The impacts of emergent pollutants on *Ruditapes philippinarum*: biochemical responses to carbon nanoparticles exposure

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Keywords:
Multi-walled carbon nanotubes
Clams
Metabolic capacity
Oxidative stress
Neurotoxicity

a b s t r a c t

Multi-walled carbon nanotubes (MWCNTs) are one of the most important carbon Nanoparticles (NPs). The production and use of these NPs are increasing rapidly and, therefore, the need to assess their presence in the environment and associated risks has become of prime importance. Recent studies demonstrated the impacts of different NPs on bivalves, a taxonomic group where species tolerance to anthropogenic stressors, such as pollutants, is widely variable. The Manila clam *Ruditapes philippinarum* is one of the most commonly used bivalve species in environmental monitoring studies and ecotoxicology tests, however, to our knowledge, no information is available on biochemical alterations on this species due to MWCNTs exposure. Thus, the present study aimed to assess the toxic effects of different MWCNT concentrations (0.01; 0.10 and 1.00 mg/L) in *R. philippinarum* biochemical (energy reserves, metabolic capacity, oxidative status and neurotoxicity) performance, after 28 days of exposure. The results obtained revealed that exposure to MWCNTs altered energy-related responses, with higher metabolic capacity and lower glycogen and protein concentrations in clams exposed to these carbon NPs. Moreover, *R. philippinarum* exposed to MWCNTs showed oxidative stress expressed in higher lipid peroxidation and lower ratio between reduced and oxidized glutathione, despite the activation of defence mechanisms in exposed clams. Additionally, neurotoxicity was observed by inhibition of cholinesterases activity in organisms exposed to MWCNTs. The present study provides valuable information regarding how these emerging pollutans could become a potential risk for the environment and living organisms.

1. Introduction

Nanoparticles (NPs) are widely used in numerous applications including various fields of medicine, chemistry and electronics (Renn and Roco, 2006). As a consequence of the fast NPs production and application increase the introduction of these materials into the environment, namely aquatic systems, is expected to occur.

Among NPs, carbon-based NPs are a broad class of materials that have a diverse range of applications (Solarskaciuk et al., 2014; Vlasova et al., 2016; Wu et al., 2013; Muller and Nowack, 2008; Köhler et al., 2008). Carbon nanotubes (CNTs), one of the most important carbon-based NPs (Sanchez et al., 2012), were already identified in aquatic systems (Scown et al., 2010; Eckelman et al., 2012) at predicted environmental concentrations of 0.97 ng/L (mean value for EU in 2020), using dynamic probabilistic material flow model (DP-MFA) (Sun et al., 2016). However, currently available data regarding their impacts on aquatic organisms, especially on invertebrate species, are still limited.

Bivalves comprise a wide range of invertebrate organisms with different tolerances to anthropogenic stressors, such as pollutants (e.g. Almeida et al., 2015; Coelho et al., 2014; Ji et al., 2015; Matozzo et al., 2016; Velez et al., 2016). In the last few years, different studies demonstrated the impacts of nanoparticles (NPs) in bivalves, a group of organisms considered to represent a particularly suitable model for investigating the effects and mechanisms of action underlying the potential toxicity of NPs to marine invertebrates (Canesi et al., 2012; Libralato et al., 2013; Rocha et al., 2015). Among others, Canesi et al. (2008, 2010) demonstrated that exposure to different NPs concentrations (0.05, 0.2, 1, 5 mg/L) (nano carbon black, NCB; Nano fullerene, C60; Nano titanium dioxide, TiO_2; Silicon

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dioxide NPs, SiO_2) for 24 h elicit significant impacts in the mussel *Mytilus galloprovincialis* immune system. Gomes et al. (2013) observed DNA damage in *M. galloprovincialis* exposed to 10 g/L of silver nanoparticles (Ag NPs) for a period of 15 days. Dai et al. (2013) exposed the clam *Macoma balthica* to Ag NPs (150–200 μg/g) during 35 days of exposure to spiked sediment, and observed burrowing activity delay. Al-Subiai et al. (2012) exposed *Mytilus* sp. adults to C60 at 0.10 and 1 mg/L for 3 days and showed histological anomalies and DNA damage in mussels exposed to these NPs.

The Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850), a species native from the Indo-Pacific region, is one of the most commonly used bivalve species in environmental monitoring studies and ecotoxicology tests. Characteristics including wide geographical distribution, long life cycle, ease of collection, high propensity to bioaccumulate pollutants and high capacity to reflect their impacts both under environmental and laboratory conditions (Bebianno et al., 2004; Liu et al., 2011), makes *R. philippinarum* a good sentinel and bioindicator species for the study of anthropogenic stressors. Different studies demonstrated the ability of this species to respond to a wide diversity of pollutants, with impacts at physiological and biochemical levels (Antunes et al., 2013; Ji et al., 2015; Matozzo et al., 2016; Velez et al., 2016). Nevertheless, the assessment of NPs toxic effects in *R. philippinarum* has been largely neglected, namely in what regards to carbon-based NPs. Marisa et al. (2015) demonstrated that TiO_2 NPs affected haemocyte phagocytosis in *R. philippinarum* at both 1 and 10 μg/mL after 60 min of exposure. Based on transmission electron microscope (TEM) data, these authors stated that the effects on haemocyte functionality are mediated (at least in part) by internalisation of NPs within haemocytes.

The use of biochemical markers can be particularly useful in detecting the early signs of possible injuries in bivalves under anthropogenic disturbance (Zuykov et al., 2013), because toxic effects are expected to occur at the subcellular level before being observable at higher levels of biological organization (i.e. individual and population level). Several studies already demonstrated that biochemical markers are sensitive to a variety of stressors such as NPs. In fact, due to the physico-chemical reactivity of carbon NPs (small size, remarkably large surface area per unit mass and high surface reactivity (Auffan et al., 2009)), these new emerging pollutans are able to trigger the formation of free radicals, directly or via activation of oxidative enzymatic pathways, leading to oxidative stress (Shvedova et al., 2012). Studies conducted by De Marchi et al. (2017) in two polychaetes species (*Diopatra neapolitana* and *Hediste diversicolor*), showed the induction of oxidative stress due to multi-walled carbon nanotubes (MWCNTs) exposure (0.01; 0.10 and 1.00 mg/L) after 28 days. Pretti et al. (2014) also demonstrated that the exposure to graphene monolayer flakes, up to the concentration of 1 mg/L, for 48 h increased antioxidant defenses in the crustacean *Artemia salina*.

Focusing on bivalves, studies show that these organsisms possess sub-cellular mechanisms that allow them to cope with the toxic effects of different pollutants. In particular, when exposed to pollutants, clams present a number of cellular responses that include antioxidant defenses (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT)), metabolization mechanisms (e.g., glutathione S-transferases (GSTs)), cellular damages (e.g. lipid peroxidation (LPO)) and neurotoxicity (e.g., acetylcholinesterase (AChE)) (Bebianno et al., 2004). While several published studies show that these biochemical markers are useful in detecting possible damages in bivalves under anthropogenic disturbances, such as metal pollution (Brock, 1993; Gomes et al., 2013; Liu et al., 2011; Velez et al., 2016; Yesudhason et al., 2013), eutrophication (Verdelhos et al., 2005) and pharmaceuticals (Antunes et al., 2013; Correia et al., 2016; Freitas et al., 2015; Freitas et al., 2016; Matozzo et al., 2016), little is known on the biochemical alterations induced by carbon NPs (Canesi and Corsi, 2015; Cattaneo et al., 2009; Matranga and Corsi, 2012; Miller et al., 2015).

Therefore, the impacts induced by chronic exposure to MWCNTs, one of the most important CNTs used in broad industrial and biomedical applications (Du et al., 2013), were evaluated in *R. philippinarum*, by measuring alterations induced in clams oxidative status, neurotoxicity and metabolic capacity.

2. Materials and methods

2.1. Sampling and experimental conditions

R. philippinarum specimens were collected in northwest Atlantic coast of Portugal (40°38′N, 8°45′W). Specimens with similar size were used to minimize differences on organisms MWCNTs accumulation and biochemical responses. The mean length and weight of clams were 33.7 ± 0.21 mm and 18.2 ± 2 g, respectively. After sampling, organisms were transported to the laboratory and distributed by 4 aquaria (20 L each) filled with artificial seawater (salinity 28), and acclimated for 7 days to laboratory conditions. Aquaria were set up by the addition of artificial sea salt (Tropic Marin® Sea Salt) to reverse osmosis water. During the acclimation period organisms were under constant photoperiod of 12 h light: 12 h dark, temperature (18 ± 1 °C) and aeration conditions. During this period every two-three days the specimens were fed with AlgaMac Protein Plus, Aquafauna Bio-Marine, Inc (150,000 cells/animal).

For the experimental assay organisms were placed in different aquaria (20 L each) and exposed for 28 days to four test conditions: control (CTL, 0 mg/L), 0.01; 0.10 mg/L and 1.00 mg/L of MWCNTs. The concentrations of MWCNT used in this study were prepared from a stock solution of 50 mg/L concentration. The concentrations selected were necessary to test i) wide range of contamination levels, and ii) determine the threshold concentrations that may affect bivalves. For each condition 18 organisms were used (3 aquaria *per* condition, with 6 organisms *per* aquarium). Each aquarium was set up at the same conditions as in the acclimation period (see above). During the experimental period, MWCNT concentrations were re-established weekly after complete water renewals to ensure the presence of the exposure concentrations during the experiment. After a sonication step, to promote stable suspension of carbon NMs in the water column (Hwang et al., 2007), MWCNTs were homogenously dispersed in the water using one submersible circulation pump *per* aquarium, which diminishes the possibility that the dynamical equilibrium between gravitational settling and Brownian motion can result in the presence of CNTs near the sediment–water interface (Vonk et al., 2009).

Before each water renewal, water samples were collected every week from each aquarium (10 mL) and used for the characterization analysis.

After exposure (28 days), for biochemical analyses 3 organisms *per* aquarium (9 *per* condition) were immediately frozen and the whole body of frozen organisms was pulverized individually with liquid nitrogen and divided in 0.5 g aliquots. For lipids content determination the remaining organisms were dried at 60 °C for 48 h.

2.2. MWCNTs characterization

Thin MWCNT materials were produced via the Catalytic Chemical Vapor Deposition (CCVD) process and characterized using Transmission electromicrographs (TEM) (Fig. 1). These carbon NPs were purchased from Nanocyl S.A. (MWCNTs: NC7000 series, http://www.nanocyl.com) and manufacturers specifications are showed in Table 1.

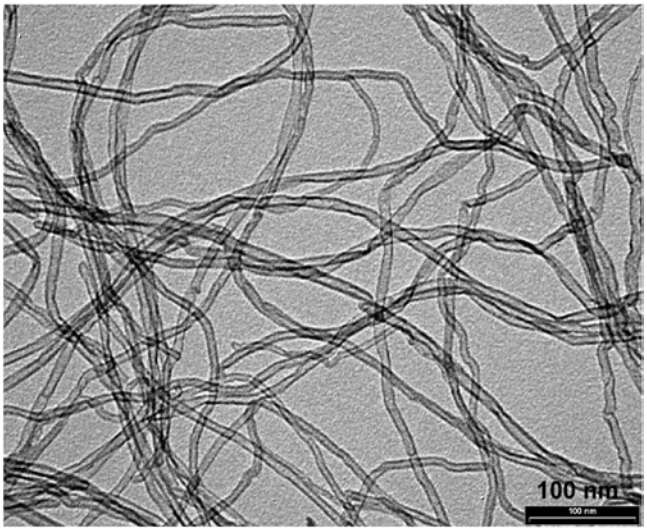

Fig. 1. Transmission electromicrographs (TEM) of the powder form of MWCNTs produced via the catalytic carbon vapor deposition (CCVD) process.

Table 1
Characterization of the powder form of MWCNTs.

Property	Unit	Value	Method of measurement
Average Diameter	nanometers	9.5	TEM
Average Length	microns	1.5	TEM
Carbon Purity	%	90	TGA
Metal Oxide	%	10	TGA
Amorphous Carbon	–	*	HRTEM
Surface Area	m^2/g	250–300	BET

* Pyrolytically deposited carbon on the surface of the MWCNTs.

The average size distribution of MWCNT suspensions in each exposure condition (0.01; 0.10 and 1.00 mg/L) collected in different exposure periods (first, second, third and fourth week of exposure), was analyzed by dynamic light scattering (DLS), using a DelsaTM NanoC Particle Size Analyzer (Beckman Coulter). Measurements were performed on 1 mL of suspension and each analysis was repeated three times.

The Zeta Potential value of MWCNT suspensions in seawater was measured with a DelsaTM NanoC Particle Size Analyzer (Beckman Coulter). Water samples were collected in each exposure condition in different exposure periods. Measurements were performed on 1 mL of suspension and each analysis was repeated three times.

2.3. Biochemical parameters

Aliquots (0.5 g) of frozen individuals were used for all biochemical analyses. Extractions were performed with specific buffers (see De Marchi et al., 2017) to determine: protein (PROT), glycogen (GLY), and lipid (LIP) concentrations; electron transport system (ETS) activity; lipid peroxidation (LPO) levels; reduced (GSH) and oxidized (GSSG) glutathione content; and the activity of antioxidant (superoxide dismutase, SOD; glutathione peroxidase, GPx), biotransformation (Glutathione S-transferases, GSTs) and neurotransmitter (cholinesterases ChEs) (Acetylcholinesterase (ATChI-ChE); Propionylcholinesterase, PTChI-ChE) enzymes. Biochemical analyses were performed in duplicate for each sample and parameter.

2.3.1. Energy reserves and metabolic activity

PROT content was determined following the spectrophotometric method of Biuret (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standard (0–40 mg/mL). Absorbance was measured at 540 nm. Concentrations of PROT were expressed in mg *per* g fresh weight (FW).

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

LIP content was determinated according Cheng et al. (2011). After 1 h of color development, the absorbance was measured at 540 nm. Results were expressed in percentage *per* g dry weight (DW).

The ETS activity was measured following King and Packard (1975) and modifications performed by De Coen and Janssen (1997). The absorbance was measured at 490 nm during 10 min in 25 s intervals. The amount of formazan formed was calculated using ϵ = 15,900 $M^{-1}\,cm^{-1}$ and the results expressed in nmol/min *per* g FW.

2.3.2. Indicators of cellular damage

According to the method described by Ohkawa et al. (1979), LPO levels were obtained by the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Absorbance was measured at 535 nm (ϵ = 156 $mM^{-1}\,cm^{-1}$) and LPO levels were expressed in nmol of MDA formed *per* g FW.

GSH and GSSG contents were measured at 412 nm (Rahman et al., 2014) and used as standards (0–60 μmol/L). GSH and GSSG concentrations were expressed in μmol *per* g FW. GSH/GSSG was calculated dividing the GSH values by 2x the amount of GSSG.

2.3.3. Antioxidant defenses and biotransformation mechanisms

SOD activity was quantified following the method of Beauchamp and Fridovich (1971). Absorbance was measured at 560 nm. A standard curve was performed with SOD standards (0.25–60 U/mL). Results were expressed in U *per* g FW where U represents the quantity of enzyme that catalyzes the conversion of 1 μmol nitroblue tetrazolium (NBT) *per* min.

GPx was quantified following Paglia and Valentine (1967). The activity was performed with 100 mM Tris-HCl (pH 8), 0,1 mM EDTA, 1% (w/v) PVP, 0,5% (v/v) Triton X-100 extraction buffers. Enzyme activity was determined using ϵ = 0,00622 $\mu M^{-1}\,cm^{-1}$ and absorbance measured at 340 nm in 10 s intervals during 5 min. The results were expressed in U *per* g FW where U represents the quantity of enzyme which catalyzes the conversion of 1 μmol nicotinamide adenine dinucleotide phosphate (NADPH) *per* min.

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

2.3.4. Neurotoxicity

Acetylthiocholine iodide (ATChI, 470 μM) and propionylthiocholine (PTChI 1880 μM) were used as substrates for the determination of ATChI-ChE and PTChI-ChE activities according to Ellman et al. (1961) and Mennillo et al. (2017). Enzyme activities were recorded continuously for 5 min at 412 nm in a Perkin Elmer double-beam spectrophotometer and the specific activity was corrected for the spontaneous hydrolysis of the substrate and expressed in nmol *per* min *per* g FW, using a molar extinction coefficient of 13.6 $\times 10^3$ $M^{-1}\,cm^{-1}$ (the yellow dianion of 5-thio- 2-nitrobenzoic acid, TNB).

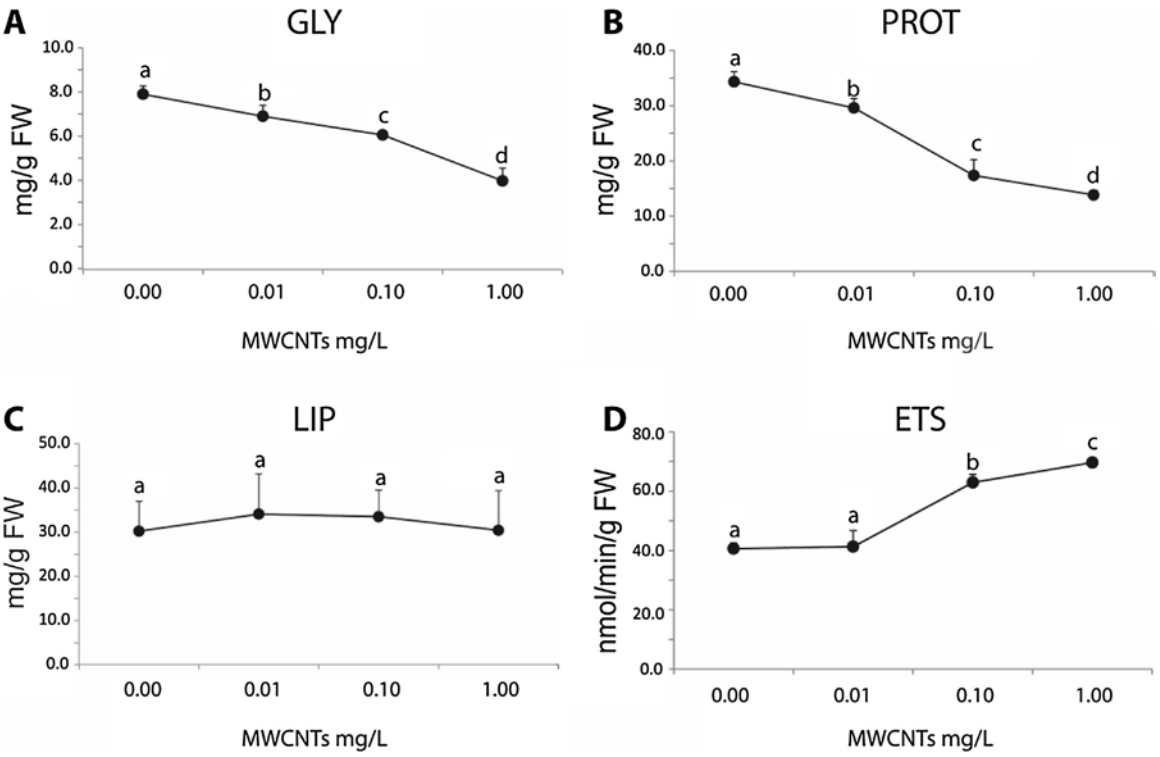

Fig. 2. A: Glycogen (GLY) content; B: Protein (PROT) content; C: Lipid (LIP) content; D: electron transport system (ETS) activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Different letters represent significant differences ($p \leq 0.05$) among conditions.

Table 2
MWCNTs characterization data in exposure medium (0.01; 0.10; 1.00 mg/L) collected in different exposure periods (first, second, third and fourth week of exposure). Dynamic Light Scattering (DLS) and Zeta potential; pH: 8.4, salinity: 28.

	7 days		14 days		21 days		28 days	
	DLS (nm)	Zeta potential (mV)	DLS (nm)	Zeta potential (mV)	DLS (nm)	Zeta potential (mV)	DLS (nm)	Zeta potential (mV)
MWCNTs (mg/L)								
0.01	3434	−8.63	3432	−8.62	3429	−8.61	3430	−8.63
0.10	1679	−14.71	1683	−14.70	1682	−14.71	1680	−14.68
1.00	2374	−13.49	2379	−13.50	2376	−13.49	2375	−13.48

2.4. Data analysis

Results on PROT and GLY content, ETS activity, LPO levels, GSH/GSSG, SOD, GPx, GSTs, and ChE activities, were submitted to hypothesis testing using permutational multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6. A one-way hierarchical design was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. If significant differences were observed using main test, subsequent pairwise comparisons were performed. Pairwise comparisons were evaluated in terms of significance and significant differences were identified for Monte-Carlo *p*-values lower than 0.05. The null hypothesis tested was: for each biomarker, no significant differences existed among exposure concentrations. Significant differences among exposure concentrations were represented in figures with different letters.

3. Results

3.1. MWCNTs characterization

The mean size and the surface charge of MWCNTs suspended particles in seawater at each tested concentrations (0.01, 0.10 and 1.00 mg/L) collected at the first, second, third and fourth week of exposure, were measured by dynamic light scattering (DLS) and Zeta potential, respectively (Table 2). DLS characterization is able to estimate the ability of NPs to form micro/nano-sized agglomerates in aqueous media under the adopted experimental conditions (Pretti et al., 2014). No significant differences were observed among samples collected in different exposure periods (first, second, third and fourth weeks of exposure) in each condition (0.01; 0.10 and 1.00 mg/L). The results also showed that the mean diameter (nm) of MWCNTs in each exposure period at the lowest concentration (0.01 mg/L) was larger compared to the mean diameter at 0.10 (mg/L) and 1.00 mg/L, indicating a greater tendency for MWCNTs to form agglomerates at lower concentrations (Table 2). These results are in agreement with the study conduced by De Marchi et al. (2017) which showed similar paticle diameters using the same carbon NPs. The absolute zeta potential is a method used to measure surface charge of carbon NMs and is a key indicator of the stability of colloidal dispersions (Miller et al., 2015). As for the DLS analysis, also the zeta potential values of MWCNT agglomerates used in the present study didn't present significant differences among samples collected in different exposure periods in each condition. The zeta potential were higher at the lowest MWCNTs concentration (0.01 mg/L) compared to those at 0.10 and 1.00 mg/L. The behaviour of MWCNTs in aqueous media was similar to those found in the literature (De Marchi et al., 2017; Miller et al., 2015).

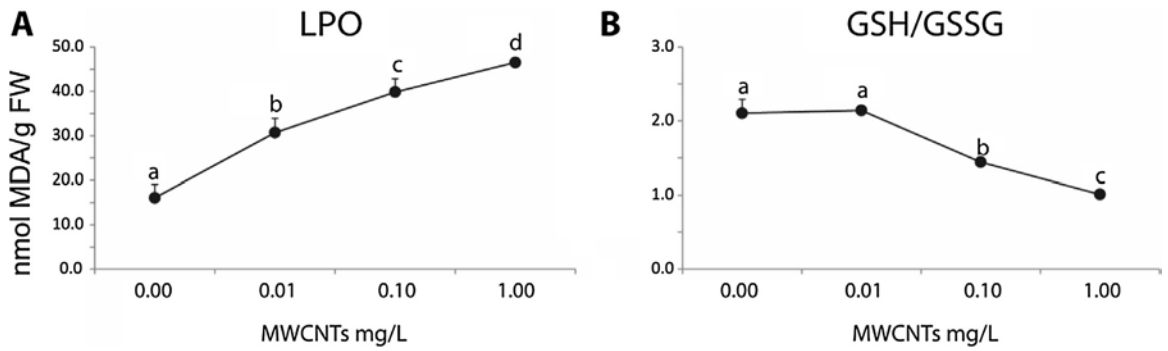

Fig. 3. A: Lipid peroxidation (LPO) levels; B: GSH/GSSG (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Different letters represent significant differences ($p \leq 0.05$) among conditions.

3.2. Mortality

R. philippinarum presented 100% of survival in all conditions except for individuals exposed to 1.00 mg/L MWCNTs (5,55% mortality).

3.3. Biochemical parameters

3.3.1. Energy reserves and metabolic activity

GLY content decreased in clams with the increase of exposure concentrations, with significant differences among all conditions (Fig. 2A).

A similar decreasing pattern was obtained for PROT content, with significant differences among clams exposed to different MWCNT concentrations (Fig. 2B).

LIP content was similar among exposure concentrations, with no significant differences among conditions (Fig. 2C).

ETS activity was significantly higher in clams exposed to 0.10 and 1.00 mg/L MWCNTs compared to organisms under control (0.00 mg/L) and exposed to the lowest concentration (0.01 mg/L). No significant differences were found between clams exposed to control and the lowest MWCNTs concentration (Fig. 2D).

3.3.2. Indicators of cellular damage

Clams exposed to MWCNTs increased LPO levels with the increase of exposure concentrations, with significant differences among all tested conditions (Fig. 3A).

GSH/GSSG was significantly lower in clams exposed to 0.10 and 1.00 mg/L in comparison to clams exposed to control and 0.01 mg/L. Clams exposed to control and 0.01 mg/L showed no significant differences in GSH/GSSG values (Fig. 3B).

3.3.3. Antioxidant defenses and biotransformation mechanisms

Clams showed significantly higher SOD activity when exposed to MWCNTs, with the highest values at the highest exposure concentration (Fig. 4A). Significant differences were observed among all MWCNTs concentrations (Fig. 4A).

The activity of GPx in clams increased with the increase of exposure concentrations up to 0.10 mg/L, with significant differences among conditions (Fig. 4B). At the highest exposure concentration (1.00 mg/L) the activity of GPx was significantly lower compared than values observed in control clams (Fig. 4B).

Clams showed a slight decrease in GSTs activity with the increase of exposure concentrations with significant differences observed between control and the remaining conditions as well as between clams exposed to 0.01 and 0.10 and those exposed to 1.00 mg/L (Fig. 4C).

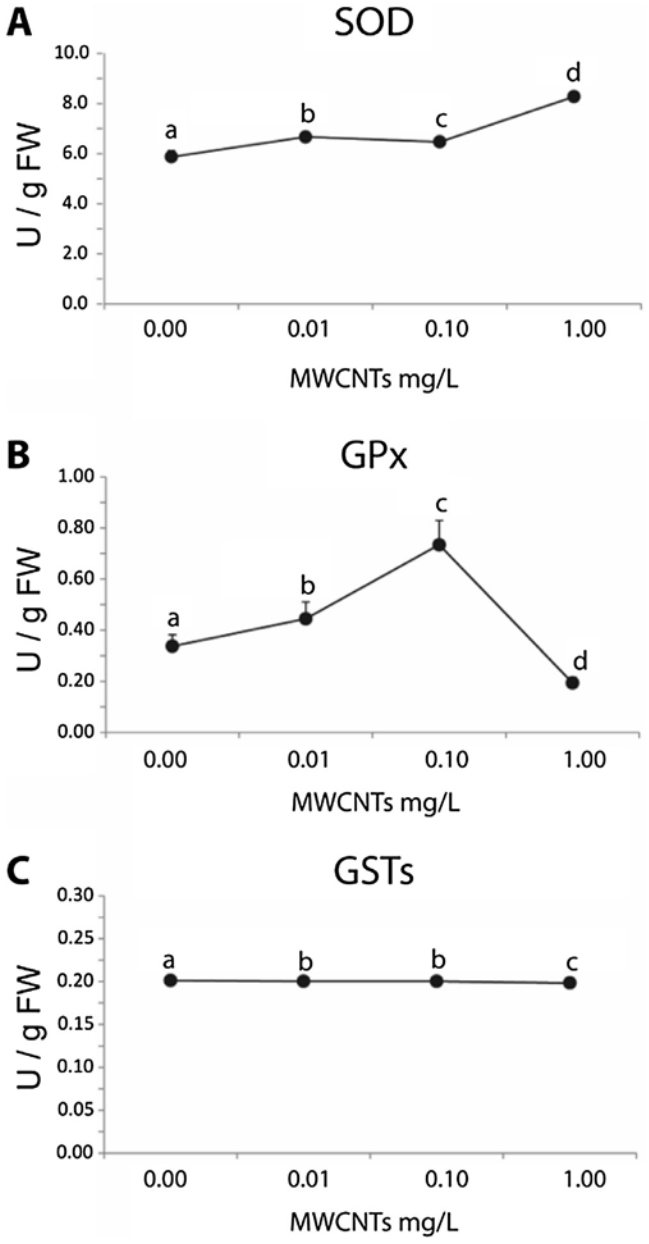

Fig. 4. A: Superoxide dismutase (SOD) activity; B: Glutathione peroxidase (GPx) activity; C: Glutathione S-transferases (GSTs) activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Different letters represent significant differences ($p \leq 0.05$) among conditions.

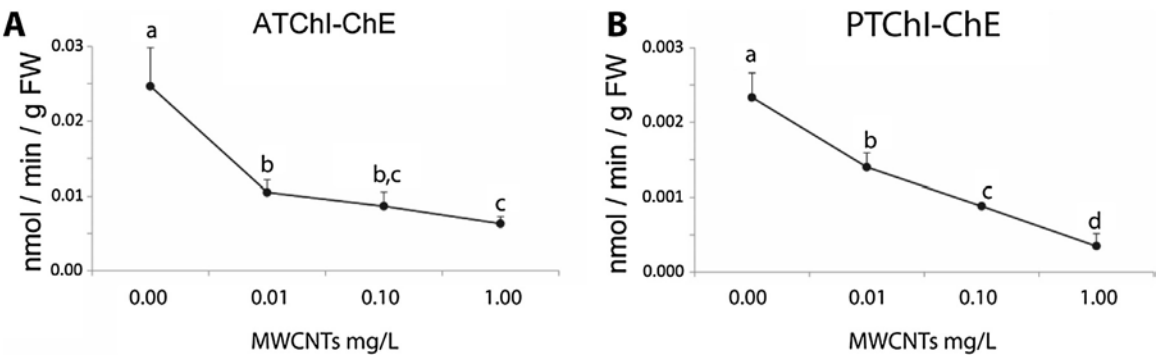

Fig. 5. A: ATChI-ChE activity; B: PTChI-ChE activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Different letters represent significant differences ($p \leq 0.05$) among conditions.

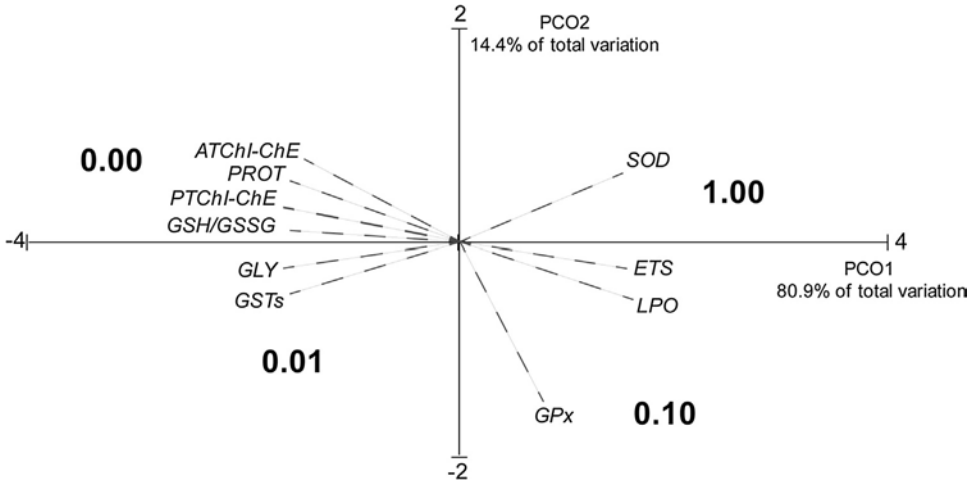

Fig. 6. Centroids ordination diagram (PCO) based on biochemical parameters, measured in *Ruditapes philippinarum* exposed to different MWCNTs concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): PROT; GLY; ETS; LPO; GSH/GSSG; SOD; GPx; GSTs; ATChI-ChE; PTChI-ChE.

3.3.4. Neurotoxicity

Results obtained showed that both ATChI-ChE and PTChI-ChE activities decreased with the increase of exposure concentrations (Fig. 5A and B). Regarding the activity of ATChI-ChE organisms exposed to MWCNTs showed significant differences to organisms under control, but no significant differences were observed between organisms exposed to 0.01 and 0.10 mg/L as well as between organisms exposed to 0.10 and 1.0 mg/L (Fig. 5A). For PTChI-ChE significant differences were observed among all tested conditions (Fig. 5B).

3.4. Multivariate analysis

Principal coordinates analysis (PCO) graph obtained for *R. philippinarum* is shown in Fig. 5. PCO axis 1 explained 80.9% total variation, while PCO axis 2 explained 14.4% (Fig. 6). PCO1 primarily separated individuals from control (0.00) and the lower MWCNTs concentration (0.01 mg/L) at the negative side from the ones exposed to 0.10 and 1.00 mg/L in the positive side. PCO2 separated individuals exposed to control and the highest concentration in the positive side from organisms exposed to 0.01 and 0.1 mg/L in the negative side. GLY and GSTs were well correlated with individuals exposed to 0.01 mg/L *R. philippinarum* exposed to 1.00 mg/L MWCNTs concentration were strongly correlated with SOD, ETS and LPO since under this condition these parameters showed the highest values. *R. philippinarum* specimens exposed to 0.10 mg/L were associated to the antioxidant defenses activity (GPx) since higher activity was observed at this MWCNTs concentration. High correlation was observed between organisms under control and ChE enzymes activity, PROT content, and GSH/GSSG, the condition where clams showed the highest values for these parameters (Fig. 6).

4. Discussion

4.1. Energy reserves and metabolic capacity alterations

It is known that organisms can increase their energy expenditure when exposed to anthropogenic environmental disturbances (Sokolova et al., 2012). In accordance, the present study demonstrated that *R. philippinarum* clams presented lower GLY and PROT content, although were able to preserve their LIP content, when exposed to MWCNTs, which may indicate that clams were using GLY and PROT to fuel their mechanisms of defence against NPs. Although no information is available on the expenditure of energy reserves in bivalves exposed to MWNCTs, different studies already demonstrated the expenditure of GLY and PROT in bivalves exposed to other pollutants. Hamza-Chaffai et al. (2003) carried out a field experiment and demonstrated that GLY was inversely correlated with Zn in the clam *R. decussatus*. Also Duquesne et al. (2004) demonstrated that the clam *Macoma balthica*, after the exposure of 5 weeks to four different Cd concentrations (10, 30, 100 and 300 ppb), presented decreased GLY content when exposed to the highest concentration (300 ppb) in comparison to the remaining

conditions. Dickinson et al. (2012) further revealed, in the juvenile eastern oysters *Crassostrea virginica* exposed to different salinities (15 and 30), no significant effect on LIP content after eleven weeks exposure.

In the present study the decrease of energy reserves (GLY and PROT) observed along the increasing exposure gradient was closely related to clams metabolic capacity measured through the electron transport system (ETS) activity. The ETS passes electrons along a series of intermediary dehydrogenases and cytochromes to a final electron acceptor, the O_2. This activity enables organisms to carry out oxidative phosphorylation and provides the energy used to generate ATP (Cammen et al., 1990). As already demonstrated, ETS can be used as a measure of metabolic capacity in different organisms (namely in invertebrates) in response to environmental disturbances (Bielen et al., 2016; Cammen et al., 1990; Freitas et al., 2016; Schmidlin et al., 2015; Simčič et al., 2014). Our results demonstrated that clams presented higher ETS activity with the increase of MWCNT concentrations, especially noticed at higher concentrations. Thus, the increase of clams metabolic capacity may explain high energy expenditure and may also be associated to the activation of defence mechanisms. Although no information is available on the impacts of CNTs on the metabolic capacity of bivalves, De Marchi et al. (2017) also observed an increase of the ETS activity in two polychaetes species (*D. neapolitana* and *H. diversicolor*) exposed to MWCNTs (1.00 mg/L) after 28 days of exposure.

4.2. Reactive oxygen species production

Several toxic impacts at the cellular level, caused by environmental pollutants, can be induced by reactive Reactive oxygen species (ROS) overproduction (Livingstone, 2003), which can cause oxidative stress (Valavanidis et al., 2006). ROS such as hydrogen peroxide, hydroxyl radicals, superoxide anions and other oxygen radicals are capable of directly oxidizing DNA, amino acids in proteins and polyunsaturated fatty acids in lipids (Yoshida et al., 2004). Under basal conditions, organisms produce ROS whose adverse effects are prevented by the antioxidant system, consisting of scavengers and antioxidant enzymes (Regoli and Giuliani, 2014). It has long been recognized that different stimuli, such as pollutants, are sources of ROS over production. When antioxidant defence mechanisms are not able to eliminate the excess of ROS damages in the lipidic membrane can occur, which is measured by lipid peroxidation (LPO).

The present study clearly demonstrated that MWCNTs induced cellular damages in *R. philippinarum*, as the LPO levels increased with the increase of exposure concentrations. In accordance to our findings, previous studies already demonstrated that LPO is induced in bivalves exposed to different pollutants, including NPs. Among others, recent studies demonstrated that LPO occurs in *Mytilusedulis* as a consequence of gold nanoparticles (Au NPs) exposure at the concentration of 750 ppb for 24 h (Tedesco et al., 2010); in *Elliptio complanata* exposed to silver nanoparticles (Ag NPs) with two different sizes (20 and 80 nm) (Gagné et al., 2013) for 48 h; in *M. galloprovincialis* submitted to 10 g/L of copper oxide nanoparticles (CuO NPs) concentration for a period of 15 days (Gomes et al., 2012); in *Corbicula fluminea* contaminated with 0.01, 0.1, 1, and 10 mg/L of nanodiamonds (NDs) throughout 14 days (Cid et al., 2015). However, no studies are known on bivalves cellular damage due to CNTs.

Scavengers neutralize ROS by direct reaction with them, such as the case of the cytosolic reduced glutathione (GSH), which directly neutralizes several reactive species through its oxidation, generating oxidized glutathione (GSSG) or, in addition, acts as a cofactor of several antioxidant glutathione dependent enzymes that eliminate ROS (Regoli and Giuliani, 2014). Thus, the ratio between GSH and GSSG is often used as a marker of oxidative stress, including in organisms exposed to NPs (Falfushynska et al., 2015; Tedesco et al., 2010; Zhu et al., 2011). In the present study GSH/GSSG results clearly demonstrate that clams exposed to MWCNTs exhibit oxidative stress, as the GSH/GSSG values decreased with the increase of exposure concentrations (especially at 0.10 and 1.0 mg/L). GSH/GSSG decrease may indicate that GSH was used to neutralize the excess of ROS that caused LPO increase, through its oxidation to GSSG, or that GSH was used as cofactor of different antioxidant glutathione dependent enzymes. Although no studies are known on the impacts of CNTs in GSH content, similar results were obtained for other NPs. Tedesco et al. (2010) showed lower GSH/GSSG values in the digestive gland of *M. edulis* exposed to Au NPs in comparison to organisms under control conditions. Falfushynska et al. (2015) demonstrated that in the mussel *Unio tumidus* the levels of GSH were not affected by Nano Zinc Oxide (nZnO) at 3.1 μM for 14 days, but GSSG levels significantly increased in response to this NP. Oxidative stress was also identified in the marine abalone *Haliotis diversicolor supertexta* exposed for 96 h to titanium dioxide nanoparticles ($nTiO_2$) (0.1, 1.0 and 10 mg/L) with significantly lower GSH content in organisms exposed to the highest NPs concentration compared to control (Zhu et al., 2011).

As mentioned above, the excess ROS produced when organisms are under stressful conditions may not only be eliminated by scavengers as GSH but also by antioxidant enzymes. Compared to scavenger molecules, which interact with more than one type of ROS, antioxidant enzymes catalyze highly specific reactions with specific substrates (Regoli and Giuliani, 2014). Superoxide dismutase (SOD) is the enzyme responsible for the removal of the superoxide anion (O_2^-) with formation of hydrogen peroxide (H_2O_2) that can be used by catalase (CAT) or glutathione peroxidases (GPx) enzymes (which uses GSH as electron donor to catalyze the reduction of H_2O_2 to H_2O). In the present study the activity of SOD increased in organisms exposed to MWCNTs, especially at the highest concentration (1.0 mg/L) which indicate an enzymatic response to eliminate ROS and to prevent LPO. Similar results were observed by Al-Shaeri et al. (2014), which demonstrated an oxidative status in *M. galloprovincialis* after 72 h exposure to 100 μg/L SWCNTs, measured as an increased SOD activity and LPO in gills.

Our results further revealed that GPx activity accompanied the increase of SOD activity except at the highest MWCNTs concentration where GPx levels were significantly lower. These findings indicate that H_2O_2 produced by SOD enzyme were probably eliminated by GPx up to a certain level of stress, however this mechanism was not effective at the highest exposure concentration (1.0 mg/L).

Therefore, despite an observable response in *R. philippinarum* clams to eliminate ROS by induced antioxidant enzymes activity, these defence mechanisms were not enough to prevent cellular damage, observed by the increase of LPO with the increasing of exposure concentration. Also Gomes et al. (2012) demonstrated that although SOD and GPx increased in mussels (*M. galloprovincialis*) exposed CuO NPs the activation of this defence strategy was not sufficient to prevent LPO. Similarly Cid et al. (2015) showed in *C. fluminea*, an incresead activity of CAT in bivalves exposed from 0.01 to 1.0 mg/L of NDs compared to control organisms, but these mechanisms were not able to eliminate the excess of ROS produced and to prevent organisms from LPO increase. Also Buffet et al. (2011) showed that the clam *Scrobicularia plana* exposed to copper nanoparticles (Cu NPs) (1 and 10 μg/L) for 16 days, presented increased activity of antioxidant enzymes SOD and CAT at the highest exposure concentrations, but still LPO increased along the increasing exposure gradient. Canesi et al. (2010), exposed *M. galloprovincialis* to different concentrations (0.05, 0.2, 1, 5 mg/L) of NP suspensions (nano carbon black, NCB; Nano fullerene, C60; Nano titanium dioxide, TiO_2; Silicon dioxide NPs, SiO_2) for 24 h, showing an inhinibition of CAT activity at highest concentrations of NCB and $nSiO_2$ as a conseguence of significant lysosomal membrane

destabilisation, which may explain GPx inhibition at the highest MWCNTs observed in the present study.

As previously discussed, GSSG is 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. When oxidative processes enhance formation of GSSG above the reducing capability of GR, the ratio GSH/GSSG is temporarily altered before the excess of GSSG is excreted through multidrug resistance-related protein 1, MRP1 (Regoli and Giuliani, 2014). This protein is able to eliminate metabolites resulting by glutathione S-transferase (GSTs) mediated conjugation of GSH to both xenobiotics and endogenous lipophilic compounds (Regoli and Giuliani, 2014). In fact, GSTs are a group of enzymes associated to Phase II metabolic processes involved in detoxification processes by catalyzing the conjugation of GSH to xenobiotic substrates (Lesser, 2006; Newman and Unger, 2003; Siddiqui et al., 1993; Wright and Welbourn, 2002). These phase II reactions result in water-soluble non-toxic products readily excreted through bile or urine (Regoli and Giuliani, 2014). In the presence of pollutants, bivalves may increase the intracellular concentrations of GSTs that allows them to function as biomarkers of cell injuries (Regoli and Giuliani, 2014).

In the present study the activity of GSTs increased with the increase of exposure concentration, revealing the capacity of clams to use these enzymes to detoxify MWCNTs. Nevertheless, along with the antioxidant responses, this mechanisms seemed to be not sufficient to prevent the occurrence of cellular damages in clams. Similar results were obtained by Cid et al. (2015) that showed that GSTs activity increased with increasing NDs concentrations in *C. fluminea* clams, although LPO still occurred. In the gills of *M. galloprovincialis* Canesi et al. (2010) demonstrated that, at the concentration of 5 mg/L, C60 fullerene induced an increase of GSTs activity due to a significant increase in lysosomal lipofuscin, an end-product of LPO. Also Ciacci et al. (2012) showed a stimulation of biotransformation enzymes activities (GSTs) as a conseguence of induction of LPO when *M. galloprovincialis* were exposed to different nano-oxides ($nTiO_2$, $nSiO_2$, nZnO and nano- Cerium dioxide ($nCeO_2$)). Nevertheless, Barmo et al. (2013) exposed *M. galloprovincialis* for 96 h to different concentrations of $nTiO_2$ suspensions demonstrating that these NPs induced a decrease in GSTs activity at 1 and 10 g/L (~49 and ~30% respectively) suggesting that low level of exposure may pose a serious risk to mussels, since alterations in functional and molecular immune parameters may impair the ability of animals to defend themselves against this emerging pollutant.

4.3. Induced neurotoxicity

Cholinesterases (ChEs) are an ubiquitous class of serine hydrolases which physiologically remove acetylcholine from the synaptic cleft (Valbonesi et al., 2003). Several studies have tried to use these neurotoxicity markers as a useful tool to evaluate the impact of pollutants among different invertebrate species (Ait Alla et al., 2006; Bocquené et al., 1997; De Marchi et al., 2017; Fossi Tankoua et al., 2012; Maranho et al., 2014; Mora et al., 1999; Pérez et al., 2004). However, even if these enzymes have been extensively studied in invertebrates, few studies regarding their activities are available on bivalves (Bebianno et al., 2004; Buffet et al., 2011; Galloway et al., 2002; Mora et al., 1999; Tim-Tim et al., 2009). In the present study, our results revealed that MWCNTs impaired the hydrolytic activity of ChEs. In fact, a significant inhibition of both enzymes (ATChI-ChE and PTChI-ChE) activity in *R. philippinarum* under MWCNTs exposure compared to control condition was demostrated. Our findings are in agreement with previous studies conducted in bivalves using other NPs, including studies by Marisa et al. (2016) that evaluated the effects of 1 and 10 μg/mL of nZnO for 7 days and demonstrated that in the gills of *R. philippinarum* clams AChE activity was significantly inibhited by exposure time and concentration/time interaction. Gomes et al. (2011) investigated the effects of 10 μg/L of CuO NPs for 15 days in the gills of *M. galloprovincialis* and showed an inhibition of AChE at the end of the exposure period. The effects in terms of ChE inhibition following MWCNTs exposure were already observed in organisms belonging to phylum Annelida. The earthworms (Lumbricidae) *Eisenia fetida*, exposed to 0.03 mg/g and 0.3 mg/g of MWCNTs-spiked soil for 14 days exhibited an AChE inhibition at the highest concentration (Calisi et al., 2016); similarly, in polychaetes such as *Diopatra neapolitana* and *Hediste diversicolor* exposed to MWCNTs ChEs inhibition was also observed (De Marchi et al., 2017).

5. Conclusions

R. philippinarum is one of the most widely used bioindicator species of environmental pollution. Several studies demonstrated the capacity of this species to reflect the impacts of a diversity of pollutants, and the present study presents novel information on the biochemical responses of this species when exposed to MWCNTs.

In particular, the present study showed for the first time that the exposure of *R. philippinarum* clams to MWCNTs caused an oxidative stress response. Although defence mechanisms were activated, such as the activity of antioxidant and biotransformation enzymes, clams were not able to prevent the occurrence of lipid peroxidation. Our findings also demonstrated that *R. philippinarum* clams under oxidative stress presented increased metabolic activity with expenditure of their energy reserves probably to fuel defence mechanisms. The results obtained further revealed that MWCNTs induced neurotoxicity in *R. philippinarum*, as the activity of cholinesterases (ATChI-ChE and PTChI-ChE) was greatly inhibited with the increase of exposure concentration.

Considering the increase of nanotechnology and industrial uses of nanomaterials and consequent release into aquatic ecosystems, the present study provides valuable information regarding the potential risk for the aquatic environment and living organisms, namely economically relevant resources as *R. philippinarum* clams.

Acknowledgments

This work was supported by CESAM (UID/AMB/50017), by FCT/MEC through national funds and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. Lucia De Marchi benefited from PhD grant (SFRH/BD/101273/2014) and Rosa Freitas benefited from a post-doc grant (SFRH/BPD/92258/2013) provided by the Portuguese FCT (Fundação para a Ciência e a Tecnologia).

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