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# Toxic effects of multi-walled carbon nanotubes on bivalves: Comparison between functionalized and nonfunctionalized nanoparticles

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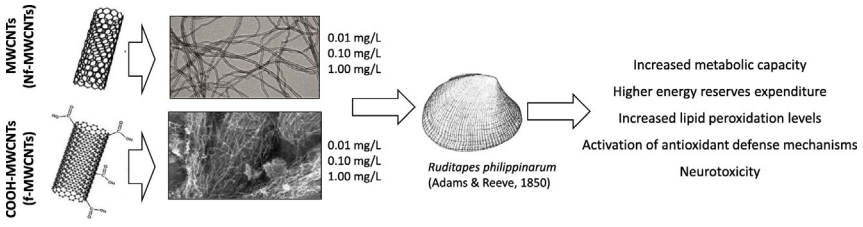
## HIGHLIGHTS

- Both Nf-MWCNTs and f-MWCNTs altered biochemical responses in *Ruditapes philippinarum*
- f-MWCNTs generated greater toxicity impacts in exposed clams compared to Nf-MWCNTs
- Inhibition of cholinesterases confirmed neurotoxicity of both MWCNTs materials
- *Ruditapes philippinarum* is a potential bioindicator to monitor a variety of carbon NMs

## GRAPHICAL ABSTRACT

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## ABSTRACT

Despite of the large array of available carbon nanotube (CNT) configurations that allow different industrial and scientific applications of these nanoparticles, their impacts on aquatic organisms, especially on invertebrate species, are still limited. To our knowledge, no information is available on how surface chemistry alteration (functionalization) of CNTs may impact the toxicity of these NPs to bivalve species after a chronic exposure. For this reason, the impacts induced by chronic exposure (28 days) to unfunctionalized MWCNTs (Nf-MWCNTs) in comparison with functionalized MWCNTs (f-MWCNTs), were evaluated in *R. philippinarum*, by measuring alterations induced in clams' oxidative status, neurotoxicity and metabolic capacity. The results obtained revealed that exposure to both MWCNT materials altered energy-related responses, with higher metabolic capacity and lower glycogen, protein and lipid concentrations in clams exposed to these CNTs. Moreover, *R. philippinarum* exposed to Nf-MWCNTs and f-MWCNTs showed oxidative stress expressed in higher lipid peroxidation and lower ratio between reduced and oxidized glutathione, despite the activation of defense mechanisms (superoxide-dismutase, glutathione peroxidase and glutathione S-transferases) in exposed clams. Additionally, neurotoxicity was observed by inhibition of Cholinesterases activity in organisms exposed to both MWCNTs.



## 1. Introduction

The use of carbon nanomaterials (CNMs) has increased rapidly in the last years, namely due to their important properties such as electromagnetic, optical, catalytic, mechanical, thermal, and pharmokinetics (Petersen and Henry, 2012).

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Currently, carbon nanotubes (CNTs) are one of the most important and commercially used CNMs (Potočnik, 2011; Sanchez et al., 2012). CNTs are hollow graphene cylinders that are microns to millimeters in length and can be divided in single-walled (SWCNTs) with a diameter of 0.7 to 3 nm, and multi-walled (MWCNTs) with a diameter of 10 to 25 nm (Baughman et al., 2002). CNTs are engineered with a wide variety of core structures and surface functionalizations that change their chemical and physical properties to enhance their suitability for different industrial applications (Arndt et al., 2013). Due to their hydrophobic and non-biodegradable characteristics, which make difficult the dispersion of CNTs in the water (Donaldson et al., 1998), once in the aquatic environment these nanoparticles can be accumulated by aquatic biota through body surface, digestive and respiratory systems (Jackson et al., 2013). Nevertheless, toxicological effects of CNTs, but in general of CNMs, depend on various factors including complex interplay between particle features (e.g., diameter, form, surface charge, and chemistry), concentration, time of exposure, nature of the materials, exposure medium composition, route of particle administration, and target species immune system (Khosravi-katuli et al., 2017). CNTs readily aggregate in solution, and even more in saltwater (Kataoka et al., 2016). For this reason they can get dispersible by functionalization which is 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 (Sun et al., 2002) and the presence of functional groups on nanotubes surface therefore increases nanotubes dispersibility (Shahnawaz et al., 2017). 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 (Shahnawaz et al., 2017). In a study conducted by Shahnawaz et al. (2017), the authors tried to compare the dispersibility and stability in aqueous solution of chemically functionalized (carboxyl groups) MWCNTs (f-MWCNTs) with unfunctionalized MWCNTs revealing that the presence of functional groups on the side wall of MWCNTs significantly increased their dispersibility. Water-dispersible CNTs have shown 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 (Arndt et al., 2013). Recently Kataoka et al. (Kataoka et al., 2016), investigated the toxicity of functionalized (SWCNTs) and unfunctionalized (N-SWCNTs) single-walled carbon nanotubes at the concentrations of 10 mg/L for 14 days on medaka embryos' behavior, and demonstrated that SWCNTs were densely adsorbed over the surface of the egg chorion further inducing acute toxicity to medaka embryos while N-SWCNTs exhibited no toxicity and were not adsorbed onto the egg chorion. Arndt et al. (2013) investigated the toxic effects of acute (48 h) and chronic exposures (21 days) of *Daphnia magna* to different types of CNMs that differed in core structure and surface functionalization such as $nC_{60}$, hydroxylated fullerenes ($C_{60}$-$OH_{24}$), SWCNTs, carboxylic acid functionalized SWCNTs (SWCNT-COOH), carboxy-amide functionalized SWCNTs (SWCNT-$CONH_2$), polyethylene glycol functionalized SWCNTs (SWCNT-PEG), and MWCNTs at concentrations of 0, 10, or 50 mg/L. These authors showed that overall nanomaterials up to 10 mg/L with a CNT core and functionalized were more toxic than $nC_{60}$ in terms of mortality, reproduction, and growth to daphnids.

Despite the large array of available CNT configurations that allow different applications of these nanoparticles, 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 (Coelho et al., 2014; Matozzo et al., 2016a; Torre et al., 2013a; Burgos-Aceves and Faggio, 2017; Torre et al., 2013b; Pagano et al., 2017; Pagano et al., 2016). Specifically, when exposed to pollutants, bivalves present a number of cellular responses that include antioxidant defences (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT)), metabolization mechanisms (e.g., glutathione S-transferases (GSTs)), cellular damage (e.g., lipid peroxidation (LPO)) and neurotoxicity (e.g., acetylcholinesterase (AChE)) (Bebianno et al., 2004). While several studies showed that these biochemical markers are useful in detecting possible damages under metal pollution (Velez et al., 2016; Wang et al., 2011; Yesudhason et al., 2013; Gomes et al., 2013) eutrophication (Verdelhos et al., 2005) and pharmaceuticals (Freitas et al., 2015; Antunes et al., 2013; Matozzo et al., 2016b; Correia et al., 2016), less is known on the biochemical alterations induced by CNMs in this group of organisms (Cattaneo et al., 2009; De Marchi et al., 2017a; Canesi and Corsi, 2015; Miller et al., 2015; Matranga and Corsi, 2012; De Marchi et al., 2017b).

The Manila clam *Ruditapes philippinarum* (Adams & Reeve, 1850), because of its considerable commercial value, was intentionally introduced and became established in several regions worldwide including the Pacific coast of North America and along the shores of Europe from the United Kingdom to the Mediterranean basin (Moura et al., 2017). This species has been already used to assess a diversity of stressors due to its wide distribution, long life cycle, ease of collection, and high capacity to bioaccumulate contaminants (Bebianno et al., 2004; Zhang et al., 2011; Savorelli et al., 2017). Nevertheless, to our knowledge, no information is available on how surface chemistry alteration (functionalization) of CNTs may impact the toxicity of these pollutants to this species after a chronic exposure. For this reason, the impacts induced by unfunctionalized MWCNTs (Nf-MWCNTs) and functionalized MWCNTs (f-MWCNTs) were evaluated in *R. philippinarum*, by measuring alterations induced in clams' oxidative status, neurotoxicity and metabolic capacity at the end of 28 days.

## 2. Materials and methods

### 2.1. Experimental setup

*R. philippinarum* specimens were collected in the northwest Atlantic coast of Portugal (40°38′ N, 8°45′ W). Bivalves with similar size (mean length: 23.2 ± 0.32 mm; mean weight: 7.9 ± 1.7) 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 sea salt (Tropic Marin® Sea Salt) (salinity 28) was added to deionized water to fill the aquaria. During the acclimation period organisms were under 12 h light: 12 h dark photoperiod, temperature (18 ± 1 °C) and aerated conditions. Every two-three days' specimens were fed with AlgaMac Protein Plus, Aquafauna Bio-Marine, Inc. (150,000 cells/animal).

After the acclimation and depuration period, 15 organisms per condition (3 aquaria/replicates *per* condition, with 5 organisms *per* aquarium/replica) were exposed during 28 days to two types of MWCNTs: unfunctionalized MWCNTs (Nf-MWCNTs) and chemically functionalized MWCNTs by introducing polar groups such as carboxyl groups (—COOH) (f-MWCNTs), both at the concentrations of 0.01; 0.10 and 1.00 mg/L. The choice of these two CNTs was based on their different physical and chemical properties, different behavior on the water media (aggregation/disaggregation, adsorption/desorption, sedimentation/resuspension and dissolution) (Arndt et al., 2013) as well as their industrial applicability. The exposure concentrations of both MWCNT were selected taking into consideration previous studies conducted by De Marchi et al. (De Marchi et al., 2017b; De Marchi et al., 2017c) which, using the same species (De Marchi et al., 2017b) or other invertebrates (polychaetes) (De Marchi et al., 2017c) and the same range of unfunctionalized MWCNTs, revealed observable biochemical responses from both these organisms, although the dynamic probabilistic material flow model (DP-MFA) showed that predicted environmental concentrations of CNTs in the water in the range of ng/L (Sun et al., 2016).

Salinity, pH, temperature and aeration conditions in each aquarium were set up as in the acclimation period (see above). Both Nf-MWCNT and f-MWCNT concentrations were re-established weekly after complete water renewals to ensure the same exposure concentrations

during the experiment. To promote stable suspension of both CNTs in the water column (Hwang et al., 2007), the Nf-MWCNTs was sonicated for 1 h using 30 Hz ultrasound bath (IKA Labortechnik IKASONIC U50), while the f-MWCNTs, (Shahnawaz et al., 2017), was sonicated by probe sonicator (55 W $cm^{-2}$) (UP 400S, hielscher Ultrasound Technology) for few minutes. The added MWCNTs (f and Nf) were homogenously dispersed in the seawater 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 bottom–water interface (Vonk et al., 2010).

### 2.2. MWCNTs characterization (Nf-MWCNTs and f-MWCNTs)

Both unfunctionalized and functionalized MWCNT materials were produced via the Catalytic Chemical Vapor Deposition (CCVD) process and characterized using Scanning Electron Microscopy (SEM) and Transmission electromicrographs (TEM) (Fig. 1A and B respectively). The Nf-MWCNTs were purchased from Nanocyl S.A. (MWCNTs: NC7000 series, http://www.nanocyl.com) while f-MWCNTs from Times Nano: Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com) and manufacturer's specifications are showed in Table 1.

The concentrations of both MWCNTs used in this study (0.01, 0.10 and 1.00 mg/L) were prepared from a stock solution of 50 mg/L concentration each. For particles characterization in the exposure medium, before water renewals, water samples (10 mL each) were collected from each aquarium at different periods: t0: time zero, immediately after medium contamination (dispersed Nf-MWCNT and f-MWCNT concentrations in artificial seawater); t7: 1 week after exposure, immediately before water renewals; t28: water samples collected after the fourth week of exposure.

Dynamic light scattering (DLS) measurements were carried out with a Delsa Nano C Beckman Coulter, Inc. (Fullerton, CA) equipped with a laser diode operating at 658 nm. Scattered light was detected at 165° angle and analyzed with a log correlator over 120 accumulations for a 2.0 mL of sample in a glass size cell. Each sample was reproducibly shaken before analysis and exposed to an appropriate number of DLS measurements needed to obtain at least three valid data. When no colloidal material was detected, result was reported as Invalid data (I.d.). The calculation of the particle size distribution and distribution averages were performed by CONTIN particle size distribution analysis routines through Delsa Nano 3.73 software. The hydrodynamic radius and polydispersity index of the analyzed dispersions were calculated on three replicates of each sample by the cumulant method. Due to the inherent heterogeneity and colloidal instability of the analyzed samples, DLS analyses were repeated five times to ensure reproducible results. The results are reported in the Table 2.

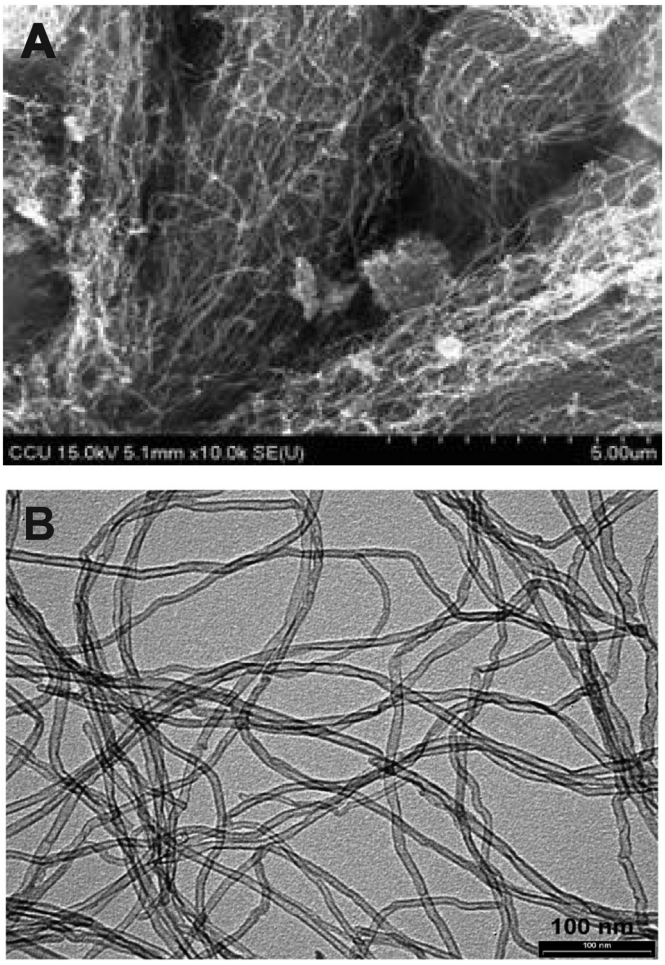


**Fig. 1.** A: Scanning Electron Microscopy (SEM) of the functionalized form MWCNTs-COOH (f-MWCNTs) produced via the catalytic carbon vapor deposition (CCVD) process; B: Transmission Electron Microscopy (TEM) of the powder form of MWCNTs produced via the catalytic carbon vapor deposition (CCVD) process.

### 2.3. Biochemical parameters

After 28 days exposure, clams used for biomarker analysis (3 *per* aquarium, 9 *per* condition) were frozen, pulverized individually with liquid nitrogen and divided in 0.5 g aliquots. Extractions were performed with specific buffers (De Marchi et al., 2017d; De Marchi et al., 2017c) for each biomarker. Biochemical analyses were performed in duplicate for each sample and biomarker with a BioTek Synergy HT micro-plate Reader. For lipids content determination the remaining organisms (2 *per* aquarium, 6 *per* condition) were dried at 60 °C for 48 h.

#### 2.3.1. Energy reserves and metabolic activity

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

Glycogen (GLY) content was quantified following the sulphuric acid method (Dubois et al., 1956), (glucose standards (0–2 mg/mL)). Absorbance was read at 540 nm and GLY expressed in mg *per* g of FW.

Lipid (LIP) content was determined following Yi et al. (2011). The absorbance was measured at 540 nm and the results were expressed in percentage *per* g dry weight (DW).

The electron transport system (ETS) activity was measured following King and Packard (1975) revised by De Coen and Janssen (1997) methods. The absorbance was measured at 490 nm during 10 min with intervals of 25 s. The results were expressed in nmol/min *per* g of FW.

#### 2.3.2. Antioxidant defences and biotransformation mechanisms

To quantify Superoxide-dismutase (SOD) activity, the method of Beauchamp and Fridovich (1971) was performed with a standard curve including SOD standards between 0.25 and 60 U/mL. Results were expressed in U *per* g of FW (U = 1.0 μmol nitroblue tetrazolium (NBT) *per* min). The absorbance was measured at 560 nm.

Glutathione peroxidase (GPx) was quantified in accordance with Paglia and Valentine (1967) methods. The results were expressed in U *per* g of FW (U = 1.0 μmol NADPH oxidized *per* min). The absorbance was read at 340 nm in 10 s intervals during 5 min.

Glutathione-S-transferase (GSTs) activity was determined using the extinction coefficient 9.6 mM $cm^{-1}$ for CDNB and the absorbance was read at 340 nm (Habig et al., 1976). Results were expressed in U *per* g of FW where U is defined as the amount of enzyme that produces 1 μmol of dinitrophenyl thioether *per* min.

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

| | Diameter (nm) | Length (μm) | 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.

*2.3.3. Indicators of cellular damage*

Lipid peroxidation (LPO), was determined according to the method described by Ohkawa et al. (1979), by the quantification of malondialdehyde (MDA) and expressed in nmol of MDA formed *per* g of FW. Absorbance was measured at 535 nm.

Reduced (GSH) and oxidized (GSSG) glutathione 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 nmol *per* g FW. The calculation of the ratio between oxidized and reduced glutathione was done dividing the GSH values by 2× the amount of GSSG.

*2.3.4. Neurotoxicity*

Acetylthiocholine iodide (ATChI, 470 μM) substrates were used for the determination of Acetylcholinesterase (ATChI-ChE) following the methods of Ellman et al. (1961) and modification by Mennillo et al. (2017). Enzyme activities were recorded continuously for 5 min at 412 nm and expressed in nmol *per* min *per* g FW.

*2.4. Data analysis*

All the biochemical results (PROT, GLY LIP, ETS, SOD, GPx, GSTs, LPO, GSH/GSSG, and ATChI-ChE) were submitted to hypothesis testing using permutational multivariate analysis of variance with the PERMANOVA + add-on using PRIMER v6 software. The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different. The null hypotheses tested were: i) for each biomarker and for each MWCNT material, no significant differences existed among exposure concentrations (0.00, 0.01, 0.10, 1.00 mg/L); ii) for each biomarker and for each exposure concentration (0.00, 0.01, 0.10, 1.00 mg/L) no significant differences existed between MWCNT materials. Significant differences concerning each biomarker among exposure concentrations were represented with different letters (lowercase letters for f-MWCNTs; uppercase letters for Nf-MWCNTs). Significant differences concerning each biomarker between the two MWCNT materials at each exposure concentration were represented with asterisks.

The matrix gathering biochemical descriptors *per* condition were used to calculate an Euclidean distance similarity matrix. The similarity matrix was simplified through the calculation of the distance among centroids matrix, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of biochemical descriptors (correlation >0.75) were provided.

**Table 2**
Dynamic Light Scattering (DLS) data of Size (nm) and Polydispersity Index (PDI) in exposure medium (0.01 mg/L f-MWCNTs; 0.01 mg/L Nf-MWCNTs; 0.10 mg/L f-MWCNTs; 0.10 mg/L Nf-MWCNTs; 1.00 mg/L f-MWCNTs and 1.00 mg/L Nf-MWCNTs) collected at different exposure periods (t0; t7 and t28). I.d.: "Invalid data" (no colloidal material detected into the analyzed sample). n.d.: absence of triplicates values for mean size calculation.

| Samples | Nanoparticles | Size (nm) | PDI |
|---|---|---|---|
| t0_0.01 mg/L | Nf-MWCNTs | 3434.0 | n.d |
| | f-MWCNTs | 2987.3 | – |
| t7_0.01 mg/L | Nf-MWCNTs | 3432.2 | n.d |
| | f-MWCNTs | n.d. (5 I.d.) | – |
| t28_0.01 mg/L | Nf-MWCNTs | 3430.0 | n.d |
| | f-MWCNTs | n.d. (5 I.d.) | – |
| t0_0.10 mg/L | Nf-MWCNTs | 2407.1 | 0.98 |
| | f-MWCNTs | 3244.8 | 1.30 |
| t7_0.10 mg/L | Nf-MWCNTs | n.d (3 I.d.) | n.d. |
| | f-MWCNTs | n.d. (5 I.d.) | – |
| t28_0.10 mg/L | Nf-MWCNTs | 4542.7 | 1.81 |
| | f-MWCNTs | n.d. (5 I.d.) | – |
| t0_1.00 mg/L | Nf-MWCNTs | 5714.4 | 1.45 |
| | f-MWCNTs | 3806.1 | 1.30 |
| t7_1.00 mg/L | Nf-MWCNTs | 3602.9 (1 I.d.) | 1.39 |
| | f-MWCNTs | n.d. (5 I.d.) | – |
| t28_1.00 mg/L | Nf-MWCNTs | 3865.2 | 1.40 |
| | f-MWCNTs | n.d. (5 I.d.) | – |

## 3. Results

*3.1. MWCNTs characterization (Nf-MWCNTs and f-MWCNTs)*

As reported in the literature, DLS measurements have been routinely carried out as an effective tool to observe the evolution of relative particle size distributions of carbon nanotubes in aqueous media as a function of time (Moon et al., 2009; Wang et al., 2010; Xu et al., 2011). In the present work, DLS measurements were carried out to obtain data regarding the tendency to aggregate and the settling behavior of suspended CNTs in aqueous media. In Table 2 results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized Nf-MWCNTs and f-MWCNTs particle aggregates in aqueous media under the adopted experimental conditions are reported. The mean size of the suspended particle aggregates was determined by application of the cumulant method. The mean diameters of f-MWCNTs particles at the time zero (t0) were found to be smaller than those calculated for Nf-MWCNTs aggregates under the same experimental conditions indicating an improved dispersion in the aqueous media (Table 2).

DLS and polydispersity index (PDI) analysis of experimental samples exposed to different concentrations of f-MWCNTs (0.01 mg/L, 0.10 mg/L, 1.00 mg/L) among collection periods (t7 and t28) did not detect measurable macro/micro/nano-sized particle aggregates (Table 2). Concerning samples prepared with 0.01; 0.10 and 1.00 mg/L of Nf-MWCNTs observed among collection periods (t0, t7 and t28), these 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 2). Furthermore, a time-dependent increase of the PDI in each condition was also possible to observe, due to the generation of large particles or aggregates in the investigated samples, with one exception for 0.01 mg/L Nf-MWCNTs, where the detection of measurable macro/micro/nano-sized particle aggregates was not possible.

*3.2. Biochemical parameters*

*3.2.1. Energy reserve and metabolic activity*

*R. philippinarum* exposed to Nf-MWCNTs presented a decrease in PROT content with the increase of exposure concentration, with significant differences among all exposure conditions (Fig. 2A). Clams exposed to f-MWCNTs presented significantly lower PROT content compared to non-exposed organisms, but in this case no significant differences were observed among clams exposed to different MWCNTs concentrations (Fig. 2A). Significant differences between the two MWCNT materials at each of the tested concentrations (control-0.00, 0.01, 0.10, 1.00 mg/L) were only detected in clams exposed to 0.01 mg/L, with higher PROT content in organisms exposed to Nf-MWCNTs.

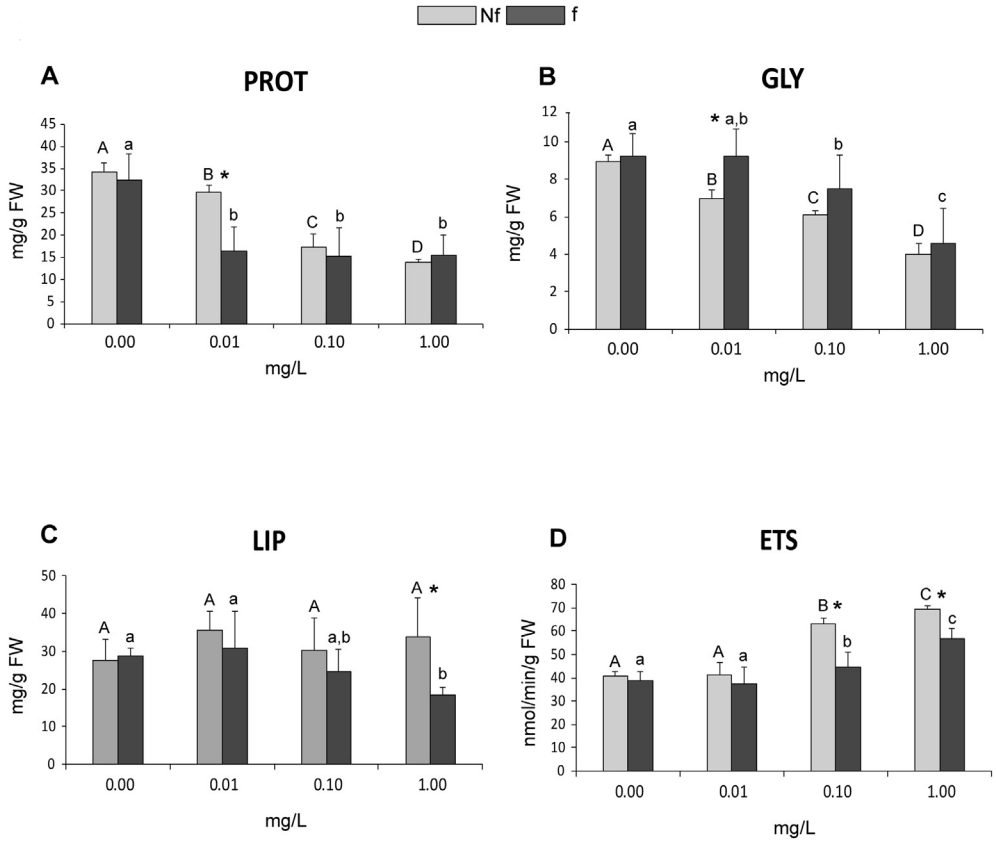


**Fig. 2.** A: Protein (PROT) content; B: Glycogen (GLY) content; C: Lipid (LIP) content; D: electron transport system (ETS) activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (lowercase letters for f-MWCNTs; uppercase letters for Nf-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNT materials at each exposure concentration were represented with asterisks.

All the clams exposed to Nf-MWCNTs presented decreased GLY content along the increase of exposure gradient, with significant differences among all tested conditions (Fig. 2B). A similar pattern was observed in organisms under f-MWCNTs, with significantly lower GLY content in clams exposed to 0.10 and 1.00 mg/L f-MWCNTs compared to control organisms (Fig. 2B). When comparing organisms exposed to two different MWCNT materials, significant differences in GLY content were observed in organisms exposed to 0.01 mg/L conditions (Fig. 2B).

The LIP content in organisms exposed to Nf-MWCNTs was similar among all exposure concentrations, with no significant differences among conditions, while the LIP content decreased at 0.10 and 1.00 mg/L in clams under f-MWCNTs, with significantly lower LIP content at the highest concentration (Fig. 2C). Significant differences in LIP content between the two MWCNT materials was observed at 1.00 mg/L (Fig. 2C).

In *R. philippinarum* exposed to Nf-MWCNTs and f-MWCNTs, the ETS activity significantly increased with the increase of exposure concentrations, with significantly higher ETS activity in organisms exposed to 0.10 and 1.00 mg/L in comparison to organisms exposed to control and 0.01 mg/L (Fig. 2D). Between MWCNTs materials, 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 (Fig. 2D).

### 3.2.2. Antioxidant defences and biotransformation mechanisms

The activity of SOD in clams exposed to Nf-MWCNTs increased with the increase of exposure concentrations with significant differences among conditions, while in organisms exposed to f-MWCNTs SOD activity was only significantly increased at 0.10 and 1.00 mg/L in comparison to organisms exposed to the remaining conditions (control-0.00 mg/L and 0.01 mg/L) (Fig. 3A). Significant differences between *R. philippinarum* submitted to different MWCNT materials for each of the tested concentrations were observed at 0.10 and 1.00 mg/L with higher SOD activity in organisms exposed to f-MWCNTs (Fig. 3A).

The activity of GPx in clams exposed to Nf-MWCNTs significantly increased at 0.01 and 0.10 mg/L in comparison to control values, while at the highest exposure concentration (1.00 mg/L) the activity of GPx was significantly lower than control levels (Fig. 3B). Different GPx activity was recorded in clams under f-MWCNTs, where significantly higher activity was observed between the two highest f-MWCNTs (0.10 and 1.00 mg/L) and the remaining conditions (control and 0.01 mg/L) (Fig. 3B). Between MWCNT materials, significant differences in GPx activity were observed at all exposure conditions, with higher values in clams exposed to Nf-MWCNTs at concentrations 0.01 and 0.10 mg/L while at the highest exposure concentration (1.00 mg/L) significantly higher GPx activity was observed in clams exposed to f-MWCNTs (Fig. 3B).

Clams exposed to Nf-MWCNTs decreased the GSTs activity with the increase of exposure concentrations with significant differences between control and the remaining conditions as well as between clams exposed to 0.10 and 1.00 and those exposed to 0.01 mg/L (Fig. 3C). The activity of GSTs also decreased with the increase of exposure concentrations in organisms under f-MWCNTs, with significant differences between control and individuals exposed to 0.10 and 1.00 mg/L f-MWCNTs (Fig. 3C). Comparing GSTs activity in *R. philippinarum* exposed to different MWCNT materials, significant differences were only recorded at the two highest concentrations (0.10 and 1.00 mg/L), with organisms under Nf-MWCNTs presenting higher enzymatic activities (Fig. 3C).

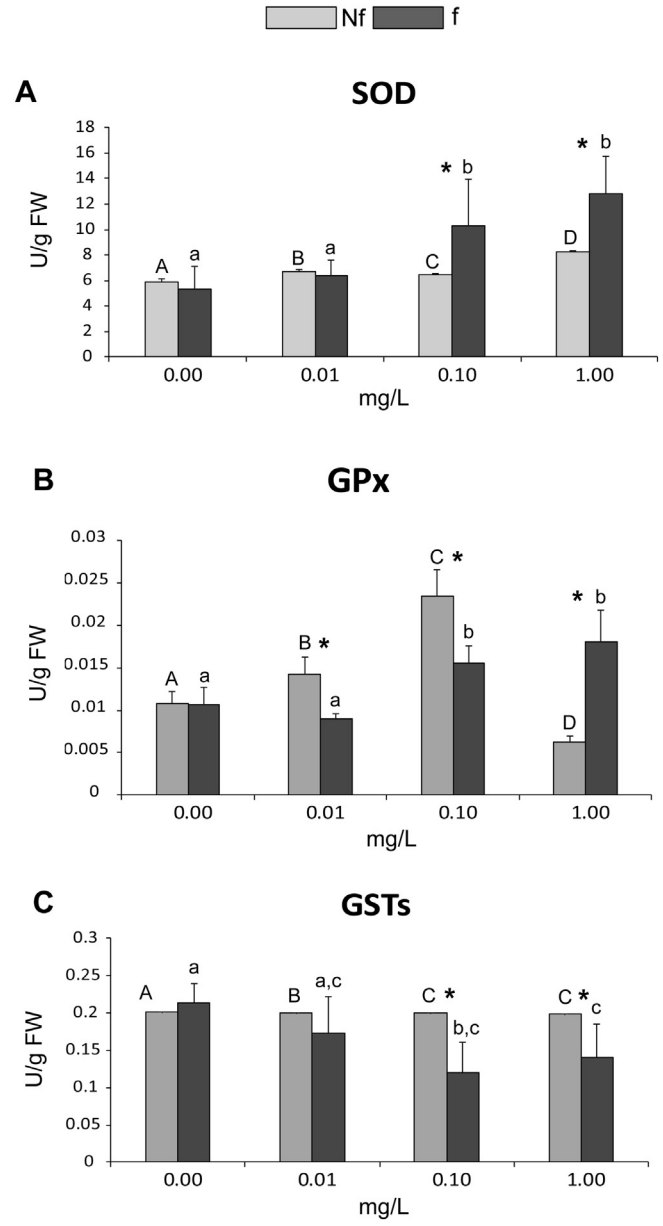


**Fig. 3.** A: Superoxide dismutase (SOD) activity; B: Glutathione peroxidase (GPx) activity; C: Glutathione S-transferases (GSTs) activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (lowercase letters for f-MWCNTs; uppercase letters for Nf-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNT materials at each exposure concentration were represented with asterisks.

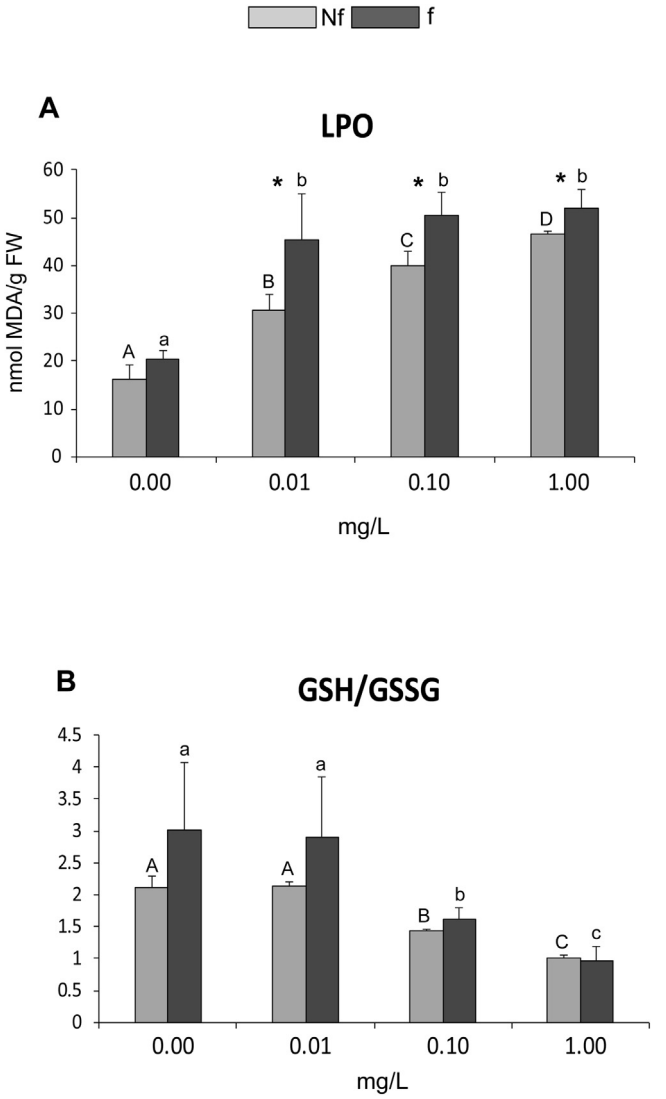


**Fig. 4.** A: Lipid peroxidation (LPO) levels; B: GSH/GSSG (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (lowercase letters for f-MWCNTs; uppercase letters for Nf-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNT materials at each exposure concentration were represented with asterisks.

### 3.2.3. Indicators of cellular damage

Specimens exposed to Nf-MWCNTs increased LPO levels with the increase of exposure concentrations, with significant differences among all tested conditions (Fig. 4A). Increased LPO was also observed in clams under f-MWCNTs, with significant differences among contaminated and non-contaminated (control) organisms and no significant differences in LPO levels among organisms exposed to different NF-MWCNT concentrations (Fig. 4A). Between MWCNT materials, significant differences were observed at all exposure concentrations (0.01, 0.10, 1.00 mg/L), with the highest LPO levels in *R. philippinarum* under f-MWCNTs (Fig. 4A).

At both tested CNTs, (Nf-MWCNTs and f-MWCNTs), GSH/GSSG was significantly lower in clams exposed to 0.10 and 1.00 mg/L in comparison to clams exposed to 0.00 (control) and 0.01 mg/L (Fig. 4B). Clams exposed to control and 0.01 mg/L showed no significant differences in GSH/GSSG values. 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 MWCNTs, although higher values were observed for f-MWCNTs organisms (Fig. 4B).

### 3.2.4. Neurotoxicity

ATChI-ChE activity presented significantly lower values in contaminated organisms exposed to Nf-MWCNTs in comparison 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. 5). Decreased of ATChI-ChE activity was also observed in organisms exposed to f-MWCNTs, with significant differences between individuals at control and all the remaining conditions with the lowest value at the highest exposure concentration. However, no significant differences were observed between clams under 0.01 and 0.10 mg/L (Fig. 5). Comparing MWCNT materials, significant differences in terms of ATChI-ChE activity were obtained at the lowest

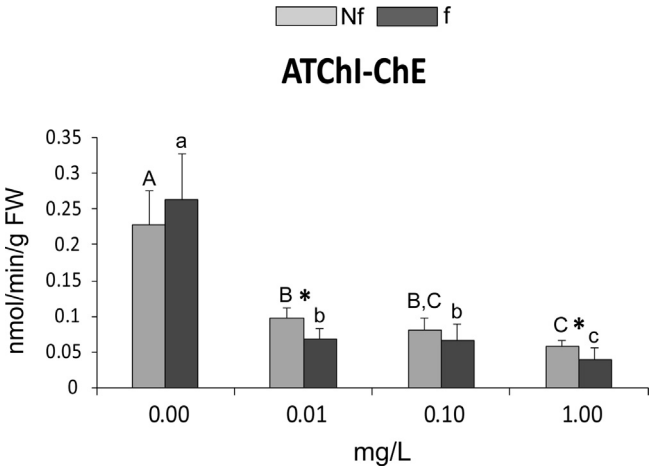


**Fig. 5.** ATChI-ChE activity in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters (lowercase letters for f-MWCNTs; uppercase letters for Nf-MWCNTs). Significant differences ($p \leq 0.05$) between the two MWCNT materials at each exposure concentration were represented with asterisks.

(0.01 mg/L) and the highest (1.00 mg/L) concentrations, with higher values in organisms exposed to Nf-MWCNTs (Fig. 5).

### 3.3. Multivariate analysis

Principal coordinates analysis (PCO) graph obtained is shown in Fig. 6. PCO axis 1 explained 56.9% total variation, while PCO axis 2 explained 18.8% (Fig. 6). PCO1 separated individuals exposed to both control conditions (Nf and f-MWCNTs) and 0.01 mg/L Nf-MWCNTs at the negative side from clams exposed to 0.10 and 1.00 mg/L of both MWCNT materials and 0.01 mg/L f-MWCNT in the positive side. PCO2 separated individuals exposed to 0.10 mg/L and 1.00 mg/L Nf-MWCNTs in the negative side from the remaining conditions in the positive side. Non-

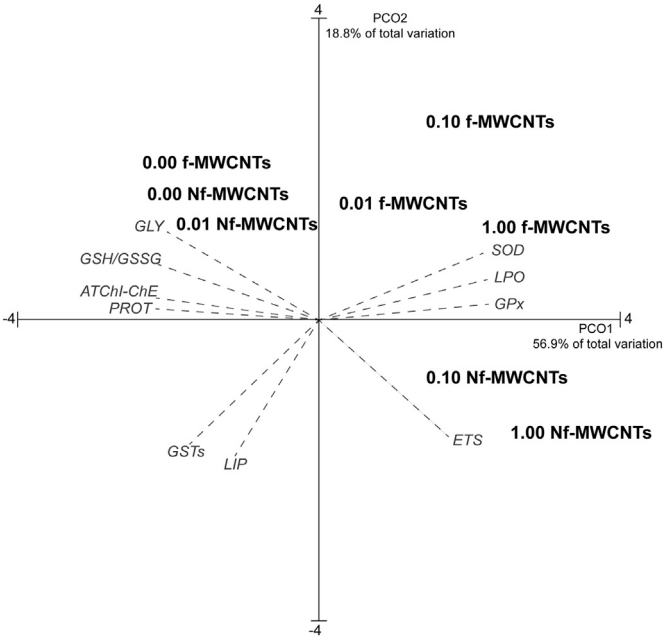


**Fig. 6.** Centroids ordination diagram (PCO) based on biochemical parameters, measured in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.01; 0.10 and 1.00 mg/L). Pearson correlation vectors are superimposed as supplementary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

contaminated organisms and organisms exposed to the lowest concentration of unfunctionalized NMs (0.01 mg/L) were associated to ATChI-ChE, PROT, GLY and GSH/GSSG as these markers presented the highest values at these conditions. Individuals exposed to f-MWCNTs (0.01, 0.10 and 1.00 mg/L) were closely related to SOD, GPx and LPO, where the highest values for these biomarkers were recorded. Organisms exposed to Nf-MWCNTS (0.10 and 1.00 mg/L) were close related to ETS values, where higher ETS values were observed.

## 4. Discussion

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). As compared to pristine and organic soluble carbon NMs, water-soluble CNTs have the highest probability of entering the human body and the aquatic environment (Klaper et al., 2010). There are two basic strategies to increase dispersibility of CNTs in water. The first one is the chemical functionalization of CNTs by introducing polar groups such as carboxyl groups and the other one is the physical functionalization of CNTs by adsorption of surfactants and polymers to decrease the van der Waals interaction (Shahnawaz et al., 2017). Carboxylation of SWCNTs, as well as MWCNTs, by introduction of polar groups such as carboxyl groups (—COOH), showed 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). Our results are in agreement with the this finding, clearly showing that both Nf-MWCNTs and f-MWCNTs were able to generate oxidative stress in the exposed clams and were also responsible for changes in organisms' metabolism (expressed in alteration of energy reserves) and neurotoxicity induction in *R. philippinarum*, however greater impacts were caused by f-MWCNTs, namely in terms of metabolic capacity (GLY, LIP and ETS), and oxidative stress responses (LPO, GSH/GSSG, SOD and GPx) compared to Nf-MWCNTs.

Energy metabolism plays a fundamental role in organisms' survival and function, as well as in stress adaptation and tolerance (Sokolova et al., 2012). It was already demonstrated that once the organisms are exposed to pollutants they can increase energy expenditure (considered a mechanism of cellular protection) (Bielen et al., 2016). Recently, different authors demonstrated expenditure of energy reserves (expressed as a decrease of glycogen (GLY) and protein (PROT) content) in invertebrates exposed to carbon NMs (De Marchi et al., 2017b; De Marchi et al., 2017a; De Marchi et al., 2017c; De Marchi et al., 2017d). In agreement with such findings, 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. Similarly, De Marchi et al. (2017b) studied the response of *R. philippinarum* exposed to two concentrations of MWCNT (0.10 and 1.00 mg/L) under pH variations (control-7.9 and 7.6) for 28 days, and observed a decrease of PROT and GLY content at both salinity conditions.

Lipid (LIP) content is considered another important reserve of stored energy and has been also examined as an indicator of sub-lethal toxicity in invertebrates (Herbes and Allen, 1983). The relationship of LIP, as energy storage, and environmental stressors has been demonstrated by analyzing LIP components in marine invertebrates (Gardner et al., 1985; Abele and Puntarulo, 2004; Dickinson et al., 2012; Oliveira et al., 2017; Faggio et al., 2016; Messina et al., 2014). In the present study clams exposed to Nf-MWCNT preserved their LIP content while using GLY and PROT as primary energy source to fuel defense mechanisms against CNTs. However, when exposed to f-MWCNTs clams showed not only a decrease in GLY and PROT concentrations but also in LIP content. These results may probably indicate that f-MWCNTs may induce greater

impacts than Nf-MWCNTs and therefore, more energy expenditure was necessary to fight against the impacts induced.

The balance between energy reserves (PROT, GLY and LIP) and mitochondrial electron transport system (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; Bielen et al., 2016; Freitas et al., 2016; Schmidlin et al., 2015; Simčič et al., 2014; Aliko et al., 2015),due to the ability to release the energy stored within the reduced hydrogen carriers in order to synthesize ATP (Liu et al., 2002). The results obtained in the present study clearly demonstrated that after 28 days exposure to both Nf-MWCNTs and f-MWCNTs, the clams presented increased metabolism (ETS) with the increase of exposure concentrations, which can be associated to energy expenditure observed at higher exposure concentrations. De Marchi et al. (2017b) also observed a slight increase of the ETS activity in *R. philippinarum* exposed to MWCNT concentrations under both pH conditions for 28 days.

The 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). Mitochondrial ROS generation is a physiologically significant process *in vivo*, and that mitochondrial Superoxide-dismutase (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 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 $H_2O$) (Regoli and Giuliani, 2014). Under stressful conditions, namely exposure to different NMs, ROS are overproduced. However invertebrate species are known to increase the activity of SOD in response to the generated oxidative stress (De Marchi et al., 2017b; De Marchi et al., 2017c; De Marchi et al., 2017d; Buffet et al., 2014a; Gomes et al., 2011; Buffet et al., 2011; Zhu et al., 2011; Mccarthy et al., 2013; Gomes et al., 2014; Gomes et al., 2012). In the present study clams exposed to Nf-MWCNTs and f-MWCNTs increased the activity of SOD, indicating the activation of this antioxidant mechanisms both when clams were exposed to Nf and f-MWCNTs, an enzymatic response to eliminate ROS and to prevent cellular damage (e.g. lipid peroxidation).

As mentioned above, despite being an antioxidant, SOD represents a source of $H_2O_2$, thus being necessary that its activity is coordinated with $H_2O_2$ reducing enzymes such as CAT or GPx (Regoli and Giuliani, 2014). 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 NMs has been demonstrated (De Marchi et al., 2017b; Volland et al., 2015; Gomes et al., 2014; Gomes et al., 2012). Considering clams exposed to Nf-MWCNTs in the present study, the antioxidant activity of GPx increased at 0.10 mg/L, but decreased at the highest Nf-MWCNTs. This discrepancy may result from $H_2O_2$ produced by SOD that could be eliminated by GPx up to a certain level of stress, but at 1.00 mg/L (the highest exposure concentration) the enzyme activity could be inhibited due to a shift in the balance between oxidants and antioxidants in favor of oxidants, resulting in the increase of pollutants-induced oxidative effect (Chatziargyriou and Dailianis, 2010). On the other hand, the activity of GPx was not inhibited in clams exposed to the two highest concentration of f-MWCNTs (0.10 and 1.00 mg/L). Thus, our findings may indicate that the hydrogen peroxide produced by SOD is possibly being converted not by GPx but by other antioxidant systems which contribute in the defense against oxidative stress, e.g. CAT.

In the presence of NMs, invertebrates may also increase the activity of Glutathione-S-transferases (GSTs), a group of enzymes used as a biomarker to evaluate the detoxification capacity of organisms (Volker et al., 2014; Garaud et al., 2014; Cid et al., 2015; Minetto et al., 2014; Minetto et al., 2016; Ciacci et al., 2012). In the present study, the GSTs activity in clams exposed to Nf-MWCNTs slightly increased up to exposure concentrations of 0.10 mg/L, indicating that these enzymes could be involved in detoxification of Nf-MWCNTs in *R. philipinarum*. In agreement with the present results, Cid et al. (2015), exposed *Corbicula fluminea* clams to 0.01, 0.1, 1, and 10 mg/L of carbon nanodiamonds (NDs) throughout 14 days, and showed an increase of GSTs activity with increasing NDs concentration. However the behavior of the antioxidant enzymes is dependent on the type and concentration of the NMs (Canesi and Corsi, 2015). Indeed, in our study clams exposed to f-MWCNTs showed a decrease of GSTs activity with the increasing exposure concentration, indicating that these group of enzymes were not involved in the biotransformation of f-MWCNTs into less toxic excreted substance. In agreement with the present results, Anisimova et al. (2015) observed a decrease of GSTs activity in *Crenomytilus grayanus* mussels exposed to 12–14 nm diameter of MWCNTs (100 mg/L) after 48 h.

Under stressful conditions, the excess of ROS produced by the organisms, may not be eliminated by defense mechanisms such as antioxidant enzymes leading to lipid peroxidation (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 NMs 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 (De Marchi et al., 2017a; De Marchi et al., 2017b; Volland et al., 2015; De Marchi et al., 2017c; De Marchi et al., 2017d; Zhu et al., 2011; Mccarthy et al., 2013; Gomes et al., 2014; Cid et al., 2015; Anisimova et al., 2015; Ayala et al., 2014; Buffet et al., 2014b; Tedesco et al., 2010). In the present study, clams exposed to Nf-MWCNTs showed a gradual increase of LPO levels with the increase of exposure concentration, while clams submitted to f-MWCNTs presented a steeper increase of LPO levels at the lowest exposure concentration after which LPO only slightly increased, which may be associated with increased SOD activity at the two highest exposure concentrations. Anisimova et al. (2015) exposed *C. grayanus* to 12–14 nm diameter MWCNTs (100 mg/L) for 48 h, and showed that CNTs were responsible for the increase of LPO levels. Using the same CNTs, De Marchi et al. (2017b) also showed increased LPO levels in organisms exposed to 0.10 and 1.00 mg/L MWCNTs under different pH levels (Correia et al., 2016; Wang et al., 2011) for 28 days.

Oxidative stress has often been associated to 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). NMs also showed to affect the processes involved in the maintenance of tissue redox balance in invertebrates, expressed as the decrease of GSH/GSSG (De Marchi et al., 2017b; De Marchi et al., 2017c; De Marchi et al., 2017d; 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 in organisms exposed to both MWCNT materials decreased with the increase of exposure concentrations (especially at 0.10 and 1.0 mg/L), indicating that the stress induced by carbon NMs led to a decrease of GSH that was oxidized to GSSG. Using nonfunctionalized MWCNTs, Anisimova et al. (2015) observed GSH increased in hemolymph of *C. grayanus* on the second day of exposure in respect to control.

Recently the inhibition of cholinesterase in invertebrates has been used as a sensitive biomarker of exposure to various NMs (De Marchi et al., 2017b; Marisa et al., 2016; De Marchi et al., 2017c; Buffet et al., 2014a; Gomes et al., 2011; Luis et al., 2016; Buffet et al., 2014c). 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. Inhibition of AChE activity by NMs has been shown, and is primarily caused by adsorption or directly interaction with AChE (Lionetto et al., 2011). Our 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. The decrease of the activity at all MWCNT exposure concentrations for both materials (Ng and f) may have been caused due to high affinity of MWCNT and SWCNT for AChE, and their ability to cause 76–88% AChE activity reductions (Wang et al., 2009).

## 5. Conclusion

In the present study, it was clearly demonstrated that nanomaterial toxicity can be attributed to 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 NMs in different fields and industrial applications and consequent release into aquatic ecosystems, the present study provides valuable information regarding the potential risk of MWCNTs in the aquatic environment and living organisms, namely economically relevant resources like *R. philippinarum*. 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 NMs. Consequently, future studies must include more realistic exposure scenarios to drive accurate safety levels toward biodiversity conservation.

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