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Effects of graphene oxide nanosheets in the polychaete *Hediste diversicolor*: Behavioural, physiological and biochemical responses

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ABSTRACT

Numerous applications exist for graphene-based materials, such as graphene oxide (GO) nanosheets. Increased concentrations of GO nanosheets in the environment have the potential to have a large negative effect on the aquatic environment, with consequences for benthic organisms, such as polychaetes. The polychaete *Hediste diversicolor* mobilises the sediments, hence altering the availability of contaminants and the nutrients biogeochemical cycle. As such, this study proposes to assess the effects of different GO nanosheets concentrations on the behaviour, feeding activity, mucus production, regenerative capacity, antioxidant status, biochemical damage and metabolism of *H. diversicolor*. This study evidenced that *H. diversicolor* exposed to GO nanosheets had a significantly lower ability to regenerate their bodies, took longer to feed and burrow into the sediment and produced more mucus. Membrane oxidative damage (lipid peroxidation) increased in exposed specimens. The increased metabolic rate (ETS) evidenced a higher energy expenditure in exposed organisms (high use of ready energy sources – soluble sugars) to fight the toxicity induced by GO nanosheets, such as SOD activity. The increase in SOD activity was enough to eliminate reactive oxygen species (ROS) induced by GO on cytosol at the lowest concentrations, avoiding the damage on proteins (lower PC levels), but not on membranes (LPO increase). This study revealed that the presence of GO nanosheets, even at the lower levels tested, impaired behavioural, physiological, and biochemical traits in polychaetes, suggesting that the increase of these engineered nanomaterials in the environment can disturb these benthic organisms, affecting the *H. diversicolor* population. Moreover, given the important role of this group of organisms in coastal and estuarine food webs, the biogeochemical cycle of nutrients, and sediment oxygenation, there is a real possibility for repercussions into the estuarine community.

1. Introduction

In recent years, both science and industry have concentrated their efforts on a diverse array of engineered nanomaterials. The use of these nanomaterials, which are defined as at least having one dimension with a size between 1 and 100 nm (ASTM, 2012), is increasing due to their wide range of uses in the fields ranging from cosmetics to food, medicine, agriculture and environmental remediation (Dong and Feng, 2007; Fabrega et al., 2011; Kachynski et al., 2008; Lens, 2009; Lopes et al., 2021; Pavasupree et al., 2006; Tungittiplakorn et al., 2004; Wei et al., 2008). One type of engineered nanomaterials that is widely used is graphene sheets, two-dimensional carbon nanomaterials formed by a single layer of carbon atoms compacted into benzene-ring structures (Chen et al., 2012). The two-dimensional single layer of carbon may be chemically functionalised to yield more hydrophilic derivatives such as graphene oxide (GO), which makes them suitable for dispersion in aqueous solutions and, therefore, expanding their applications (Wang et al., 2011). GO nanosheets are particularly interesting in the field of nanomedicine, where they are used for drug-delivery, photo-thermal therapy, bio-imaging, and cancer therapy (Dykman and Khlebtsov, 2012; Lytton-Jean and Mirkin, 2005; Wang et al., 2011; Zhang et al., 2010), but are also widely used in electronics and energy storage (Park and Ruoff, 2009; Wang et al., 2011; Zhao et al., 2012). Recently, environmental applications have also been developed on wastewater treatment for metal ions, dyes and organic micro-pollutants absorption (Bessa et al., 2020; Khan et al., 2017; Yin et al., 2020). However, because of their increased production and use, GO waste can be unintentionally released in the marine environment, the final destination of many contaminants (Baker et al., 2014; Thit et al., 2015a). Due to limitations in detection methods, there are few investigations on the con-

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centration of graphene in the environment. Nevertheless, previous studies predicted environmentally relevant concentrations of GO in aquatic environments between 0.001 and 1 mg/L (Hu et al., 2016; Zhang et al., 2017).

Nonetheless, even at low concentrations, it is expectable that bioaccumulation and biomagnification across the food web could increase their impact on marine communities. GO NP tend to agglomerate in marine and estuarine waters (Adeleye et al., 2020). The level of agglomeration and the size of aggregates depend on the physical characteristics of the particles, their concentration, and environmental system conditions, influencing their settlement in the sediment (Adeleye et al., 2020; Girão et al., 2020). Thus, benthic organisms will be exposed to GO NP over a long period of time, becoming relevant to investigate the potential toxicity of GO in these organisms (Adeleye et al., 2020).

Although some works have demonstrated that graphene and its derivatives negatively impact marine organisms (De Marchi et al., 2017, 2019a; Urban-Malinga et al., 2021), our knowledge of the ecological threats related to GO exposure to polychaetes is reduced. Besides, previous studies demonstrated that they could go through cell membranes in invertebrates and fish (Lammel and Navas, 2014; Katsumiti et al., 2017). Mesarič et al., 2015a reported alterations in swimming behaviour and enzyme biomarkers in Artemia larvae and mortality at the highest concentration (700 mg/L) after 48 h of GO exposure. In vitro GO nanoplatelets exposure of the mussel *Mytilus galloprovincialis* haemocytes harmed membrane integrity, cell viability and enhanced the formation of reactive oxygen species (ROS) (Katsumiti et al., 2017). After exposure for 14 days to a GO nanomaterial, the oyster *Crassostrea virginica* showed elevated lipid peroxidation and glutathione-S-transferase (GST) activity (Khan et al., 2019). Morphological anomalies were observed in *Paracentrotus lividus* embryos and larvae developed after egg fertilisation with sperm exposed to carbon based nanomaterials (carbon black and graphene oxide, from 0.0001 mg/L to 1.0 mg/L) (Mesarič et al., 2015b). Additionally, the same authors observed a decrease in the activity of cholinesterases AChE and PChE in gastrula tissues.

Elevated lipid peroxidation and increased catalase and glutathione and peroxidase activity have also been shown in the marine crustacean *Artemia salina* exposed to two commercial formulations of pristine graphene (nanoparticles and monolayer flakes) (Pretti et al., 2014). De Marchi et al. (2019b) also demonstrated that carboxylated and non-carboxylated multi-walled carbon nanotubes (MWCNTs) induced oxidative stress and neurotoxicity in *H. diversicolor* exposed during 28 days. Moreover, the same authors stated that carboxylated MWCNTs induced more toxicity than non-carboxylated MWCNTs.

Polychaetes are frequently the marine invertebrate group more abundant in benthic communities (Dorgham et al., 2014), living in a wide array of marine habitats (Scaps, 2002), and being important species in coastal and estuarine food webs (Thit et al., 2015a). Polychaetes are usually used to study the toxicity of several contaminants, *via* sediments and pore water, due to their intimate contact with sediments (Banta and Andersen, 2003; Scaps, 2002). The polychaete *Hediste diversicolor* is the most used species in ecotoxicological studies (Dean, 2008). This species lives in the sediments and is a bioturbator, creating galleries. Due to its burrowing behaviour affects nutrients biogeochemical cycle, the availability of contaminants, and promotes sediment oxygenation and a more diverse fauna (Banta and Andersen, 2003; Scaps, 2002).

Thus, this research intended to assess the effects of GO nanosheets in *H. diversicolor*, by evaluating behaviour (burrowing and feeding activity), physiological (regeneration, mucus production), and biochemical (antioxidant status, membrane and protein damage and metabolism) endpoints of this polychaete species.

2. Materials and methods

2.1. Test organisms

H. diversicolor polychaetes were harvested from a reference site in Ria de Aveiro, Western Portugal (40.6331°N, - 8.7367°W) (Pires et al., 2016). In controlled laboratory conditions (salinity 28, pH 8.0 and temperature 19 ± 1 °C), constant photoperiod (12h light: 12h dark) and under continuous aeration, *H. diversicolor* were allowed to reproduce. Offspring grew in the laboratory in the same conditions. Polychaetes were fed with commercial dry fish food (protein 46.0%, lipids 11.0%), every 2–3 days, *ad libitum* and water was renewed every week. Adult organisms were carefully selected for the assay, according to (Silva et al., 2020a).

2.2. Graphene oxide nanosheets

The commercial graphene oxide (GO) nanosheet water dispersion (0.4 wt % concentration) was purchased from Graphenea (San Sebastian, Spain) and was used as received. The GO nanosheets lateral size is < 10 μm and the monolayer content >95%, according to the supplier. We previously confirmed this data by AFM analysis (Girão et al., 2020). To mention that there is a high variability on the GO lateral size with flakes with a mean size of GO of ~790 nm, having a typical sheet-like morphology, and the presence of monolayers (typically 0.97 nm) together with and few-layered nanosheets (Girão et al., 2020).

2.3. Experimental design

2.3.1. Experimental assay

Following the procedure described by Silva et al. (2020a), a total of 100 organisms were selected and cut after being anaesthetised using ice. After amputation, polychaetes were haphazardly distributed per experimental conditions (0, 0.01, 0.1, 1 and 10 mg GO/L). The GO concentrations were chosen based on earlier research using graphene nanoparticles and marine invertebrates, including polychaetes (De Marchi et al., 2017; De Marchi et al., 2019a; Oliva et al., 2020). Moreover, looking at the literature, the predicted environmental concentrations of this emerging contaminant in aqueous systems were projected to be approximately 0.001–1 mg/L (Hu et al., 2016; Zhang et al., 2017). Additionally, GO concentrations used in this study were essential to examine a broad range of contamination levels and ascertain the polychaete-toxic concentrations. Each condition comprised 20 polychaetes, distributed by four glass aquaria (1L) filled with sediment and artificial seawater (1:2), with five organisms each. *H. diversicolor* organisms were exposed during 28 days to GO nanosheet concentration at salinity 28, pH 8.0, temperature 19 ± 1 °C, constant photoperiod (12h light: 12h dark) and under continuous aeration. Commercial fish food was used to fed polychaetes *ad libitum* every 2–3 days. Every 6 days, artificial seawater was renewed to remove metabolism products, and test media was reestablished.

At the end of the exposition, 30% of the organisms of each condition were randomly selected to evaluate the regenerative capacity, 20% of organisms to test mucus production and the remaining organisms were used for the burrowing assay and at the end were frozen at −80 °C for biochemical analysis.

2.3.2. Mortality

Mortality was determined for each condition by dividing the number of dead polychaetes after exposure (28 days) by the number of polychaetes utilised at the beginning of the exposure and expressed in percentage.

2.3.3. Regenerative capacity

After 28 days of exposure, regenerated segments, characterised by their smaller width when contrasted to the rest of the polychaete's body and/or their lighter colour, were examined. It was counted the number of regenerated segments, and it was assessed the percentage of the regeneration of the posterior body part (Silva et al., 2020a).

2.3.4. Mucus production

The production of mucus in exposed organisms was determined following a procedure adapted from (DuBois et al., 1956). Organisms were immersed for 30 s in 15 mL of ASW, salinity 28, at 40 °C. After this, ASW with the mucus was conserved at −20 °C for further analysis. Mucus levels were quantified by the method of phenol-sulphuric acid, following DuBois et al. (1956) and were expressed in mg of g FW.

2.3.5. Feeding activity

The time that polychaetes needed to feed was determined 14 days after exposure. For this assay, commercial fish food was added to the aquaria, and the time needed to polychaetes catch the food and enter again in the sediment was recorded in video. Results were then expressed in seconds (sec).

2.3.6. Burrowing assay

Burrowing tests were performed with individuals of each condition (adapted from Bonnard et al., 2009). The time that organisms needed to bury themselves completely was counted over a period of 20 min. Results were then expressed as the percentage of unborrowed organisms over time (min).

2.3.7. Biochemical analysis

Frozen organisms were cut longitudinally, and half of the organisms was dried for total lipidic content (LIP) determination and the other half was homogenised with potassium phosphate buffer (0.1 M, pH 7.4) for determination of the remaining biochemical parameters. Homogenates were separated into four aliquots: for lipid peroxidation (LPO); Glycogen (GLY); Electron Transport System (ETS), was centrifuged during 10 min, at 3000g, at 4 °C; and for the rest of biochemical parameters (protein content, sugars content, superoxide dismutase activity, glutathione-S-transferases activity and protein carbonylation content) was centrifuged at 10000g and at 4 °C during 20 min, (Silva et al., 2020b).

2.3.8. Energy related parameters

The activity of Electron transport system (ETS) was determined following King and Packard (1975) methodology with some modifications (Coen and Janssen, 1997). Absorbance was read every 25 s, during 5 min, at 490 nm. Formazan formed was calculated using $\mathcal{E}$ = 15,900 M^{-1} cm^{-1} and results were expressed as nmol per minute per gram of Fresh Weight (g FW).

Glycogen (GLY) and sugars were quantified by the method of phenol-sulphuric acid, following DuBois et al. (1956) and absorbance was read at 492 nm. Gly and sugars levels were expressed in mg of g FW.

Lipids (LIP) were determined following Folch et al. (1957) methodology, modified by Cheng et al. (2011) using cholesterol as standards (0–100%). Absorbance was measured at 520 nm and results were expressed in mg of mg DW.

The Biuret method (Robinson and Hogden, 1940) was used to determine protein (PROT), using as standard the bovine serum albumin (BSA). Protein results were expressed in mg of g FW.

2.4. Antioxidant enzyme response

The enzyme Superoxide dismutase (SOD) activity was determined following Beauchamp & Fridovich (1971) methodology. SOD activity was quantified at 560 nm. SOD activity was expressed in units (U) per g FW, where U corresponds to the quantity of enzyme that inhibited NBT diformazan formation by 50%.

The activity of the enzyme Glutathione-S-transferases (GSTs) was assessed following Habig et al. (1974) methodology, adapted to microplate. During 5 min and every 25 s was read the absorbance at 340 nm. GSTs activity were expressed in nmol per minute per g FW, using ϵ = 9.6 x 103 M^{-1} cm^{-1}.

2.4.1. Oxidative damage

Lipid peroxidation (LPO) was assessed spectrophotometrically according Buege and Aust (1978) methodology. The thiobarbituric acid reactive substances (TBARS) produced were read at 532 nm. LPO levels were determined using ϵ = 1.56 × 105 M^{-1} cm^{-1} and were expressed as nmol per g FW.

Protein carbonylation (PC) levels were determined following the alkaline method of Mesquita et al. (2014) with some modifications (Udenigwe et al., 2016). Results were calculated using ϵ = 22,308 m M^{-1} cm^{-1} and were expressed as nmol of CG per g FW.

2.5. Statistical analysis

For each GO nanosheet concentration, feeding activity, regenerative capacity, mucus production and biochemical descriptors (ETS, GLY, Sugars, Prot, LIP, SOD, GST, LPO, PC) were submitted to hypothesis tests applying permutational multivariate analysis of variance, using the PERMANOVA + add-on in PRIMER v6 software (Anderson et al., 2008). Obtained data was evaluated following a one-way hierarchical design, applying as the main fixed factor the exposure concentrations of GO nanosheets. The null hypothesis tested was. no significant differences were among GO nanosheets exposure concentrations. In the PERMANOVA main tests, the pseudo-F values were calculated in terms of significance between different concentrations. When statistically significant differences ($p \leq 0.05$) were obtained by the main test, pairwise comparisons were done. Among concentrations, levels of significance ($p \leq 0.05$) were represented with different letters. Burrowing behavioural data was analysed by linear regression using the IBM SPSS Statistics 26 software.

In order to analyse if feeding activity, regeneration, bioturbation, mucus production and biochemical responses of *H. diversicolor* were influenced by GO nanosheets, the data (square root transformed, normalised and the resemblance matrix normalisation (Euclidean distance)) were submitted to an ordering analysis performed by Principal Coordinates (PCO), using the PRIMER 6 & PERMANOVA+ (Anderson, Gorley, and Clarke, 2008). Pearson correlation vectors of physiological, behavioural and biochemical descriptors (correlation >0.9) were provided as supplementary variables being overlaid on the PCO graph.

3. Results

3.1. Mortality

H. diversicolor mortality was high (around 40%) in the GO concentration of 1 mg/L, while in the two lowest concentrations (0.01 and 0.1 mg/L) was 30%. In the highest concentration (10 mg/L) the mortality was 5%. No mortality was observed in the control (Fig. S1).

3.2. Physiological and behavioural parameters

3.2.1. Regeneration assay

The number of regenerated segments and the body regeneration percentage after 28 days of exposure is shown in Fig. 1A and B. All *H. diversicolor* organisms exposed to GO nanosheets demonstrated a significant reduction in the number of regenerated segments (between 5.4 ± 1.21 and 6.5 ± 1.69) when compared to control (12 ± 0.58). In terms of percentage of body regeneration, organisms exposed to the ex-

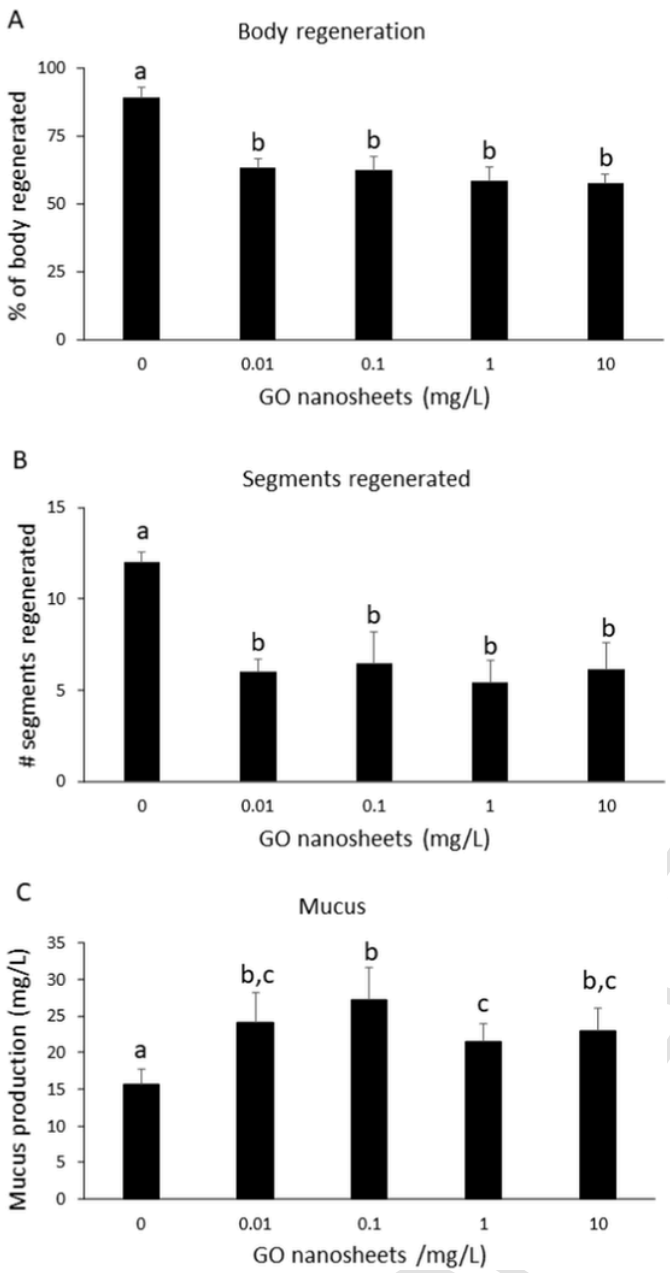

Fig. 1. Physiological parameters: Body regenerative capacity (A), number of segments regenerated 28 days after amputation (B) and Mucus production (C) measured in *Hediste diversicolor* after exposure to graphene oxide nanosheets (0–10 mg/L). Statistically significant differences ($p < 0.05$) are marked with letters (a–c) between experimental conditions.

perimental conditions demonstrated a significant decrease between 29 and 35% when compared to control.

3.2.2. Mucus production

Mucus production of *H. diversicolor* organisms is presented in Fig. 1C. The segregation of mucus was significantly higher in all organisms exposed to GO nanosheets, corresponding to an increase of 37–54% compared to control. Organisms exposed to the concentration of 0.1 mg/L produced the highest concentration of mucus.

3.2.3. Feeding activity

The feeding activity of *H. diversicolor* was compromised in exposed organisms since organisms exposed to GO nanosheets significantly needed more time to grab the food and get back into the sediment compared to the control (Fig. 2A). The feeding time was especially long at the highest concentration (10 mg/L), being 3-fold longer than the control.

3.2.4. Burrowing assay

The burrowing behaviour of polychaetes overtime was presented in Fig. 2B. After 6 min, all the organisms from control were buried. Exposed organisms showed a significantly slower burrowing rate than the control, particularly in the three highest concentrations (0.1–10 mg/L), where 20–35% of the organisms could not burrow in the sediment by the end of the assay (30 min).

3.3. Biochemical status

3.3.1. Energy related parameters

In terms of energy metabolism, *H. diversicolor* showed significantly higher ETS activity in relatively to control conditions (Fig. 3A).

Concerning glycogen content, organisms demonstrated a significant increase in 0.1 and 10 mg/L, having 36.9 and 77.3% more glycogen than control organisms, respectively (Fig. 3B).

Sugars showed a decreasing tendency along the concentrations tested, with significant differences from control at higher concentrations (0.1 and 10 mg/L) (Fig. 3C).

Regarding lipid content, a significant increase was observed in organisms exposed at 0.1 and 1 mg/L (Fig. 3D).

Only polychaetes exposed to the highest GO nanosheets concentration (10 mg/L) showed a significant increase in the protein content (Fig. 3E).

3.3.2. antioxidant response and oxidative damage

SOD activity increased (16–47%) in organisms exposed to GO nanosheets in all concentrations (0.01–10 mg/L) compared to control, being only significant at 0.1 mg/L, (Fig. 4A).

GSTs activity significantly decreased in organisms exposed to the two lowest GO concentrations (0.01 and 0.1 mg/L) (Fig. 4B).

Protein carbonylation levels significantly decreased (43.3–63.9%) when compared to control conditions in organisms exposed from 0.01 to 1 mg/L (Fig. 5A).

Lipid peroxidation levels increased between 7 and 34% in all concentrations tested, in relation to control, being significant at 0.1 and 1 mg/L (Fig. 5B).

3.3.3. Multivariate analysis

Principal coordinates analysis (PCO) graphs obtained for *H. diversicolor* physiological and behavioural parameters is shown in Fig. 6A. PCO1 described 86.8% of the total variation, and PCO2 explained 10.4%. PCO1 primarily separated control individuals from the GO nanosheets exposed organisms, with a high correlation between the percentage of regenerated body width of *H. diversicolor* and the number of regenerated segments. This was explained by the higher regenerative capacity observed in *H. diversicolor* from control compared to organisms exposed to GO. Exposed organisms were strongly correlated with mucus production, mainly at lower concentrations (0.01 and 0.1 mg/L), and with burrowing and feeding activity (mainly organisms exposed at higher concentrations).

The principal coordinates analysis (PCO) graph attained for biochemical parameters of polychaetes is shown in Fig. 6B. PCO1 explained 56.4% of the total variation, while PCO2 explained 29.3%. PCO1 primarily separated individuals from control and the highest GO nanosheets concentration (10 mg/L) at the positive side from polychaetes exposed at 0.01, 0.1 and 1.0 mg/L in the negative side. PCO2 separated individuals exposed to control and the lowest exposure concentration (0.01 mg/L) on the positive side from organisms exposed at concentrations 0.1 mg/L and 1.0 mg/L near PCO2 axis and 10 mg/L on

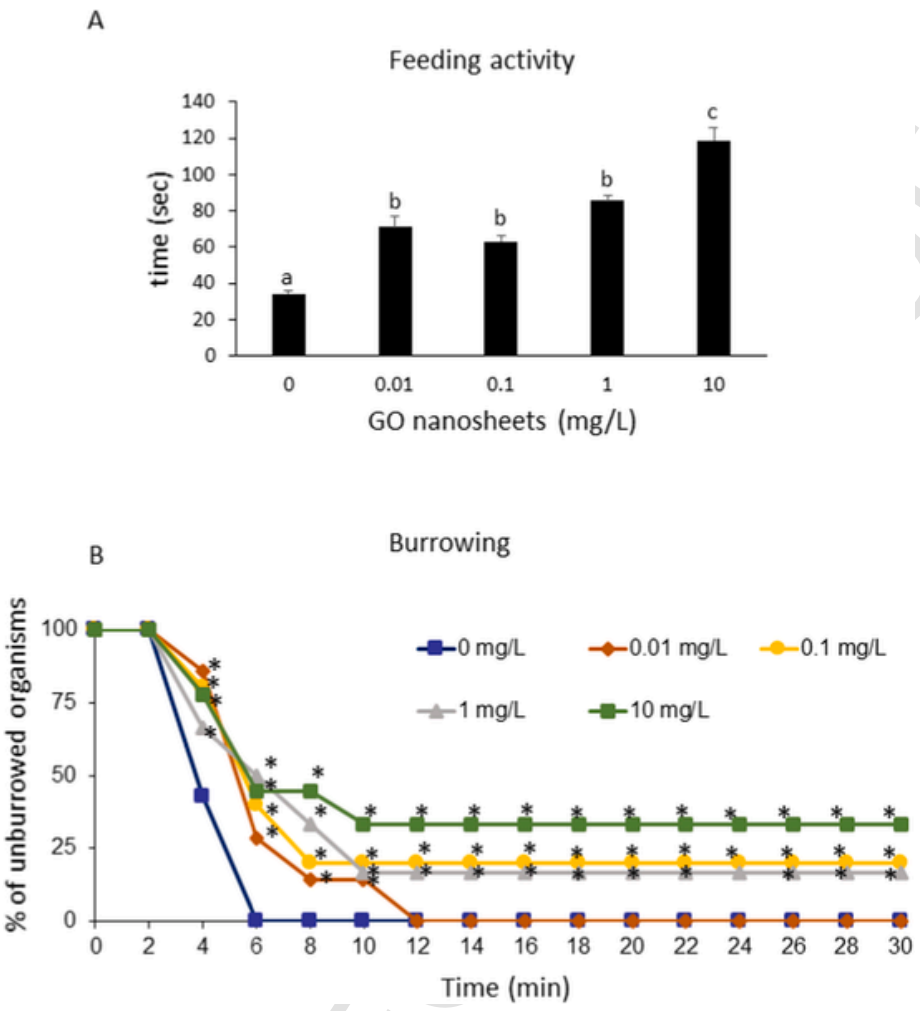

Fig. 2. Behavioural parameters measured in the polychaete *Hediste diversicolor* after exposure to graphene oxide nanosheets: Feeding activity 14 days after exposure (A) and and Burrowing activity 28 days after exposure (B). Statistically significant differences ($p < 0.05$) are marked with letters (a–c) between experimental conditions or with * comparing to the control.

the negative side. From PCO analysis, it was observed that sugars were strongly correlated ($r > 0.9$) with organisms from control and exposed to 0.01 mg/L, while LIP, SOD and ETS were more correlated ($r > 9$) with organisms exposed to 0.1 and 1.0 mg/L, evidencing a higher metabolic activity and antioxidant response. Organisms from control and exposed to the highest concentration were strongly correlated with PROT, PC and GSTs.

4. Discussion

4.1. Toxicity and physiological responses

The highest mortality rate (around 40%) was observed when *H. diversicolor* was exposed to 1.0 mg/L. Mortality was also observed in the other GO concentrations 30% for 0.01 and 0.1 mg/L and 5% for the highest concentration (10 mg/L), evidencing that mortality was not linearly related with GO concentration. However, considering that graphene materials tended to aggregate along the time and with increasing concentrations due to the high ionic strength of seawater and pH (Adeleye et al., 2020; Girão et al., 2020), altering their reactive surface area and consequently their mode of cellular uptake, and influencing the biological responses of organisms (Hotze et al., 2010; Khan et al., 2019). Urban-Malinga et al. (2021) observed for the same species a mortality rate between 12.5% at the lowest graphene nanoflakes (5–30 nm) concentration (4 mg/L) and 37.5% at higher concentration (40 mg/L), after 24 days of exposure. De Marchi et al. (2017) also observed an increased mortality rate in *H. diversicolor* exposed during 28 days to higher concentrations (11% and 22% at concentrations of 0.1 mg/L and 1.0 mg/L, respectively) of carbon nanotubes (diameter 9.5 nm; length 1.5 μm). These results may indicate the physical properties of the graphene-based material, as shape and size may influence organisms responses. In fact, De Marchi et al. (2019b) observed that carboxylated carbon nanotubes (CNT) increased oxidative stress and neurotoxicity than pristine CNT. Mesarič et al. (2015a,b) did not observe lethal effects in *Artemia* exposed to multiwall carbon nanotubes, but the same species exposed 48h to GO showed mortality higher than 90% at the highest concentration (700 mg/L), but no effects at 600 mg/L. Thus, nanomaterials' most severe effect (mortality) on macroinvertebrates appears to be a complex process governed by not yet fully identified factors. However, results from our and herein referred work evidence that the type of graphene material and the period of exposure are determining factors in the observed mortality rates.

Regarding the regenerative capacity, our findings clearly demonstrated that organisms exposed to GO nanosheets regenerated fewer body segments and thus had a lower percentage of body regeneration than those in the control condition. Our results suggest that *H. diversicolor* organisms did not use their reserves of energy to regenerate their body but to fuel up detoxification mechanisms, compromising the regenerative process. The regenerative capacity of *H. diversicolor* was also affected by polystyrene nanoplastics (100 nm) (Silva et al., 2020a), and

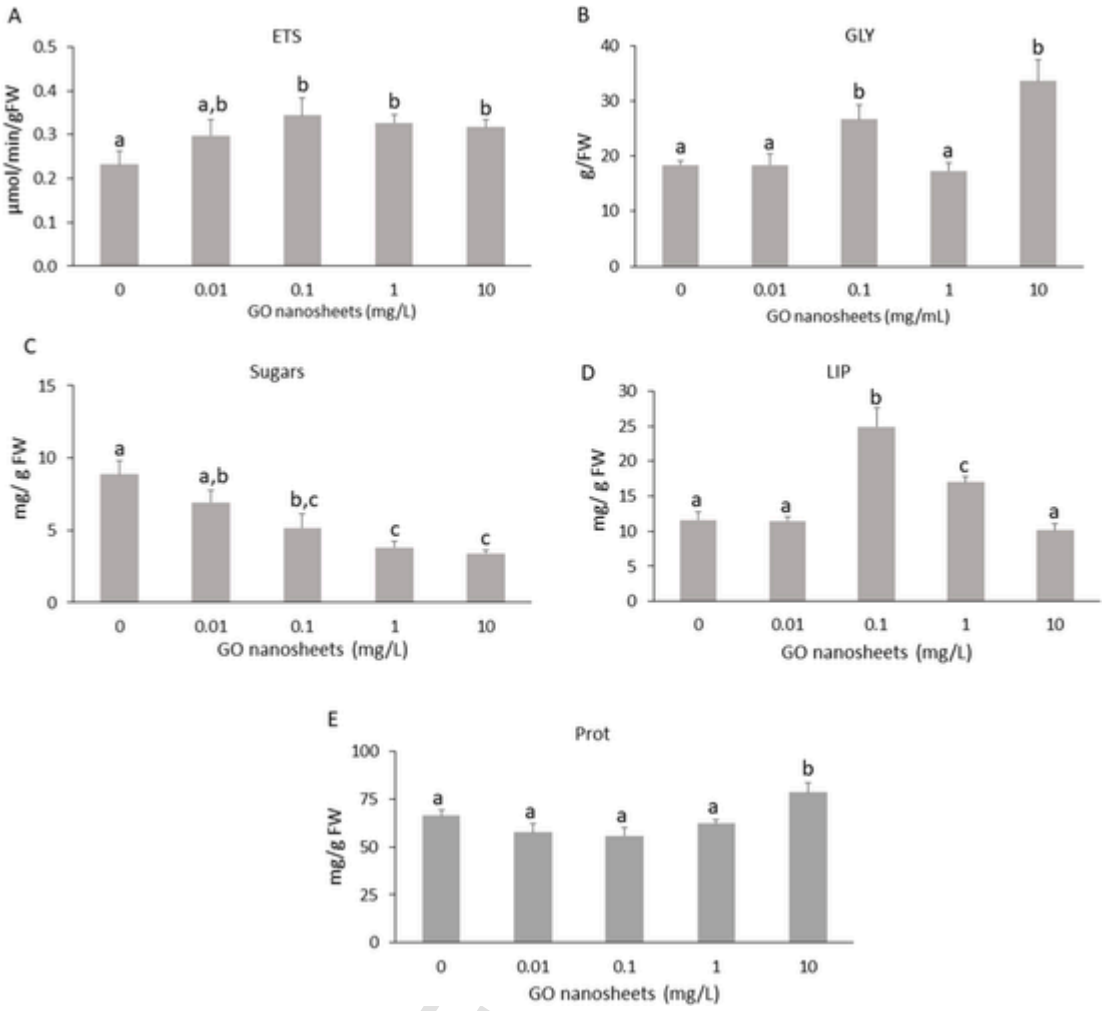

Fig. 3. Energy related parameters: Electron transport chain, ETS (A); Glycogen, GLY (B); Dissolved sugars (C); Lipids, LIP, (D) and total protein, PROT (E) measured in *Hediste diversicolor* after 28 days of exposure to graphene oxide nanosheets (0–10 mg/L). Statistically significant differences ($p < 0.05$) are marked with letters (a–c) between experimental conditions.

the regenerative ability of the polychaete *D. neapolitana* was severely impacted when exposed to carbon nanotubes, being more severe at the highest concentration tested (1.0 mg/L) (De Marchi et al., 2017). The crustacea *Artemia salina* GO significantly impaired growth after 48h of exposure, negatively correlated with GO concentration (1–500 mg/L).

Some polychaetes species produce mucus used for various functions related to locomotion, feeding, reproductive strategies and toxin elimination (Murray et al., 2012; Alain et al., 2002). The increase of mucus production by *H. diversicolor* in the presence of pollutants, as metals and pharmaceuticals and its relation with a defence mechanism, functioning as a protective layer over the surface of the tegument against the intake of pollutants is well documented (Mouneyrac et al., 2003; Gomes et al., 2019; Rodrigues et al., 2017). These observations are in accordance with results obtained in this study since *H. diversicolor* organisms exposed to GO nanosheets significantly secreted more mucus than control, possibly constituting a barrier against GO nanosheets direct contact and body injury. Indeed, due to material shape and size, it is expected that GO cause body injury when in contact with polychaetes, and harmed worms may increase mucus production as a protection mechanism to prevent not only future injuries but, given the antiseptic and immunomodulation properties of mucus, potential infections by pathogens. In fact, other studies reported that fish mucus contains components related to immune responses, including antibacterial agents as lysozyme, immunoglobins, lectins, and antimicrobial polypeptides that have the function of inhibit or lysis different pathogens (Cordero et al., 2015; Fernández-Montero et al., 2020; Pérez-sánchez et al., 2017).

4.2. Behavioural responses

H. diversicolor are important organisms in the ecosystems they inhabit since their functional traits, such as burrowing and feeding behaviour, provide sediment irrigation, oxygenation and particle mixing (Banta and Andersen, 2003; Scaps, 2002) and alterations on these bioturbation activities can provide ecological consequences (Bonnard et al., 2009). In the present study, burrowing and feeding activity seemed to be significantly impaired by exposure to increasing concentrations of GO nanosheets. Exposed organisms took longer to full burrow, or were not able to fully burrow, in the sediments and needed more time to detect, grab the food and get back into the sediment. Previous studies demonstrated that burrowing behaviour is a sensitive parameter of polychaetes exposed to contaminants, as silver nanoparticles (Cong et al., 2014), polystyrene nanoplastics (Silva et al., 2020b), zinc oxide nanoparticles (Buffet et al., 2012), cadmium sulfide nanoparticles (Buffet et al., 2014), copper (Buffet et al., 2011) and CuO nanoparticles (Thit et al., 2015b). In all these studies, an increase in burrowing time of exposed *H. diversicolor* organisms was observed, even at lower concentrations. Other studies also indicated that the increase in burrowing time might be associated with an inhibition of ChE activity (an enzyme associated with normal muscle and behav-

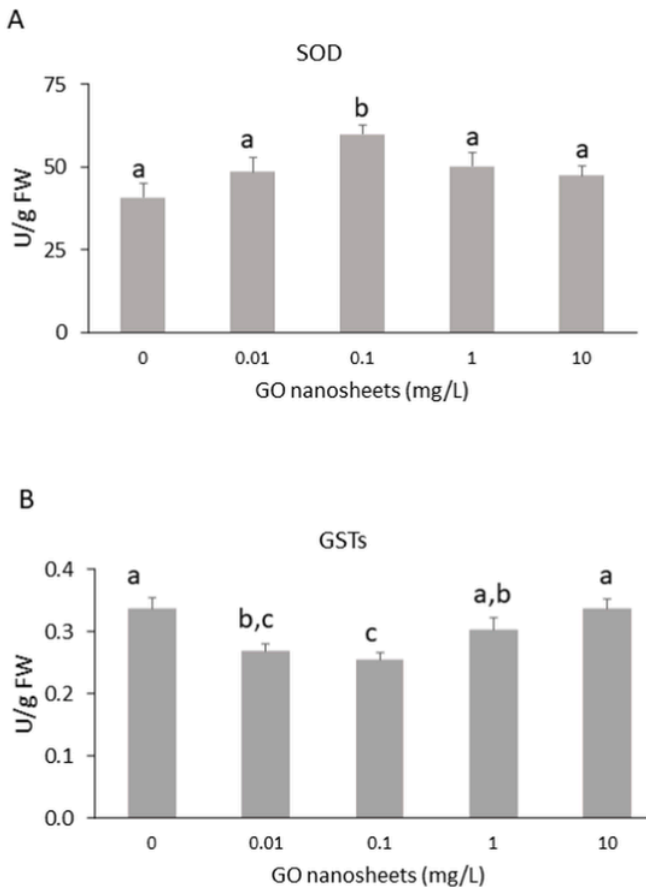

Fig. 4. Antioxidant and biotransformation enzymes response: Superoxide dismutase, SOD (A) and glutathione-S- transferases, GSTs (B) measured in polychaetes after exposure to graphene oxide nanosheets (0–10 mg/L). Statistically significant differences ($p < 0.05$) are marked with letters (a–c) between experimental conditions.

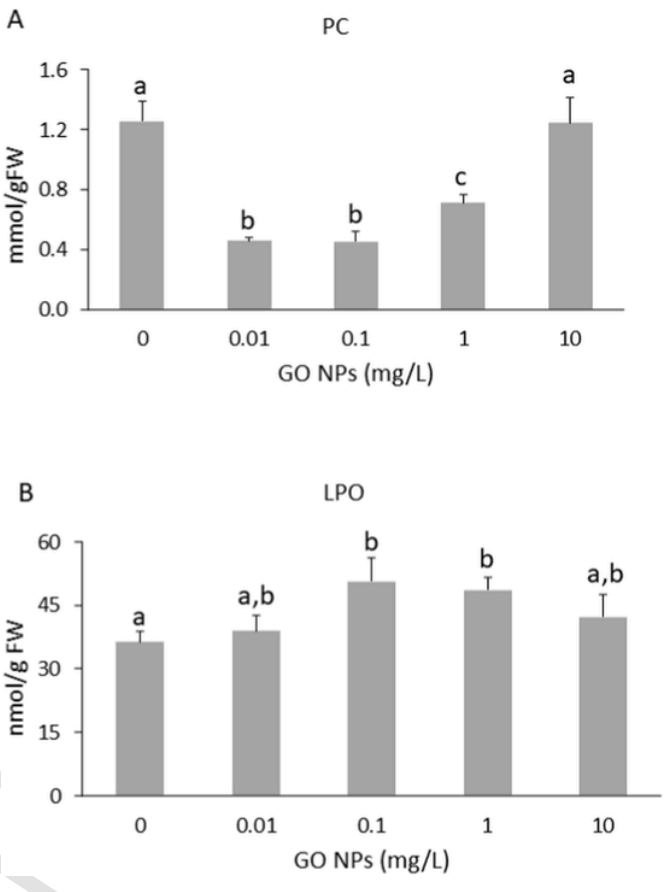

Fig. 5. Oxidative damage: Protein carbonylation, PC (A) and lipid peroxidation, LPO (B) measured in *Hediste diversicolor* after 28 days of exposure to graphene oxide nanosheets (0–10 mg/L). Statistically significant differences ($p < 0.05$) are marked with letters (a–c) between experimental conditions.

ioural functions) (Cajaraville and Bebianno, 2000; Fonseca et al., 2017)). Besides, in our study, we did not determine ChE activity, previous studies indicated that the exposure to graphene nanoparticles inhibited the activity of this enzyme (Mesarič et al., 2015; De Marchi et al., 2017).

Additionally, Urban-Malinga et al. (2021) demonstrated that *H. diversicolor* exposed to graphene nanoflakes resided deeper in the sediment than controls, suggesting an escape response to graphene. Thus, besides this parameter had not been measured in our study, if organisms are deeper in the sediment, it could explain the increased time that polychaetes needed to detect food. Moreover, it is expected that a lower feeding activity may decrease energy assimilation and thus compromise polychaetes fitness (Wright et al., 2013), like burrowing activity, as we demonstrated. Additionally, the reduction of feeding and burrowing activities will conduct to a decrease in sediment oxygenation, which is essential for maintaining infauna diversity (Banta and Andersen, 2003).

The integration of the physiological and behavioural endpoints (PCO, Fig. 5A) brings out the impact of exposure to GO nanosheets on polychaetes, expressed by the lower regenerative ability, feeding and burrowing time increase and produced more mucus, indicating that these physiological and behavioural parameters were sensitive to GO nanosheets exposure.

4.3. Energy related responses

Energy metabolism has an essential role in the survival and function of organisms, being also important for adaptation and tolerance to stressful situations (Sokolova, 2013). Additionally, organisms can increase energy expenditure under pollutants exposure, being considered a cell protection mechanism (Bielen et al., 2016). In fact, previous studies demonstrated that invertebrates exposed to carbon nanomaterials (De Marchi et al., 2019a) decreased their energy reserves, as glycogen content. However, the present study only demonstrated that *H. diversicolor* decreased sugars when exposed to GO nanosheets, which may indicate polychaetes are using an immediately available energy source to fuel their defence mechanisms against GO nanosheets toxicity. Moreover, contrary to previously cited studies with graphene, stored energy, like lipids and glycogen, were preserved. Urban-Malinga et al. (2021) did not also observe significant alterations in energy content of *H. diversicolor* exposed for 24 days to GO nanoflakes, and Van Cauwenberghe et al. (2015) did not observe alterations in sugar, carbohydrates and lipids content in the polychaete *Arenicola marina* exposed to polystyrene microspheres (10, 30 and 90 μm). Additionally, these two authors also stated that the smallest particles (10 and 30 μm) were retained more easily within the animals than larger particles. Nevertheless, on the contrary, studies by Wright et al. (2013) demonstrated a reduction in lipid content of *A. marina* organisms exposed to microplastics (130 μm mean diameter), theorising that it may, in part, be due to a reduction in feeding rate. The results reported by these studies suggest that size and probably the shape of the particles play a role in polychaetes feeding behaviour. In fact, Urban-Malinga et al. (2021) detected the presence of graphene flakes in polychaetes gut, indicating that worms swallowed the particles during feeding, but since these authors detected no alterations in energy content, they suggested that graphene flakes were easily egested from polychaetes intestines and due to their nano-dimension.

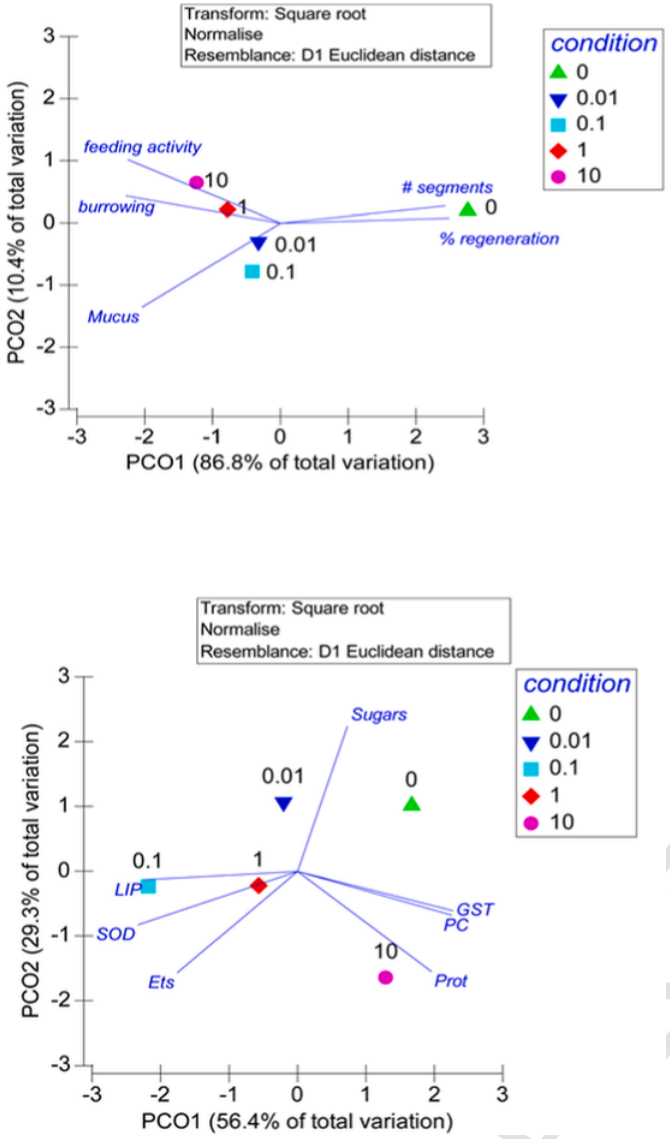

Fig. 6. Centroids ordination diagrams (PCO) based on physiological and behavioural parameters (A) and biochemical parameters (B) measured in *Hediste diversicolor* exposed to different graphene oxide nanosheets (0–10 mg/L). Pearson correlation vectors are superimposed as supplementary variables ($r > 0.85$).

Electron transport system activity may be used to measure the metabolic capacity in response to environmental disturbances, such as abiotic alterations and anthropogenic stress (Bielen et al., 2016; Schmidlin et al., 2015; Simčič et al., 2014). ETS activity can also be used as a proxy for the respiratory potential of cells (Berridge et al., 2005). In this study, ETS activity was significantly increased in the exposure conditions (0.1–10 mg/L), which may indicate an increase of the metabolic rate to provide energy towards the oxidant defence system, which may also explain the significant reduction of sugars on exposed organisms. Previous studies also demonstrated an increase in ETS activity when polychaetes were exposed to carbon nanotubes (De Marchi et al., 2017) and other nanomaterials, such as PS nanoplastics (Silva et al., 2020b).

4.4. Antioxidant defences and oxidative damage

Previous studies demonstrated that contamination by graphene induced several harmful biochemical effects, such as oxidative stress in acute (Urban-Malinga et al., 2021) and chronically exposed organisms (De Marchi et al., 2017, 2019a), which result from the overproduction and accumulation of reactive oxygen species (ROS). In order to prevent these injuries, organisms are able to activate their defences, as antioxidant (SOD) and biotransformation (GSTs) enzymes, to eliminate ROS and toxic compounds formed from the metabolism of oxidised molecules such as lipid hydroperoxides, avoiding cell and protein damage.

The results obtained suggest that *H. diversicolor* exposed to GO nanosheets slightly activated defence mechanisms to diminish cellular damage caused by ROS, namely by slightly increasing SOD activity with significance at 0.1 mg/L. Moreover, our results showed an increase in LPO levels, with significance in organisms exposed at 0.1 and 1.0 mg/L, indicating that the excess of ROS produced were not able to be eliminated by antioxidant enzymes, leading to membrane damage in worms exposed to GO nanosheets. De Marchi et al. (2017) demonstrated that after the exposure of *H. diversicolor* and *Diopatra neapolitana* organisms to carbon nanotubes, both species increased SOD activity and LPO levels. Buffet et al. (2014) also reported a slight increase of SOD activity in *H. diversicolor* after exposure to silver nanoparticles, and Silva et al. (2020b) also demonstrated the capacity of polystyrene nanoparticles to enhance SOD activity.

GSTs are responsible for detoxifying the xenobiotics and metabolites produced from oxidative stress as lipid peroxides by-products and is widely used to assess the capacity of organisms to detoxify. Although previous studies demonstrated that graphene materials are inducers of GSTs (De Marchi et al., 2017; De Marchi et al., 2019a), in this study, GSTs activity was inhibited at the lower concentrations (0.01 and 0.1 mg/L), which may indicate a slight inhibitory effect of GO nanosheets on these biotransformation enzymes. Previous studies have demonstrated that some contaminants, as polystyrene nanoparticles (Silva et al., 2020b), copper and cadmium (Cunha et al., 2007; Salazar-Medina et al., 2010) and propranolol and acetaminophen (Solé et al., 2010), have also lead to a reduction in GSTs activity in polychaetes, gastropods, shrimps and mussels species, respectively.

No damage in proteins was observed in exposed organisms. On the contrary, in lower concentrations (0.01–1.0 mg/L), the PC levels were significantly lower compared with the control. These results suggest that *H. diversicolor* was able to counteract the GO effects on proteins by induction of antioxidant defences, such as SOD. Urban-Malinga et al. (2021) also did not observe protein and membrane damage in *H. diversicolor* exposed to GO nanoflakes after 24 days of exposure. These authors reported an increase in CAT activities for a short period of exposure (36h), but did not observe significant differences after a more prolonged period (24 days), suggesting an initial opposite response of polychaetes antioxidant defence to eliminate ROS, and inferred that the long period of defence mechanisms action for longer exposure periods was enough to eliminate ROS production. However, other studies also revealed a later induction of defence mechanisms that are activated during longer exposures to contaminants and that replace those responding more promptly to stress (Pamplona and Costantini, 2021). Chen et al. (2016) also reported a significant increase of oxidative stress biomarkers in *Danio rerio* exposed to graphene oxide for a short period (1–4 days) and the lack of such an effect after more prolonged exposure (8–14 days), suggesting that may have occurred a rebalancing of ROS generation and its elimination with time.

PCO analysis (Fig. 5B) attained for biochemical parameters of polychaetes evidenced SOD activity, ETS and lipids as the biochemical endpoints that were most correlated with GO exposed organisms at environmentally relevant concentrations (0.01–1 mg/L) already reported (Hu et al., 2016; Zhang et al., 2017).

5. Conclusion

This study evidenced that exposure to GO nanosheets induced physiological, behavioural and biochemical alterations in *H. diversicolor*, with effects visible even at the lowest doses studied. In terms of biochemical endpoints, exposed organisms exhibited an increase in the

metabolic rate (ETS), evidencing a greater energy expenditure fuelled by readily available energy sources (sugars) to fight the toxicity caused by GO nanosheets. At low doses, the oxidative stress caused by GO nanosheets exposure was mitigated by an increase of SOD activity, which effectively contained proteins damage, but not membrane destruction. The behavioural changes generated (longer time to burrow and feed) may have intrinsic ecological significance since slower organisms are more susceptible to predators. Additionally, sediment oxygenation may be reduced, affecting organisms that rely on bioturbation. Moreover, our data also demonstrated that the regeneration process was retarded, with already described (Zajac, 1985) effect on the reproduction success and consequently negatively impacting *H. diversicolor* population and the ecosystem functions it provides.

Credit author statement

Adília Pires: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. M.S.S. Silva: Formal analysis, Investigation, Data curation, Writing – original draft. Carina Sá; : Investigation, Formal analysis. Etelvina Figueira: Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. Paula Marques: Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.118869.

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