs under both pH levels generated negative effect on the regenerative capacity of *D. neapolitana*, especially at the highest exposure concentration, showing a lower percentage of body width as well as the number of new chaetigers compared to the other conditions after 18^{th} and 28^{th} days of exposure. Moreover, the polychaetes increased their metabolism, and this response was similar under control and low pH conditions, indicating that the impacts induced by both MWCNTs were not altered by acidified conditions and assuming that sublethal stress caused by both CNT materials had generated compensatory changes in the organism's energy metabolism. Moreover, the present findings further revealed that although polychaetes metabolic capacity was enhanced in all contaminated organisms and under both pH levels, they were able to increase (PROT) or at least maintain (GLY) their energy reserves. Similar results were also observed when *D. neapolitana* was exposed to the same contaminants acting alone and under different salinity levels, indicating that this species increased their energy reserves when exposed to CNTs regardless the form of the materials (carboxylated or pristine) as well as their state of aggregation. This results could be justify by the successful CNT uptake and bioaccumulation in the body of the exposed organisms, which in turn have caused the generation of stresfull condition and the prevention of energy expenditure by the organisms (e.g. limiting their use for polychaetes regeneration).

Looking to the oxidative status results, similarly to *H. diversicolor* (previously described) exposed to the same conditions, *D. neapolitana* also presented higher LPO under the combined exposure (low pH + both CNTs). However, contrary to the results obtained for *H. diversicolor*, in the present study the increase of LPO was also observed when the polychaetes were maintained at control pH and contaminated with both CNTs, an indication of higher cellular injury induced by the presence of both CNT materials regardless the different pH levels. Moreover, analyzing the antioxidant systems, alterations of antioxidant enzymes (SOD and GPx) as well as non-enzymatic antioxidants (GSH/GSSG) were observed. Specifically, significant higher SOD and GPx activity was observed in polychaetes exposed to low pH and both CNTs and control pH and f-MWCNTs, indicating that these organisms were subjected to higher oxidative stress which induced antioxidant enzymes to mitigate enhanced ROS formation. However, the possible excessive ROS production, especially under the highest exposure concentration, may lead to oxidative damage and a loss of

compensatory mechanisms which may contribute to higher LPO levels recorded under these conditions. Interestingly, different behaviour was observed in *D. neapolitana* when exposed to Nf-MWCNTs at pH control, where SOD and GPx activites were similar to values observed in control, evidencing a differentiated antioxidant response in this condition. Several factors could explain these results such as enzyme activity impairment, antioxidant response dynamics or lower rates of ROS production in this condition. However, considering the high impairment of the membrane recorded under this condition, most likely the excessive ROS production may lead to oxidative damage and a loss of compensatory mechanisms of the antioxidant activities. Another important result was that observed concerning GSTs activity. GSTs are important enzymes involved in cellular detoxification, and the results showing that while under Nf-MWCNTs the GSTs activity was maintained similar to control condition, lower GSTs activity in *D. neapolitana* exposed to f-MWCNTs under both pH levels was detected, suggesting that the carboxylated MWCNTs generated a supression biotransformation capacity in these organisms regardless the different pH levels.

For what concern the neuro status, the inibhition of AChE activity was observed when polychaetes were exposed to f-MWCNTs under both pH levels, where the dimension of the aggragates were smaller compared to those detected for the pristine form of MWCNTs both at control and low pH. These findings confirmed the results presented by Šinko et al. (2014), that suggested that the inhibition effect of the NPs may be attributed to smaller size of the NPs which caused the adsorption or interaction with AChE protein.

3.3.2.2.2. Impacts of pH variations on the sensitivity of the organisms to MWCNTs

Ruditapes philippinarum (Adams & Reeve, 1850)

II) In the present study, it seems that the pH variations did not alter directly the the sensitivity of the clams but most likely that the acidified pH may modify the availability of the f-MWCNTs. In detail, both in terms of metabolic activity as well as oxidative stress the responses of the clams to Nf-MWCNTs when exposed to both pH levels were similar and changed only when exposed to f-MWCNTs, showing higher toxic action caused by these NPs under acidify condition. The fact that these variations were observed in organisms exposed to f-MWCNTs under acidify pH and not in individuals exposed to Nf-MWCNTs, could suppose most likely that the low pH may modify the availability of the f-MWCNTs, changing as a consequence the sensibility of the organisms to this contaminant.

Hediste diversicolor (O.F. Müller, 1776)

II) In the present study was it also confirmed that pH variations may alter the sensitivity of the individuals to the CNTs. Looking the results of the energy reserves and metabolic activity under acidify condition, no differences were observed between CNT materials, suggesting a possible higher impact of pH variations in polychaetes in comparison to the impacts caused by CNTs, highlighting a possible increase of the sensitivity to the contaminants caused by the low pH. It has been demonstrated that the low pH negatively affected the efficiency of organism's metabolism (Sun et al., 2017). When pCO_2 levels increase in seawater, dissolved CO_2 more readily diffuses across animal surfaces, crossing biological membranes and entering the intracellular spaces causing obvious impact on the physiological condition and functionality of the organisms (Fabry et al., 2008) and the suppression of metabolism is considered a "sublethal" reversible process, with the reductions of the energy reserves expressed as growth and reproductive output which effectively diminish the survival of the species on longer time-scales (Fabry et al., 2008). This response could explain why in the presence of acidified condition the organisms showed a decrease of metabolic rate preventing the consumption of energy reserves. Moreover, in agreement with the present results, Sun et al. (2017), exposing the mussels *M. edulis* to pH levels mimicking near future OA (pH 7.7), showed that low pH negatively affected the efficiency of the mitochondrial electron transport system (ETS) by increasing the electron slip in the ROS-generating mitochondrial complexes I and III and/or by partially inhibiting the flow through the downstream ETS complexes.

Diopatra neapolitana (Delle Chiaje, 1841)

II) From the results presented above, it seems that the low pH did not alter directly the the sensitivity of the polychaetes to the contaminants. In fact, similar biomarkers trend in terms of energy reserves and metabolic activity as well as oxidative status were observed between control and acidify conditions. However, the different behavior observed in the antioxidant systems and the neuroactivity could be traced back to the different availability of the materials. In detail, the fact that these variations were observed in organisms exposed to f-MWCNTs under acidify pH and not in individuals exposed to Nf-MWCNTs, could suppose most likely that the low pH may modify the availability of the f-MWCNTs, changing as a consequence the sensibility of the organisms to this contaminant.

3.3.2.2.3. Impacts of pH variations on the toxicity of MWCNTs

Ruditapes philippinarum (Adams & Reeve, 1850)

III) Looking to the present results, it seems that acidify pH modified the physical-chemical structure of f-MWCNTs which in turn caused higher toxic effects in exposed clams both in terms of energy reserves and metabolic capacity as well as oxidative stress compared to the Nf-MWCNTs. These results may suggest a higher bioavailability in the water media and a possible major uptake of the carboxylated form of MWCNTs compared to the pristine one. A recent study by Xia et al. (2018) showed that decreased pH can facilitate the dissolution of metal NPs in aquatic medium increasing their bioavailability in the water media. Considering carbon NPs, a study conducted by Nepal and Geckeler (2006), showed that preparing CNTs in an aqueous solution using a combination of ultrasonication, the product was a pH-sensitive dispersion, which remained in a highly dispersed state at pH<8 and pH>11 while in an aggregated state at pH 8–11. Our results are in line with the present finding, showing through DLS analysis less aggregate state of f-MWCNTs especially under pH 7.6 compared to pH 8.0. Therefore, we hypothesized that OA could alleviate aggregation and agglomeration of the used f-MWCNTs in comparison to normal pH, which may increase the uptake and bioaccumulation into the organisms generating some synergistic and more toxic interactive effects of pH and NPs.

Hediste diversicolor (O.F. Müller, 1776)

III) Looking the results obtained under pH control, differences in terms of energy reserves and metabolic activity were detected between materials, suggesting that under this condition the observed behaviour could be attributed to the surface functionalization of the CNTs. Due to the presence of higher amorphous carbon fragments in comparison to pristine MWCNTs, carboxylated MWCNTs induced higher levels of toxicity to biological systems (Arndt et al., 2013) causing higher cellular damage with the activation of antioxidant mechanisms (Freixa et al., 2018). The confirmation that the two different pH levels may alter the chemical behaviour of both materials and consequent fate in exposed polychaetes, was also observed in terms of oxidative and neuro status of the exposed individuals. In fact, higher cellular damage and neurotoxicity were observed in individuals exposed to f-MWCNTs compared to Nf-MWCNTs. While Nf-MWCNTs, due to their insolubility, as also demonstrated by DLS analysis, could be less available for the organisms, f-MWCNTs were more dispersible in the water column probably increasing their mobility and thus may intensify the risk of exposure and toxicity and possible uptake (Jackson et al., 2013). Moreover, higher damage was observed under low pH from both materials, indicating higher toxicity of CNPs at acidified conditions.

Diopatra neapolitana (Delle Chiaje, 1841)

III) As already detected in the other two invertebrate species exposed to the same conditions, it seems that the differences observed in terms of biochemical responses also in *D. neapolitana* could be attributed to the surface functionalization of the CNTs. Different studies already demonstrated that the toxicity of the MWCNTs depends on their dispersion state, showing higher cytotoxic and oxidative effects when organisms were exposed to the more soluble form of the NPs (Karlsson et al., 2008). Such findings could explain why f-MWCNTs, which are more dispersed in water media at both pH levels compared to the Nf-MWCNTs under the same conditions, generated higher toxic effects in comparison the pristine form.

3.3.3. Final considerations

In the presented studies, physiological and biochemical responses in the three invertebrate species *(R. philippinarum, H. diversicolor* and *D. neapolitana)* exposed of two concentrations of Nf-MWCNTs and f-MWCNTs maintained at two different pH levels were investigated. For all the studies the three main hypotheses assessed were: **I)** both MWCNT materials generated toxic impact on the organisms after 28 days of exposure under different pH levels; **II)** different pH may alter the sensitivity of the individuals exposed to the CNTs; **III)** the alteration induced by different pH levels on the chemical behaviour of both materials changed the toxicity of the MWCNTs and consequent fate in exposed organisms.

I) The present results demonstrated alterations in terms of physiological and biochemical responses in all three species caused by both MWCNTs, especially at the highest exposure concentration, concluding that both materials were able to generate toxic action effects in all exposed organisms proving that the three species were sensitive to both compounds. In general, in all invertebrate species a dose-dependent increase of the toxicity caused by both CNT materials under both pH levels was detected. As for the salinity alteration, these results were more evident in terms of cellular damage and antioxidant defence systems, confirming that those responses are the common effects caused by these materials in the exposed organisms (Rocha et al., 2015). These results can be justified by the successful CNT uptake, translocation and retention in the exposed organism. It has been already demonstrated that CNTs are ingested by invertebrate organisms (Jackson et al., 2013) and when into the organisms hydrophobic NPs (such as CNTs) agglomerate readily and interact with other hydrophobic residues of proteins or peptides thus promoting internalization (Kettiger et al., 2013). Moreover, because CNTs may exist in the size range of proteins (e.g. the hydrodynamic radius is close to 5 nm) they are able to interact with the cellular machinery in a similar way to macromolecules (Kettiger et al., 2013). Overall, these findings could justify the biochemical responses obtained in the presented studies.

II) The findings presented here in general demonstrated that Nf-MWCNTs and f-MWCNTs under low pH generated greater toxic impacts in the organisms compared to individuals maintained under pH control. However, the results also showed a species-dependent sensitivity to contaminants under the two pH tested. The results of *R. philippinarum* and *D. neapolitana* demonstrated clearly alterations of organisms' oxidative status but also changes in organisms' metabolism and neurotoxicity induction when exposed to both CNT materials. However, while under low pH conditions the toxicity of Nf-MWCNTs was similar to the impacts measured under actual pH, which may indicate that predicted seawater acidification scenarios may not change the toxicity of these NPs, major toxicity was caused by f-MWCNTs under low pH, suggesting that mechanism of

enhanced toxicity in the exposed organisms should be attributed to slighter aggregation and more suspended f-MWCNTs in acidified seawater and concluding that abiotic factors such as pH may influence more the bioavailability of water dispersible NPs compared to pristine ones. In what regards to *H. diversicolor*, in the presence of both MWCNTs the organisms prevented the consumption of energy reserves under acidified conditions, while when polychaetes were exposed to pH control, the used concentrations were not high enough to result in metabolic depression. Looking at oxidative stress status, in organisms exposed to pristine MWCNTs under pH control, the lipid peroxidation did not increase along with the increasing exposure concentrations while under acidified pH the level was higher in the contaminated organisms in comparison to non-contaminated ones, indicating higher impact of pH in polychaetes in comparison to the impacts caused by the CNTs despite the activation of antioxidant enzymes. The present results further demonstrated the neurotoxicity caused by both NPs, especially noticeable at acidified conditions.

III) Comparing the toxic effects of both CNTs, in all invertebrate species major cellular damage was induced by carboxylated forms of MWCNTs in comparison to the pristine one. These results supported the theory that while raw CNTs do not readily cross biological barriers, water dispersible MWCNTs due to the presence of higher amorphous carbon fragments in comparison to pristine MWCNTs, induced higher levels of toxicity to biological systems (Arndt et al., 2013).

Overall, we observed that the mechanism of enhanced toxicity in the exposed organisms should be attributed to slighter aggregation and more suspended CNTs in acidified seawater. Therefore, ocean acidification may cause a higher risk of CNTs to marine ecosystems. Based on the results here presented, it is possible to confirm again that nanomaterials toxicity was not only attributed to the core structure and surface functionalization, but also to the physico-chemical parameters of the media which alter the behaviour of the CNTs and consequently the toxicity in the exposed organisms.

CHAPTER 4. CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Concluding remarks and future perspective

The first step in assessing a potential toxic action of a contaminant is to understand both the properties of a nanomaterial and its physical-chemical nature. The CNTs used in the present studies were the pristine form of multi-walled CNTs (MWCNTs) (Nf-MWCNTs) and the carboxylated form of MWCNTs (COOH-MWCNTs) (f-MWCNTs). The selection of the CNTs were based considering their different physical and chemical properties, industrial applicability as well as their safe testing procedures and risk assessment. Despite the existing literature on CNTs toxicity, environmental safety guidelines concerning exposure scenarios of CNTs to aquatic biota are still undefined, as well as their administration, concentrations and exposure time (Khosravi-katuli et al., 2017). As such, there are no specific standardized protocols or certified reference materials for CNTs eco-testing probably due to the low sensitivity of the detection methods for the low environmental concentrations, or due to the many experimental challenges and issues faced when assessing the toxicity of nanomaterials. Most of the methods used for toxicity assessment have been designed without considering all the different properties of these NPs. CNTs display several unique physico-chemical properties that can interfere or pose challenges to the use of classical toxicity assays, and therefore an incomplete CNTs characterization will interfere in the interpretation of any correlation between biological effects and particle properties. For this, in the presented thesis, both pristine and carboxylated MWCNTs were characterized during the experimental period at different times of exposure using dynamic light scattering (DLS) for the liquid matrix and thermogravimetric analysis (TGA) for the solid matrix. DLS, which is an established laser-based non-microscopic and non-imaging technique (Dzakpasu and Axelrod, 2004), is one of the common method used to determine particle size in colloidal suspensions (De Marchi et al., 2018d) and generally it is commonly used and referred to as photon correlation spectroscopy (PCS), which is a particle suspended in a liquid solvent that undergoes a random Brownian motion (Hoo et al., 2008). Regarding TGA, it has been used for the first time in the thesis as an innovative method to assess the presence of pristine MWCNTs aggregates in the sediments exposed to CNTs dispersions. To the best of our knowledge, no reports describing the use of TGA for such purpose are reported in the literature. Nevertheless the results obtained represent an effective method to detect the presence of CNTs. In this thesis, the results of the characterization analyses revealed that the mean recorded hydrodynamic diameter of carboxylated MWCNT aggregates was smaller than those calculated for pristine MWCNT aggregates under the same experimental conditions indicating higher dispersion of the functionalized form of CNTs in salt aqueous media.

Considering the NP concentrations, many studies are done at high levels of exposure concentrations and currently still little is known about the subtle physiological and biochemical responses of organisms at lower dose exposure, or the mechanism by which CNTs produce an effect at these lower concentrations. Even though it can logically predict potential sources of NPs pollution,

there appear to be no measurements of concentrations of manufactured NPs in field-collected samples and no routine environmental monitoring programs for manufactured NPs are reported yet (Handy et al., 2008). Nonetheless, simple models based on estimated product usage and NP concentrations in the products are used as a starting point to predict worse case scenarios. In order to make the studies the most environmentally relevant, the concentrations tested in the presented thesis have reflected the expected future releases in the environment, following the predicted exposure concentrations (PECs) (0.001-1000 µg/L) reported in the literature for the CNTs in aquatic systems (Zhang et al., 2017).

In the environment, organisms are not only exposed to contaminants but to a wide range of different abiotic variables that can change by anthropogenic intervention (Beketov and Liess, 2012; Mearns et al., 2017). For these reasons, the influence of climate change-related factors, namely salinity and pH alterations, on the physical-chemical structure of both CNTs was investigated in the present thesis. Although a research community is already able to describe some of the fundamental physical-chemical behaviour of colloids and other particles, recognising that generally the bioavailability and the ecotoxicology of chemicals (and particles) is altered by abiotic factors such as pH, salinity, water hardness, temperature, dissolved organic matter in the water etc. However, this is an area where research is particularly lacking for CNTs. Looking the results of both CNTs aggregation state under two different salinity levels, it was observed that the particle diameter and sedimentation rate increased at increasing salt concentrations (higher at salinity 28 compared to salinity 21), due to the effect of ionic strength, confirming that at high salinity agglomeration was promoted by the presence of salt ions, which shield the NP charge reducing the repulsive effect among NPs (Rotini et al., 2017). Referring to pH, a recent study by Xia et al. (2018) reported that the decrease of the pH can facilitate the dissolution of NPs in aquatic medium increasing their bioavailability in the water media. The results presented here were in line with the present findings, showing through DLS analysis less aggregate state of both MWCNTs under pH 7.6 compared to pH 8.0, hypothesizing that Ocean Acidification could alleviate aggregation and agglomeration of the used materials in comparison to normal pH.

The second step to evaluate a toxic action of the contaminants represents the interpretation of biomarker responses which remains the question of species selection. In the literature it is rare to see a comparison of different biomarker responses in different organisms. Most biomarkers have been validated in only one species. This is a disadvantage to their widespread application for monitoring as a chosen indicator species may occur in only a limited number of habitat types and its biomarker responses might not reflect the sensitivity of other species or functional groups within a community. It is known that species' sensitivities to different contaminants can vary by several orders of magnitude depending on various factors including geographic distribution, taxonomic group, or functional feeding group. Such information is fundamental in risk assessment and to understand why

some species appear to be more tolerant to contaminants than others. Differences would clearly be important for the assessment of results from environmental monitoring and the determination of species-dependent threshold values for contaminant effects. For all these reasons, in the present thesis, the use of the different species with different trophic behaviour was necessary to fully understand the fate of the contaminants in all different natural matrices. In these studies, considering that the CNTs stay initially in the aqueous phase, but then they tend to aggregate and settle down persisting in sediments matrix and benthic organisms should be a particular exposed to these contaminants (Handy et al., 2008), the selection of these three invertebrate species was appropriate due to the presence of a filter-feeder bivalve (which is able through their gills to capture particulate matter and particles greater than ca. 6 µm are captured with an efficiency >90% (Ward and Kach, 2009)) and the two polychaetes species (which the ingestion of nano-contaminated sediment is crucial for uptake and cellular internalization of NPs by polychaetes (Magesky et al., 2018)).

Looking to the obtained findings, regarding to single exposures (Chapter 3.1.), the results showed that when the organisms were exposed to both CNT materials, all three species appeared susceptible to the test compounds. However, inter-species differences in sensitivity to environmentally relevant concentrations of both materials were reflected in the biomarker responses of the organisms. In detail, both CNT materials caused alteration of the energy reserve contents and metabolism in *R. philippinarum* and *D. neapolitana*, while in the *H. diversicolor* the used concentrations of Nf-MWCNTs were not high enough to result in metabolic depression or alteration of the energy contents. These results may be due to higher tolerance responses by the polychaetes to the pristine form of MWCNTs or the lower uptake of these materials by the organisms. This was also observed in terms of oxidative stress. In the presence of Nf-MWCNTs, *H. diversicolor* seemed to be able to tolerate oxidative stress being able to increase their defense mechanisms and, therefore, preventing cellular damages under these exposure concentrations. Differently, in the organisms contaminated with f-MWCNTs, the observed impairment of metabolic activity could be attributed to membranes cellular damage despite the activation of antioxidant enzymes. Regarding *R. philippinarum* and *D. neapolitana*, both CNTs generated toxic impacts in terms of oxidative status although the activation of the antioxidant systems. Considering the neuro status, in the two polychaete species, it was possible to observe an inhibition of the neurotransmitter only when exposed to the carboxylated MWCNT, while in *R. philippinarum* both MWCNTs were able to generate neurotoxicity. Overall, when comparing the toxic effects of both CNTs, in all invertebrate species major cellular damage was induced by the carboxylated forms of MWCNTs in comparison to the pristine one. Water-dispersible MWCNTs, due to the presence of higher amorphous carbon fragments in comparison to pristine one, can induced higher levels of toxicity to biological systems, as also demonstrated by Arndt et al. (2013), causing higher cellular damage with the activation of antioxidant mechanisms (Freixa et al., 2018). In fact, the release of ions resulting from the dissolution of the NPs caused higher oxidative stress mediated by ROS generation at the NPs surface, a

process already suggested to be a major responsible factor for NPs toxicological effects (Freixa et al., 2018). Moreover, the results of the present studies demonstrated clearly that nanomaterial toxicity not only has been attributed to core structure and surface functionalization, which have been shown to alter the level of toxicity to biological systems, but also by salinity and pH variations, which altered the dispersion and consequently the detection of CNTs in the media: aggregation/disaggregation, adsorption/desorption, sedimentation/resuspension and dissolution. Furthermore, under both salinity and pH stress, the biochemical responses to both MWCNTs showed to be species-dependent responsive.

Looking to the salinity results (Chapter 3.2.), in all invertebrate species was observed a dose-dependent increased of the toxicity caused by both CNT materials under both salinities, especially in terms of oxidative status. However, when we evaluated if the alteration induced by salinity shifts could modify the sensitivity of the polychaetes and/or the toxicity of the CNTs, it was observed species-dependent sensitivity to contaminants under the two salinities tested showing *R. philippinarum* and *D. neapolitana* more susceptible to the contaminant exposure in comparison to *H. diversicolor,* where the biochemical responses observed between the two salinities were similar. These results confirmed that the susceptibility detected in these species would, however, be expected not only to depend on the characteristics of the compounds, but also on the physiology of that particular species.

Considering the results obtained under pH variations (Chapter 3.3.), the findings presented here in general demonstrated that Nf-MWCNTs and f-MWCNTs under low pH generated greater toxic impacts in the organisms compared to individuals maintained under pH control. However, the results again showed a species-dependent sensitivity to contaminants under the two pH tested. The results of *R. philippinarum* and *D. neapolitana* demonstrated that while under low pH conditions the toxicity of Nf-MWCNTs was similar to the impacts measured under actual pH, which may indicate that predicted seawater acidification scenarios may not change the toxicity of these NPs, major toxicity was caused by f-MWCNTs under low pH, suggesting that mechanism of enhanced toxicity in the exposed organisms should be attributed to slighter aggregation and more suspended f-MWCNTs in acidified seawater and concluding that abiotic factors such as pH may influence more the bioavailability of water dispersible NPs compared to pristine ones. In what regards to *H. diversicolor*, similar biochemical responses were observed between the two CNT materials under different pH levels, indicating higher impact of pH in polychaetes in comparison to the impacts caused by the CNTs.

Together, it was possible to understand that *H. diversicolor* was the most tolerant species to both CNT materials acting alone and under future climate change scenario since both salinity and pH variations induced less subcellular changes in comparison to the other polychaete species *D. neapolitana* and the filter-feeding bivalve *R. philippinarum.* These results may be explained by the fact that *H. diversicolor* is able to live in sediments very high in trace metal content and organic

contaminants and the effects of these pollutants on life history characteristics of these species may provide a more tolerance when exposed to toxicological tests in laboratory conditions (Dean, 2008).

The last important step is trying to understand what type of biomarkers can provide a 'diagnosis of stress' in the chosen organisms exposed to different conditions. Much time and effort have been spent standardising individual biomarkers and defining the range of responses that can be considered 'normal' for a particular organism (Wells and Balls, 1994; Viarengo et al., 2000), which is particularly important for comparing data between laboratories. There are, however, drawbacks to this validation approach as biomarker responses are known to vary considerably with environmental factors (Hauton et al., 1998), as well as a sensibility of the organism (Depledge, 1993; Sukhotin et al., 2002). For multi-biomarker studies, it may be more useful to use a suite of biomarker responses to provide a 'diagnosis of stress', whereby, effects at the molecular level can be used to interpret the level of physiological or biochemical impairment of an organism (Downs et al., 2001). Therefore, to predict the potential of pollutants to damage ecosystems, laboratory data should be obtained linking the effects of biomarkers at the biochemical, cellular and physiological level (Viarengo et al., 2000). In such a holistic approach to environmental assessment, standardisation of individual markers of contaminant effects may be less important than interpreting how combinations of different biomarkers reflect the integrated toxic effect of a contaminant to an organism. For these reasons, a battery of biomarkers was used in the presented studies reporting possible variations in terms of energy reserves and metabolic activity as well oxidative and neuro status when the organisms were exposed to both CNT materials under climate change scenario.

Regarding single exposures (Chapter 3.1) as well as the combination with salinity (Chapter 3.2) and pH (Chapter 3.3), one the most specificity and sensitivity marker used for these conditions in all three species was the ETS acivity which is considered an important indicator of metabolic capacity. Mitochondrial dysfunction observed in all invertebrate species when exposed to both CNT materials under different environmental conditions may hold significant informative value of mitochondrial disorders. Moreover, observing that NPs availability disturbed normal functioning of organisms' metabolism, with the results presented here it may be possible to conclude a successful uptake of both NPs by the organisms and interaction with membrane receptors with subsequently taken up by receptor-mediated endocytosis as already demonstrated by Kettiger et al. (2013). Based on the obtained results, LPO levels and the antoxidant enzymes (SOD and GPx) activity can also be considered a relieble markers for CNTs exposure. In fact, in all conditions LPO levels were observed as well as activation or inhibition of the antioxidant systems. These results are in line with the information provided by the literature which confirmed that among the oxidative damage induced by NPs, the breakdown of the antioxidant defence system, as well as lipid peroxidation, are the common harmful effects caused by these materials in the exposed organisms (Rocha et al., 2015).

The present results highlight the fact that a primary consideration for hazard and risk assessment is the need to understand both the properties of the starting nanomaterial and the physical-chemical nature of the test and potential receptor systems before commencing an ecotoxicological study. Although the temptation to launch into an ecotoxicological test is great, the present study has the objective to underline the importance to understand the intrinsic characteristics of the starting material (e.g., particle size and ligand chemistry) and how the material will behave in the study medium. These answers are fundamental to understanding the likely fate, behavior, uptake and ecotoxicity of the material under environmental conditions. This knowledge must be used to inform the design of the study. The interaction between the exposure aspects of eco-hazard assessment (e.g., characterization in test media and exposed organisms; associated abiotic factors that influence behavior) and how such exposure data are then reported in studies are critical areas where collaboration involving the materials science, particle chemistry and ecotoxicological areas could be highly profitable in terms of developing consensus and good practice. Moreover, despite that most (eco)toxicity studies with CNTs observed some degree of adverse effects, it is still unclear which physical and/or chemical characteristics of CNTs are main driver of toxicity and since a very limited number of studies are made in the field of environmental fate of CNTs, their behavior in the environment is still largely unexplored. For these reasons, it is very important to study their environmental fate in order to understand their pathways of environmental as well as human exposure. Another urgent research needed in regard to the environmental exposure of CNTs is to establish the degree of their environmental mobility and bioavailability. Understanding the environmental fate of these materials would greatly help to assess exposure of ecosystems and conseguently toxicity in biota. Moreover, due to the scarce information presented in the literature, the impact of CNTs under current and future exposure scenarios on communities, ecosystems, ecosystem functions deserves special attention.

Finally, although the studies performed focused mainly on cellular biomarkers, it seems important to integrate traditional chemical and biomarker measures with omics technologies (such as proteomics and metabolomics), behavioral biomarkers and histopathological analyses to redefine the surveillance of emerging pollutions and assess the climate change effects in model organisms, such as bivalves and polychaetes, to understand the complete picture of stressful events.

CHAPTER 5. REFERENCES

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