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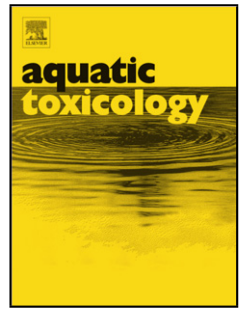

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Effects of multi-walled carbon nanotube materials on *Ruditapes philippinarum* under climate change: the case of salinity shifts

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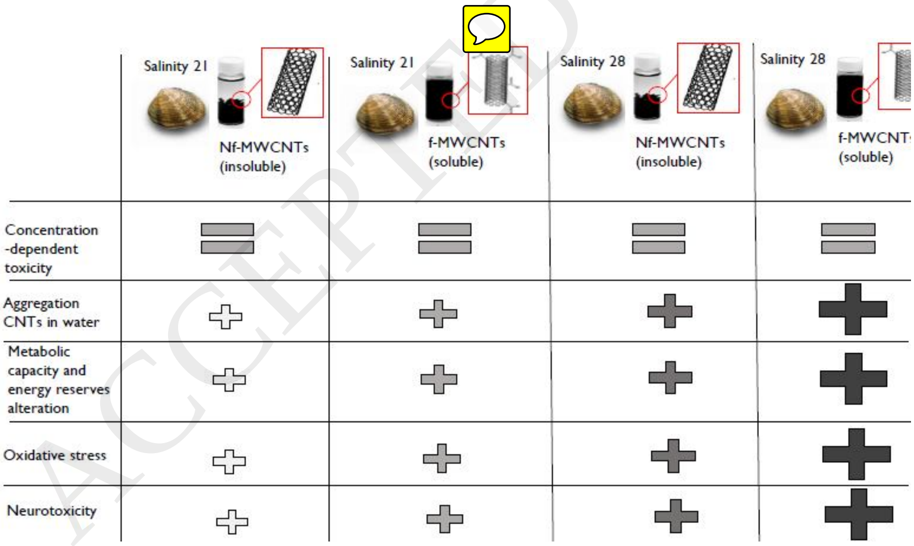
Graphical abstract



Highlights

- Concentration-dependent toxicity was observed in clams exposed to both CNTs and both salinities
- Major toxicity was caused by salinity 28 on the chemical behavior of CNTs and on their effects in exposed clams in comparison to salinity 21
- Greater toxic impacts were induced in exposed organisms by f-MWCNTs compared to Nf-MWCNTs

Abstract

The toxicity of carbon nanotubes (CNTs) is closely related to their physico-chemical characteristics as well as the physico-chemical parameters of the media where CNTs are dispersed. In a climate change scenario, changes in seawater salinity are becoming a topic of concern particularly in estuarine and coastal areas. Nevertheless, to our knowledge no information is available on how salinity shifts may alter the sensitivity (in terms of biochemical responses) of bivalves when exposed to different CNT materials. For this reason, a laboratory experiment was performed exposing the Manila clams *Ruditapes philippinarum*, one of the most dominant bivalves of the estuarine and coastal lagoon environments, for 28 days to unfunctionalized multi-walled carbon nanotube MWCNTs (Nf-MWCNTs) and carboxylated MWCNTs (f-MWCNTs), maintained at control salinity (28 PSU) and low salinity 21 (PSU). Concentration-dependent toxicity was demonstrated in individuals exposed to both MWCNT materials and under both salinities generating alteration of energy reserves and metabolism, oxidative stress biomarker responses and neurotoxicity induction compared to non-contaminated clams. Moreover, our results showed greater toxic impacts induced in exposed clams by f-MWCNTs compared to Nf-MWCNTs. In the present study it was also demonstrated how salinity shifts altered the toxicity of both MWCNT materials as well as the sensitivity of *R. philippinarum* exposed to these contaminates in terms of clam metabolism, oxidative status and neurotoxicity.

Keywords: *Ruditapes philippinarum*; salinity shifts; carboxylated Multi-Walled Carbon Nanotubes; unfunctionalized Multi-Walled Carbon Nanotubes; oxidative stress

INTRODUCTION

In recent years, the rapid development of nanomaterials (NMs) in different fields, has increased their application and consequently their production and commercialization (Montagner et al. 2016). Among NMs, the carbon-based ones (CNMs) are among the most widely researched and used due to their unique combination of chemical and physical properties (Cha et al. 2013), with a predominant role occupied by carbon nanotubes (CNTs) (Qiu et al. 2010). The diversity of CNT properties such as aspect ratio, mechanical strength, electrical and thermal conductivity, high tensile strength, high flexibility and elasticity, low thermal expansion coefficient and them being good electron field emitters (Liu & Cheng 2013), make these materials very attractive to different consumer products (see the Woodrow Wilson database: http://www.nanotechproject.org/inventories/consumer/) (Petersen & Henry 2012). A study published by Lawal (2015) showed that the CNT market is expected to grow from an estimated $ 3.43 billion in 2016 to $ 8.70 billion by 2022. This growth, however, needs to be accompanied by an interest in the nanosafety of CNTs, in order to reduce possible risks to the environment, especially to the aquatic environment, where they can ultimately accumulate.

The toxicity of CNTs is closely related to their physico-chemical characteristics (Lanone et al. 2013). Among the various determinants known to influence the behavior of CNT, functionalization has been considered and investigated (Allegri et al. 2016). Functionalization is a chemical modification of the structure such as amidation and esterification of the nanotube-bound carboxylic acids (Sun et al. 2002). As an example, the chemical functionalization of CNTs by introducing polar groups such as carboxyl groups (-COOH) in order to improve better dispersibility in the media (Shahnawaz et al. 2017) is one of the most common approaches. Hydrophobic nanoparticles tend to aggregate in water system, while hydrophilic nanoparticles are likely to be stable in the water media for long periods (Brar et al. 2010). Moreover, water-dispersible CNTs have been shown to have an increased amount of 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).

Nevertheless, the toxicity of CNTs is not only dependent on their physico-chemical characteristics, but also on the physico-chemical parameters of the media where the NMs are dispersed (Jastrzębska et al. 2012) and salinity is one of the main factors influencing NM behavior (Chinnapongse et al. 2011). Changes in the salinity of the aqueous environment can influence the stability of nanoparticles, which might change their toxicity to organisms (Jastrzębska et al. 2012). It has already been demonstrated that NMs transferred from fresh water to seawater decreased their zeta potential (because of the higher ionic strength of seawater due to salinity), thus causing aggregation and precipitation (Wong et al. 2013).

Salinity plays a fundamental role in aquatic systems, and may be pronouncedly affected by environmental factors related to climate change, including the occurrence of extreme weather events (Lapresta-Fernández et al. 2012; IPCC 2013). Changes in salinity are of especial concern in estuarine and coastal areas (Cardoso et al. 2008) impairing growth and reproduction of inhabiting estuarine population, as well as impacts on functioning of food webs (Calliari et al. 2008). Estuarine bivalves are often exposed to short-term (tidal) and long-term (rain periods) changes in salinity. However, recently, the increased stress may have lead to mortality episodes (Verdelhos et al. 2015).

One of the most dominant bivalves of the estuarine and coastal lagoon environments is the clam species *Ruditapes philippinarum* (Adams & Reeve, 1850) (Jensen et al. 2005). Due to relatively high fecundity and growth rates, this species has become widespread all over the world. The current worldwide distribution of *R. philippinarum* is mostly based on the intentional introduction of the clam for economic exploitation during the twentieth century, including both fisheries and aquaculture. (http://www.fao.org/fishery/culturedspecies/Ruditapes_philippinarum/en). Furthermore, studies showed that *R. philippinarum* possess sub-cellular mechanisms (which include antioxidant defenses, metabolization mechanisms, tolerance of cellular damages and neurotoxicity) (Bebianno et al. 2004) that allow them to cope with the toxic effects of different stressors such as pollutants (metal pollution (Liu et al. 2011; Wang et al. 2011; Ji et al. 2015; Cátia Velez et al. 2016; Oaten et al. 2016), pharmaceuticals (Antunes et al. 2013; Freitas et al. 2015; Almeida et al. 2015; Matozzo et al. 2016; Correia et al. 2016), pesticides (Barreira et al. 2007; Zhang et al. 2011; Tao et al. 2013) and recently NMs (Garcia-Negrete et al. 2013; Volland et al. 2015; Marisa et al. 2015; 2016; De Marchi et al. 2017a,b)) as well as environmental changes including salinity shifts (Kim et al. 2001; Coughlan et al. 2009; Wu et al. 2013) and seawater acidification (Gazeau et al. 2013; Catia Velez et al. 2016; Xu et al. 2016). Nevertheless, to our knowledge no information is available on how salinity shifts may alter the sensitivity of *R. philippinarum* when exposed to different CNT materials. For this reason, in the present study, a laboratory experiment was performed exposing the clam *R. philippinarum* for 28 days to unfunctionalized MWCNTs (Nf-MWCNTs) and carboxylated MWCNTs (f-MWCNTs), maintained at control salinity 28 and low salinity 21. Organism responses were assessed by measuring alterations induced in sub-cellular mechanisms of clams such as metabolic capacity, oxidative status and neurotoxicity.

MATERIALS AND METHODS

MWCNT material characterization

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

The concentrations of both MWCNTs used in this study (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 renewal, water samples (10 mL each) were collected from each aquarium at different periods along the experimental period: t0, t7, t21 and t28. t0: time zero, immediately after the dispersion of both CNTs in a water medium; t7: water samples collected after 1 week of exposure before water renewal; t21: water samples collected after the third week of exposure before water renewal; t28: samples collected at the end of the fourth week of exposure. The choice of these two CNTs was based on: i) their different physical and chemical properties; ii) different behavior in the water medium (aggregation/disaggregation, adsorption/desorption, sedimentation/resuspension and dissolution) (Arndt et al. 2013) and iii) their industrial applicability. The exposure concentrations of both MWCNT were selected considering previous studies conducted by De Marchi et al. (2017a; b; c) which, using the same species (De Marchi et al. 2017a; c) or other invertebrates (polychaetes) (De Marchi et al. 2017b) and the same range of concentrations of CNTs, observed biochemical alterations.

To observe the evolution of relative particle size distributions of CNTs in aqueous media as a function of time, dynamic light scattering (DLS) measurements were carried out by using 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 using a log correlator over 120 accumulations for a 2.0 mL of sample in a glass size cell. Each sample was 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 was performed using CONTIN particle size distribution analysis routines through Delsa Nano 3.73 software. The hydrodynamic radius and polydispersity index (PDI) of the analyzed dispersions were calculated on three replicates of each sample by using the cumulant method.

Bioassays

R. philippinarum specimens were obtained from the Ria de Aveiro (northwest Atlantic coast of Portugal (40º38' N, 8º45' W)) and individuals with similar size (mean length: 23.2 ± 0.32 mm; mean weight: 7.9 ± 1.7) were used for the experiment to prevent differences on biochemical responses of unexposed organisms. Animals were then acclimated in a tank of 100 L of artificial seawater (salinity 28) set up by the addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water for two weeks prior to the beginning of the experiment. Clams were fed every two-three days with AlgaMac Protein Plus, Aquafauna Bio-Marine, Inc (150000 cells/animal) under laboratory conditions (12 h light: 12 h dark photoperiod, temperature 18 ± 1 ºC, pH 8.0 ± 1 ºC and aeration). After acclimation period, 15 organisms for each condition (3 aquaria per condition, with 5 organisms per aquarium) were exposed for 28 days to two different salinities (21 and 28-control), each one combined with two different concentrations (0.10 and 1.00 mg/L) of both MWCNT materials (f-MWCNTs and Nf-MWCNTs).

Prior to experiment initiation, the salinity was progressively decreased (2 units) every 2 days until testing value was reached (salinity 21) while the other parameters (pH, temperature and aeration conditions) in each aquarium were set up as in the acclimation period (see above). The used salinities were selected according to the environmental salinity range where specimens were collected (Santos et al. 2007).

During the exposure period, MWCNT concentrations were re-established weekly after complete water renewals to ensure the same exposure concentrations throughout the experiment. To promote stable suspension of both CNTs in the water column (Hwang et al. 2007), the Nf-MWCNTs were sonicated for 1 h using 30 Hz ultrasound probe (IKA Labortechnik IKASONIC U50), while the f-MWCNTs were sonicated by a probe sonicator (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. 2009).

Biochemical analyses

After 28 days of exposure, clams were frozen, pulverized individually with liquid nitrogen and divided in 0.5 g aliquots. Extractions were performed with specific buffers for each biomarker. Biochemical analyses were repeated in duplicate for each sample and biomarker.

Regarding energy reserves and metabolism, due to the high-energy demand in invertebrates when under stressful conditions (Azeez et al. 2014), protein (PROT), glycogen (GLY) contents and electron transport system (ETS) activity were evaluated.

Reactive oxygen species (ROS) are normally produced during endogenous oxidative reactions in aerobic cells, which contributes to mitochondrial damage reacting with the polyunsaturated fatty acids of lipid membranes inducing lipid peroxidation (LPO). The protection against the potential toxicity of oxyradicals towards biological molecules is done by naturally occurring scavengers (mainly reduced glutathione (GSH), which is oxidized to GSSG by oxyradicals), and antioxidant enzymes which include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferases (GSTs) (Viarengo et al. 1991). All the mentioned biomarkers were investigated in the present study.

Another basic mechanism of the toxic action by pollutants in invertebrates is the inhibition of cholinesterase activity of nervous tissue (Nunes et al. 2017). A biomarker approach using cholinesterase (ChE), specifically Acetylcholinesterase (ATChI-ChE), inhibition as effect criterion has been presented in the present study.

All the details regarding the methods used for each biomarker determination are described in Almeida et al. (2017) and De Marchi et al. (2017c). Biochemical analyses were performed in duplicate for each sample and biomarker with a BioTek Synergy HT micro-plate Reader.

Energy reserves and metabolism

Protein (PROT) content was determined following the spectrophotometric method of Biuret (Robinson and Hogden 1940) with bovine serum albumin (BSA) as standard (0-40 mg/mL). Absorbance was measured at 540 nm. PROT was expressed in mg per g of FW.

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

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

Cellular damage

Lipid peroxidation (LPO) was measured according to Ohkawa et al. (1979) with modifications by Carregosa et al. (2014). The absorbance was measured at 535 nm and LPO levels were determined using ε = 156mM−1 cm−1. LPO levels were expressed in nmol of MDA equivalents formed per g of 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 nmol per min per g FW. Reduced to oxidised glutathione ratio (GSH/GSSG) was calculated dividing GSH content by 2x the amount of GSSG.

Antioxidant and biotransformation enzyme activities

The activity of SOD was determined using the method described in Beauchamp and Fridovich (1971) with adaptations by Carregosa et al. (2014). The standard curve was determined using SOD standards (0.0001-60 U mL^{-1}). Absorbance was measured at 560 nm. The enzymatic activity was expressed in U per g FW, where U corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

The activity of CAT was quantified according to Johansson and Borg (1988) with the modifications by Carregosa et al. (2014). The standard curve was determined using formaldehyde standards (0-150 µM). The absorbance was measured at 540 nm. The enzymatic activity was expressed in U per g FW, where U represents the amount of enzyme that caused the formation of 1.0 nmol formaldehyde.

The activity of GPx was quantified following Paglia and Valentine (1967). The absorbance was measured at 340 nm in 10 s intervals during 5 min and the enzymatic activity was determined using ε=6.22 $mM^{-1}cm^{-1}$. The results were expressed as U per g of FW, where U represent the number of enzymes that caused the formation of 1.0 µmol NADPH oxidized per min.

The activity GSTs was determined according to Habig et al. (1976). The absorbance was measured at 340 nm and the activity of GSTs was determined using the extinction coefficient 9.6 mM cm^{-1} for CDNB. Results were expressed in U per g of FW where U is defined as the amount of enzyme that catalysis the formation of 1 µmol of dinitrophenyl thioether per min.

Neurotoxicity

Acetylthiocholine iodide (ATChI, 470 µM) substrates were used for the determination of Acetylcholinesterase (ATChI-ChE) activity following the method of Ellman et al. (1961) with 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.

Data analysis

All the biochemical results 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 executed. Values lower than 0.05 were considered as significantly different. The null hypotheses tested were: A) for each MWCNT material (f and Nf) and for each salinity (28 and 21), no significant differences existed among Nf-MWCNT and f-MWCNT exposure concentrations (0.10 and 1.00 mg/L) (represented in all figures with letters); B) for each salinity (21 and 28) and for each exposure concentration no significant differences exist between MWCNT materials (f and Nf) (represented in table 3); C) for each exposure concentration (0.10 and 1.00 mg/L) and for each MWCNT material (f and Nf), no significant differences exist between salinities (21 and 28) (represented in all figures with asterisks).

The matrix gathering biochemical descriptors per condition was used to calculate the Euclidean distance similarity matrix. This 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.

RESULTS

MWCNT material characterization

In Table 2 the results of the Dynamic Light Scattering (DLS) characterization, used to detect the presence of macro/micro/nano-sized, and Polydispersity Index (PDI), used as measure of the molecular weight distributions, of both concentrations of Nf-MWCNTs and f-MWCNTs particle aggregates and in aqueous media under control salinity (28) and low salinity (21) are reported. In the present work, DLS measurements were carried out to obtain data regarding the tendency of CNTs to aggregate and the settling behavior of suspended CNTs in aqueous media. Due to the inherent heterogeneity and colloidal instability of the analyzed samples, DLS analyses were repeated several times to ensure reproducible results (Table 2). The mean size of the suspended particle aggregates was determined by applying the cumulant method, which is particularly recommended for the analysis of polydisperse colloidal systems. The DLS analysis carried out on the control samples did not reveal the presence of suspended micro-sized particle aggregates.

DLS and polydispersity index (PDI) analysis of experimental samples exposed to different concentrations of Nf-MWCNTs (0.10 mg/L, 1.00 mg/L) among collection periods (t0, t7, t21 and t28) under salinity 28 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, it was also possible to observe a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples. DLS analysis of samples exposed to Nf-MWCNTs at salinity 21 at t0 evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 2). The mean dimensions of the particle aggregates recorded after different exposure periods (t7, t21 and t28) showed a general decrease in the hydrodynamic radius of the aggregates at both tested concentrations. This could be due to the fractional deposition of larger particles, occurring during the period of exposure. The decrease of the PDI was directly correlated with the detected aggregates in the investigated samples.

DLS and PDI analysis of samples exposed to different concentrations of f-MWCNTs (0.10 mg/L, 1.00 mg/L) at salinity 28 did not allow for the detection of measurable macro/micro/nanosize particle aggregates observed among collection periods (t7, t21 and t28), however at t0 it was evidenced the presence of micro-sized particle aggregates whose hydrodynamic radius was directly correlated with the nominal concentrations of the samples (Table 2). The time evolution of the mean values of the dimension of the suspended f-MWCNTs aggregates exposed to salinity 21 was similar to that recorded for plain Nf-MWCNTs at the same experimental condition.

In conclusion, the mean recorded hydrodynamic diameter of f-MWCNT aggregates were smaller than those calculated for Nf-MWCNT aggregates under the same experimental conditions indicating higher dispersion of f-MWCNTs in aqueous media (Table 2). Comparing the aggregates of both MWCNT materials under salinity 21 and 28, it was possible to observe bigger mean diameters of both carbon NMs under salinity 21 compared the ones under control salinity 28. Under salinity 28, through a visual observation the presence of floatin macro-particle with larger particle sizes was identified compared to the ones at salinity 21, which the DLS was not able to record.

Biochemical analysis

All the results were discussed considering three main topics: i) understanding the effects of exposure concentrations of both MWCNTs maintained under both salinity levels; ii) understanding the effects of salinity shifts in organisms exposed to both MWCNT materials in each exposure concentration; iii) understanding the effects of the carboxylation of the surface of MWCNTs in organisms maintained under both salinity levels for each exposure concentration.

Energy reserves and metabolism

i) Considering the effects of exposure concentrations, results of PROT content in *R. philippinarum* showed that for both MWCNT materials (f and Nf) and for both salinities (28 and 21) significantly lower PROT content was observed in contaminated organisms in comparison to control organisms (Figure 2A).

ii) For each MWCNTs (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were only observed at control condition for Nf-MWCNTs, with higher PROT content in individuals maintained under salinity control 28 (Figure 2A).

iii) When comparing organisms exposed to the same salinity and exposure concentration, no significant differences were observed in PROT content between organisms exposed to different MWCNTs (Table 3).

i) Along the increasing Nf-MWCNTs and f-MWCNTs exposure concentrations, all the clams maintained at control salinity (28), decreased their GLY content, with significant differences among all tested treatments (Figure 2B). *R. philippinarum* under salinity 21 showed the lowest GLY content when exposed to the highest Nf-MWCNT concentration (1.00 mg/L), with significant differences compared to control individuals (Figure 2B).

ii) For each MWCNTs (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were observed in all tested concentrations for individuals exposed to Nf-MWCNTs, and only at highest concentration for specimens under f-MWCNTs, with lower content in organisms maintained to control salinity 28 compared to the ones under salinity 21 (Figure 2B).

iii) Comparing organisms under each salinity (28 and 21) and each exposure concentration, no significant differences were observed between organisms exposed to different MWCNTs (Table 3).

i) The ETS activity significantly increased with increasing exposure concentrations of Nf-MWCNTs and f-MWCNTs in *R. philippinarum* maintained at salinity 28, while at salinity 21, the activity of ETS was significantly higher in clams exposed to 0.10 and 1.00 mg/L relative to non-contaminated organisms, with no significant differences between these two concentrations (Figure 2C).

ii) For each MWCNT (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were observed in individuals exposed to Nf-MWCNT (0.10 and 1.00 mg/L) concentrations compared to non-exposed organisms with higher values at control salinity. Individuals exposed to f-MWCNTs showed significant differences between salinities only at the highest exposure concentration, with higher ETS at salinity 28 (Figure 2C).

iii) When comparing *R. philippinarum* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed only in clams exposed to 0.10 and 1.00 mg/L under salinity 28 showing in an increase of the activity for individuals contaminated with Nf-MWCNTs (Table 3).

Cellular damage

i) Under salinity 28 the level of LPO in clams exposed to Nf-MWCNTs increased with increasing exposure concentrations with significant differences among all treatments, while in organisms under low salinity 21 the LPO at 0.10 and 1.00 mg/L was significantly higher than values observed in non-exposed organisms, and no significant differences were observed between individuals exposed to these two concentrations (Figure 3A). Regardless of the salinity tested (control-28 and 21), increased LPO levels were also observed in clams under f-MWCNTs, with significant differences between all exposed (0.10 and 1.00 mg/L) and non-exposed (control) conditions (Figure 3A).

ii) For each MWCNT (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were observed in non-contaminated clams and in clams exposed to the highest MWCNT concentration (both f and Nf), with higher levels in individuals maintained at control salinity 28 compared to individuals under salinity 21 (Figure 3A).

iii) Comparing organisms under the same salinity and exposure concentration, significantly higher LPO levels in all tested concentrations were observed in clams exposed to f-MWCNTs compared to Nf-MWCNTs under both salinities (Table 3).

i) Significantly lower ratio of GSH and GSSG was observed in contaminated *R. philippinarum* in comparison to control organisms maintained under both salinities (28 and 21) for both MWCNT materials (f and Nf) (Figure 3B).

ii) For each MWCNT (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were observed only at higher exposure concentration for specimens under Nf-MWCNTs, with significantly lower GSH/GSSG in organisms maintained in control salinity 28 in comparison to clams under salinity 21 (Figure 3B).

iii) When comparing clams exposed to the same salinity and exposure concentration, significant differences between MWCNT materials (f and Nf) were observed only in clams exposed to 1.00 mg/L at salinity 21, showing a decrease of the ratio in individuals contaminated with f-MWCNTs (Table 3).

Antioxidant and biotransformation enzyme activities

i) Under salinity 28 the activity of SOD increased in clams along the increasing exposure gradient of Nf-MWCNTs, with significant differences among exposure concentrations. At salinity 21, significantly higher enzyme activity was observed in contaminated compared to non-contaminated clams (Figure 4A). When exposed to f-MWCNTs, higher SOD activity was observed in clams maintained at salinity 28 at 0.10 and 1.00 mg/L compared to control individuals, while under salinity 21, significantly higher activity was recorded only at 0.10 mg/L in comparison to the remaining conditions (Figure 4A).

ii) For each MWCNTs (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were only observed at 1.00 mg/L f-MWCNTs, showing higher SOD activity in individuals maintained at salinity 28 in comparison to organisms under low salinity 21 (Figure 4A).

iii) Comparing organisms under the same salinity and exposure concentration, significantly greater SOD activity in all tested concentrations (0.10 and 1.00 mg/L) have been observed in clams exposed to f-MWCNTs compared to Nf-MWCNTs under both salinities (21 and 28) (Table 3).

i) At salinity 28 *R. philippinarum* presented a significant increase of CAT activity only at 1.00 mg/L Nf-MWCNTs, while at salinity 21, significantly higher values were found in contaminated compared to non-contaminated clams (Figure 4B). Considering clams exposed to f-MWCNTs under salinity 28, significant increase of the activity with increasing exposure concentrations was recorded, while significant differences in CAT activity between exposed and non-exposed clams were observed under salinity 21 (Figure 4B), showing greater CAT activity in contaminated compared to non-contaminated individuals.

ii) For each MWCNT (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were identified in organisms maintained at control condition, with higher activity in clams under salinity 28, and in *R. philippinarum* exposed to 0.10 mg/L f-MWCNTs, with the highest enzyme activity observed under the lowest salinity (21) (Figure 4B).

iii) When comparing organisms exposed to the same salinity and exposure concentration, no significant differences were observed in CAT activity between organisms exposed to different MWCNTs (Table 3).

i) In clams maintained at 28 salinity, the activity of GPx increased significantly, when the animals were exposed to 0.10 mg/L Nf-MWCNTs, but at the highest exposure concentration (1.00 mg/L) the enzyme activity significantly decreased to values lower than control levels. Under salinity 21, significantly higher GPx activity was observed in contaminated clams compared to non-contaminated individuals (Figure 4C). Similar patterns were also identified in clams submitted to f-

MWCNTs and salinity 28, while under low salinity (21), the activity of GPx showed no significant differences among all exposure concentrations (Figure 4C).

ii) For each MWCNT (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were observed only in organisms submitted to Nf-MWCNTs, where at salinity 28 the highest enzyme activity was observed at 0.10 mg/L, while at salinity 21 the highest activity was recorded at 1.00 mg/L (Figure 4C).

iii) When comparing organisms exposed to the same salinity and exposure concentration, significant differences between clams exposed to different MWCNTs were observed only at 1.00 mg/L, with higher GPx activity in clams exposed to f-MWCNTs under salinity 28 compared to individuals exposed to Nf-MWCNTs (Table 3).

i) In *R. philippinarum* maintained at salinity 28, GST activity significantly decreased in clams exposed to Nf-MWCNTs and f-MWCNTs compared to non-exposed organisms, while, at salinity 21, no significant differences were observed among exposure concentrations (Figure 4D).

ii) For each MWCNT (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were observed between organisms exposed to 0.10 and 1.00 mg/L Nf-MWCNTs, showing higher GST activity in individuals under salinity 28 compared to individuals under salinity 21. Significant differences between salinities were also recorded in individuals submitted to 0.10 mg/L f-MWCNTs, with higher enzyme activity in individuals under salinity 21 in comparison to control salinity (28) (Figure 4D).

iii) When comparing organisms exposed to the same salinity and exposure concentration, significantly higher GST activity was observed in individuals exposed to all Nf-MWCNT concentrations compared to the functionalized ones only under salinity 28 (Table 3). No significant differences were found in individuals exposed to salinity 21.

Neurotoxicity

i) In clams maintained under both salinities 28 and 21, the ATChI-ChE activity was significantly lower in Nf-MWCNT contaminated compared to non-contaminated clams. Similar trend was also identified in organisms exposed to f-MWCNTs under salinity 21, while for individuals maintained at salinity 28, ATChI-ChE activity decreased in clams along the increasing exposure gradient of f-MWCNTs, with significant differences among exposure concentrations. (Figure 5).

ii) For each MWCNT (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were observed in clams maintained under control condition, with lower activity in individuals under salinity 28, and in clams exposed to 0.10 mg/L Nf-MWCNTs, showing lower activity in individuals under salinity 21 compared to organisms maintained at control salinity (Figure 5).

iii) When comparing organisms exposed to the same salinity and exposure concentration but different MWCNTs, significantly higher enzyme activity was only recorded in organisms contaminated with 1.00 mg/L Nf-MWCNTs at salinity 28 in comparison to f-MWCNTs (Table 3).

Multivariate analysis

Principal coordinate analysis (PCO) graphs obtained for *R. philippinarum* exposed to f-MWCNTs and Nf-MWCNTs both under salinity control 28 and low salinity 21 are shown in Figure 6. The PCO axis 1, which explained 65.8% total variation, separated non-contaminated individuals maintained under both salinities (28 and 21) and individuals exposed to 0.10 mg/L (f-MWCNTs and Nf-MWCNTs) under salinity 21 at the positive side of the axis from the remaining conditions at the negative side. The PCO axis 2 explained 13.1% and separated at the positive side of the axis non-exposed individuals and clams submitted to 1.00 mg/L, both conditions maintained under salinity control (28), and the remaining conditions at the negative side (Figure 6). High correlation was observed between GSTs, ATChI-ChE, PROT as well as GLY and GSH/GSSG in clams maintained in uncontaminated conditions (0.00 mg/L Nf and f-MWCNTs) under both salinities (21 and 28) ($p > 0.88$). The values of GPx, SOD and LPO were closely correlated ($p > 0.92$) in *R. philippinarum* contaminated with 0.10 mg/L of both carbon CNMs combined with salinity 28 as well as clams exposed to 1.00 mg/L Nf and f-MWCNTs under salinity 21, with the highest values for these biomarkers observed under these conditions. High correlation ($p > 0.88$) between CAT and ETS in specimens exposed to 1.00 mg/L of both Nf-MWCNTs and f-MWCNTs maintained under control salinity (28) was observed.

DISCUSSION

The results of the present the present study demonstrated A) concentration-dependent toxicity in *R. philippinarum* exposed to both MWCNT materials and under both salinities; B) that salinity shifts altered the toxicity of both MWCNT materials as well as the sensitivity of *R. philippinarum* exposed to these contaminants; C) that greater toxic impacts were induced in exposed clams by carboxylated MWCNTs compared to pristine MWCNTs.

A) For each MWCNT material (f and Nf) and for each salinity (28 and 21), significant differences between exposure concentrations in organisms exposed to Nf-MWCNT and f-MWCNT were found. Specifically, despite the different salinities and NMs, the present study demonstrated that *R. philippinarum* presented a concentration-dependent decrease of GLY and PROT content when exposed to both f-MWCNTs and Nf-MWCNTs, which may indicate that clams were using GLY and PROT as defense mechanisms against high CNT concentrations. Analysis of biochemical composition in these clams indicated that PROT as well as GLY constituted the main energy reserves (Beninger & Lucas 1984) and it was already demonstrated that once the organisms are exposed to pollutants they can increase their energy expenditure (considered a mechanism of cellular protection) (Klaper et al. 2010). In our previous study, *R. philippinarum,* which were exposed to raw MWCNTs at two different pH levels (7.9 and 7.6) for 28 days, increased their energy expenditure with increasing exposure concentration to fight the oxidative stress induced by MWCNTs, which resulted in the consumption of energy reserves (De Marchi et al. 2017c). The present results reported that the clams increased their metabolism (ETS) with increasing exposure concentration of both nonfunctionalized and functionalized MWCNT under both salinities. Due to the ability to allow the energy stored within the reduced hydrogen carriers in order to synthesize ATP (Liu et al. 2002), the ETS was already used as a measure of metabolic capacity in bivalves in response to different stressors showing higher ETS activity in contaminated organisms (Bielen et al. 2016; De Marchi et al. 2017a; c). When organisms are exposed to different pollutants, oxidative stress may occur as a consequence of ROS generation, causing partial damage to the inner mitochondria membranes by lipid peroxidation (LPO), thus impairing ETS activity (Choi et al. 2001; Bielen et al. 2016). In the present study, oxidative conditions upon exposure to both MWCNT materials under both salinities were evidenced by an increase in LPO level, and decrease of glutathione (GSH) / glutathione disulfide (GSSG), the major variables detecting oxidative disturbances in cells (Mocan et al. 2010), with increasing exposure concentration. Various studies have been already reported higher levels of LPO in bivalves with the increase of NM concentration (Kádár et al. 2010; Tedesco et al. 2010; Gomes et al. 2011; Gomes et al. 2012; Gagné et al. 2013; Trevisan et al. 2014; Anisimova et al. 2015; Volland et al. 2015; Cid et al. 2015; De Marchi et al. 2017a; c) and a consequent decrease of GSH/GSSG (Tedesco et al. 2010; De Marchi et al. 2017a; c), confirming a concentration-dependent increase of lipid damage in organisms exposed to these contaminants. When organisms are under stressful conditions, ROS are overproduced and bivalves are able to increase the activity of antioxidant enzymes (SOD, CAT and GPx) in response to the generated cellular oxidative stress. These antioxidant abilities are found to be associated with NM exposure concentrations, showing increased activity of antioxidant enzymes in response to an increase of ROS production at the highest exposure concentration (Buffet et al. 2011; Gomes et al. 2012; Mccarthy et al. 2013; Gomes et al. 2014; Volland et al. 2015; De Marchi et al. 2017a; c). Our results supported this idea, showing an activation of SOD and CAT when *R. philippinarum* was exposed to both CNTs and under both salinities. Same behavior was also observed for the activity of GPx in individuals exposed to f-MWCNTs, while, when clams were exposed to Nf-MWCNTs under salinity 28, the activity of GPx increased at 0.10 mg/L, but then the activity decreased at the highest concentration, showing in this case that the behavior of GPx did not depend on exposure concentration, but may depend on other variabilities such as different salinities and NMs. The cytosolic glutathione S-transferase enzymes (GSTs) serve as biomarkers of cellular damage as they exhibit many of the required characteristics, i.e. specific localisation, high cytosolic concentration and relatively short half-life (Pérez et al. 2004). The results of the present study showed an increase of the activity of GSTs in *R. philippinarum* exposed to Nf-MWCNTs, and a decrease of the activity in clams exposed to f-MWCNTs both under salinity control (28), confirming concentration-dependent activation (increase) or inhibition (decrease) of these biotransformation enzymes, while under salinity 21, no differences were found between concentrations in organisms exposed to either MWCNT material, indicating that this group of enzymes was not involved in the biotransformation process under these conditions. In agreement with the present results, Cid et al. (2015) exposing *Corbicula fluminea* clams to 0.01, 0.1, 1, and 10 mg/L of carbon nanodiamonds (NDs) for 14 days, showed an increase of GST activity with increasing ND concentration, while Anisimova et al. (2015) observed a decrease of GST activity in *Crenomytilus grayanus* mussels exposed to MWCNTs (100 mg/L) with 12-14 nm diameter after 48h. In the recent years, the number of the studies which investigated the interactions between cholinesterases and NMs are increasing, demonstrating an inhibition of cholinesterase activity in invertebrates as a consequence of NM exposure concentration-dependently (Gomes et al. 2011; Buffet et al. 2014; Marisa et al. 2016; Luis et al. 2016; De Marchi et al. 2017a; b; c). Cholinesterases are esterases that lyse choline-based esters, several of which serve as neurotransmitters (Mennillo et al. 2017), and can be divided in specific cholinesterase (acetylcholinesterase (AChE)) and non-specific cholinesterase (or pseudocholinesterase). In the present study, despite the different salinities and NMs, the AChE activity decreased concentration-dependently. The decrease of the activity in organisms exposed to both materials (Nf and f) under both salinities may have been caused because MWCNTs had high affinity for AChE, and they are able to cause 76–88% AChE activity reductions (Wang et al. 2009).

B) The ability of NMs to act as carriers of toxic contaminants seems to be affected by their dispersion in exposure media (Canesi & Corsi 2015). The present results showed that, for each exposure concentration and for each MWCNT material, the salinity shifts altered the toxicity of both MWCNT materials as well as the sensitivity of *R. philippinarum* exposed to these contaminants in terms of metabolism, oxidative status and neurotoxicity of clams. Although estuarine bivalves are often exposed to short-term (tidal) and long-term (rain periods) changes in salinity, recently, the increased stress may have led to episodes of increased mortality (Verdelhos et al. 2015), and different studies revealed that bivalves exhibited physiological and morphological abnormalities with ensuing mortalities when exposed to low salinity (Coughlan et al. 2009; Sarà et al. 2008; Munari et al. 2011) However, in the present study, both Nf-MWCNTs and f-MWCNTs under salinity 28 generated greater alterations of energy reserve (PROT) and metabolic activity (ETS), oxidative stress biomarker responses (LPO) and antioxidant enzymes activities (SOD, CAT, GPx and GSTs) as well as alteration of the neurostate (ATChI-ChE) compared to individuals maintained under salinity 21, demonstrating that the alteration induced by salinity shifts in the chemical behavior of both MWCNTs and consequent fate in exposed clams had greater effect on toxicity in comparison to the sensitivity of the clams to low salinity. These results may be explained by relationships among physicochemical characterization of the nanomaterials, salinity and the consequent toxicity of the materials. In detail, looking at the DLS and PDI analysis of experimental samples exposed to different concentrations of Nf-MWCNTs and f-MWCNTs among collection periods under salinity 28 and 21, the results showed larger mean diameters on both CNTs under salinity 21 compared the ones under control salinity 28. However, under salinity 28, it was noted, through a visual observation, the presence of floating macro-particles with larger particle sizes compared to the ones at salinity 21, which the DLS was not able to record. In fact, it has been already demonstrated in the literature that the higher salinity causes the formation of large-size aggregates, which will increase the chance of physical retention, such as gravitational sedimentation, interception and straining of NMs (Hu et al. 2017). Aggregation of NMs can alter their biological effects by affecting ion release from the surface and their reactive surface area, affecting the mode of cellular uptake of NMs together with subsequent biological responses in the organisms (Hotze et al. 2010). Ward & Kach (2009) found that the bigger aggregates can considerably increase the uptake and bioavailability of NMs to suspension filter-feeding bivalves. These authors, exposing mussels *Mytilus edulis* and oysters *Crassostrea virginica* to polystyrene NMs at a concentration of ca.1.3 x 104 particles mL^{-1}, which were either dispersed or embedded within aggregates, showed that both organisms more efficiently captured and ingested NMs that were incorporated into aggregates compared to those freely suspended. Also, Gagné et al. (2008) mentioned that cadmium-telluride quantum dots tended to aggregate at medium (4 mg L^{-1}) and high (8 mg L^{-1}) concentrations. If so, then the aggregated quantum dots probably were ingested by mussels at a higher rate than those not aggregated (i.e., at 1.6 mg L^{-1}). This idea is in agreement with our results, showing major toxic impacts in organisms exposed to the higher salinity 28.

C) For each salinity and for each exposure concentration, our results demonstrated clearly that nanomaterial toxicity has been attributed also to the surface functionalization showing greater toxic impacts in clams exposed to f-MWCNTs compared to Nf-MWCNTs. Specifically, the highest ETS activity after exposure to f-MWCNTs compared to Nf-MWCNTs may be related to an activation of respiratory chains due to an increase of energy needs associated with chemical detoxification under this condition (Choi et al. 2001). In fact, besides mitochondria and chloroplasts, eukaryotes have ETS in other membranes, such as the plasma membrane (PM) redox system, or the cytochrome P450

(CYP) system. These systems have fewer complexes and simpler branching patterns than those in energy-transforming organelles, and they are often adapted to non-bioenergetic functions such as detoxification or cellular defense (Berry 2003). Focusing on our results, this hypothesis was confirmed by a greater antioxidant enzyme activities such as those of SOD and GPx in organisms exposed to f-MWCNTs compared to Nf-MWCNTs, demonstrating that these enzymes could be indicators of compensatory cellular response to this NM exposure. These results are in line with the PCO analysis observing high correlation between detoxification enzymes, and ETS in specimens exposed to both CNTs. However, when the individuals were exposed to Nf-MWCNTs under salinity 28, the antioxidant activity of GPx was increased at 0.10 mg/L, but then the activity decreased at the highest concentration. These findings may indicate that H_2O_2 produced by SOD enzyme was probably eliminated by GPx up to a certain level of stress, but at 1.00 mg/L, the activity was inhibited. Studies confirmed that the antioxidant defense systems could be remarkably induced under a certain level of stress, with a decreasing tendency of their activity with increasing exposure time and concentration of pollutants (Hao & Chen 2012). Moreover, it has been already demonstrated that the behavior of the antioxidant enzymes is dependent also on the type of NMs (Canesi & Corsi 2015). As a consequence, the bioavailability as well as biodistribution and consequent biological responses are dependent on the interactions of NMs inside the body of the organism. This hypothesis may explain the different responses of the antioxidant enzyme GPx in clams exposed to two different NMs. As mentioned above, the GST enzymes were also activated in organisms exposed to the two different NMs. However, controversial behavior of this enzyme was observed, demonstrating that the behavior of the antioxidant enzymes not only depend of the exposure concentrations but also on the type of NM (Lehman et al. 2011). In detail, *R. philippinarum* exposed to Nf-MWCNTs showed an increase of the GST activity, revealing the capacity of bivalves to use these enzymes to detoxify NMs into less toxic excreted substance (Ciacci et al. 2012¸Cid et al. 2015), while clams submitted to f-MWCNTs showed a decrease of the activity, indicating that these mechanisms were not sufficient to prevent the occurrence of cellular damages at the higher concentration (Garaud et al. 2014; Anisimova et al. 2015; De Marchi et al. 2017a; c). In agreement with the present results, Canesi et al. (2010) exposed *Mytilus galloprovincialis* to different carbon-based NMs (nano carbon black-nNCB, C60 fullerene) (0.05, 0.2, 1, 5 mg/L) for short-term (24 h), showing that both induced changes in GST activities, with increases and decreases of the activity, depending on NM type and concentration. Although increased antioxidant enzyme activities in *R. philippinarum* exposed to both MWCNTs under both salinities were observed, the present results showed that these mechanisms were not enough to eliminate the excess of ROS, and LPO increased with the increasing exposure concentration of both NMs under both salinities, with major lipid membrane destruction in clams exposed to f-MWCNTs. The carboxylation of SWCNTs, as well as MWCNTs, introducing polar groups such as carboxyl groups (-COOH) in order to achieve better dispersibility in water, is shown to cause more amorphous carbon fragments to be formed as a result of increased oxidation of carbon, and these amorphous fragments can induce higher levels of toxicity (expressed as cellular damage) to biological systems (Arndt et al. 2013).

CONCLUSION

CNTs are increasingly being used and introduced in different fields and are attracting increased attention for several industrial sectors. This growth, however, needs to be accompanied by an interest in the nanosafety of CNTs, in order to reduce possible risks to the environment, especially on the aquatic environment, where they can finally accumulate. Most studies assessing the effects of NMs in aquatic invertebrates have focused on freshwater species invertebrates (mainly crustaceans, and *Daphnia* in particular) and vertebrates (fish), while less information is available on species from estuarine and marine environments, where the chemical behavior of NMs and their consequent fate may be different from fresh water and consequently their effects on organisms may also be different. The results of the present study demonstrated clearly that nanomaterial toxicity not only has to be attributed to core structure and surface functionalization, which have been shown to alter the level of toxicity to biological systems, but also to the physico-chemical parameters of the medium, which alter the dispersion and consequently the detection of CNTs in the media: aggregation/disaggregation, adsorption/desorption, sedimentation/resuspension and dissolution. In a future climate change scenario, it is necessary to focus on their fate into the environment, which is in certain cases unknown. They could eventually end up in water treating systems and their effluents, consequently affecting and/or modifying aquatic communities.

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Figure captions

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

Figure 2. **A**: Protein (PROT) content; **B:** Glycogen (GLY) content; **C:** electron transport system (ETS) activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) at different concentrations (0.00; 0.10 and 1.00 mg/L) and different salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNT at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and italic letters for f-MWCNT at salinity 28; lowercase and italic letters for f-MWCNT at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks.

Figure 3. A: Lipid peroxidation (LPO) levels; **B:** GSH/GSSG (mean + standard deviation) in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) at different concentrations (0.00; 0.10 and 1.00 mg/L) and salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNT at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and italic letters for f-MWCNT at salinity 28; lowercase and italic letters for f-MWCNT at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks.

Figure 4. **A:** Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione peroxidase (GPx) activity; **D:** Activity of glutathione S-transferases (GSTs) (mean + standard deviation) in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) at different concentrations (0.00; 0.10 and 1.00 mg/L) and salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNT at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and italic letters for f-MWCNT at salinity 28; lowercase and italic letters for f-MWCNT at salinity 21. Significant

differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks.

Figure 5. ATChI-ChE activity in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) at different concentrations (0.00; 0.10 and 1.00 mg/L) and salinities (control-28 and low-21). Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNT and salinity were represented with different letters: uppercase and regular letters for Nf-MWCNT at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and italic letters for f-MWCNT at salinity 28; lowercase and italic letters for f-MWCNT at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNT and exposure concentration were represented with asterisks.

Figure 6. Centroid ordination diagram (PCO) based on biochemical parameters in *Ruditapes philippinarum* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) at different concentrations (0.00; 0.10 and 1.00 mg/L) and salinities (control-28 and low-21). Pearson correlation vectors are superimposed as supplementary variables to biochemical data ($r > 0.75$): PROT; GLY; ETS; LPO; GSH/GSSG; SOD; CAT; GPx; GSTs; ATChI-ChE.

Fig 1

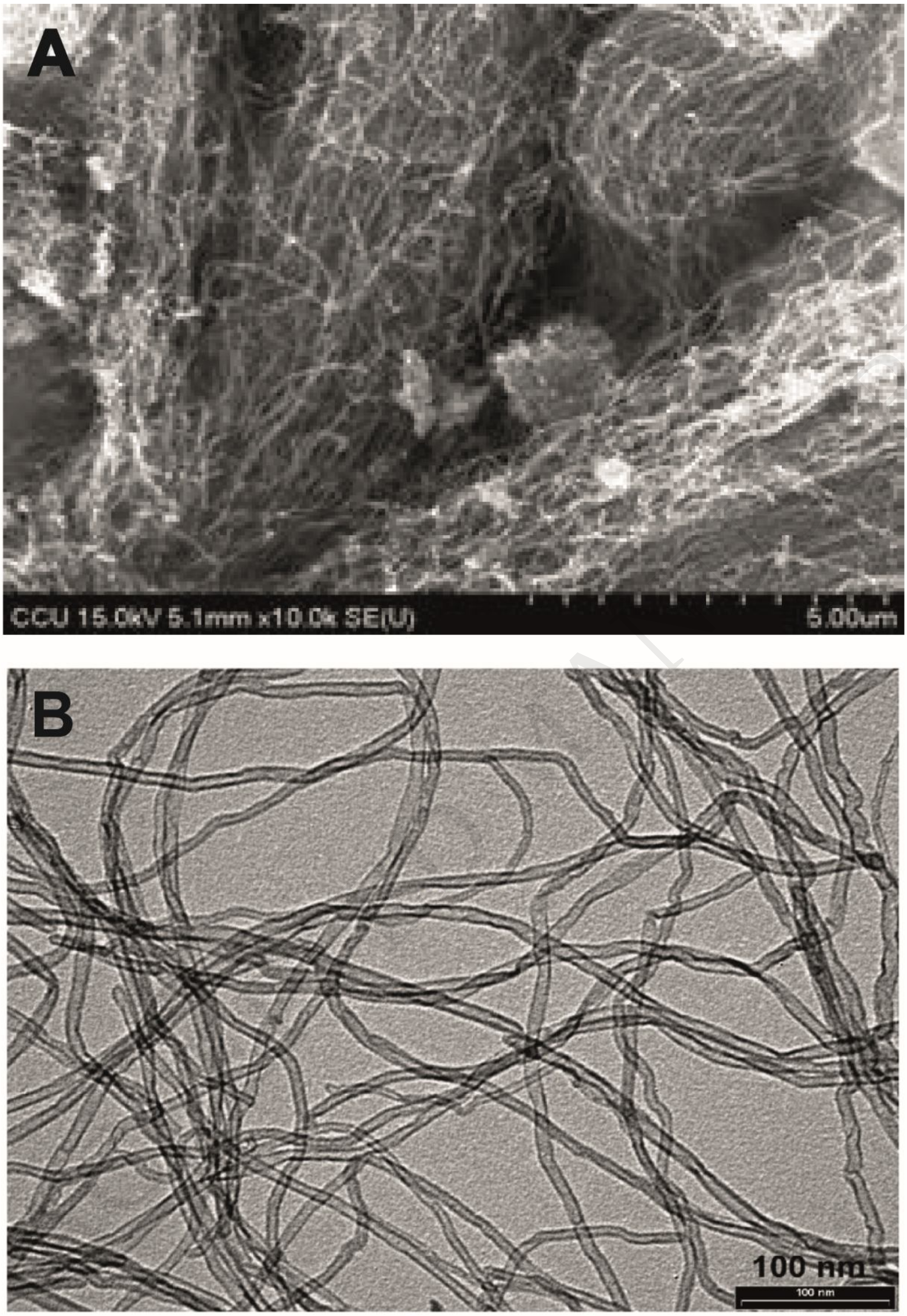
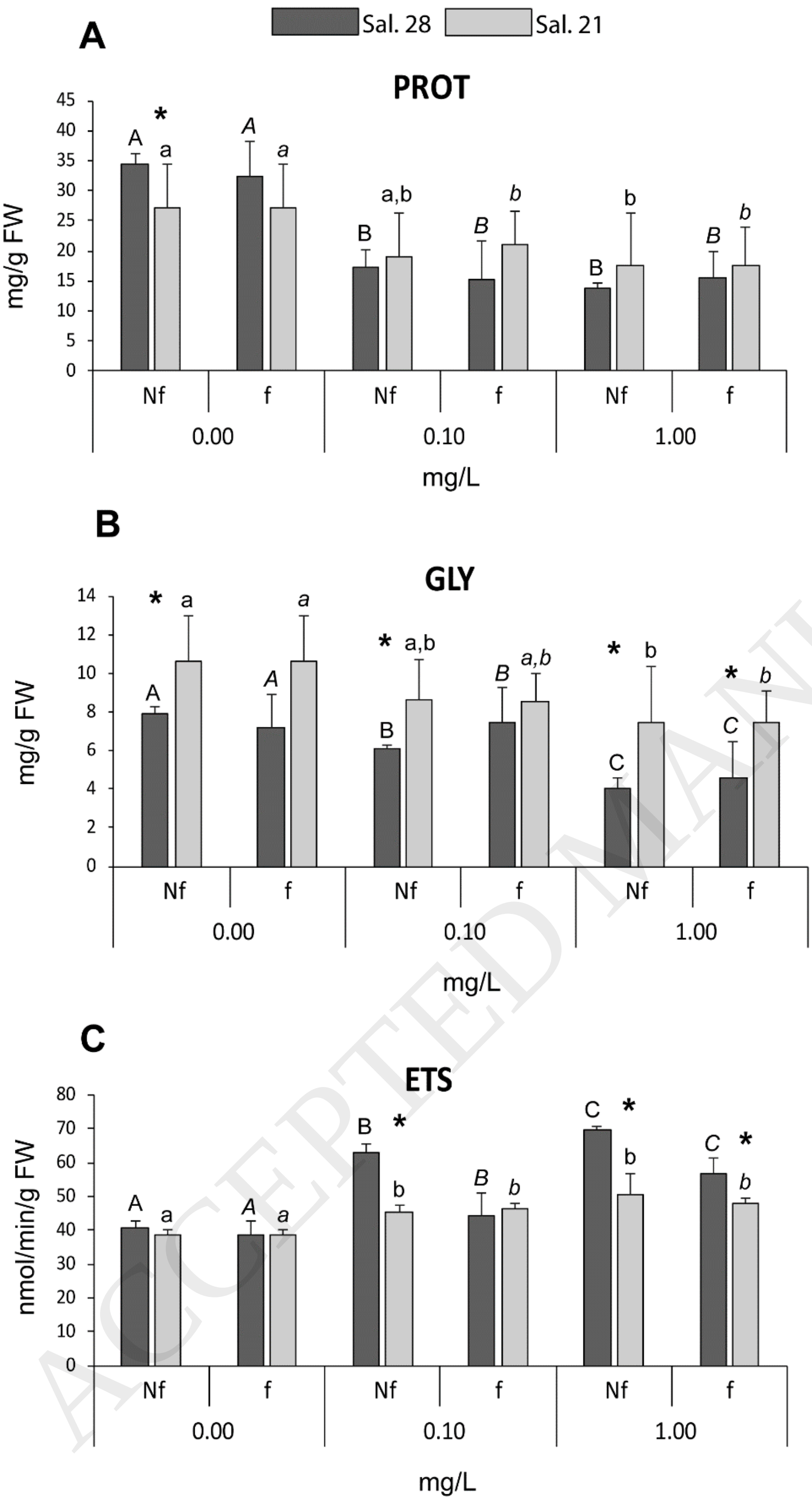
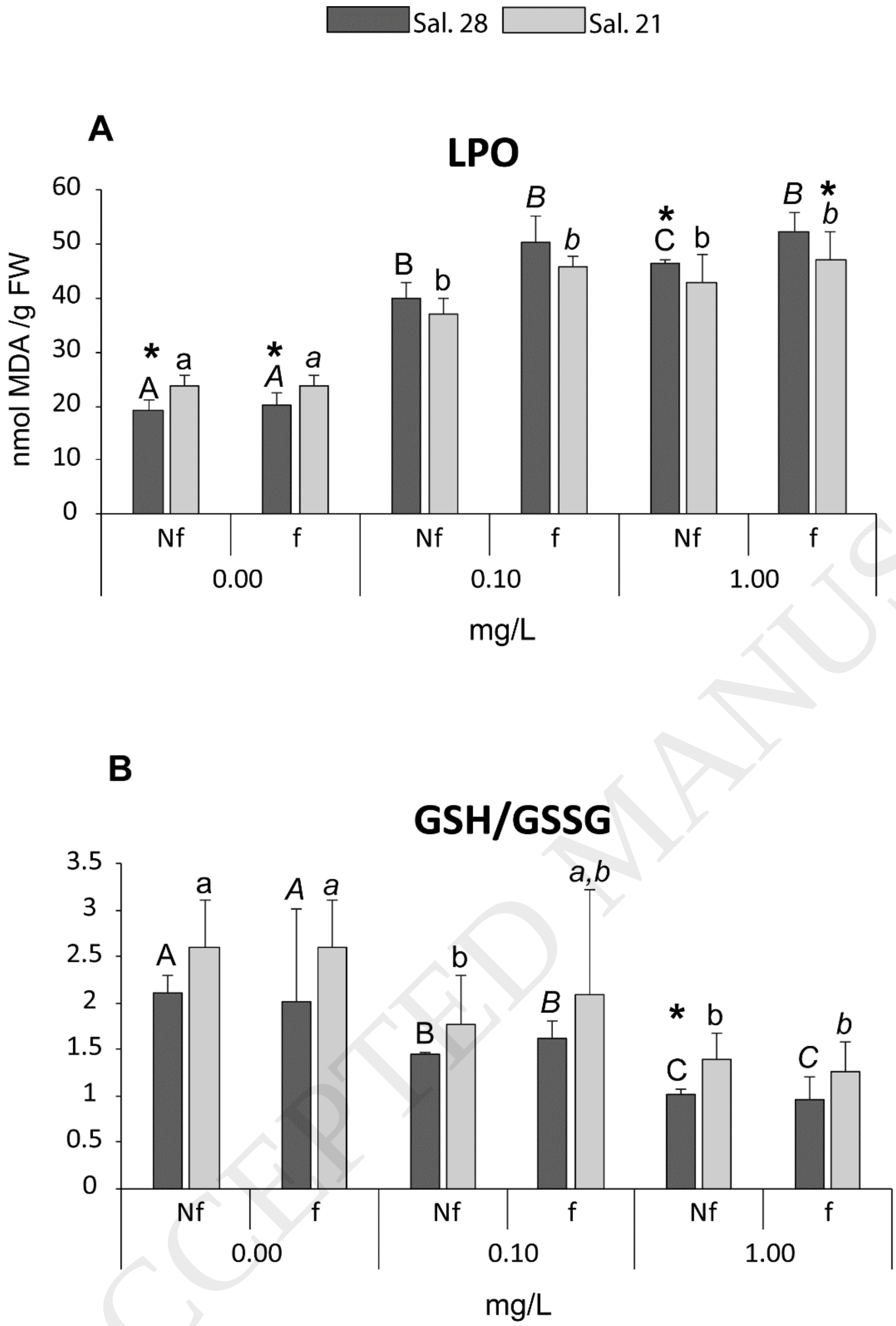


Fig 2



Sal. 28
Sal. 21
A
PROT
mg/g FW
Nf
f
0.00
0.10
1.00
mg/L
B
GLY
mg/g FW
C
ETS
nmol/min/g FW

Fig 3


Sal. 28
Sal. 21
A
LPO
nmol MDA /g FW
60
50
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Nf
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GSH/GSSG
3.5
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2.5
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1
0.5
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Fig 4

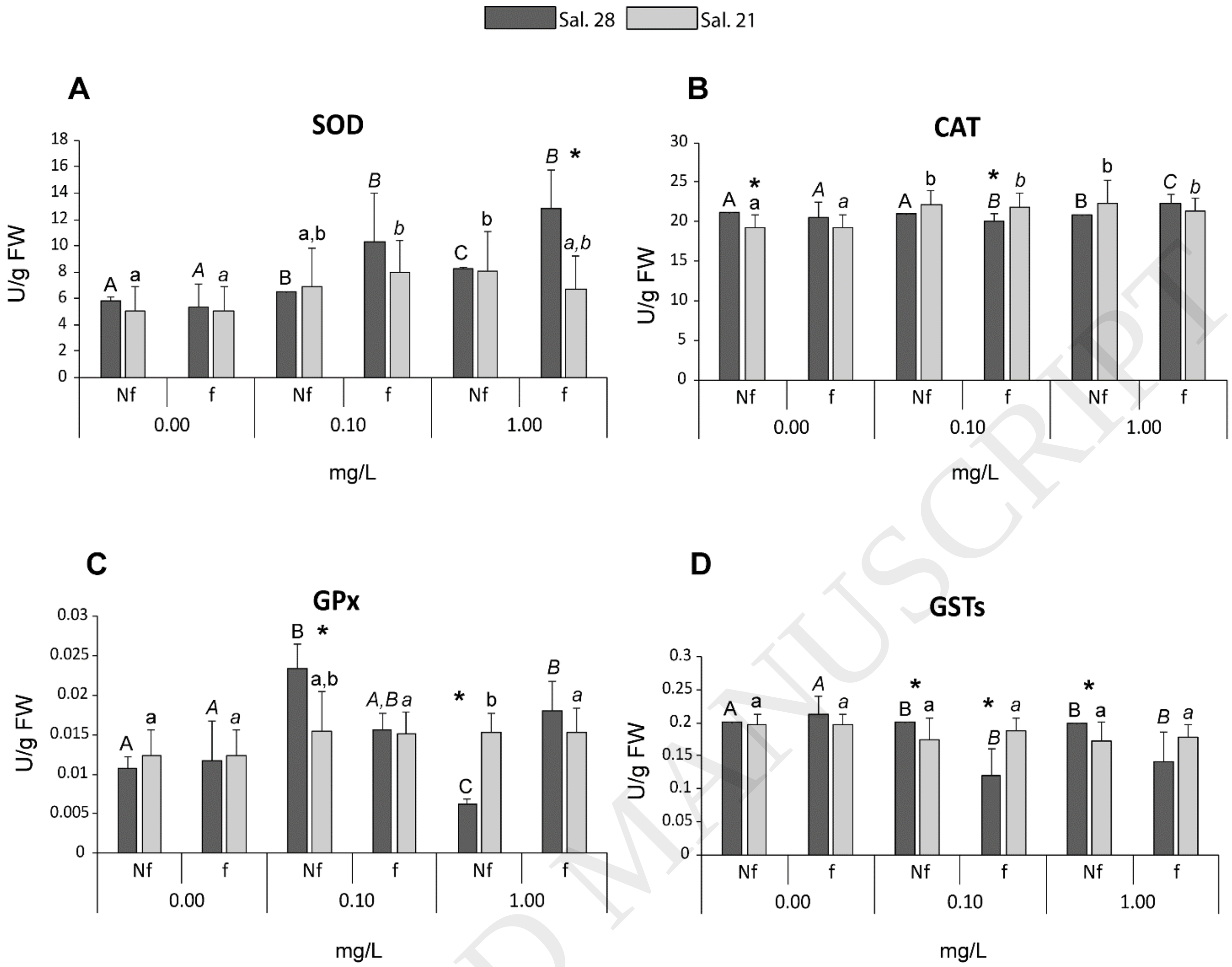
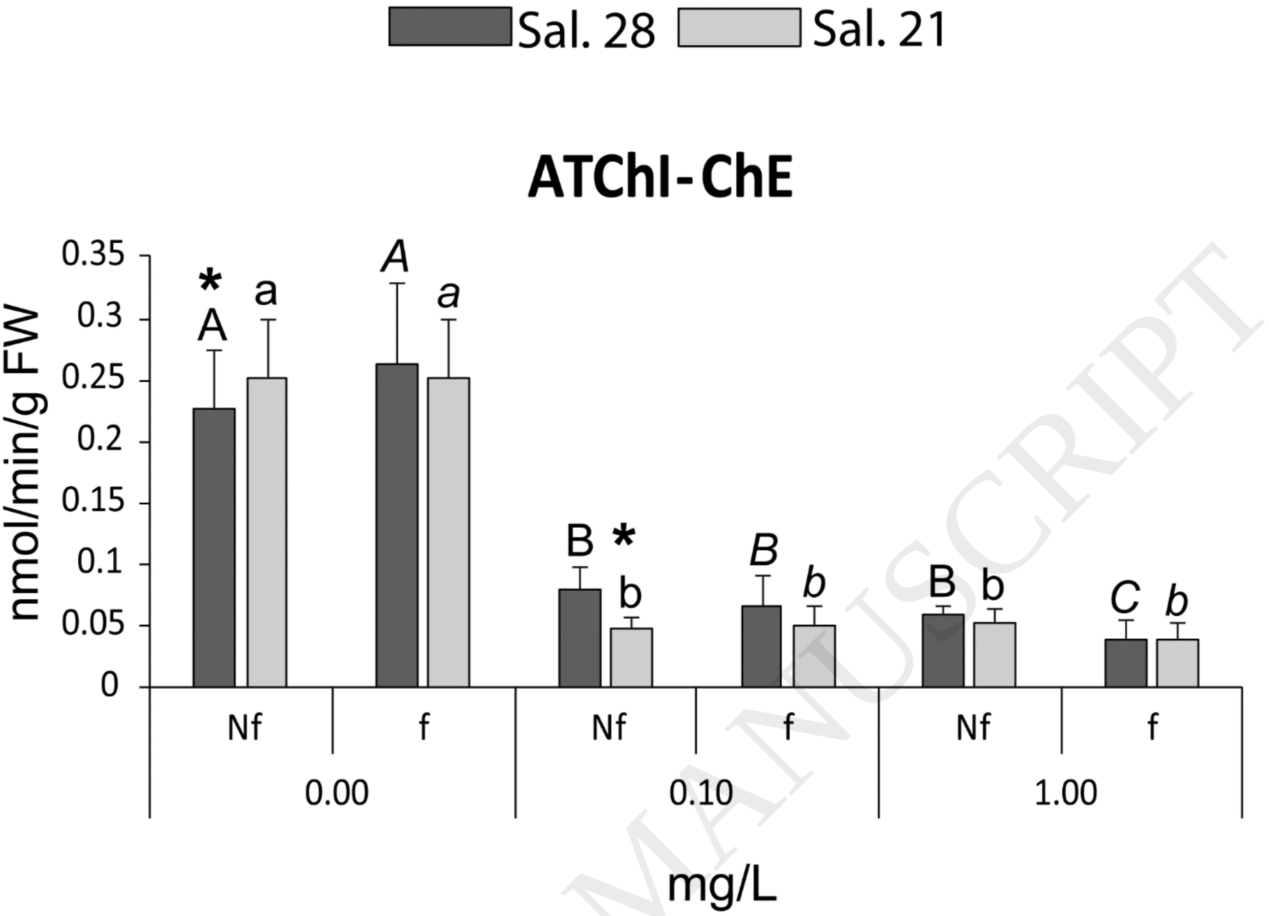


Fig 5


Sal. 28
Sal. 21
ATChI-ChE
nmol/min/g FW
0.35
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*
A
a
A
a
B
*
b
B
b
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Nf
f
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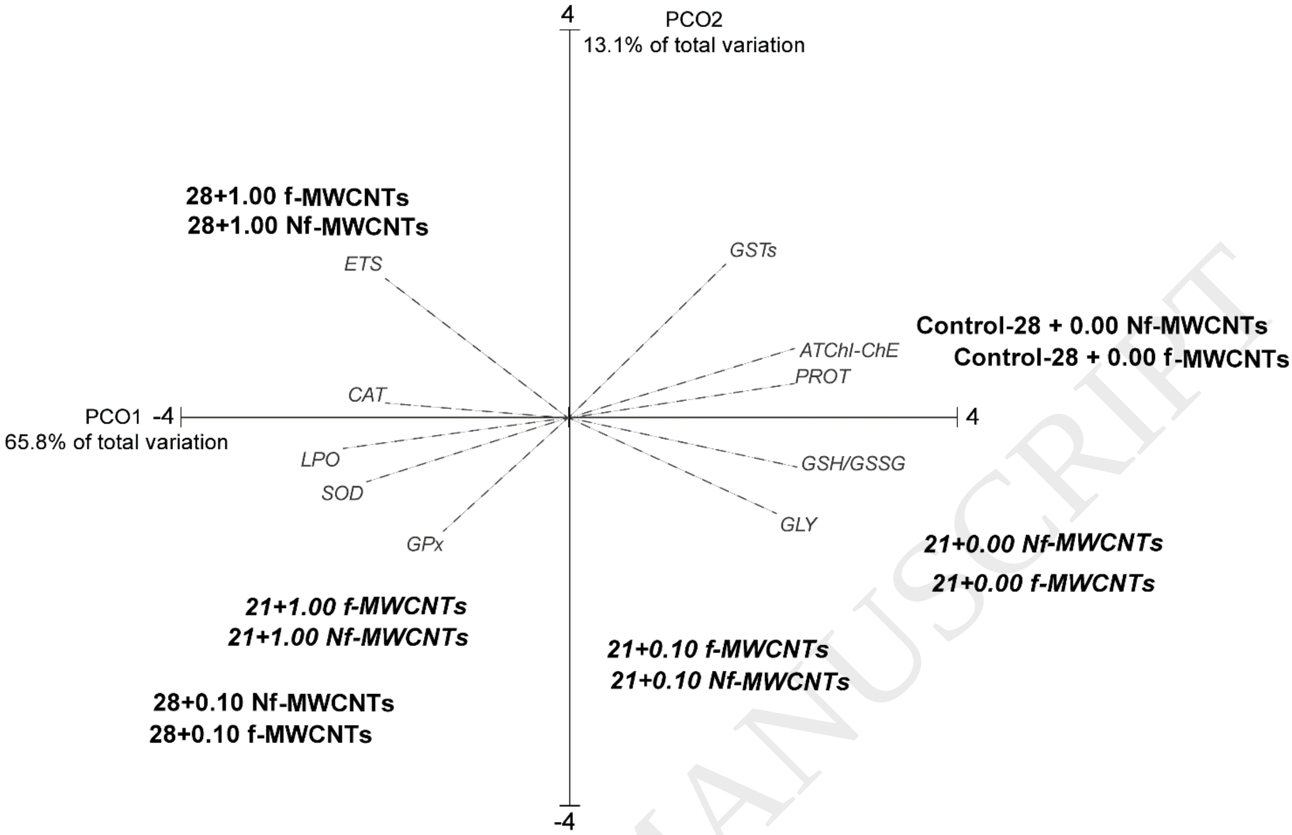
f
0.00
0.10
1.00
mg/L

Fig 6


4
PCO2
13.1% of total variation
28+1.00 f-MWCNTs
28+1.00 Nf-MWCNTs
ETS
GSTs
Control-28 + 0.00 Nf-MWCNTs
Control-28 + 0.00 f-MWCNTs
ATChl-ChE
PROT
CAT
PCO1 -4
65.8% of total variation
4
LPO
SOD
GSH/GSSG
GLY
GPx
21+0.00 Nf-MWCNTs
21+0.00 f-MWCNTs
21+1.00 f-MWCNTs
21+1.00 Nf-MWCNTs
21+0.10 f-MWCNTs
21+0.10 Nf-MWCNTs
28+0.10 Nf-MWCNTs
28+0.10 f-MWCNTs
-4

Tables

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

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

* Pyrolytically deposited carbon on the surface of MWCNTs

Table 2. Dynamic Light Scattering (DLS) data of Size (nm) and Polydispersity Index (PDI) in exposure medium **f-MWCNTs**: Control-salinity 28 + 0.10 mg/L f-MWCNTs; Control-salinity 28 + 1.0 mg/L f-MWCNTs; Salinity 21 + 0.10 mg/L f-MWCNTs; Salinity 21 + 1.00 mg/L f-MWCNTs and **Nf-MWCNTs**: Control-salinity 28 + 0.10 mg/L Nf-MWCNTs; Control-salinity 28 + 1.0 mg/L Nf-MWCNTs; Salinity 21 + 0.10 mg/L Nf-MWCNTs; Salinity 21 + 1.00 mg/L Nf-MWCNTs. All the samples were collected at different exposure periods (t0; t7; t21 and t28). I.d.: "Invalid data" (no colloidal material detected into the analyzed sample). n.d.: absence of triplicates values for mean size calculation

0.10 mg/L

	f-MWCNTs		**Nf-MWCNTs**		**f-MWCNTs**		**Nf-MWCNTs**		**f-MWCNTs**		**Nf-MWCNTs**		**f-MWCNTs**		**Nf-MWCNTs**	
	T0				**T7**				**T21**				**T28**			
salinity	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**
Size (nm)	3244.8	4551.8	2407.1	5330.4	3 I.d.	5 I.d.	3 I.d.	3938.3	5 I.d.	1661.8	n.d.	3841.9	5 I.d.	5 I.d.	4542.7	5 I.d.
PDI	1.30	1.86	0.98	1.79	n.d.	n.d.	n.d.	1.23	n.d.	0.10	n.d.	1.09	n.d.	n.d.	1.81	n.d.

1.00 mg/L

	f-MWCNTs		**Nf-MWCNTs**		**f-MWCNTs**		**Nf-MWCNTs**		**f-MWCNTs**		**Nf-MWCNTs**		**f-MWCNTs**		**Nf-MWCNTs**	
	T0				**T7**				**T21**				**T28**			
salinity	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**	**28**	**21**
Size (nm)	5714.4	6264.2	6714.4	7845.3	5 I.d.	5548.8	3602.9	8824.0	5 I.d.	2953.8	n.d.	6230.6	5 I.d.	5 I.d.	3865.2	4270.5
PDI	1.45	2.17	1.75	2.83	n.d.	1.74	1.39	1.83	n.d.	0.75	n.d.	2.29	n.d.	n.d.	1.40	1.40

Table 3. Effect on oxidative stress biomarkers (PROT, GLY, ETS, SOD, CAT, GPx, GSTs, LPO, GSH/GSSG, ATChI-ChE) in *Ruditapes philippinarum* by f-MWCNTs and Nf-MWCNTs at each of the tested concentrations (control-0.00, 0.10, 1.00 mg/L) under control-salinity 28 and low-salinity 21. Significant differences ($p \leq 0.05$) between f-MWCNT and Nf-MWCNTs within each salinity at each exposure concentration were represented with asterisks

	0.00 mg/L				**0.10 mg/L**				**1.00 mg/L**			
	28		**21**		**28**		**21**		**28**		**21**	
	f-MWCNTs	Nf-MWCNTs	f-MWCNTs	Nf-MWCNTs	f-MWCNTs	Nf-MWCNTs	f-MWCNTs	Nf-MWCNTs	f-MWCNTs	Nf-MWCNTs	f-MWCNTs	Nf-MWCNTs
PROT	32.39 ±5.85	34.31 ±1.84	27.10 ±7.33	27.10 ±7.34	15.23 ±6.33	17.36± 2.87	21.00 ±5.58	19.09 ±7.33	15.56 ±4.46	13.83 ±0.83	17.63 ±6.39	17.67 ±8.50
GLY	7.22± 1.72	7.93± 0.37	10.60 ±2.33	10.62 ±2.33	7.46± 1.82	6.09±0.20*	8.51± 1.51	8.63± 2.05	4.56± 1.87	4.01± 0.57*	7.44± 1.61	7.46± 2.93
ETS	38.59 ±4.04	40.67 ±1.86	38.77 ±1.59	38.77 ±1.57	44.38 ±6.40	62.92± 2.66*	46.44 ±1.51	45.19 ±1.98*	56.71 ±4.45	69.61 ±1.09*	48.02 ±1.67	50.64 ±6.14*
LPO	20.27 ±2.00	19.98 ±2.03	23.63 ±1.83	23.67 ±1.84	50.39 ±4.79	39.83± 3.02	45.77 ±1.83	36.88 ±2.95	52.09 ±3.71	46.50 ±0.68	47.07 ±5.28	42.76 ±5.33*
GSH/ GSSG	2.00± 0.99	2.10± 0.18	2.60± 0.51	2.61± 0.52	1.62± 0.17	1.44±0.02	2.08± 1.13	1.77± 0.53	0.96± 0.23	1.01± 0.05	1.27± 0.31	1.39± 0.27*
SOD	5.33± 1.76	5.41± 0.24	5.08± 1.85	5.08± 1.85	10.28 ±3.67	6.46±0.07*	7.93± 2.50	6.92± 2.93*	12.81 ±2.91	8.27± 0.05*	6.73± 2.53	8.04± 3.02
CAT	20.57 ±1.83	21.11 ±0.10	19.28 ±1.60	19.25 ±1.61	20.10 ±0.85	21.04± 0.04	21.87 ±1.81	22.22 ±1.71	22.37 ±1.10	20.82 ±0.11	21.37 ±1.58	22.36 ±2.81
GPx	0.01± 0.00	0.01± 0.00	0.02± 0.00	0.02± 0.00	0.02± 0.00	0.02±0.00	0.01± 0.00	0.01± 0.00	0.01± 0.00 *	0.006 ±0.00*	0.02± 0.00	0.02± 0.00
GSTs	0.21± 0.02	0.20± 0.00	0.19± 0.01	0.19± 0.01	0.12± 0.03	0.20±0.00*	0.18± 0.01	0.17± 0.03	0.14± 0.04	0.19± 0.00	0.17± 0.02	0.17± 0.02
ATChI-ChE	0.26± 0.06	0.23± 0.04	0.25± 0.04	0.25± 0.04	0.06± 0.02	0.07±0.01	0.04± 0.01	0.04± 0.01	0.04± 0.01	0.05±* 0.00	0.04± 0.01	0.05± 0.01