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M.S.S. Silva, Miguel Oliveira, Helena Almeida, A. Dick Vethaak, Concepción Martínez-Gómez, Etelvina Figueira, Adília Pires

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[1]Centre for Environmental and Marine Studies (CESAM), Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Departament of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

[3]Department of Environment and Health, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

[4]Deltares, Marine and Coastal Systems, Delft, The Netherlands

[5]Instituto Español de Oceanografía (IEO), CSIC, Centro Oceanográfico de Murcia, C/Varadero, 1, San Pedro del Pinatar, Murcia 30740, Spain

*Email contact: adilia@ua.pt

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[1]Centre for Environmental and Marine Studies (CESAM), Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Departament of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

[3]Department of Environment and Health, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

[4]Deltares, Marine and Coastal Systems, Delft, The Netherlands

[5]Instituto Español de Oceanografía (IEO), CSIC, Centro Oceanográfico de Murcia, C/Varadero, 1, San Pedro del Pinatar, Murcia 30740, Spain

*Email contact: adilia@ua.pt

Abstract

Plastic pollution is a serious problem in aquatic systems throughout the world. Despite the increasing number of studies addressing the impact of macro- and microplastics on biota, there is still a significant knowledge gap regarding the effects of nanoplastics alone and in combination with other contaminants. Among the aquatic contaminants that may interact with nanoplastics is arsenic (As), a metalloid found in estuarine and coastal ecosystems, pernicious to benthic organisms. This study aimed to understand how a parental pre-exposure to 100 nm polystyrene nanoplastics (PS NPs) would influence the response of *Hediste diversicolor* to exposure to arsenic in terms of behaviour, neurotransmission, antioxidant defences and oxidative damage, and energy metabolism. The obtained data revealed an increase in burrowing time and a significant inhibition in cholinesterase activity in all polychaetes exposed to As, regardless of the pre-exposure to PS NPs. Oxidative status was altered particularly in parentally exposed organisms, with damage detected in terms of lipid peroxidation at 50 µg/L and protein carbonylation at 50 and 250 µg As/L exposed organisms when compared to control. Overall, data shows that parental pre-exposure to plastics influences the response of aquatic organisms, increasing their susceptibility to other contaminants. Thus, more studies should be performed with other environmental contaminants, to better understand the potential increased risk associated with the presence of nanoplastics may pose to aquatic ecosystems.

Keywords: polychaetes; arsenic; nanoplastics; pre-exposure; behaviour; biochemical parameters

1. Introduction

Discharges of substances/materials to aquatic environments have increased over the years, leading to an accumulation of contaminants in the marine environment. Sediments, in particular, may act as sinks and sources of contaminants (Breton and Prentiss, 2019; Bryan and Langston, 1992; Leslie et al., 2017; Pan and Wang, 2012). Benthic organisms, such as polychaetes, frequently the most abundant group in a wide range of marine/estuarine sediment types (Dorgham et al., 2014; Scaps, 2002; Silva et al., 2020a), are particularly vulnerable, being in constant contact with the sediment, pore water and the water-sediment interface (Banta and Andersen, 2003; Scaps, 2002). For some benthic macroinvertebrates, such as deposit-feeding polychaetes (e.g.: *Arenicola marina* and *Hediste diversicolor*), feeding is also an important *via* of exposure (Jumars et al., 2015; Weston et al., 2000) due to strategies that involve the ingestion of sediment particles, potentiating the accumulation of contaminants like metals (Fan et al., 2002; Jumars et al., 2015; Wang and Fisher, 1999) and plastics.

Metals and metalloids have been recognized as a relevant class of contaminants for many years. These contaminants are found at higher concentrations in the sediment than in the water column due to their affinity for sediment particles, like arsenic (As) (Casado-Martinez et al., 2012). Metals have been reported to accumulate in the tissue of macroinvertebrates and along higher trophic levels (Gaion et al., 2014; Golovanova, 2008; Has-Schön et al., 2015; Kiser et al., 2010), and alter the expression of genes associated with antioxidant enzymes and their activities, in macroinvertebrates (Amiard et al., 2006; Breton and Prentiss, 2019; English and Storey, 2003; Fang et al., 2010;

Golovanova, 2008; Lee et al., 2008; Won et al., 2012). Arsenic is frequently found in sediments, and in organisms along the food web (Boyle et al., 2008), with predators accumulating one of the non-toxic forms of As, arsenobetaine (Maher et al., 2009; Neff, 1997). However, fish ingestion of *H. diversicolor* contaminated with the inorganic forms arsenate and arsenite has been suggested to affect its reproductive capacity (Boyle et al., 2008). Various studies have reported the effects of metals and As on important endpoints in polychaetes, such as regenerative capacity, behaviour, antioxidant defences, and oxidative damage. In a field study analysing the impacts of sediment contamination by chromium (Cr), nickel (Ni), copper (Cu), lead (Pb), cadmium (Cd), mercury (Hg), and As, on the regeneration of the polychaete *Diopatra neapolitana*, on various sites in Ria de Aveiro, authors demonstrated that the higher the metal contamination of the site the more time worms needed to fully regenerate and the fewer segments were regenerated (Pires et al., 2017). In the same study, it was also demonstrated that polychaetes collected in more contaminated sites had higher levels of oxidative stress (Pires et al., 2017). When *H. diversicolor* was exposed to different metals (e.g., Cu, zinc (Zn), Cd, and silver (Ag)) behavioural alterations were found, with organisms needing more time to fully burrow into the sediments, when compared to control conditions. Endpoints related to oxidative status (catalase, superoxide dismutase, glutathione peroxidase activities), apoptosis (caspase activity), neurotransmission (cholinesterase activity), cell damage (thiobarbituric acid reactive substances levels), and immunomodulation (acid phosphatase and laccase-type phenoloxidase) were measured and found altered in the exposure conditions (Buffet et al., 2014a, 2014b, 2012a, 2012b, 2011a; Thit et al., 2020).

Regarding As exposure, to the authors' knowledge, only one study analysed the speciation of this metalloid on *H. diversicolor*, confirming that trimethyl-arsine was the predominant form found in the tissues of this polychaete (Gaion et al., 2014), but no studies analysed the effects of As on this polychaete species.

Plastics have emerged in recent years as a serious environmental problem due to their increased production in the last decades associated with low cost of production and a wide range of applications, from cosmetics to biomedical applications, that have promoted single-use materials, easily discarded (Avio et al., 2017; da Costa, 2018; de Sá et al., 2018; Li et al., 2016). Despite the restrictions implemented by various countries (e.g., Portugal, Spain, and the United Kingdom, in the European Union) (Lam et al., 2018), plastic production has increased exponentially in the last few years (PlasticsEurope, 2021). As a result plastic materials reach the marine environments, where slow degradation processes (Rios Mendoza et al., 2018) influenced by biotic and abiotic factors (Oliveira and Almeida, 2019) lead to micro- and nanoplastics (NPs). The definition of NPs is not consensual with some authors considering NPs particles of sizes up to 1000 nm (Gigault et al., 2018), while others those up to 100 nm (similar size range of, for example, metallic nanoparticles) (Lambert and Wagner, 2016; Oliveira and Almeida, 2019; Silva et al., 2020b; Thit et al., 2015). With a decrease in size, the role of surrounding media on plastic behaviour increases. Previous studies have demonstrated that NPs tend to aggregate in highly ionic strength media, like seawater, which leads to an increase in particle size (Brandts et al., 2018; Browne et al., 2007; da Costa et al., 2016; Gigault et al., 2018; Oliveira and Almeida, 2019; Silva et al., 2020b). The wide variety of sizes and shapes of plastic fragments makes them available to a wide range of marine organisms, from pelagic to benthic (de Sá et al., 2018; Ferreira et al., 2019; Silva et al., 2020b).

Polystyrene (PS) is among the most produced and most frequently found polymers in marine environments (Eriksen et al., 2014; PlasticsEurope, 2021). A recent study, using waterborne 100 nm PS NPs, demonstrated the capacity of these particles to promote an increase in burrowing time in the marine polychaete *H. diversicolor*, a decrease in cholinesterase activity, and damage at the protein level (Silva et al., 2020b). These same particles were also demonstrated to decrease the regenerative capacity of *H. diversicolor*, in which organisms regenerated fewer segments with the increase in concentrations (Silva et al., 2020c).

Considering that marine worms can affect the biogeochemical cycle of nutrients and have the potential to also influence the distribution of contaminants, due to their bioturbation activity (Banta and Andersen, 2003; Gebhardt and Forster, 2018; Scaps, 2002), they should be used as a valuable model organism in the study of the effects of environmental contaminants. Although there are available studies addressing the individual effects of PS NPs on *H. diversicolor*, no studies have addressed the effects of parental exposure to PS NPs on the response to other environmental contaminants. The present study aimed to evaluate the effects of two environmentally relevant As concentrations on *H. diversicolor*, that had parental exposure to PS NPs 100 nm and that had never been exposed to contaminants. The assessed endpoints included burrowing behaviour, energy metabolism, and oxidative stress and damage.

2. Methods and materials

2.1. *Test organisms*

Specimens of *H. diversicolor* were collected from a reference site in Ria de Aveiro (40.6331ºN, -8.7367ºW) (Pires et al., 2016) and allowed to depurate for two weeks under laboratory conditions with artificial seawater (pH 8.00 and salinity 28) and sediment (at a ratio of 3:1) on a temperature-controlled room (16±1ºC), under continuous aeration (Silva et al., 2020b).

2.2. *Experimental design*

For this study, two groups of six-months-old organisms were selected: one that had never been exposed to contaminants (NPEO – non-parentally exposed organisms) and another that had their parents exposed for 28 days to 0.005 mg/L of 100 nm PS NPs (PEO – parentally exposed organisms), with test water medium renewal twice a week (Silva et al., 2020b). Parents from both groups (PEO and NPEO) were transferred to new tanks with clean water and sediment where reproduction was induced by increasing the temperature of the corresponding tanks, according to Bartels-Hardege and Zeeck (1990) with some modifications. Offspring were allowed to grow for six months (size of organisms: 6-8 cm) under laboratory conditions with clean artificial seawater (pH 8.00 and salinity 28) and clean sediment (at a ratio of 3:1), in a temperature-controlled room (16±1ºC), under continuous aeration (Silva et al., 2020b). Polychaetes were fed *ad libitum* twice a week with commercial fish food (Protein 46.0%, Lipids 11.0%) (Santos et al., 2016; Silva et al., 2020b).

After the growth period, both groups of polychaetes were exposed for 28 days to two environmentally relevant As concentrations (0, 50, and 250 µg/L), which were chosen based on previous studies (Coppola et al., 2016). A stock solution of sodium arsenate (Na_3AsO_4) (CAS no. 10048-95-0, Sigma-Aldrich, Missouri, USA) was prepared in ultra-pure water and spiked in aquaria to achieve nominal As concentrations of 50 and 250 µg/L. Specimens of each group were randomly distributed per experimental condition (18 per condition; 3 per replicate) in 1 L glass aquaria. For each condition, the corresponding aquaria were filled with artificial seawater and sediment (2:1 ratio), with corresponding As concentrations. The water was renewed every week to remove products of metabolism and re-establish As concentrations. The polychaetes were fed *ad libitum* every 2-3 days with commercial fish food (Silva et al., 2020b).

At the end of the exposure period, six specimens per condition were randomly selected and used for burrowing tests, according to Bonnard et al. (2009) and Silva et al. (2020), and all animals were frozen at – 80 °C for biochemical analysis.

2.3. As quantification

The concentration of As bioaccumulated in the animals was analysed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), after acid digestion, at an accredited laboratory of the Department of Geochemistry, at University of Aveiro. The tissue of polychaetes was dried at 40 °C, and then 1.5 mL of hydrochloric acid (HCl) and 4.5 mL of nitric acid (HNO_3) were added in Teflon vessels. After 24 h, the Teflon vessels were placed on a heating plate at 115 °C and, after 6 h, the contents were transferred to a centrifuge tube. After adding 20 mL of ultrapure water, tubes were centrifuged for 20 minutes at 4500g. Total concentration of As was determined using an Agilent 7700 ICP-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an octopole collision cell and autosampler. A rigorous quality control was performed during these analyses, which included the analysis of blanks, duplicate samples, and certified reference materials (CRMs). Accuracy of the ICP-MS and digestion method was evaluated by the analysis of a certified reference material, Till-2, for polychaetes tissues. The values obtained for all the CRMs analysis ranged from 90% to 110% of the concentration defined for these materials. The precision and bias error of the chemical analysis was less than 10%.

2.4. *Burrowing behaviour*

Burrowing behaviour was assessed according to the procedure described by Bonnard et al. (2009) with some modifications (Silva et al., 2020b). Briefly, each polychaete was gently placed in an aquarium containing 8 cm of clean sediment and 2 cm of clean water and the time each animal took to completely burrow into the sediment was recorded.

2.5. *Biochemical analysis*

For biochemical measurements, samples were weighed and homogenized in 0.1 M Potassium Phosphate Buffer (pH 7.4). Homogenates were separated into three aliquots: one for lipid peroxidation levels and glycogen content assessment; another for cholinesterase and electron transport system activities determination, which was centrifuged for 3 minutes, at 3300 g, at 4 °C; and the remaining sample was centrifuged for 20 minutes, at 10000 g, at 4 °C, for Post-Mitochondrial Fraction (PMS) isolation (Oliveira et al., 2015) to determine superoxide dismutase and glutathione S-transferases activities, and protein carbonylation.

2.5.1. *Cholinesterase activity*

The Ellman's method (Ellman et al., 1961) was used to determine Cholinesterase (ChE) activity, as described by Oliveira et al. (2015). The rate of acetylthiocholine degradation was determined every 25 seconds for 5 minutes at 412 nm by measuring the increase in the yellow colour due to the binding of the thiocholine with 5,5-dithio-bis (2-nitrobenzoic acid). Results were expressed in micromole of thiocholine formed per minute per gram of fresh weight (FW) ($\mathcal{E}$ = 1.36 x 10^4 M^{-1} cm^{-1}), using acetylthiocholine as substrate.

2.5.2. *Energy related parameters*

The activity of the Electron Transport System (ETS) was assessed following the methodology of King and Packard (1975) with the adjustments of Coen and Janssen (1997). Absorbance was read at 490 nm, every 25 seconds for 5 minutes. Using $\mathcal{E}$ = 15,900 M^{-1} cm^{-1} it was possible to calculate the amount of formazan formed. The results were expressed as micromole per minute per gram of FW.

Glycogen (GLY) content was quantified using the phenol-sulphuric acid method, as described by DuBois et al. (1956). After 30 minutes of incubation, absorbance was read at 492 nm and results were expressed as milligram per gram of FW.

2.5.3. *Antioxidant defences*

The activity of Superoxide Dismutase (SOD) was measured based on the method described by Beauchamp & Fridovich (1971). After 20 min incubation, SOD activity was measured at 560 nm. One unit of enzyme activity (U) corresponds to a 50 % reduction of nitro blue tetrazolium. Results were expressed as micromole per minute per gram of FW.

Glutathione *S*-Transferases (GST) activity was assessed following the protocol described by Habig et al. (1974), adapted to the microplate. Absorbance was read at 340 nm every 25 seconds for 5 minutes. Results were expressed as nanomole per minute per gram of FW ($\mathcal{E}$ = 9.6 x 10^3 M^{-1} cm^{-1}).

2.5.4. *Oxidative damage endpoints*

Lipid peroxidation (LPO) levels were assessed based on the method described by Buege & Aust (1978) by quantifying thiobarbituric acid reactive substances (TBARS) at 532 nm. The molar extinction coefficient of malondialdehyde (MDA) ($\mathcal{E}$ = 1.56 × 10^5 M^{-1} cm^{-1}) was used to calculate LPO levels and results were expressed as nanomole per gram of FW.

Protein Carbonylation levels were measured by quantifying carbonyl groups (CG) through the 2,4-Dinitrophenylhydrazine (DNPH) alkaline method described by Mesquita et al. (2014), with revisions performed by Udenigwe et al. (2016). Absorbance was read at 450 nm and results were expressed as nanomoles of CG per gram of FW ($\mathcal{E}$ = 22,308 mM^{-1} cm^{-1}).

2.6. *Statistical Analysis*

For each condition, burrowing activity and biochemical descriptors (ChE, ETS, GLY, SOD, GST, LPO and Protein Carbonylation) were submitted to hypothesis testing using permutational multivariate analysis of variance, with PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008) by following a one-way hierarchical design to analyse the data, with exposure concentration as the main fixed factor. The following null hypotheses were tested: a) no significant differences in biomarker responses (dependent variable) existed between NPEO and PEO exposures to each As concentration (0, 50 and 250 µg/L); b) no significant differences in biomarker responses existed between As concentrations (0, 50 and 250 µg/L) for NPEO; c) no significant differences on biomarker responses existed between As concentrations (0, 50 and 250 µg/L) for PEO. The significance of pseudo-F values, between different concentrations, in the PERMANOVA main tests were evaluated. When the main tests revealed statistically significant differences ($p \leq 0.05$), pairwise comparisons were performed.

To analyse if burrowing activity and the overall biochemical response of *H. diversicolor* were influenced by As, the data (square root transformed, normalized, and with the resemblance matrix normalization (Euclidean distance)) were submitted to an ordering analysis performed by Principal Coordinates (PCO), using the PRIMER 6 & PERMANOVA+ (Anderson et al., 2008). Pearson correlation vectors of burrowing activity and biochemical descriptors (correlation >0.85) were provided as supplementary variables being superimposed on the PCO graph.

3. Results

3.1. Arsenic bioaccumulation

Significant differences were found between all tested conditions (Table 1) regarding tissue concentration of As. In terms of control polychaetes, a concentration of 0.83 ± 0.41 (mg/Kg) was found for NPEO, and of 0.94 ± 1.44 (mg/Kg) for PEO. The lowest concentration of As, 50 µg/L, demonstrated significant differences when compared to respective control, for both groups, where NPEO had a tissue concentration of As of 10.45 ± 4.67 mg/Kg, and PEO 5.83 ± 2.61 mg/Kg. In the specimens exposed to 250 µg/L, As levels were significantly different from respective controls. In NPEO a concentration of As of 11.91 ± 5.33 mg/Kg was found, whereas PEO exposed to 250 µg As/L had a tissue concentration of 10.51 ± 4.70, significantly higher than 50 µg/L exposed polychaetes. Significant differences were found between the two groups (NPEO and PEO) when exposed to 50 µg/L.

3.2. Burrowing assay

After 28 days of exposure to As, *H. diversicolor* specimens exhibited a significant increase in the time needed to burrow into the sediments, regardless of parental exposure, when exposed to As (Fig. 1A). NPEO had an increase of over 1.5 times when exposed to 50 µg/L and were almost two times slower than control worms when exposed to 250 µg/L. PEO were over two times slower than control polychaetes when exposed to 250 µg/L, but only 0.3 times slower when exposed to 50 µg As/L. No differences between NPEO and PEO were found regarding behaviour.

3.3. *Cholinesterase activity*

A significant decrease in ChE activity was observed in NPEO and PEO exposed to As (Fig. 1B). Thus, when compared to the control group, a 13.7 and 18.6% lower activity was observed at 50 and 250 µg/L, respectively. PEO displayed a decrease in ChE activity of 12.5 and 13.2% at 50 and 250 μg/L, respectively. No significant differences were found between NPEO and PEO in terms of ChE activity for each As concentration.

3.4. *Energy related parameters*

An increase in ETS activity, when compared to controls, was observed in NPEO (Fig. 2A), corresponding to 15.7% (50 μg/L) and 16.9% (250 μg/L) increases. PEO, when exposed to 50 μg/L, displayed a 28.3% increase in ETS activity. However, a 4.7% decrease was observed in the polychaetes exposed to 250 μg As/L. The parental exposure to PS NPs had a significant effect on promoting higher levels in animals exposed to 50 μg As/L.

GLY content (Fig. 2B) increased, although not significantly, after exposure to As, only in NPEO. However, for PEO exposed to 250 μg/L GLY levels were significantly lower compared to the respective control. This parameter also proved sensitive to the parental exposure to PS NPs, with significant differences found between exposure groups NPEO and PEO (50 and 250 μg As/L). The most significant effect was found at 50 μg/L, where NPEO demonstrated to have higher GLY content.

3.5. Antioxidant defences

In NPEO, SOD activity (Fig. 3A) was increased at 50 μg/L (9.9%) and significantly increased at 250 μg/L (12.2%) exposed polychaetes compared to the respective control. In PEO, SOD activity was increased significantly in specimens exposed to 50 μg/L (23.3%) and 250 μg/L of As (21.9%). No differences in SOD activity were found between NPEO and PEO groups for each As concentration.

In NPEO, GST activity (Fig. 3B) significantly increased in polychaetes exposed to both As concentrations, whereas PEO only displayed an increase in enzyme activity in animals exposed to 250 μg/L. No differences in As concentrations tested were found between NPEO and PEO in GST activity.

3.6. Oxidative damage endpoints

LPO levels (Fig. 4A) in PEO exposed to 50 μg/L of As showed an increase of 44% compared to the respective control group. The remaining conditions showed no significant differences from their respective controls. No differences between NPEO and PEO in LPO levels were found for each As concentration.

Protein carbonylation levels (Fig. 4B) were only significantly different from respective controls in NPEO exposed to 250 μg As/L, which displayed significantly higher damage. In PEO, a significant increase in protein carbonylation levels was found at both As concentrations tested (50 and 250 μg/L). Significant differences between NPEO and PEO were found at 50 μg/L As exposed polychaetes.

3.7. PCO

Axis 1 of the PCO (Fig. 5) explained 62.9% of the total data variation, separating the controls of both groups (NPEO and PEO) on the positive side, from the organisms exposed to As concentrations, regardless of parental exposure condition, on the negative side. This separation is mainly due to the increase in ChE activity in the control groups.

Axis 2 of the PCO explained 19.2% of the total data variation, separating the PEO exposed to 50 and 250 μg of As/L on the positive side, from the remaining conditions on the negative side, but PEO exposed to 50 μg/L is near the origin of the axis. This axis highlighted especially the increase in SOD activity, bioaccumulation of As in the tissues of polychaetes, and protein carbonylation levels, and the decrease of ChE.

Overall, PCO analysis demonstrated a clear separation between controls and the remaining As exposure treatments, and also between the two exposure groups, NPEO and PEO.

4. Discussion

Polychaetes can provide important insights into contamination levels and the impacts it can have on the ecosystems (Fan et al., 2014). In this respect, the increase in burrowing time observed in this study highlights that, regardless of parental exposure, polychaetes exposed to As could be more vulnerable to predators and unable to promote proper sediment oxygenation, since the excavating behaviour of *H. diversicolor* impacts the ecosystems it inhabits (Banta and Andersen, 2003; Scaps, 2002). The increase in burrowing time found in our study may be associated with an inhibition of ChE activity (an enzyme associated with normal muscle and behavioural functions), as it has been suggested by other authors (Cajaraville et al., 2000; Fonseca et al., 2017; Payne et al., 1996)), whose activity was inhibited in exposed polychaetes. This alteration in behaviour may also be influenced by the bioaccumulation of As in the tissue of polychaetes. Previous studies analysing the effects of metals and metal-associated nanoparticles (between 5 and 100 nm) have also demonstrated their ability to impact the behaviour of *H. diversicolor*, however, with no reported effects on ChE. In a study analysing the effects of Cu and copper oxide nanoparticles (CuO NPs) (10 - 100 nm), Cu also impaired the burrowing behaviour of *H. diversicolor* in acute tests (7 days) in low concentrations (10 µg/L)), but CuO NPs did not impact polychaetes burrowing at all (Buffet et al., 2011b). Thit et al. (2015) demonstrated similar results when exposing this species to sediment spiked with Cu, in which specimens buried less into the sediment with concentration increase (7, 70, 140 µg/g). A study exposing *H. diversicolor* to 10 µg/L of Ag also reported that organisms took longer to bury into the sediments (Buffet et al., 2014b). The increase in burrowing time may be also related to avoidance behaviour observed in various invertebrate species (Amiard-Triquet, 2009), an increase in metabolism due to the detoxification systems associated with As exposure, or an overwhelmed detoxification capacity, that may affect the behaviour of the polychaetes (Amiard-Triquet, 2009; Buffet et al., 2011b). A recent study conducted by Silva, et al. (2020b) demonstrated that exposure to low concentrations (0.005-0.5 mg/L) of PS NPs 100 nm promoted not only an increase in the time that organisms remain on the sediment surface, not burying into it, but also a decrease in ChE activity. These results, as well as the data provided in this study, demonstrate the sensitivity of this endpoint which may reveal an impact on the individual and on the ecosystem.

In this study, both groups of organisms exposed to the lowest As concentration tested (50 µg/L) showed an increase in ETS activity. Since ETS activity has been demonstrated to be a good endpoint to evaluate the metabolic capacity of organisms exposed to environmental disturbances (Bielen et al., 2016; De Marchi et al., 2017; Freitas et al., 2016; Schmidlin et al., 2015; Simčič et al., 2014) and act as a proxy of cellular potential in organisms (Berridge et al., 2005), it can be suggested that the organisms in this study increased the metabolic activity to counteract the effects of As. However, in this study, the parental exposure to PS NPs only demonstrated a significant impact at the lowest As concentration tested. This increase may indicate a higher metabolic rate of exposed polychaetes to provide energy towards the oxidant defence system, which may suggest that lower As concentrations are more harmful. Similar responses have also been found in *H. diversicolor* and other invertebrate species exposed to various stressors, such as low mercury concentrations (5 μg/L) (Freitas et al. 2017); and 100 nm PS NPs (0.005 to 50 mg/L), which increased ETS activity with particles concentration increase (Silva et al., 2020b). A study analysing the effects of PS microplastics (< 1 mm) in the natural environment revealed that they promote an increase in ETS activity in *Mytilus edulis* (Van Cauwenberghe et al., 2015). In a previous study analysing the effects of carbamazepine (0.3 μg/L), caffeine (0.5 μg/L), and a combination of these two stressors (0.3 μg/L of carbamazepine + 0.5 μg/L of caffeine; 6.0 μg/L carbamazepine + 3.0 μg/L caffeine) on *H. diversicolor* it was also found that ETS activity increases in the lower concentrations of exposure to these contaminants (Pires et al., 2016).

GLY content was significantly different between NPEO and PEO in both As concentrations tested, which also demonstrates how important parental exposure can be for organisms in the natural environment. PEO demonstrated a decreasing tendency, which may be related to the increase in ETS activity, meaning that organisms were using GLY as an energy source. This has also been observed in this species when exposed to other contaminants: multi-walled carbon nanotubes (MWCNTs) (De Marchi et al., 2018b), and carbamazepine and caffeine (Pires et al., 2016). Even though it is not significant, this decrease in GLY content may indicate an allocation of the energy reserves to the antioxidant defence system. Regarding NPEO exposed to both As concentrations, polychaetes demonstrated an increase in reserves. These findings may indicate that animals may have decreased their metabolism under stress conditions or are using other energy sources for the antioxidant defence system, which is supported by the ETS activity. When analysing the effects of a chronic exposure to MWCNTs, De Marchi et al. (2018b) reported that *H. diversicolor* decreases GLY content, suggesting that this decrease is the cost of cellular protection. Previous studies on the effects of carbamazepine (0.3 to 6.0 µg/L) and caffeine (0.5 and 3.0 µg/L) have demonstrated that these two compounds can also decrease GLY content, associated with higher energy expenditure, corroborated by the increase in ETS activity (Pires et al., 2016). In a study where *H. diversicolor* and *D. neapolitana* were exposed to MWCNTs, an increase in energy reserves was observed (De Marchi et al., 2017b). In a 14-days study with *H. diversicolor* exposed to carbamazepine (0.05 to 500 ng/g of sediment), it was demonstrated that this drug promotes an increase in GLY content (Maranho et al., 2014).

SOD activity, an enzyme responsible for converting the superoxide anion into hydrogen peroxide (Sun et al., 1988), increased in PEO when exposed to both As concentrations tested (50 and 250 µg/L), whereas in NPEO this enzyme was only increased in activity when exposed to the highest concentration tested. Biotransformation enzyme GST, which plays an important role in the conjugation reactions of active metabolites and also in the antioxidant defence (Oliveira et al., 2008), demonstrated similar results. However, for NPEO this enzyme appeared to be more sensitive to the effects of As contamination since significant differences were found for both concentrations when compared to PEO, which only had significant differences in GST activity in the highest As concentration. Previous studies, analysing the effects of different classes of contaminants, have also demonstrated their effects on the antioxidant defence system. It has been demonstrated that *H. diversicolor* had higher activities of SOD, GST and even catalase after exposure to soluble Ag (10 µg/L) (Buffet et al., 2014b). However, in *H. diversicolor* exposed to PS NPs (0.005 – 50 mg/L), only SOD activity (between 0.5 and 50 mg/L) and catalase activity (5-50 mg/L) were increased, while GST and non-protein thiols, that are also part of the antioxidant defence system, demonstrated no alterations to their activities (Silva et al., 2020b).

Despite the observed activation of antioxidant defences, polychaetes were not able to prevent oxidative damage. Lipid peroxidation damage significantly increased only in PEO exposed to the lowest As concentration (50 µg/L), demonstrating a higher susceptibility of PEO to this type of damage, compared to the respective control. These results may indicate that lower concentrations of As caused more damage to cell membranes than higher concentrations. A previous study, where *H. diversicolor* was chronically exposed to Ag and Ag NPs, found that 10 µg/L of soluble Ag promoted oxidative damage *via* TBARS, even though Ag NPs did not lead to this type of damage (Buffet et al., 2014b). However, protein carbonylation levels were significantly increased, particularly in the highest concentration tested (250 µg/L of As). PEO demonstrated an increased sensitivity to As exposure, exhibiting more damage promoted by the As concentrations tested than NPEO. Protein carbonylation measures protein oxidation promoted by reactive oxygen species, which can lead to irreversible damage, and even cell death, if not eliminated (Fedorova et al., 2014; Rodríguez-Cavallo et al., 2018; Suzuki et al., 2010). Other studies have found protein carbonylation to be a more sensitive endpoint than LPO levels (Silva et al., 2020b). A previous study has demonstrated that PS NPs increased, in a concentration-dependent manner, protein carbonylation levels, in *H. diversicolor* specimens exposed for 28 days (Silva et al., 2020b).

5. Conclusions

In this study it was demonstrated that no significant differences in As bioaccumulation, behavioural and biochemical parameters were found between the control conditions for each group, an indication that parental exposure had no significant impacts on offspring if polychaetes are not submitted to additional stressors. This data allows the hypothesis that parental exposure may have no consequences if the offspring can grow in clean media and are subjected to additional stressors in the environment. However, the impacts of parental exposure can exacerbate the effects of exposure to an environmentally relevant contaminant, such as As. The consequences that As exposure can have on the behavioural and biochemical levels demonstrated that PEO had a higher sensitivity, particularly regarding oxidative damage, compared to NPEO. Changes in behaviour and ChE activity may suggest possible consequences for the *H. diversicolor* population since slower polychaetes may be more susceptible to predators. Additionally, the functions of the ecosystem may also be altered due to the decrease in burrowing activity, since organisms may also not promote proper sediment oxygenation. Taking into consideration the increasing production of plastic worldwide and, consequently, the increasing concentration of plastic in the oceans, as well as the possible interactions that plastic particles may have with other environmentally relevant contaminants, such as metals, pharmaceuticals, and natural substances, like organic matter, present in the natural environments, it becomes highly important to understand the possible consequences that these interactions can have on benthic organisms. Future studies should also focus on the effects of lower concentrations of NPs as well as the effects of parental pre-exposure to more than one contaminant, for example, the combination of NPs and As, on the offspring.

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Fig. 1. Burrowing behaviour (A) and Cholinesterase activity (ChE) (B) of *Hediste diversicolor* from parental exposure to polystyrene nanoplastics (PS NPs) 100 nm (PEO – parentally exposed organisms) and non-parental exposure (NPEO – Non-parentally exposed organisms) exposed for 28 days to arsenic (As). Statistically significant differences ($p \leq 0.05$) are within NPEO are marked with letters a-c, within PEO with A-B.

Fig. 2. Electron Transport System (ETS) activity (A) and Glycogen (GLY) content (B) of *Hediste diversicolor* from parental exposure to polystyrene nanoplastics (PS NPs) 100 nm (PEO – parentally exposed organisms) and non-parental exposure (NPEO – Non-parentally exposed organisms) exposed for 28 days to arsenic (As). Statistically significant differences ($p \leq 0.05$) are within NPEO are marked with letters a-b, within PEO with A-C, and within arsenic concentrations *.

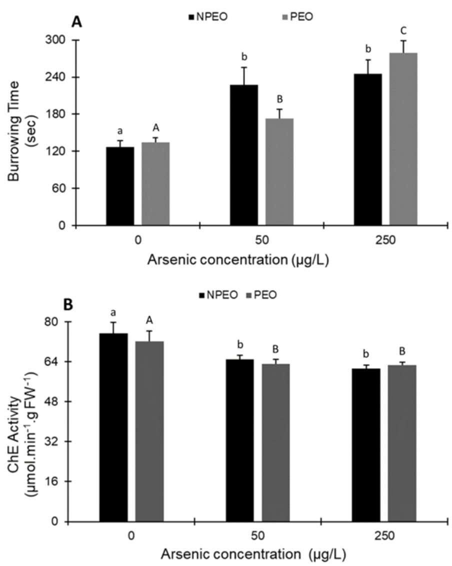
Fig. 3. Superoxide dismutase (SOD) (A) and Glutathione S-Transferases (GST) (B) activities of *Hediste diversicolor* from parental exposure to polystyrene nanoplastics (PS NPs) 100 nm (PEO – parentally exposed organisms) and non-parental exposure (NPEO – Non-parentally exposed organisms) exposed for 28 days to arsenic (As). Statistically significant differences ($p \leq 0.05$) are within NPEO are marked with letters a-b, within PEO with A-B.

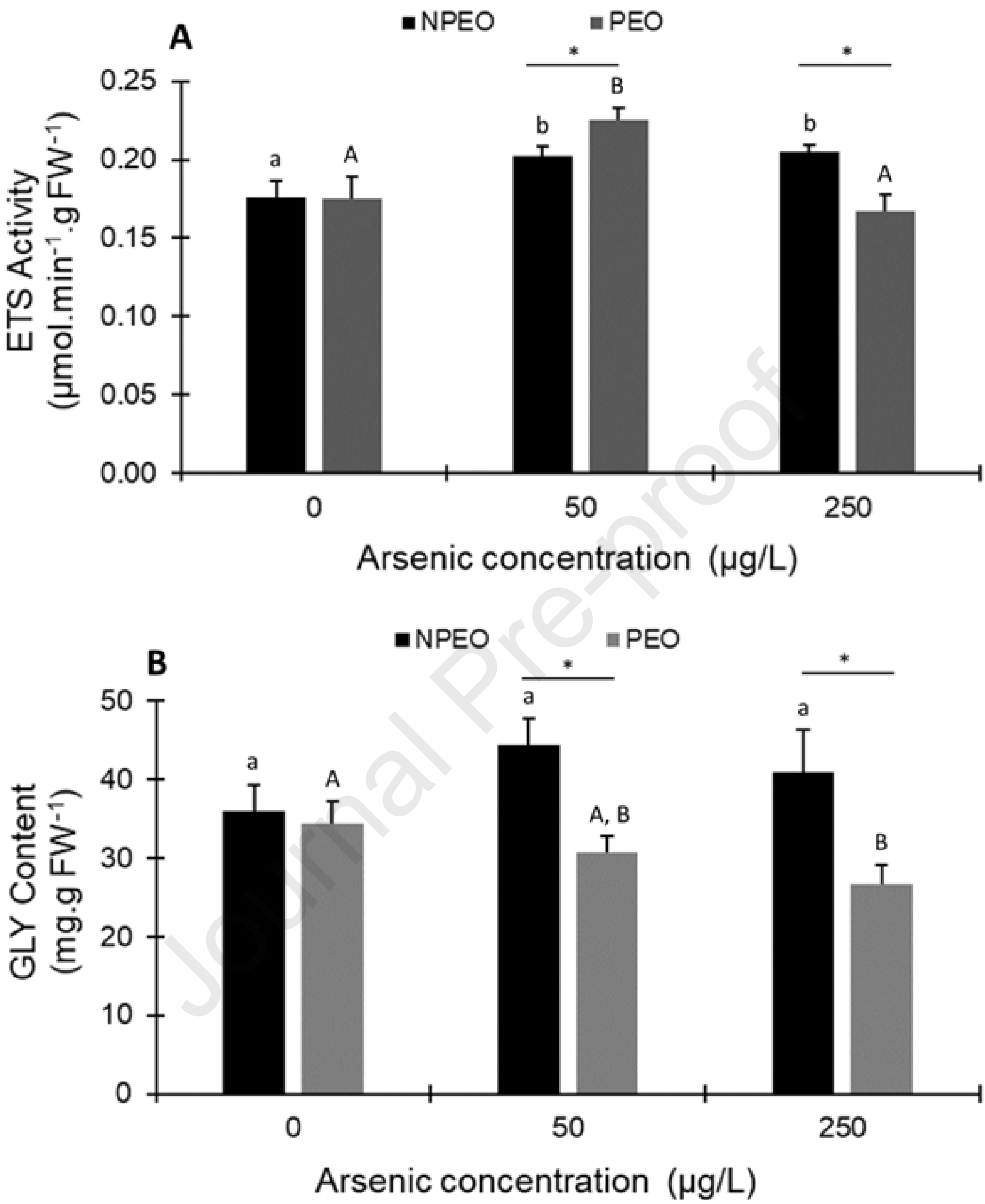
Fig. 4. Lipid Peroxidation (LPO) (A) and Protein Carbonylation (B) levels of *Hediste diversicolor* from parental exposure to polystyrene nanoplastics (PS NPs) 100 nm (PEO – parentally exposed organisms) and non-parental exposure (NPEO – Non-parentally exposed organisms) exposed for 28 days to arsenic (As). Statistically significant differences ($p \leq 0.05$) are within NPEO are marked with letters a-b, within PEO with A-B, and within arsenic concentrations *.

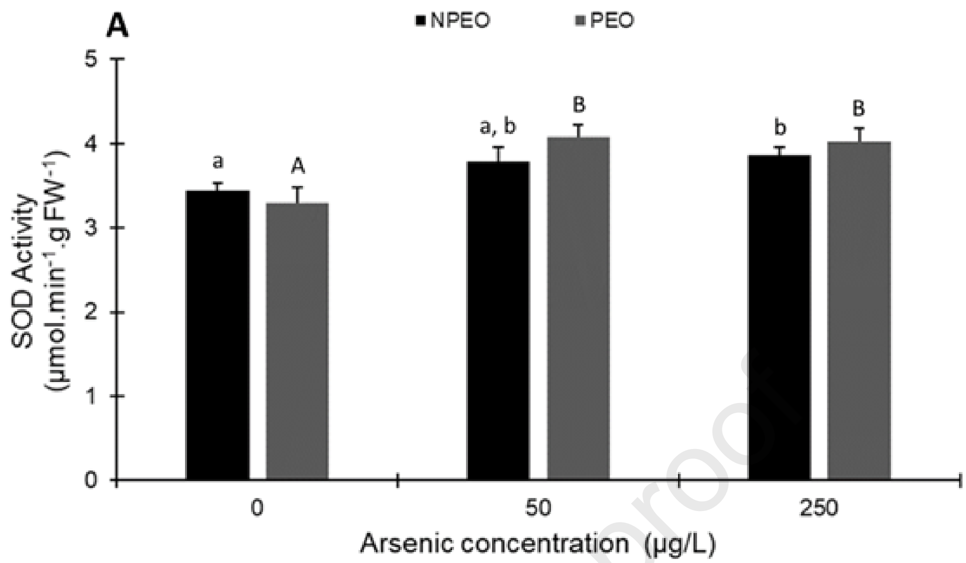
Fig. 5. Centroids ordination diagram (PCO) based on the mean of behavior and biochemical parameters, measured in *Hediste diversicolor* from parental exposure to polystyrene nanoplastics (PS NPs) 100 nm (PEO – parentally exposed organisms) (full) and non-parental exposure (NPEO – Non-parentally exposed organisms) (outlined) exposed for 28 days to Arsenic (As). Pearson correlation vectors are superimposed as supplementary variables, namely bioturbation and biochemical data ($r \geq 0.80$): burrowing; cholinesterase (ChE); electron transport system (ETS) activity; glycogen (GLY) content; superoxide dismutase (SOD) activity; glutathione S-transferases (GSTs) activity; lipid peroxidation (LPO) levels; protein carbonylation (Prot. C).

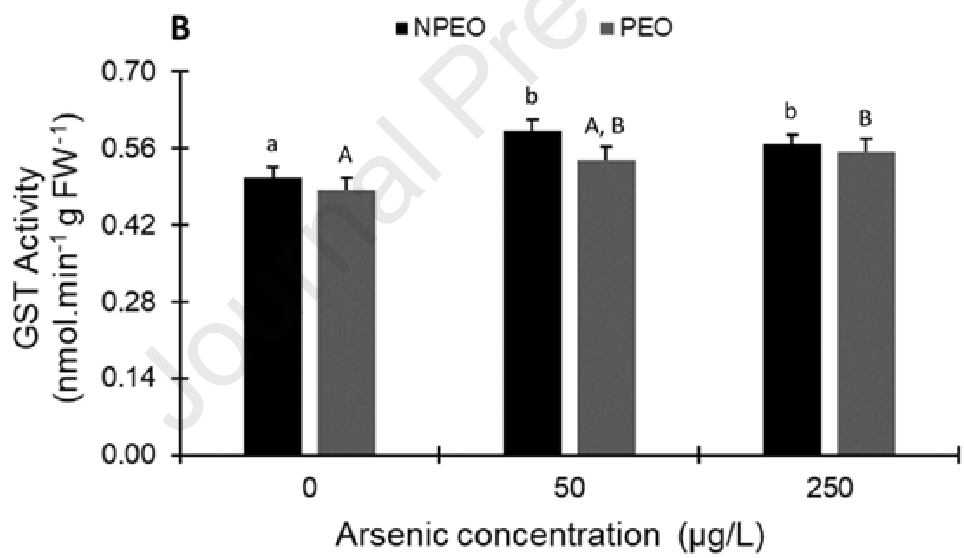
Table 1. Total Arsenic (As) levels (mg/Kg dry weight) in *Hediste diversicolor* from parental exposure to polystyrene nanoplastics (PS NPs) 100 nm (PEO – parentally exposed organisms) and non-parental exposure (NPEO – Non-parentally exposed organisms) exposed for 28 days to Arsenic (As). Statistically significant differences ($p \leq 0.05$) within NPEO are marked with letters lower case letters (a-b) whereas within PEO with capital letters (A-C), and within arsenic concentrations *.

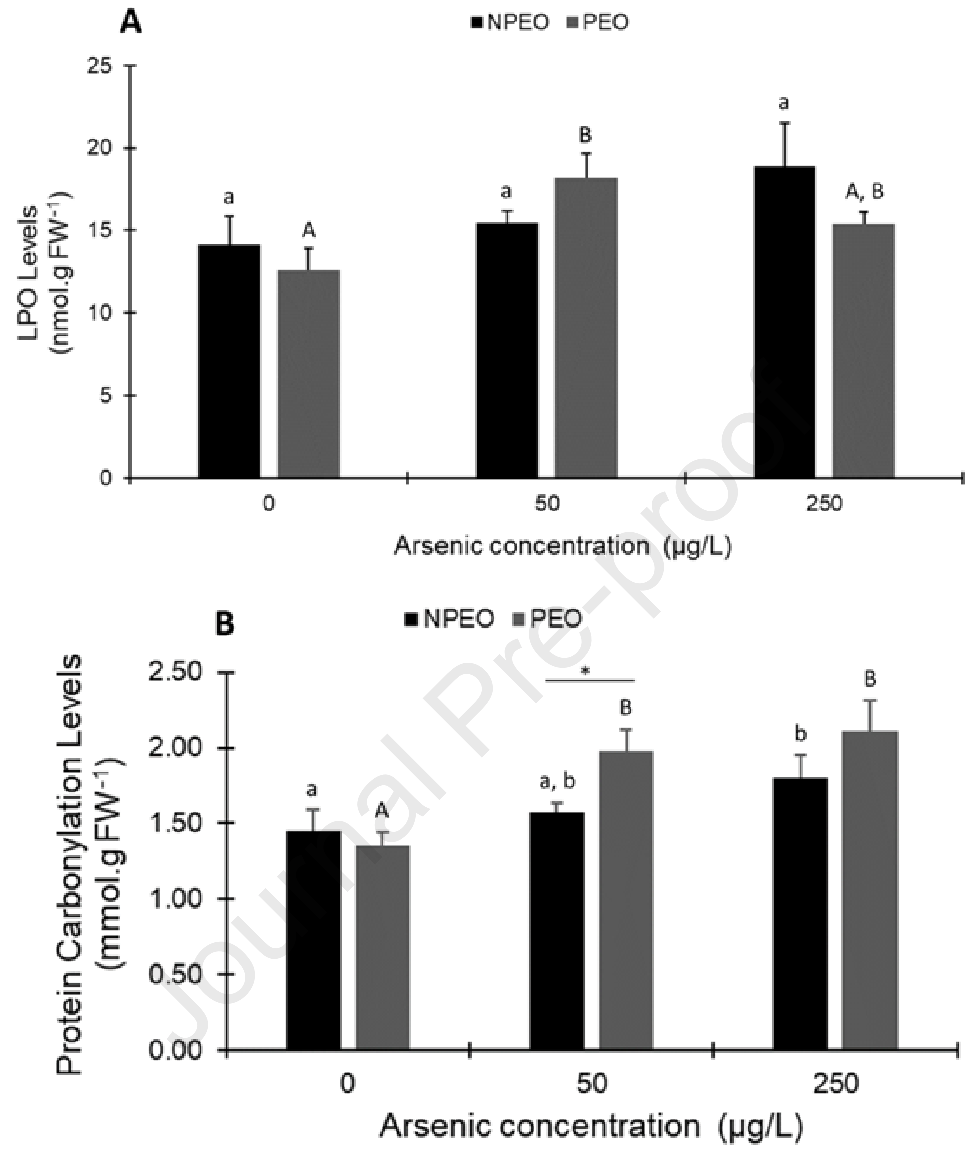
Conditions	PEO	NPEO
Control	0.94 ± 1.44^{A}	0.83 ± 0.41^{a}
50 µg As	$5.83 \pm 2.61^{B*}$	$10.45 \pm 4.67^{b*}$
250 µg As	10.51 ± 4.70^{C}	11.91 ± 5.33^{b}

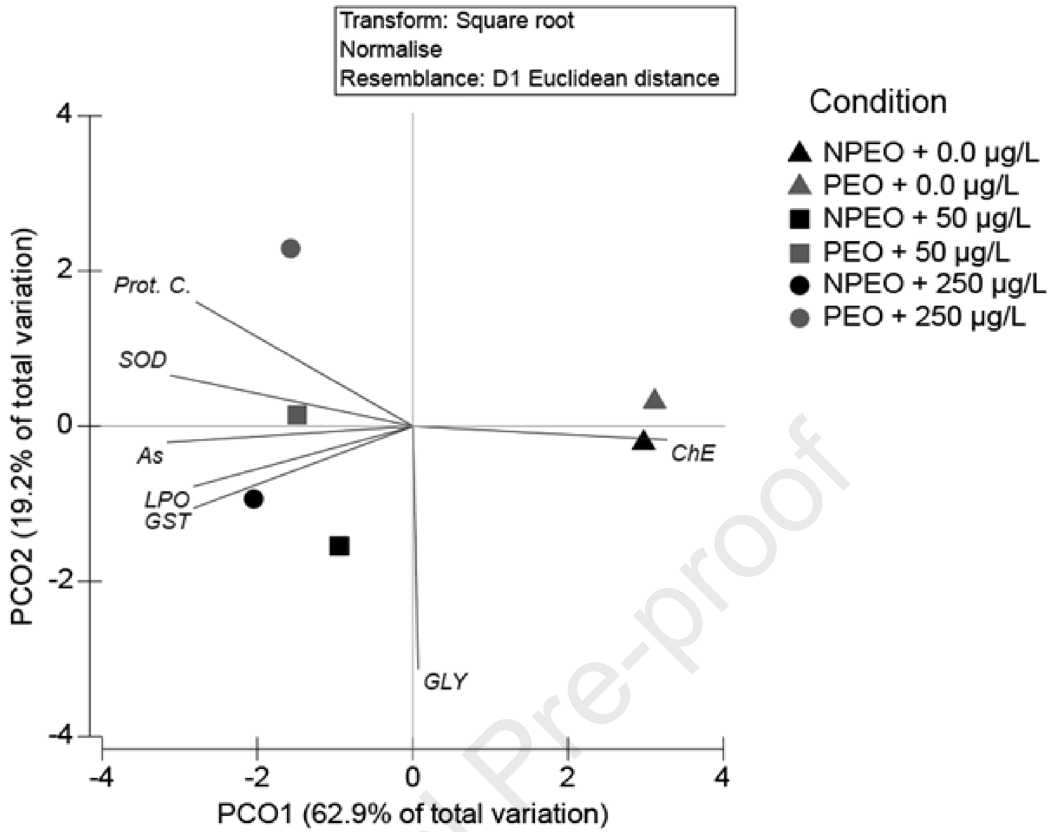

A
NPEO
PEO
Burrowing Time (sec)
300
240
180
120
60
0
a
A
b
B
b
C
0
50
250
Arsenic concentration (µg/L)
B
NPEO
PEO
ChE Activity (µmol.min⁻¹.gFW⁻¹)
80
64
48
32
16
0
a
A
b
B
b
B
0
50
250
Arsenic concentration (µg/L)


A
NPEO
PEO
ETS Activity
(µmol.min-1.g FW-1)
0.25
0.20
0.15
0.10
0.05
0.00
a
A
b
B
b
A
*
*
0
50
250
Arsenic concentration (µg/L)
B
NPEO
PEO
GLY Content
(mg.g FW-1)
50
40
30
20
10
0
a
A
a
A, B
a
B
*
*
0
50
250
Arsenic concentration (µg/L)


A
NPEO
PEO
SOD Activity (µmol.min-1.g FW-1)
5
4
3
2
1
0
a
A
a, b
B
b
B
0
50
250
Arsenic concentration (µg/L)


B
NPEO
PEO
GST Activity (nmol.min-1 g FW-1)
0.70
0.56
0.42
0.28
0.14
0.00
a
A
b
A, B
b
B
0
50
250
Arsenic concentration (µg/L)


A
NPEO
PEO
LPO Levels (nmol.g FW-1)
25
20
15
10
5
0
a
A
a
B
a
A, B
0
50
250
Arsenic concentration (µg/L)
B
NPEO
PEO
Protein Carbonylation Levels (mmol.g FW-1)
2.50
2.00
1.50
1.00
0.50
0.00
*
a
A
a, b
B
b
B
0
50
250
Arsenic concentration (µg/L)



Transform: Square root
Normalise
Resemblance: D1 Euclidean distance
Condition
NPEO + 0.0 μg/L
PEO + 0.0 μg/L
NPEO + 50 μg/L
PEO + 50 μg/L
NPEO + 250 μg/L
PEO + 250 μg/L
Prot. C.
SOD
As
LPO
GST
GLY
ChE
PCO2 (19.2% of total variation)
PCO1 (62.9% of total variation)
4
2
0
-2
-4

Declaration of interests

xThe authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: