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The influence of Arsenic on the toxicity of carbon nanoparticles in bivalves

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ABSTRACT

Although an increasing number of studies have been published on the effects of emergent pollutants such as carbon nanoparticles, there is still scarce information on the impact of these contaminants on marine organisms when acting in combination with classical pollutants such as meta(loid)s. The present study evaluated the impacts of Arsenic and Multi-Walled Carbon Nanotubes (MWCNTs) in the clam *Ruditapes philippinarum*, assessing the effects induced when both contaminants were acting individually (As, NP) and as a mixture (As + NP). Metabolic capacity (electron transport system activity), oxidative stress (antioxidant and biotransformation enzymes activity and cellular damage) and neurotoxicity (Acetylcholinesterase activity) biomarkers were evaluated in clams after a 28 days exposure period. The results obtained showed that the accumulation of As was not affected by the presence of the NPs. Our results demonstrated that higher injuries were noticed in clams exposed to NPs, with higher metabolic depression and oxidative stress, regardless of the presence of As. Furthermore, higher neurotoxicity was observed in clams exposed to the combination of both contaminants in comparison to the effects of As and NPs individually.

1. Introduction

Coastal zones have always attracted humans, with recent studies indicating that population will further growth in these areas (among others [1]). This will lead to aquatic pollution rise derived from industry, agriculture and urban wastes, including mixtures of newly developed and classical pollutants. Metals and polyaromatic hydrocarbons are the toxic contaminants most commonly found in the limelight, but a vast variety of substances arrives currently the aquatic environment, including newly developed chemicals and products [2–6]. In particular, aquatic systems such as estuaries and coastal lagoons can present mixtures of natural and anthropogenic chemicals with wide diversity of origins [7–10]. Therefore, organisms living in these environments are typically exposed to a complex mixture of contaminants that may cause toxic effects, even though their concentrations are lower than the No Observable Effect Concentration (NOEC) [11–13].

In the aquatic environment metals and metalloids are frequent and persistent contaminants [14–16]. Over the years these contaminants have become concentrated on the Earth's surface mainly by mining, smelting and industrial products, reaching aquatic systems worldwide [17,18]. Due to their toxicity and persistence, mercury (Hg), copper (Cu), lead (Pb), cadmium (Cd) and arsenic (As) are among the most hazardous elements present in aquatic ecosystems (ATSDR 2017 Substance Priority List). Although adverse effects of such elements have been known for a long time, exposure to metals and metalloids still occurs, with known effects on marine invertebrates inhabiting coastal areas [19–22]. Regarding As, this metalloid is ranked in the first position of the ATSDR [23] 2017 Substance Priority List, corresponding not only to a list of the most toxic substances but rather to a prioritization of substances based on a combination of their frequency, toxicity, and potential for human exposure. Arsenic is widely distributed throughout the environment, occurring both naturally and as a result of human activity, in organic and inorganic (the most toxic) forms (see for review [24]). In aquatic environments long-term accumulation of As has been associated with a range of impacts, including organisms biochemical alterations [25–27]. Laboratory studies also demonstrated that As causes injuries to marine organisms, including generation of oxidative stress [28–31].

Besides metals and metalloids, recent studies have shown the increasing presence of a diversity of nanoparticles (NPs) in aquatic

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ecosystems [32–34]. Among these NPs are carbon nanotubes (CNTs), currently identified as one of the most important and commercially used nanomaterials, due to their superior electrical and thermal conductivity as well as higher mechanical strength over conventional materials [35–37]. The commercial production and use of CNTs have increased over the last years, with a wide variety of industrial applications, including electronic devices, composite materials, plastics, paints, batteries, touch screens, and drug delivery. During manufacturing, use, or disposal, CNTs are expected to end up in soil, water, or air that, due to their non-biodegradability, can result in toxic effects in different organisms [38]. Nevertheless, the potential toxic impacts of NPs, namely CNTs, to the environment has been a topic of concern and the knowledge concerning this issue has significantly increased in the last years. Recently, studies with aquatic organisms showed that Graphene oxide (GO) induced negative effects on the regenerative capacity, energy-related responses and oxidative stress of *Diopatra neapolitana* polychaetes after 28 days of exposure [39]. Additionally, increased energy expenditure and toxic impacts at the cellular level (oxidative stress and neurotoxicity) has been observed in *Ruditapes phylippinarum* clams exposed to Multi-Walled Carbon Nanotubes (MWCNTs) [40]. Also, alterations of the histological structure of different tissues (mantle, gills, gut, and the digestive gland) as well as activation pof oxidative stress molecular biomarkers have been described in *Crenomytilus grayanus* mussels exposed to MWCNTs [41].

However, the current lack of information on the composition of environmental mixtures and the limited understanding of the combined effects resulting from exposure to chemical mixtures still lack investigation to perceive the risks induced in exposed organisms. Previous studies revealed that the coexistence of NPs with Cu ion enhances the toxicity of this element to daphnids [42,43]. Furthermore, information on the impacts of NPs on the bioavailability of other contaminants is limited, despite studies showing that Titanium dioxide nanoparticles (TiO_2 NPs) induced the accumulation of Arsenate in the common carp *Cyprimus carpio* ([44]). Therefore, the present study aimed to investigate the influence of As on the toxicity of functionalized Multi-Walled Carbon Nanotubes (f-MWCNTs) (by introducing carboxyl groups (-COOH)). For this, *Ruditapes philippinarum* clams were chronically exposed to As, f-MWCNTs and the combination of both contaminants, and the As accumulation as well as the biochemical parameters associated with clams metabolism, oxidative status and neurotoxicity were evaluated.

2. Materials and methods

2.1. Experimental conditions

Ruditapes philippinarum specimens with weight (17.4 ± 1.5 g), were collected from the Ria de Aveiro lagoon (northwest Atlantic coast of Portugal). In particular, clams were collected from the Mira channel, considered as a reference area, with As concentrations lower than 10 μg/L in water, and in bivalves tissues at concentrations lower than 2 μg/g (wet weight, WW) [45–47].

After sampling, clams were maintained in the laboratory for 15 days to depurate and acclimate to laboratory conditions. Previous studies developed by Freitas et al. [45] demonstrated the capacity of bivalves to depurate under laboratory conditions, by presenting decreased metal (oid)s content. During this period clams were kept in artificial seawater (salinity 35 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Center) under continuous aeration, temperature 17 ± 1 °C; pH at 7.9 ± 0.1, and photoperiod at 12 light: 12 dark. The acclimatization temperature (17 °C) and salinity (35) were chosen considering the mean values in September in the sampling area (temperature = 16–19 °C, IPMA, 2016; salinity = 34 ± 1 [48]). Seawater was renewed 2-3 times per week and organisms were fed with AlgaMac Protein Plus, Aquafauna Bio-Marine, Inc (150,000 cells/animal).

After acclimation, organisms were distributed in different aquaria, to test the impacts induced by exposures to Arsenic (As), functionalized multi-walled carbon nanotubes (f-MWCNTs) and the mixture of both contaminants, testing the following conditions: A) **CTL**, control, with 0 mg/L of As and 0 mg/L of f-MWCNTs; B) **As**, 0.1 mg/L of As and 0 mg/L of f-MWCNTs; C) **NP**, 0.1 mg/L of f-MWCNTs and 0 mg/L of As; D) **As + NP**, 0.1 mg/L of As and 0.1 mg/L of f-MWCNTs. Sodium arsenate dibasic heptahydrate was used to prepare a stock solution of 1000 mg/L.

Per condition 3 aquaria with 6 L of seawater were used, with 5 organisms per replicate.

Arsenic was selected for this study considering its wide spatial distribution in aquatic systems as well as its relative abundance in the environment, with concentrations ranging from 0.5 μg/L to 5 mg/L in water and from 25 mg/kg to 3000 mg/kg in sediments [47,49–52]. Studies conducted in Portugal identified As concentrations ranging from 5 to 15 μg/g WW in clams [47] and from 2.1 to 2.4 μg/g WW in cockles [46] from the Ria de Aveiro; while concentrations ranging from 2.3 to 21.8 μg/g were observed in clams from the Tagus estuary [53]. The concentration used (0.1 mg/L) was based on previous studies where similar concentrations revealed toxic effects in bivalves *Mytilus galloprovincialis* [54]. Considering the concentration of 100 μg/L of As and the volume of water per aquarium (6 L), as well as the weekly renewal of water contaminated during the experiment (4 times), each one of the 5 clams in the aquarium would bioaccumulate a maximum of 480 μg of As (100*6*4/5 = 480). This would lead to a concentration of As in clams whole tissue of 28 μg/g (WW), assuming the average weight of each clam of approximately 17 g WW. This concentration is higher than concentrations observed in clams from the sampling area (Ria the Aveiro), but still ecologically relevant considering values of As identified in clams from other aquatic systems such as in the Venice lagoon where As concentrations ranged between 40 and 45 μg/g WW [55].

The f-MWCNTs were selected for this experiment due to of their wide range of industrial applications (including display technologies, electronics, nutrition, cosmetics, and medical drug designing [56,57]), and because they present high probability to reach the aquatic environments [58]. Also, functionalized nanoparticles were selected for this study considering their higher dispersibility in salt water compared to non-functionalized nanoparticles, which will increase the bioavailability to the organisms. The exposure concentration of f-MWCNTs was selected: i) to obtain observable effects considering previous study conducted by [59] that revealed observable biochemical responses using bivalves and the same f-MWCNTs ii) following the predicted environmental concentrations (PECs) of CNTs in aqueous systems. Although the environmentally relevant concentrations (ERCs) of CNTs in water, based on a stochastic/probabilistic material-flow computer model, are in the μg/L or ng/L range [60], the PECs of CNTs in aqueous systems reported from the most recent literature (see for example [61,62]) were projected to approximately 0.001–1000 μg/L.

During the experiment (28 days) clams were maintained at constant aeration. Temperature, pH and salinity were daily checked and maintained at the same conditions of the acclimation period.

Along the exposure period clams were fed with Algamac protein plus (150,000 cells/animal) twice a week, and seawater was renewed weekly, after which the experimental conditions were re-established, including As and f-MWCNTs concentrations, to ensure the same exposure concentrations during the experiment, as well as water salinity, temperature and pH levels.

After exposure, organisms were individually frozen and manually homogenized with a mortar and a pestle under liquid nitrogen. Each homogenized organism was divided into 0. 3 g aliquots, that were used for biomarkers analyses and As quantification.

2.2. f-MWCNTs characterization

Commercially available MWCNTs-COOH (f-MWCNTs) were purchased from Times Nano: Chengdu Organic Chemicals Co.Ltd., Chinese

Table 1
Characterization of the powder form of MWCNTs-COOH (f-MWCNTs) provided by Times Nano: Chengdu Organic Chemicals Co.Ltd., Chinese Academy of Sciences (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com).

	MWCNTs-COOH
OD (Outer Diameter (nm))	< 8
ID (Inner Diameter (nm))	2–5
Length (um)	10–30
Carbon Purity (%)	98
Amorphous Carbon (mol%)	8–10
-COOH content (wt%)	3.86
SSA (Special Surface Area (m^2/g))	400
Tap density (g/cm^3)	0.27
True density (g/cm^3)	~2.1
EC (Electric Conductivity (s/cm))	> 100

Academy of Sciences (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com) and all the technical data of the NMs are specified in Table 1.

The f-MWCNTs were weighed (stock solution of 50 mg/L), suspended in artificial seawater (28 salinity) and sonicated using a ultrasonic processor (UP 400S, Hielscher Ultrasound Technology) for few minutes to promote stable suspension in the water column [63] without compromising the physical integrity of the nanoparticles [64]. In the present study f-MWCNTs were used directly without any chemical processing before use.

The average size distribution and the polydispersity index (PDI) of f-MWCNT suspensions were analysed in each exposure condition (0.10 mg/L f-MWCNTs; 0.10 mg/L f-MWCNTs + 0.10 mg/L As) and at different exposure periods: t0: time zero, immediately after the dispersion of f-MWCNTs in a water medium; t7: water samples collected after the first week of exposure before water renewal; t14: water samples collected after the second week of exposure before water renewal; t28I: samples collected at the start of the fourth week of exposure and t28F: samples collected at the end of the fourth week of exposure (Table 2). As reported in the literature Dynamic Light Scattering (DLS) measurements have been routinely carried out as an effective tool to observe the evolution of relative particle size distributions of CNTs in aqueous media as a function of time [65–67]. In the present work, DLS measurements were carried out by using a Delsa Nano C from Beckman Coulter, Inc. (Fullerton, CA) equipped with a laser diode operating at 658 nm. Scattered light was detected at 165° angle and analysed by using a log correlator over 120 accumulations for a 1.0 mL of sample in a UV cuvette semi-micro. Each sample was reproducibly shaken before analysis and exposed to a minimum of DLS measurements needed to obtain at least three valid data. The calculation of the particle size distribution and distribution averages was performed by using CONTIN particle size distribution analysis routines through Delsa Nano 3.73 software. The hydrodynamic radius and polydispersity index of the analysed dispersions were calculated on three replicates of each sample by using the cumulant method. Undetected colloidal material at the end of each measurement is indicated as Invalid data (I.d.).

The mean size (nm) and the PDI of functionalized MWCNTs-COOH particle aggregates (f-MWCNTs, 0.1 mg/L) and functionalized MWCNTs-COOH particle aggregates (f-MWCNTs, 0.1 mg/L) + As (0.1 mg/L) in simulated seawater (salinity 28) were measured by DLS (Table 2). Each condition was tested both in absence (sample CTRL 1 and 2) and in presence (samples A, B, C) of *Ruditapes philippinarum* individuals. DLS characterization was used to detect the presence of macro/micro/nano-sized particle aggregates in aqueous media under the adopted experimental conditions. DLS analyses were repeated several times due to the inherent heterogeneity and colloidal instability of the analysed samples. 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.

Table 2
Dynamic light scattering (DLS) data of mean size (nm) and standard deviation values (nm) of samples exposed to medium (0.10 mg/L MWCNTs-COOH and MWCNTs-COOH (0.10 mg/L) + As (0.10 mg/L)) either in absence (CTRL 1, 2) or in presence (A, B, C) of *Ruditapes philippinarum* individuals collected in different exposure periods (t0; t7; t14; t28 beginning of the week (t28I); t28 end of the week (t28 F)).

MWCNTs-COOH (0.10 mg/L)		
t0		
Samples	Mean Size (nm)	Standard deviation (nm)
CTRL1,2	1830.0	927.5
A,B,C	2406.8	1045.1
t7		
Samples	Size (nm)	Standard deviation (nm)
CTRL1,2	3410.9	108.7
A,B,C	2072.2	138.3
t14		
Samples	Size (nm)	Standard deviation (nm)
CTRL1,2	4030.8	1977.3
A,B,C	2918.2	884.2
t28I		
Samples	Size (nm)	Standard deviation (nm)
CTRL1,2	3600.7	850.1
A,B,C	3172.6	868.7
t28F		
Samples	Size (nm)	Standard deviation (nm)
CTRL1,2	3655.5	467.0
A,B,C	2629.2	382.9
MWCNTs-COOH (0.10 mg/L) + As (0.10 mg/L)		
t0		
Samples	Mean Size (nm)	Standard deviation (nm)
CTRL1,2	2121.3	1569.2
A,B,C	2454.6	1164.4
t7		
Samples	Size (nm)	Standard deviation (nm)
CTRL1,2	3790.2	48.0
A,B,C	2358.3	159.1
t14		
Samples	Size (nm)	Standard deviation (nm)
CTRL1,2	3855.0	1414.4
A,B,C	3798.3	226.7
t28I		
Samples	Size (nm)	Standard deviation (nm)
CTRL1,2	3206.8	242.5
A,B,C	3055.9	820.3
t28F		
Samples	Size (nm)	PDI
CTRL1,2	2550.4	239.6
A,B,C	4409.7	1517.0

2.3. As quantification in clams

Total As concentration in clams whole soft tissue was quantified by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS), performed on a Thermo ICP-MS XSeries equipped with a Burgener nebulizer, as described by Henriques et al. [68].

The aliquots of 0.3 g WW of previously homogenized freeze-dried tissue (3 organisms per condition) were digested with 4 mL of nitric acid (HNO_3 65%) at 60 °C for 18 h. After that, 2 mL of hydrogen peroxide (H_2O_2 37%) were added and left sit for 1 h at 80 °C. At the end of this time the mixture was evaporated almost to dryness, 0.4 mL of HNO_3 65% added and left sit for 15 min. The solution was collected into 10 mL of ultrapure water.

The limit of quantification of the method was 0.34 μg/g WW (1.1 μg/g dry weight, DW), with an acceptable coefficient of variation among replicates of 5%. The quality control was assured by running procedural blanks (reaction vessels with only HNO_3 and H_2O_2) and certified reference material TORT-2 (Lobster Hepatopancreas; 21.6 ± 1.8 mg As kg^{-1}) in parallel with samples. Blanks were always below the quantification limit and mean percentage of recovery for As was 110 ± 4% (n = 4).

2.4. Biochemical parameters

After 28 days exposure, tissue aliquots used for biomarker analysis (2 *per* aquarium, 6 *per* condition) were extracted with specific buffers for each biomarker [69–72]. Biochemical analyses were performed in duplicate for each sample and biomarker with a BioTek Synergy HT micro-plate Reader.

2.4.1. Metabolic capacity and energy reserves

The electron transport system (ETS) activity was measured following King and Packard [73] and De Coen and Janssen [74] methods. The absorbance was measured at 490 nm during 10 min with intervals of 25 s. ETS activity was expressed in nmol/min *per* g WW.

Protein (PROT) content was determined following the spectrophotometric method of Biuret [75] whit bovine serum albumin (BSA) as standard (0–40 mg/mL). Absorbance was measured at 540 nm. PROT was expressed in mg *per* g WW.

Glycogen (GLY) content was quantified following the sulphuric acid method [76], using glucose standards (0–2 mg/mL). Absorbance was measured at 540 nm and GLY expressed in mg *per* g WW.

2.4.2. Antioxidant and biotransformation defences

The activity of SOD was determined using the method described in Beauchamp and Fridovich [77] and adaptations performed by Carregosa et al. [78]. 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 WW, where U corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

The activity of CAT was quantified according to Johansson and Borg [79] and the modifications performed by Carregosa et al. [78]. 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 WW, 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 [80]. The absorbance was measured at 340 nm in 10 s intervals during 5 min and the enzymatic activity was determined using $\varepsilon = 6.22\ mM^{-1}cm^{-1}$. The results were expressed as U *per* g WW, where U represents 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. [81]. 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 WW where U is defined as the amount of enzyme that catalysis the formation of 1 μmol of dinitrophenyl thioether *per* min.

2.4.3. Indicators of cellular damage

Lipid peroxidation (LPO) was measured according to Ohkawa et al. [82] and modifications referred by Carregosa et al. [78]. The absorbance was measured at 535 nm and LPO levels were determined using $\varepsilon = 0.156\ mM^{-1}\ cm^{-1}$. LPO levels were expressed in nmol of MDA equivalents formed *per* g WW.

2.4.4. Neurotoxicity

Acetylthiocholine iodide (ATChI, 470 μM) substrates were used for the determination of Acetylcholinesterase (AChE) activity, following the methods of Ellman et al. [83] and modification by Mennillo et al. [84]. Enzyme activities were recorded continuously for 5 min at 412 nm and expressed in nmol *per* min *per* g WW.

2.5. Data analysis

All the biochemical results (ETS, PROT, GLY, CAT, GPx, GSTs, LPO, and AChE) 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 were considered as significantly different.

The null hypothesis tested was: i) for each biomarker response, no significant differences existed among exposure conditions (CTL, As, NP, As + NP). Significant differences concerning each biomarker among exposure conditions were represented with different letters.

3. Results

3.1. f-MWCNTs characterization

The comparison of the results on MWCNTs characterization did not evidence a correlation of the mean radius of the suspended particles with the time of sample collection, the presence of As or *R. philippinarum* organisms. To better evaluate the differences among the tested conditions, the mean values calculated for the controls (CTRL 1 and 2) and the *R. philippinarum* containing samples (A, B, C) were compared, but no consistent differences were evidenced due to the large variability of the calculated standard deviations. The randomly distributed hydrodynamic radius values recorded during the analysis, which did not depend on the different tested conditions, could be attributed to the constant presence of a white particulate material dispersed in the analysed samples which negatively interfered with f-MWCNTs aggregates determination.

3.2. As concentrations in clams

Arsenic concentration was higher in clams exposed to this metalloid (As and As + NP conditions, 25.5 ± 6.4 μg/g DW) in comparison to values recorded in clams exposed to control (16.8 ± 2.5 μg/g DW) and NP (17.8 ± 5.3 μg/g DW), but no statistically significant differences were observed among tested conditions.

3.3. Biochemical parameters

3.3.1. Metabolic capacity and energy reserves

In comparison to control (CTL), the ETS activity was significantly higher in clams exposed to As while significantly lower in clams exposed to f-MWCNTs (NP) and the combination of both contaminants (As + NP) (Fig. 1A).

The GLY content was significantly higher in contaminated clams

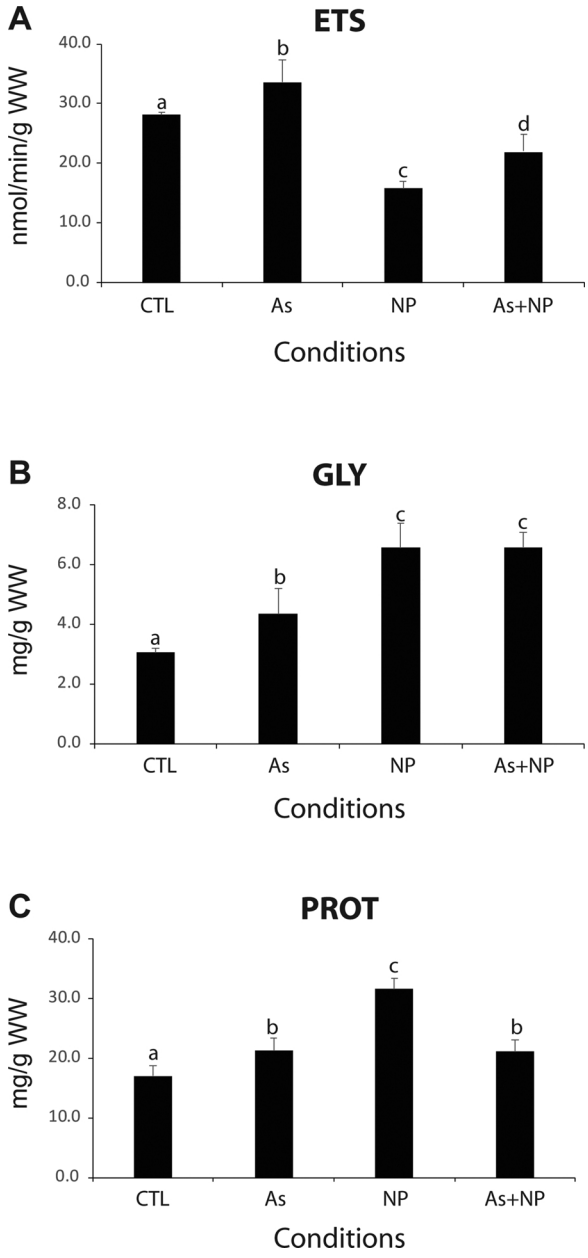

Fig. 1. A: Electron transport system (ETS) activity; **B**: Glycogen (GLY) content; **C**: Glycogen (GLY); **C**: Protein (PROT) content (mean + standard deviation), in *Ruditapes philippinarum* exposed to control (CTL), Arsenic (As) (0.1 mg/L), functionalized multi-walled carbon nanotubes (NP) (0.1 mg/L) and the combination of both contaminants (As + NP). Significant differences ($p \leq 0.05$) among exposure conditions were represented with different letters.

compared to non-contaminated ones (CTL) (Fig. 1B). The highest values were observed in clams exposed to NP and the combination of both contaminants (As + NP), with no significant differences between clams exposed to these conditions (Fig. 1B).

The PROT content was significantly higher in contaminated clams compared to non-contaminated ones (CTL) (Fig. 1C). Significantly higher values were observed in clams exposed to NP in comparison to organisms exposed to As and the combination of both contaminants (As + NP) (Fig. 1C).

3.3.2. Antioxidant and biotransformation defences

The activity of SOD was significantly higher in contaminated clams compared to control condition (CTL), with no significant differences among clams exposed to As, NP and As + NP conditions (Fig. 2A).

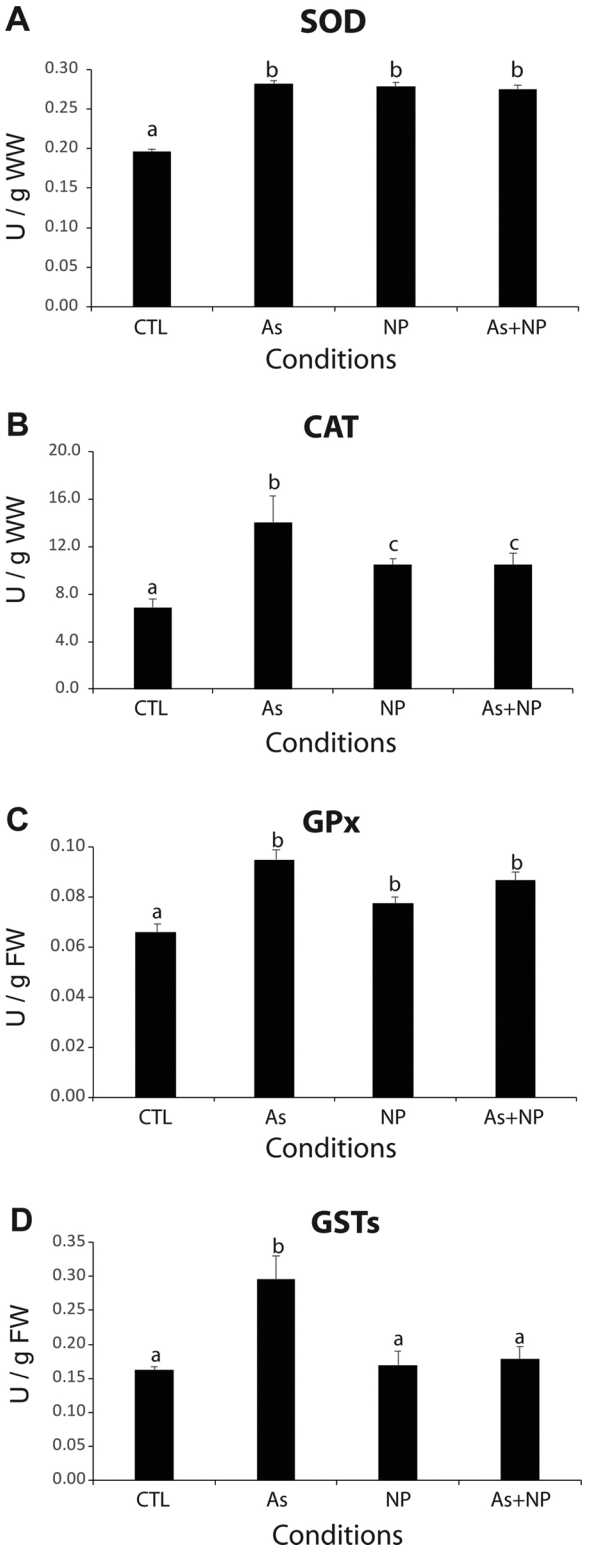

Fig. 2. A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Glutathione peroxidase (GPx) activity; **D**: Glutathione S-transferases (GSTs) activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to control (CTL), Arsenic (As) (0.1 mg/L), functionalized multi-walled carbon nanotubes (NP) (0.1 mg/L) and the combination of both contaminants (As + NP). Significant differences ($p \leq 0.05$) among exposure conditions were represented with different letters.

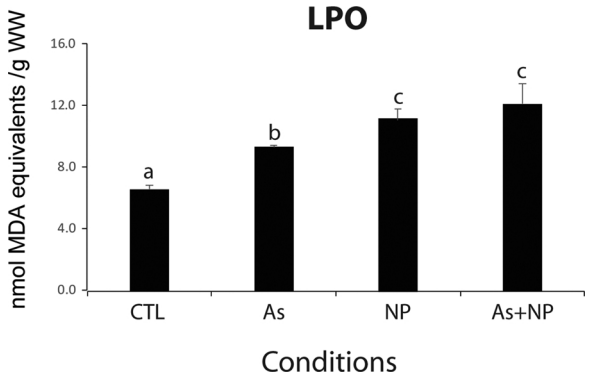

Fig. 3. Lipid peroxidation (LPO) level (mean + standard deviation), in *Ruditapes philippinarum* exposed to control (CTL), Arsenic (As) (0.1 mg/L), functionalized multi-walled carbon nanotubes (NP) (0.1 mg/L) and the combination of both contaminants (As + NP). Significant differences ($p \leq 0.05$) among exposure conditions were represented with different letters.

The activity of CAT was significantly higher in contaminated clams in comparison to control organisms (Fig. 2B). The highest CAT activity was observed in clams exposed to As compared to clams exposed to NP and As + NP conditions (Fig. 2B). No significant differences were observed between clams exposed to NP and As + NP (Fig. 2B).

The activity of GPx was significantly higher in contaminated clams compared to control organisms (CTL), with no significant differences among clams exposed to As, NP and As + NP conditions (Fig. 2C).

The activity of GSTs was significantly higher in clams exposed to As in comparison to values observed in clams exposed to the remaining conditions (CTL, NP, As + NP) (Fig. 2D). No significant differences in GSTs activity was observed among clams exposed to CTL, NP and As + NP (Fig. 2D).

3.3.3. Indicators of cellular damage

LPO levels were significantly higher in contaminated clams compared to control organisms (CTL), with significant differences between clams exposed to As and clams exposed to NP and As + NP conditions (Fig. 3).

3.3.4. Neurotoxicity

The activity of AChE was significantly lower in contaminated clams compared to control organisms (Fig. 4). The lowest enzyme activity was observed in clams exposed to As + NP, with significant differences among As, NP and As + NP conditions (Fig. 4).

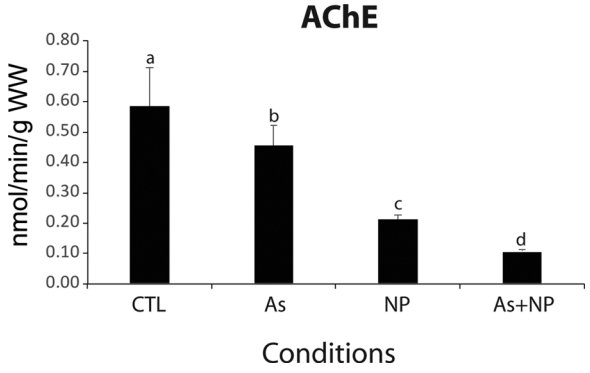

Fig. 4. Acetylcholinesterase (AChE) activity (mean + standard deviation), in *Ruditapes philippinarum* exposed to control (CTL), Arsenic (As) (0.1 mg/L), functionalized multi-walled carbon nanotubes (NP) (0.1 mg/L) and the combination of both contaminants (As + NP). Significant differences ($p \leq 0.05$) among exposure conditions were represented with different letters.

4. Discussion

The capacity of NPs, in particular carbon nanotubes (CNTs), to interact with other pollutants and influence their bioavailability and toxicity is still largely unclear. These interactions were investigated in the present study.

The capacity of adsorption of both organic and inorganic toxicants by various NPs was evaluated and summarized by several authors (e.g. [85,86]). Regarding metals, previous studies identified the 'Trojan horse' effect consisting of NPs capacity to adsorb these pollutants and thus enabling for their uptake by organisms, which may increase their toxic impacts ([87,42]). Studies have demonstrated that CNTs have a great affinity for contaminants, including metals [88]. In fact, their external surfaces, interstitial pore spaces, inner cavities, and peripheral grooves are four possible adsorption sites. Most of these sites are accessible and allow the adsorption and desorption of contaminants and the bioaccumulated CNTs-contaminant complexes can release the contaminant and thereby cause toxicity as demonstrated by Wang et al. [89] where in the snail *Bellamya aeruginosa*, MWCNTs enhanced the oxidative damage of Cd^{2+}. However, our findings showed that As accumulation in *Ruditapes philippinarum* was not affected by the presence of f-MWCNTs, with organisms revealing similar As concentrations both when exposed to As or the combination of As and f-MWCNTs. Therefore, our findings may highlight that impacts on the bioavailability and accumulation of contaminants are contaminant mixture and species specific, indicating that in the case of As the accumulation of this metalloid is not facilitated by the presence of CNTs in *R. philippinarum*.

Regarding organisms' biochemical performance, previous studies [90–92] demonstrated that the ETS activity can be used as a measure of metabolic capacity since it represents a proxy of the cellular respiratory potential of a given organism. The ETS activity assay was already used to assess aerobic potential in invertebrates, including the mussel *Dreissena polymorpha* [93]. Considering this information, the present findings indicate that clams' metabolism was reduced in NP exposures (NP and As + NP), as clams presented lower ETS activity under these conditions compared to clams exposed to As. Therefore, by reducing metabolic capacity (lower filtration rates associated to valves closure), clams exposed to As + NP may have limited the accumulation of As and the Trojan Horse effect, resulting in similar As accumulation patterns observed in clams exposed to As and to As + NP. Among others Ortmann and Grieshabe [94] demonstrated that the bivalve *Corbicula fluminea* presented a pronounced metabolic depression during valve closure, supporting the hypothesis that clams exposed to As + NP may have limited the accumulation of As by lowering their ETS activity.

Our findings further highlighted that higher reduction of ETS activity in clams exposed to NP and As + NP in comparison to clams exposed to As, resulted in lower energy expenditure under these conditions (NPs and As + NPs), with higher PROT and especially GLY content in calms exposed to NPs in comparison to As. The capacity of clams to limit the expenditure of energy reserves by reducing their metabolic capacity under stressful conditions was demonstrated in previous studies with bivalves, namely exposed to metals [54,70,95] and CNTs [59]. Nevertheless, the expenditure of energy reserves may depend on the stress level, that depends on contaminant type and concentration as well as on species physiological and biochemical performance. Our results may indicate that clams are more prone to save their energy reserves when exposed to NPs in comparison to As, which is associated to clams capacity to reduce their metabolism (identified by lower ETS activity) when exposed to NPs. Nevertheless, to the best of our knowledge no information is available on the energy related markers in bivalves exposed to a mixture of NP with metals or metalloids.

Under different stressful conditions, organisms may be able to reduce their metabolism up to a certain extent to prevent higher injuries namely by limiting the accumulation of pollutants, which is not only dependent on species but also on the contaminant. Nonetheless, in most

cases, metabolism reduction is not sufficient to prevent organisms from injuries and an overproduction of reactive oxygen species is observed [96]. In these cases, organisms activate defense mechanisms by increasing the activity of antioxidant enzymes such as superoxide-dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). In the present study, antioxidant enzymes activity increased in contaminated organisms compared to non-contaminated ones, regardless of the contaminant (As or NP). Furthermore, our study also revealed that the combination of both contaminants (As + NP) induced similar antioxidant defenses in clams compared to the responses observed in the organisms exposed to each contaminant individually. Our findings demonstrated that clams were able to activate their antioxidant enzymes to eliminate the excess of ROS due to the contaminants and that the exposure to As, NP or the combination of both contaminants may have induced the same antioxidant defense capacity, indicating that the combination of contaminants did not have an additive effect on enzymes activity. Also, although clams presented reduced metabolism in the presence of f-MWCNTs our results indicated that this decrease was not enough to limit the capacity of clams to activate their defense mechanisms when exposed to this NP. In accordance to our results, previous studies showed that metals and NPs induced the activation of antioxidant enzymes in bivalves [30,31,97–102] to prevent higher oxidative levels but to our knowledge no information is available on the effects of the combined exposure to As and MWCNTs. However, similar antioxidant enzymes activity in clams exposed to NPs (NP and As + NP) and As do not necessarily indicate similar stress levels induced by these contaminants, but could be related to the capacity of clams exposed to NPs (NP and As + NP) to reduce ROS production by decreasing ETS activity. The reduction of the metabolic activity in clams exposed to NP (NP and As + NP) may indicate that f-MWCNTs have the capacity to diminish the activity of electron transport system in this species and therefore less ROS were generated. Studies have shown that the mitochondrial electron system activity is one of the major cellular generators of ROS [103–105]. Therefore, our results may suggest that the capacity of clams to reduce their ETS activity when exposed to NPs (NPs and As + NPs) could have contributed to the reduction of ROS production leading to an overall ROS level similar to the one resulting from As exposure and thus limiting the increase of antioxidant defenses.

Regarding biotransformation defense mechanisms, the present findings clearly demonstrated clams' capacity to increase the activity of GSTs when exposed to As but not in the presence of f-MWCNTs, indicating that this group of enzymes was not involved in the biotransformation of these NPs into less toxic excreted substances. Previous studies revealed that GSTs are activated by the presence of As in bivalves including clams [30,106] and mussels [15,54,107]. Nevertheless, Cid et al. [97] exposed *Corbicula fluminea* clams to carbon nanodiamonds (NDs) throughout 14 days, and revealed an increase of GSTs activity with increasing NDs concentration, while De Marchi et al. [59] showed that clams exposed to f-MWCNTs presented lower GSTs activity compared to non-contaminated organisms. These findings may suggest that the behavior of GSTs is dependent on the type and concentration of the NPs. Furthermore, the present results showed that the combination of both contaminants (As + NP) did not induce the activity of GSTs which may indicate that MWCNTs could have inhibited their action when combined with As since clams exposed to As + NP showed no increase on these enzymes activity in comparison to As acting alone. Because clams exposed to NP and As + NP presented GSTs values similar to control values this may also indicate that NPs were not able to activate GSTs when acting alone and As interacted with f-MWCNTs when in combination with this NP, making this metalloid unavailable and therefore not sufficiently toxic to clams to activate GSTs detoxification defense mechanisms. Anisimova et al. [41] observed a decrease of GSTs activity in *Crenomytilus grayanus* mussels after 48 h exposure to 12–14 nm diameter MWCNTs.

In the present study clams showed the highest cellular damages (measured by LPO levels) when exposed to the combination of both contaminants (As + NP), followed by clams exposed to NP and As, a result that is in agreement with antioxidant and biotransformation responses. In particular, the lowest LPO levels observed in clams exposed to As could be related to the highest GSTs, GPx and CAT activity recorded at this condition. On the other hand, higher LPO levels observed in clams exposed to NP and the combination of both contaminants (As + NP) was associated to the inefficiency of defense mechanisms in clams under these conditions that were not able to activate efficiently their defense mechanisms. Studies with As demonstrated the capacity of this contaminants to induced cellular damages in calms [30,31,106,108,109]. Also studies with MWCNTs showed that these NPs induced high LPO levels in the clams *R. philippianrum* exposed to MWCNTs for 28 days [59] and a similar response was observed by Anisimova et al. [41] in *C. grayanus* exposed to MWCNTs for 48 h. Nevertheless, up to our knowledge no information is available on the effects due the combination of MWCNTs with metals or metalloids.

Besides oxidative stress, neurotoxicity may occur in bivalves exposed to pollutants. To evaluate the neurotoxic impacts due to pollutants exposure the inhibition of cholinesterase in aquatic invertebrates has been used as a sensitive biomarker of exposure to NPs and metals [110–114,40,59,115]. The results here presented demonstrated that higher neurotoxic effects were observed in clams exposed to NP, clearly indicating higher capacity of f-MWCNTs to produce neurotoxicity in clams in comparison to As. These findings are in agreement with the number of studies which investigated the interactions between cholinesterases and NMs, demonstrating an inhibition of cholinesterase activity in invertebrates as a consequence of NPs exposure [111–114,40,59,115].

5. Conclusion

Our findings showed that similar As concentrations were observed in clams exposed to As and As + NP, indicating that MWCNTs did not promote higher accumulation of this metalloid in *R. philippinarum* specimens.

The present study clearly revealed that in terms of metabolic capacity, energy reserves and oxidative stress f-MWCNTs induced higher toxicity in clams in comparison to As, and the combination of both contaminants (As + NP) revealed no additive or synergistic effects in comparison to NP acting alone. The presence of f-MWCNTs (NP and As + NP conditions) induced metabolic depression in clams, avoiding energy reserves expenditure, despite antioxidant defenses activation. However, although the antioxidant capacity increased in clams exposed to f-MWCNTs, the activity of antioxidant enzymes was not able to eliminate the excess of ROS produced by clams contaminated with NPs and cellular damages occurred, which may also be related to the inefficient detoxification capacity of GSTs under these conditions.

Neurotoxicity was also observed in clams exposed to both contaminants but especially to the mixture of As and NP, evidencing that the combination of these contaminants could result in greater neurotoxic damages to clams. These findings clearly reveal synergistic effects of As and MWCNTs in terms of neurotoxicity.

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