



Environmental relevant levels of the cytotoxic drug cyclophosphamide produce harmful effects in the polychaete *Nereis diversicolor*



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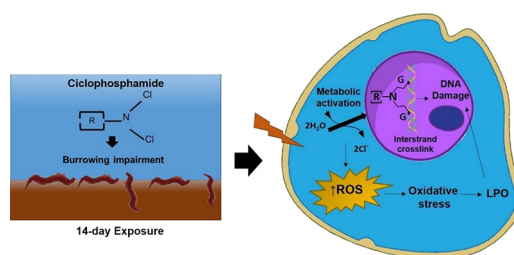
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HIGHLIGHTS

- *Nereis diversicolor* exposed to the cyclophosphamide (CP) at trace levels in seawater.
- Burrowing behavior showed a non-monotonic profile over increasing CP levels.
- Oxidative stress was observed at higher CP concentrations.
- Genotoxicity was detected as a result of prodrug activation.
- *N. diversicolor* showed to metabolize the drug according to its mode of action.

GRAPHICAL ABSTRACT



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ABSTRACT

Cytotoxic drugs applied in chemotherapy enter the aquatic environment after patient's metabolism and excretion, in both main compounds and their respective metabolites. The increased consumption and discharge of these drugs raise concern on the genotoxic burden to non-target aquatic species, due to their unselective action on DNA. Settlement and adsorption of cytotoxic drugs to aquatic sediments pose risks to benthic species through chronic exposure. The aim of the present study was to assess the effects induced by the anticancer drug cyclophosphamide (CP) on the polychaete *Nereis diversicolor*, after 14 days of exposure to environmental relevant concentrations (10, 100, 500 and 1000 ng L⁻¹). Burrowing impairment, neurotoxicity (Acetylcholinesterase - AChE activity), oxidative stress (superoxide dismutase - SOD; catalase - CAT; glutathione peroxidases - GPXs activities), biotransformation (glutathione-S-transferases - GST), oxidative damage (lipid peroxidation - LPO) and genotoxicity (DNA damage) were assessed. Burrowing impairments were higher at the lowest CP concentrations tested. The higher CP levels tested (500 and 1000 ng L⁻¹) induced a significant inhibition on the enzymatic antioxidant system (SOD, GPx) and on GST activity. DNA damage was also significant at these concentrations as an outcome of CP metabolism, and high levels of oxidative damage occurred. The results showed that the prodrug CP was metabolically activated in the benthic biological model *N. diversicolor*. In addition to the potential cytotoxic impact likely to be caused in aquatic species with similar metabolism, *N. diversicolor* proved to be reliable and vulnerable to the cytotoxic mode of action of CP, even at the lower doses.

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1. Introduction

Anticancer drugs (or antineoplastic drugs) are a group of pharmaceuticals routinely and widely administered in chemotherapy all over the world, whether individually or in combination with other cytotoxic

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agents. In 2012, 14.1 million new cancer cases were reported around the world, and the World Health Organization (WHO) estimates an enhancement of 57% of new cases until 2030 (WHO, 2014). Besides the subsequent increase of cytotoxic prescriptions, approximately 80% of oncology patients receive treatment at oncology wards and go home after drug administration (*i.e.* out-patients). Additionally to conventional elimination into hospital sewage systems, metabolites and unchanged fractions of the administered drug are excreted through urban effluents, following to municipal wastewater treatment plants (WWTPs), where elimination of these substances is incomplete (Johnson et al., 2008; Kosjek and Heath, 2011). Furthermore, these chemicals may also be released directly to the water bodies, in regions not properly attended by sewage collection and treatment systems (Abessa et al., 2005; Pessatti et al., 2016).

Consequently, anticancer drugs are detected at low levels ($\mu\text{g}\cdot\text{L}^{-1}$ range) in aquatic systems (Crane et al., 2006; Johnson et al., 2008; Kosjek and Heath, 2011; Booker et al., 2014; Mater et al., 2014) and long-term exposure to non-target species has led to the concern of genotoxic burden of cytotoxic drugs to aquatic species (Steger-Hartmann and Kiimmerer, 1996; Steger-Hartmann et al., 1997; Buerge et al., 2006; Ferrando-Climent et al., 2014). In general, the conventional chemotherapy drugs act directly on the DNA double strand, in order to avoid the synthesis and proliferation of tumor cells (Farber, 1973). Such covalent interaction primarily alters the DNA helical structure, influences the binding of chromatin protein and induces DNA strand breaks, subsequently producing genotoxic effects, followed by the death of the tumor cells (Harris, 1976; Ou and Lien, 1985). However, once these

“targets” are not specific drivers of cancer cells, normal growing cells are also affected through the same mode of action (MoA) by which they may be killed or left unrepaired, followed by genomic instability with neoplastic transformation and eventual carcinogenic mutations (Johnson et al., 2008; Kosjek and Heath, 2011; Ferrando-Climent et al., 2014). Acting unselectively on DNA, it has been hypothesized that anticancer drugs may harm all eukaryotic organisms by cytotoxic, genotoxic, mutagenic and carcinogenic effects (Johnson et al., 2008; Vyas et al., 2014). The biological risk posed by these anticancer molecules and their metabolites in aquatic species is not well elucidated (Mater et al., 2014), with few data on acute and chronic ecotoxicological assessments in algae *Tetrahymena pyriformis* (Bonnet et al., 2003), *Pseudomonas putida* (Zouneková et al., 2007) and *Pseudokirchneriella subcapitata* (Zouneková et al., 2007; Brezovsek et al., 2014; Česen et al., 2016); polychaete *Nereis diversicolor* (Fonseca et al., 2017); bivalve mollusc *Mytilus galloprovincialis* (Trombini et al., 2016); cladocera crustaceans *Daphnia pulex* (DellaGreca et al., 2007; Borgatta et al., 2015, 2016), *D. magna* (Zouneková et al., 2007; Parrella et al., 2014a, 2014b, 2015) and *Ceriodaphnia dubia* (DellaGreca et al., 2007; Parrella et al., 2014a, 2014b, 2015); crustacean amphipod *Ampelisca brevicornis* (Moreira et al., 2016); and the fishes *Danio rerio* (Kovacs et al., 2015), *Pimephales promelas* (Winter et al., 2007) and *Oryzias latipes* (Sun et al., 2011). Limited information exists regarding Environmental risk assessment (ERA) for anticancer drugs. ERA guidelines are assigned only for newly authorized pharmaceuticals (since 2006) and do not include molecules prone to cause genotoxicity impairments in aquatic ecosystems (Johnson et al., 2008; Aguirre-Martínez et al., 2016).

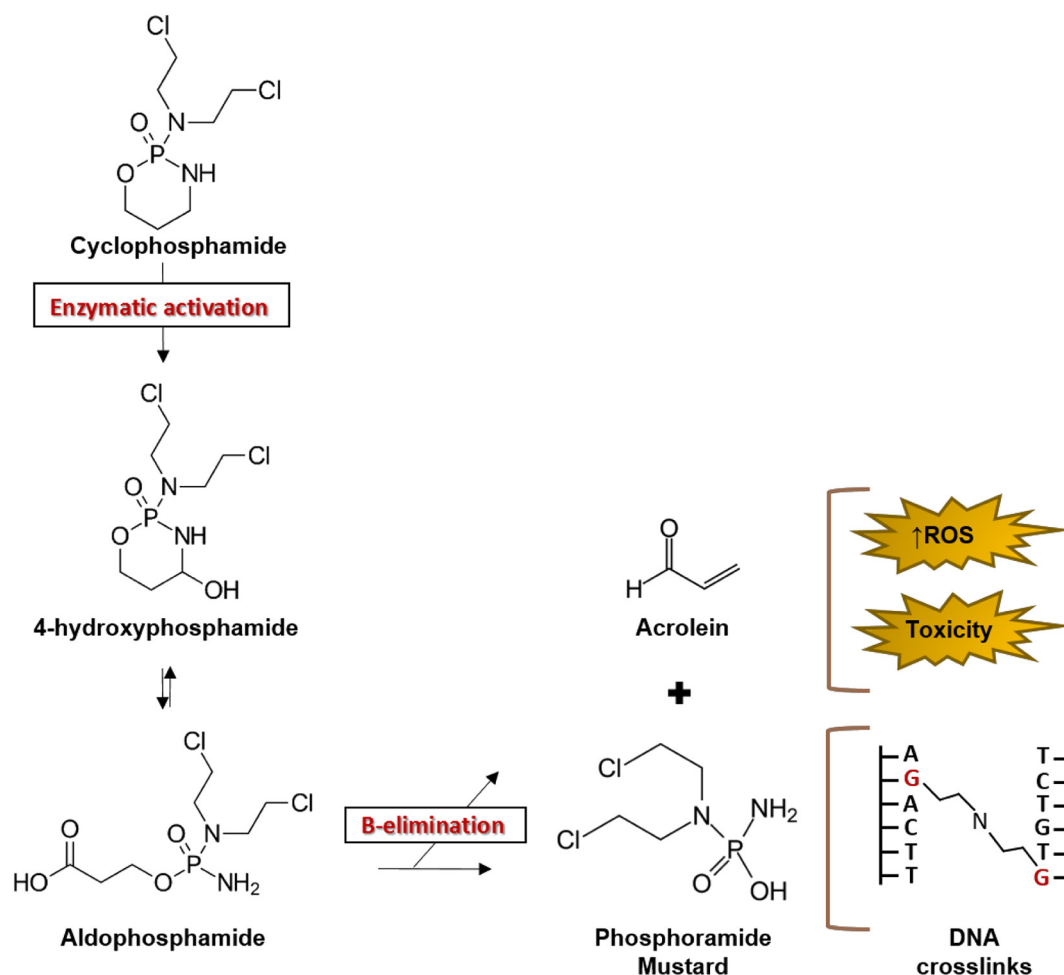


Fig. 1. CP metabolism pathway. Enzymatic activation by cytochrome P-450 enzyme system generates 4-hydroxycyclophosphamide, coexisting with the non-cytotoxic aldophosphamide metabolite. It decomposes into acrolein, that is toxic, and the DNA-binding phosphoramidate mustard.

Cyclophosphamide (CP) is one of the oldest and most frequently prescribed cytotoxic and alkylating agent used in cancer treatment, approved since 1960's (Gilard et al., 1994; Buerge et al., 2006; Česen et al., 2015). It consists of a phosphoramidate ring linked to a bifunctional moiety containing two chloro-ethyl groups. CP is a prodrug and its activation consists in the oxidation by cytochrome P450 mixed function oxidase to form 4-hydroxy-CP (4-OHCP), undergoing subsequent transformations to yield the major cytotoxic species, the phosphoramidate mustard (PAM) and acrolein (Fig. 1).

After administration, around 80% of the dose is excreted as metabolites, which accounts for substantial amounts of metabolites disposed into sewage systems (Bagley et al., 1973; Steger-Hartmann and Kiimmerer, 1996). In the absence of metabolic activating systems, CP fails to bind to DNA and its genotoxic activity is prevented. Even without metabolic activation by S9-rat extracted fraction, the parental drug CP caused mutagenic effects in the bacteria *Escherichia coli* and *Salmonella typhimurium*, and in the cyanobacteria *Synechococcus leopoliensis* (320 mg L⁻¹) (Česen et al., 2016), indicating that not only the metabolites are involved in cellular impairments (Benedict et al., 1977; Mohn and Ellenberger, 1976; Balbinder et al., 1981; Winckler et al., 1984). Research concerning CP and its ecotoxicological effects in aquatic organisms is scarce and do not comprise exposure routes by which species would uptake the drug. Data available addresses the drug as a toxic positive control in *in vivo* short term bioassays assessing mortality, immobilization, growth and reproduction inhibition, larval development and genotoxicity as end-points with CP levels far from environmental relevant concentrations (Matsumoto and Cólus, 2000).

Considering that most of cytotoxic drugs are highly polar, with low K_{ow} , the fraction of drugs adsorbed into sewage sludge could be neglected and distribution is assumed to be mainly in the water column (Kosjek and Heath, 2011; Seira et al., 2016). Negligible biodegradability, volatilization and adsorption of CP to biosolids confirms this assumption (Buerge et al., 2006; Steger-Hartmann and Kiimmerer, 1996; Steger-Hartmann et al., 1997). However, pharmaceuticals are multifunctional ionizable compounds and conventional partitioning models are not suited for a straightforward prediction of their environmental fate (Kwon and Armbrust, 2008). Once in the aquatic environment, hydrophobic interactions providing adsorbance of polar compounds to

particulates may not be disregarded, especially for organic cations, which are formed during intra-molecular cyclizations for cytotoxic drugs activation (e.g. CP aziridinium cation) (Williams et al., 2000; Emadi et al., 2009; Kosjek and Heath, 2011). Therefore, specific transformations in coastal environments may allow their adsorption, transfer and persistence to the sediments (Kosjek and Heath, 2011; Maranho et al., 2014; Moreno-González et al., 2015).

Sediments are the ultimate sink for contaminants in the marine environment, posing benthic species at risk through chronic exposure and bioaccumulation (Buruaem et al., 2012; Araujo et al., 2013; Vethaak et al., 2016). Nevertheless, aquatic ecotoxicological assessment of anticancer drugs involving sediments is lacking (Fonseca et al., 2017). In addition, to the best of our knowledge, only two studies exist on the occurrence of anticancer drugs in river sediments, where levels ranged below the detection limit (Zuccato et al., 2000) and 391 ng kg⁻¹ (bicalutamide), 392 ng kg⁻¹ (doxifluridine), and 250 ng kg⁻¹ (tamoxifen) (Azuma et al., 2017). So far, no screening approach was directed to the coastal environment. The levels of CP detected in hospital effluents, WWTPs influents and effluents, surface waters and sediments are indicated in Table 1. Data suggests that this drug and its metabolites are likely to contribute to overall toxicity in receiving waters (Kiffmeyer et al., 1998; Yasunaga et al., 2006; Kosjek and Heath, 2011; Česen et al., 2016).

Polychaetes are widely used in marine environmental health assessment due to their ecological key role in benthic dynamics (Solé et al., 2009), and their rapid and easy response to disturbances induced by different stressors (Sivadas et al., 2010; Cong et al., 2011; Buffet et al., 2014; Maranho et al., 2015). The species *Nereis (Hediste) diversicolor* is highly recommended for ecotoxicological assessment regarding their physiological and biochemical responses after exposure to different classes of pharmaceuticals (e.g. anti-inflammatory; oral contraceptive; anticonvulsant; antidepressant drugs) (Aguirre-Martínez et al., 2013; Maranho et al., 2014, 2015; Fonseca et al., 2017; Pires et al., 2016a). Exposure to the cytotoxic drug cisplatin, at trace concentrations (0.1; 10 and 100 ng Pt L⁻¹) in seawater, caused behavioural and biochemical impairments in this species, after 14 days of exposure (Fonseca et al., 2017).

The aim of the present study is to assess the effects induced by the anticancer drug CP in the polychaete *N. diversicolor*, exposed to a

Table 1
Concentrations of CP in water (ng L⁻¹) and sediments (ng g⁻¹).

Study area	Hospital effluent	STP influent	STP effluent	Surface water		River sediments	Detection method	Reference
				Upstream	Downstream			
Australia	–	–	<125	–	<100	–	SPE; LC-MS/MS (ESI)	Busetti et al. (2009)
Canada	–	<4–22	<4–21	–	–	–	SPE; LC-MS/MS (ESI)	Rabii et al. (2014)
China	6–2000	–	–	–	–	–	SPE; UPLC-MS/MS (ESI)	Yin et al. (2010)
Thailand	–	–	–	–	1907	–	SPE; HPLC-MS/MS	Usawanuwat et al. (2014)
England	–	–	0.19–0.37	–	–	–	SPE; LC-MS/MS (ESI)	Llewellyn et al. (2011)
Germany	19–4500	<6–143	<6–17	–	–	–	SPE; GC-MS (EI)	Steger-Hartmann et al. (1997)
Germany	146	–	–	–	–	–	SPE; GC-MS (EI)	Steger-Hartmann and Kiimmerer (1996)
Germany	–	–	nd	–	nd	–	LC-MS/MS (ESI)	Ternes (1998)
Italy	–	–	–	–	nd (8 rivers)	–	SPE; HPLC-MS/MS (ESI)	Calamari et al. (2003)
Italy	–	–	nd - 9	–	–	–	SPE; HPLC-MS/MS (ESI)	Castiglioni et al. (2005)
Italy	–	–	0.6	–	nd	–	SPE; HPLC-MS/MS	Zuccato et al. (2005)
Italy	–	–	–	–	2.2–10.1	<12	SPE; HPLC-MS/MS	Zuccato et al. (2000)
Japan	–	–	–	–	nd	nd	SPE; UPLC-MS/MS	Azuma et al. (2017)
Norway	<2–21	<2	<2	–	–	–	SPE; LC-TOF/MS (ESI)	Thomas et al. (2007)
Romania	–	–	–	–	<30–64.8	–	SPE; GC-MS (EI)	Moldovan (2006)
Spain	<1.1–43	8–26	7–25	<0.9	<0.9–20	–	SPE; UPLC QqLiT	Ferrando-Climent et al. (2014)
Spain	–	–	–	–	<3	–	SPE; HPLC-MS/MS	Valcárcel et al. (2011)
Spain	–	–	–	–	7.8–13.7	–	SPE; LC-MS (ESI)	Franquet-Griell et al. (2017)
Spain	5.73	<3.1–13,100	<3.1	–	–	–	SPE; LC-MS (ESI)	Gómez-Canela et al. (2012)
Spain	–	<2.1	<2.3	–	<1.7	–	SPE; HPLC-MS/MS (ESI)	Martín et al. (2011)
Spain	–	nd	nd	–	–	–	SPE; HPLC-MS/MS (ESI)	Martín et al. (2014)
Spain	–	nd - 43.8	nd - 25	–	–	–	SPE; LC-MS/MS (ESI)	Negreira et al. (2014)
Slovenia	14–22,000	19–27	17	–	–	–	SPE; GC-MS (EI)	Česen et al. (2015)
Switzerland	–	2–11	~2–10	–	0.15–0.17	–	SPE; LC-MS/MS (ESI)	Buerge et al. (2006)
Switzerland	0.161	–	–	–	–	–	SPE; HPLC-MS/MS (ESI)	Kovalova et al. (2012)

– STP: sewage treatment plant.

range of environmental relevant concentrations of CP detected in water compartments. For this purpose, the following biomarkers were assessed (1) behavioural impairment (burrowing kinetic impairment), (2) neurotoxicity (Acetylcholinesterase - AChE activity), (3) oxidative stress (superoxide dismutase – SOD; catalase – CAT; glutathione peroxidases - GPXs), (4) Phase II conjugation reaction (glutathione-S-transferases - GST activity), (5) oxidative damage (Lipid peroxidation LPO) and (6) genotoxicity (DNA damage), after exposure for 14 days to a contaminated water-sediment system.

2. Materials and methods

2.1. Chemicals

Cyclophosphamide monohydrate (Cytoxan) (CAS 0768) was purchased from Sigma-Aldrich (Portugal). For safety handling of the cytotoxic drug, experimental work was performed using class II biological safety cabinet, with appropriate clothing (open-back, impervious chemotherapy protection gown, double powder-free latex gloves and safety goggles). CP stock solution (40 mg L^{-1}) was diluted in ultrapure Milli-Q water to prepare test solutions.

2.2. Experimental setup

Sediments and specimens of *N. diversicolor* were handpicked during summer, at low tide at the intertidal estuarine mudflat in Mira River estuary (Vila Nova de Milfontes), located in the Southwest coast of Portugal (37.729031 N, -8.751585 W). The site is considered undisturbed and was used as a reference site for sediment quality assessment (Ferreira et al., 2003; Moreira et al., 2006; Fonseca et al., 2017). Sediments were mainly composed of silt and clay (73% of particles $<63 \mu\text{m}$), with a organic matter content of 7.8%. Animals were transported alive to the laboratory with sediments and water from the site of origin. The organisms were acclimated for 5 days in aerated aquaria filled with natural filtered seawater (salinity 35) from the Ria Formosa lagoon (Faro, Portugal) and sediments from the sampling site.

The sediments were wet-sieved through a 2-mm mesh for removal of large debris and other living organisms, followed by drying at 80°C (Thain and Bifield, 2001; ASTM, 2009; Maranhão et al., 2014), to remove volatile compounds and water. Sediments were then re-hydrated with the same amount of water (w.w./d.w.). Dried aliquots of the sediments were used to determine grain size distribution by the method proposed by Royse (1970). Organic matter content was determined by loss on ignition (550°C , for 5 h), as described by Gross (1971).

The 14-day bioassay was carried out in 20-L glass aquaria, with a proportion of 1:4 sediment/seawater (Faro, Ria Formosa), under constant aeration, controlled temperature ($19 \pm 2^\circ\text{C}$), salinity (35 ± 1.8) and light period (12:12 h). Each treatment was performed in triplicate, including seawater controls (day 0: CT0; day 14 CT14), solvent control (0.001% DMSO) and CP at a range of concentrations based on reported levels in the environment (Table 1). Polychaetes were divided into groups of 75 organisms per treatment in a triplicate design (25 per aquarium). Over the 14 days of exposure, water was renewed every 48 h avoiding sediment resuspension, with redosing of the drug in the water phase (0, 10; 100; 500 and 1000 ng L^{-1}) in order to simulate the input of pharmaceuticals in aquatic system. After 14 days of exposure, all animals were collected. Those used in the burrowing and comet assays were immediately handled for respective analysis, while those regarding biochemical end-points were rinsed with clean seawater and stored at -80°C until further use.

2.3. Burrowing assay

Worms of control conditions (day 0: CT0; day 14: CT14) and those exposed to the different CP concentrations were submitted to a burrowing test according to Bonnard et al. (2009). Fifteen animals of each treatment

were carefully placed individually in 150 mL-plastic containers, filled with natural seawater and 5 cm of sediments. Over a period of 30-minute, the position of the polychaetes was recorded every two minutes, to assess the time for fully burrowing. The results are expressed as the percentage (%) of unburrowed specimens, over time (min).

2.4. Biochemical analysis

2.4.1. Neurotoxicity

Polychaetes (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment) were individually homogenized in 100 mM Tris-HCl buffer (pH 8.0) and 0.1% Triton. The homogenates were centrifuged at 12,000g, for 30 min, at 4°C , and further separated in aliquots for total protein determination (Bradford, 1976) and AChE activity analysis (Ellman et al., 1961). AChE activity was measured through the increase of the absorbance of the yellow compound resulting from the production of 5-mercapto-2-nitrobenzoate ($\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$) formed by the reaction of thiocholine, a product of acetylcholine cleavage by AChE with 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB), at 405 nm (Ellman et al., 1961; Colovic et al., 2013). AChE activity is expressed as $\text{ATC} \cdot \text{min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.4.2. Tissue preparation for enzyme activities analysis

Whole organisms (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment), were homogenized in 20 mM Tris-HCl buffer (0.5 M sucrose, 0.075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6), according to the protocol described by Geret et al. (2002). The homogenates were centrifuged at 500g, for 15 min, at 4°C , and the supernatants obtained centrifuged again (12,000g, 45 min, 4°C). Aliquots (150 μL) of the cytosolic fraction were separated for determination of each antioxidant enzyme activity (SOD, CAT, GPX) and biotransformation (GST). In addition, total proteins concentrations ($\text{mg protein g}^{-1} \text{ tissue}$) were determined following the method described by Bradford (1976), adapted for microplate reader using bovine serum albumin (BSA) as a standard.

2.4.3. Antioxidant enzymes activities

SOD activity was assessed by measuring the decrease of absorbance of the substrate cytochrome-c by xanthine oxidase/hypoxanthine system, at 550 nm (McCord and Fridovich, 1969), and the results are expressed as $\text{U mg}^{-1} \text{ protein}$. CAT activity was determined spectrophotometrically by measuring the decrease of absorbance at 240 nm (Greenwald, 1985). GPX activity was measured at 340 nm by using cumene hydroperoxide and H_2O_2 as substrates for T-GPx and Se-GPx, respectively (adapted from Lawrence and Burk, 1976). The activity of CAT, Se- and T-GPx are expressed in $\text{nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.4.4. Biotransformation

GST activity was measured in the cytosolic fraction by the conjugation of 0.2 mM reduced glutathione (GSH) with 0.2 mM CDNB (molar coefficient of extinction = $0.6 \text{ mM}^{-1} \text{ cm}^{-1}$) in a reaction mixture of 0.2 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{PO}_4$ buffer (pH 7.9), at 340 nm (adapted from Habig et al., 1974). The results are expressed in $\text{nmol CDNB min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.4.5. Oxidative damage

LPO was determined in whole organisms (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment), by the absorbance of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations, at 540 nm (adapted from Erdelmeier et al., 1998). LPO is expressed in $\text{nmol MDA} + 4\text{-HNE mg}^{-1} \text{ protein}$.

2.5. Genotoxicity assay

DNA damage was assessed by the alkaline Comet assay, adapted from Singh et al. (1988) and described by Gomes et al. (2013). Slides were previously cleaned in alcohol/ether and coated with 0.65% normal melting point agarose (NMA) in Tris-acetate EDTA. Coelomocytes,

present in the coelomic fluid of *N. diversicolor* (5 specimens per aquaria, in triplicate; total of 15 organisms per treatment), were extracted from the posterior region of the polychaete body into 20 μL of PBS buffer in a 0.5 mL-syringe fitted with hypodermic needle, based on the procedure described by Fonseca et al. (2017). The mixture was centrifuged at 835 g (3 min, 4 °C) and the pellet suspended in 0.65% low melting point agarose (LMA, in Kenny's salt solution; 0.4 M NaCl, 9 mM KCl, 0.7 mM KH_2PO_4 , 2 Mm NaHCO_3 , 1000 mL Milli-Q water) and casted on the microscope slides. Subsequently, slides were immersed in a lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, 10% Dimethylsulfoxide, 1% Sarcosil, pH 10, 4 °C), over 1 h, for the diffusion of cellular components and DNA immobilization in agarose. Slides were then placed in electrophoresis chamber, embedded with buffer (300 mM NaOH, 1 mM EDTA, adjusted at pH 13, 4 °C) and left for 15 min to permit DNA unwinding. The electrophoresis was performed at 25 V and 300 mA, over 5 min. Afterwards, slides were neutralized with appropriate buffer (0.4 mM Tris, pH 7.5), rinsed with distilled water and left to dry overnight.

Slides were analysed with an optical fluorescence microscope Axiovert S100 (total magnification of $\times 400$), coupled with a camera, with an aid of 4,6-diamidino-2-phenylindole (DAPI, 1 mg mL^{-1}). Image analysis was made with the software Komet 5.5 (Kinetic Imaging Ltd.) by classifying 50 randomly chosen cells from each slide. The amount of DNA in the comet tail (DNA tail %) was used as an end-point and results are expressed as mean \pm SEM.

2.6. Statistical analysis

Data was tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) in order to determine whether they satisfy the assumptions associated with parametric tests. One-way analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis were applied and Tukey's or Dunn's post-hoc tests performed to compare treatment effects. Results were significant when $p < 0.05$. Burrowing behavioural data was analysed by linear regression. Principal Component Analysis (PCA) were applied to evaluate the relationship among biomarkers responses and between pharmaceuticals levels. Statistical analysis was carried out using the Statistica 8.0 software (Statsoft Inc., 2007, USA).

3. Results

3.1. Behavioural assay

Results of burrowing assessment are indicated in Fig. 2. Slopes of trend lines depicting by linear regression analysis showed significant statistical differences between all treatments ($p < 0.05$). Both control

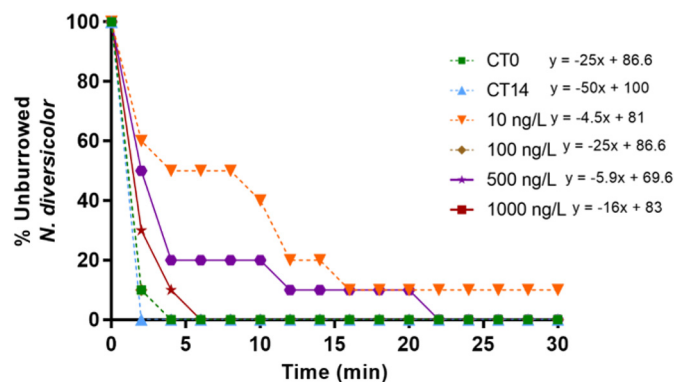


Fig. 2. Burrowing behaviour of *N. diversicolor* from control conditions (day 0: CT0; day 14: CT14) and those exposed to CP-contaminated systems (10, 100, 500 and 1000 ng L^{-1}), expressed as percentage of unburrowed organisms over time (lines with symbols). Continuous lines represent least-square best-fit regression lines, with respective equations.

conditions (CT0 and CT14) indicate similar trend, as well as for animals exposed to the concentration of 100 ng L^{-1} , that was entirely overlapped by CT0. Perturbation in burrowing, however, was higher with the decrease of cyclophosphamide levels. Polychaetes exposed to 1000 ng L^{-1} were fully buried after the first 5 min, whereas at 500 ng L^{-1} , animals lasted 20 min at surface until complete burrowing while among those exposed to 10 ng L^{-1} not all the animals were buried.

3.2. Biochemical analysis

3.2.1. Neurotoxicity

AChE activity did not change between controls over time ($p > 0.05$). A slight decreasing in AChE activity was observed in polychaetes exposed to the lowest CP concentrations (10 ng L^{-1}), although not significant ($p > 0.05$). With the increase of CP concentrations AChE activity was similar to the controls ($p > 0.05$) (Fig. 3).

3.2.2. Antioxidant and biotransformation enzymes

Antioxidant enzymes activities were similar between controls over time ($p > 0.05$). Despite no significant statistical differences between CP-treatments and controls, it was observed an increase in SOD activity in ragworms exposed to 10 and 100 ng L^{-1} of CP, followed by an inhibition in organisms exposed at the higher CP levels (i.e. 500 and 1000 ng L^{-1}) (Fig. 4A). A significant 2-fold increase in CAT activity was detected in worms exposed to concentrations of 10 and 500 ng L^{-1} (Fig. 4B), whereas no significant changes were observed for GPx Se-dependent activity with CP increasing levels ($p > 0.05$). On the other hand, a clear decreasing trend in T-GPx activity was detected with increasing CP concentrations, significantly different from controls only in polychaetes exposed to 500 and 1000 ng L^{-1} ($p < 0.05$). Likewise, GST activity decreased with the increasing CP concentrations, significant at 100, 500 and 1000 ng L^{-1} ($p < 0.05$) (Fig. 5).

3.2.3. Oxidative damage

An increase of oxidative damage was clear in polychaetes with the increase of CP concentrations with significant differences at 500 and 1000 ng L^{-1} compared to controls ($p < 0.05$) (Fig. 6).

3.3. Genotoxicity

Data of DNA tail (%) and DNA damage grade after 14 days of exposure are presented in Table 2. Despite all polychaetes exposed to CP treatments indicated DNA damage compared to its respective control

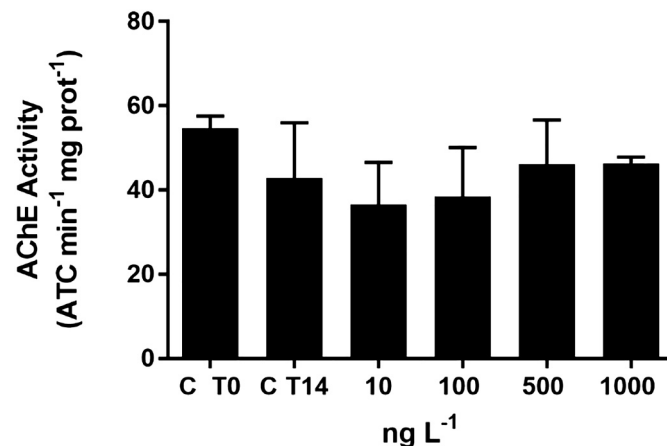


Fig. 3. AChE activity (mean \pm S.D.) ($\text{ATC} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) in *N. diversicolor* unexposed (CT 0 and CT 14) and exposed to CP (10, 100, 500 and 1000 ng L^{-1}) for 14 days. Absence of letters indicates no significant differences among treatments (Kruskal-Wallis, $p > 0.05$).

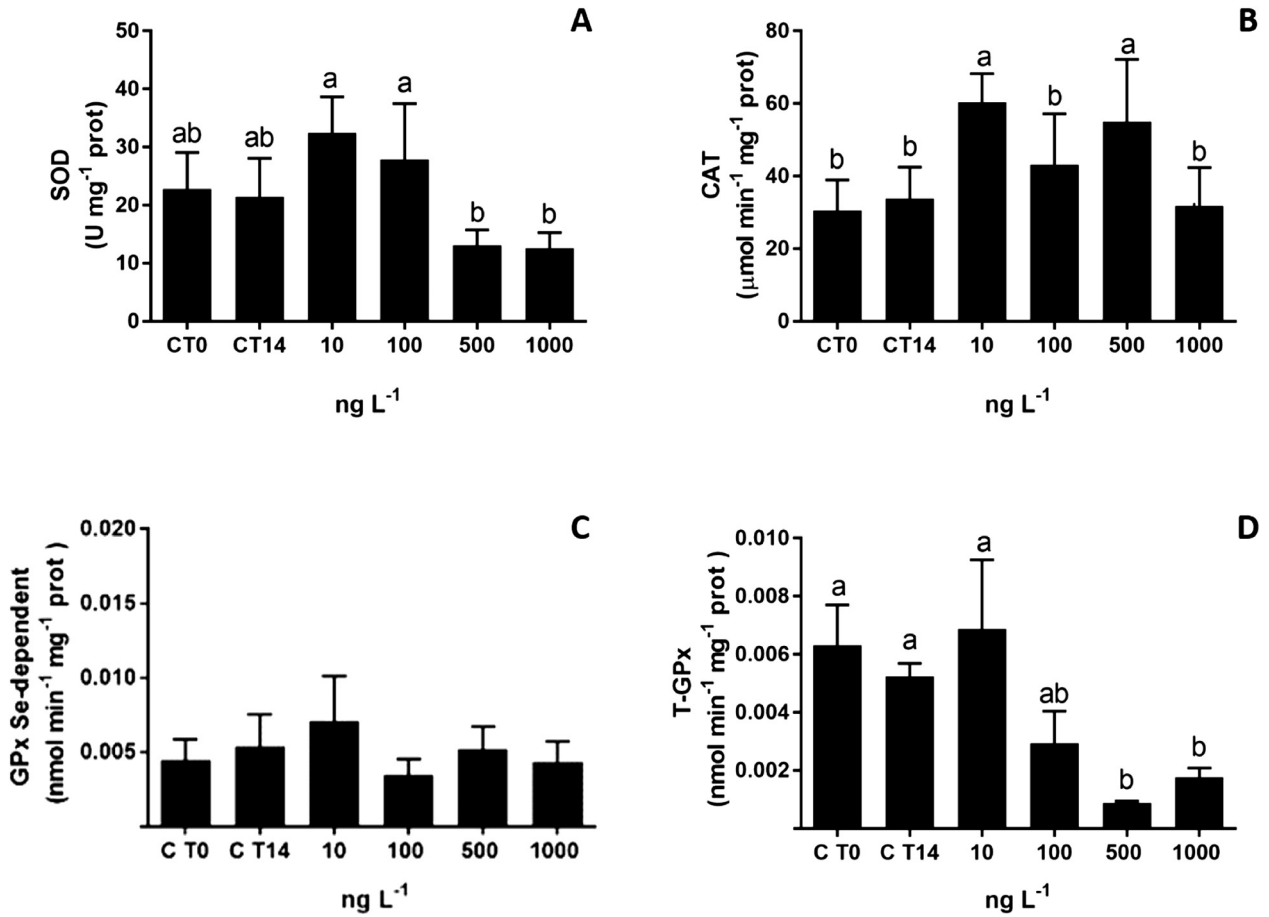


Fig. 4. Antioxidant enzymes activities (mean ± S.D.) of (A) SOD, (B) CAT, (C) GPx Se-dependent, (D) T-GPx in *N. diversicolor* of control conditions (CT0 and CT14) and exposed to CP (10, 100, 500 and 1000 ng L⁻¹) for 14 days. Different letters indicate significant difference among treatments (ANOVA: SOD, CAT; Kruskal-Wallis: GPx Se-dependent, T-GPx; $p < 0.05$). Absence of letters indicates no significant differences among treatments ($p > 0.05$).

($p < 0.05$), the grade of impairment was always between minimal to mid damage.

3.4. Principal Component Analysis (PCA)

PCA results indicate a two-dimensional pattern explaining 81.4% of the total variance (PC1 = 46.3%; PC2 = 36.2%) (Fig. 7). Overall,

the plot score indicated a clear separation between organisms from control conditions and contaminated treatments, in which CT0 and CT14 organisms are close-related in both axis projections. The first axis mainly explains the decline in SOD, T-GPx, GST, in addition to the induction of oxidative and significant DNA damage as detrimental effects of suppression of the lines of defense, in animals exposed to the two highest CP levels.

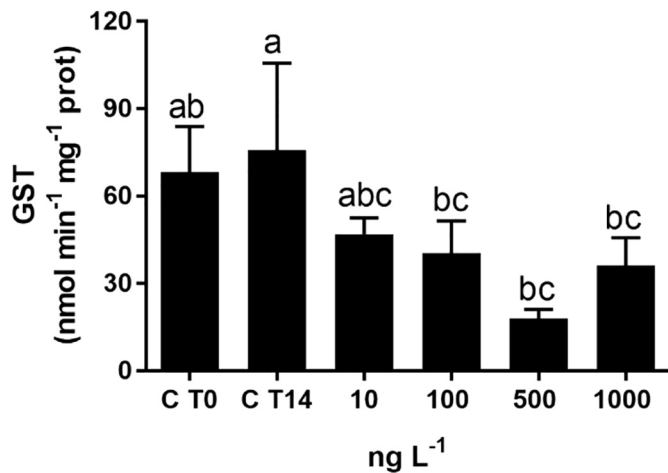


Fig. 5. Biotransformation enzyme activity (mean ± S.D.) in *N. diversicolor* of control conditions (CT0 and CT14) and exposed to CP (10, 100, 500 and 1000 ng L⁻¹) for 14 days. Different letters indicate significant differences among treatments (Kruskal-Wallis, $p < 0.05$).

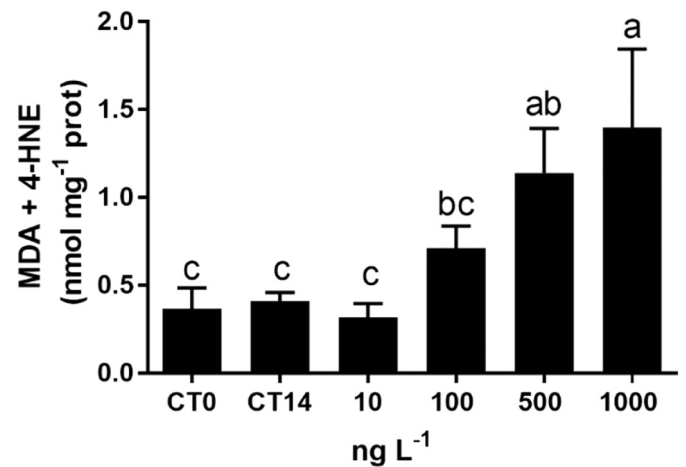


Fig. 6. LPO activity (mean ± standard deviation) (MDA+ 4-HNE nmol·mg⁻¹ protein) in animals of control conditions (CT0 and CT14) and exposed to CP at 10, 100, 500 and 1000 ng L⁻¹ for 14 days. Different letters indicate significant differences among treatments (ANOVA, $p < 0.05$).

Table 2

Genotoxic effects in polychaetes *N. diversicolor* unexposed and after 14 days of exposure to CP. DNA damage (average \pm SEM) is expressed as tail DNA %. Frequency of coelomocytes distributed by grade of DNA damage (%). Different letters represent statistical differences between treatments, during the exposure period. Percentage of coelomocytes distributed through damage criteria (*i.e.* minimal to extreme).

Treatment	DNA tail (%)	DNA damage grade (%)				
		Minimal	Low	Mid	High	Extreme
Control	7.5 ^a (± 0.4)	69.2	30.8	0	0	0
10 ng CP L ⁻¹	10.26 ^b (± 0.57)	59	30.1	10.9	0	0
100 ng CP L ⁻¹	10.59 ^b (± 0.36)	51.5	46.2	2.3	0	0
500 ng CP L ⁻¹	9.97 ^b (± 0.42)	57.2	40.0	2.8	0	0
1000 ng CP L ⁻¹	11.83 ^b (± 0.55)	50.8	41.2	8.0	0	0

In the second component it is highlighted the divergence of bio-markers profile between control groups and the lowest CP concentration (*i.e.* 10 ng L⁻¹). The non-monotonic behavior of burrowing in such exposed group elicited a reduced number of animals excavating. Likewise, at the lower CP level, SOD, CAT and GPX-Se are triggered, although no significant differences were detected ($p > 0.05$).

4. Discussion

CP is a widely used chemotherapy agent applied worldwide, with diffuse release in aquatic systems through hospital and domestic wastewater discharges. Despite that its partition is preferential through the water phase, geochemical and hydrodynamic data indicate that CP may settle in the sediments (Xie, 2012; Kosjek and Heath, 2011). Coastal polychaetes have been increasingly applied as bioindicators in ecotoxicological assessments evaluating biochemical alterations caused by pharmaceuticals (Maranho et al., 2014; Pires et al., 2016a, Fonseca et al., 2017). The species *Nereis diversicolor* indicates, as addressed in previous studies, high sensitivity to low levels (ng L⁻¹ and g⁻¹) of pharmaceuticals containing different physicochemical properties and MoA, translated by disturbances in behavior, energy status, oxidative stress, neurotoxicity, oxidative damage, and genotoxicity (Maranho et al., 2014, 2015; Pires et al., 2016a; Fonseca et al., 2017).

In the present study, burrowing activity seemed to be non-monotonically over CP levels, in which animals were most impaired at lower concentrations, whereas at the highest concentrations (*i.e.* 1000 ng L⁻¹) all animals were fully buried after 30 min (Fig. 2). As stated by Doyotte et al. (1997), it is not a general rule that an increase in contaminant concentrations induces the expected harmful effect, as proposed by the dose-response curve. Lagarde et al. (2015) reviewed different profiles of non-monotonic dose responses for various physiological and behavioural effects, in several organs or systems. Accordingly, the present burrowing profile corroborated with a bell-shaped dose-effect relationship in which minimal effects are induced at the two extreme doses (control and 1000 ng L⁻¹), although the mechanisms involved in the burrowing at the highest CP concentration may not be similar to the observed in controls. Hence, at very high doses, un-specific effects will, for instance, occur because the organism would be completely overwhelmed by the substance, through non-specific biological mechanisms (Lagarde et al., 2015). Many components of signaling pathways and their cross-talk could contribute to understand the non-linearity and dynamical monotonicity in response to stimuli (Van Wijk et al., 2015).

Contrarily to the present results, animals remained emerged at the highest concentration of the cytotoxic drug cisplatin (100 ng Pt L⁻¹), together with neurotoxic effects associated to AChE inhibition (Fonseca et al., 2017). CP has been widely reported to inhibit brain and retinal AChE activity in different biological models (Al-Jafari, 1993; Al-Jafari et al., 1995; Kamal et al., 2010) with hydrophobic interactions providing CP positioning within the acyl pockets and catalytic site of the enzyme (Shakil et al., 2011). However, herein, no effects of neurotoxicity were observed (Fig. 3), as a potential result of docking between the enzyme and CP that may not be well accomplished in the present biological model (Valasani et al., 2013). Besides, CP levels applied herein are too low to observe neurotoxic effects, compared to concentration range (mg-g L⁻¹) used in experiments with vertebrates (Santos and Pacheco, 1995).

In general, cancer cells are characterized by increased aerobic glycolysis, followed by high levels of reactive oxygen species (ROS), which need to be counteracted by expression of ROS-scavenging systems for the success of the first stage of tumor formation (Gorrini et al., 2013).

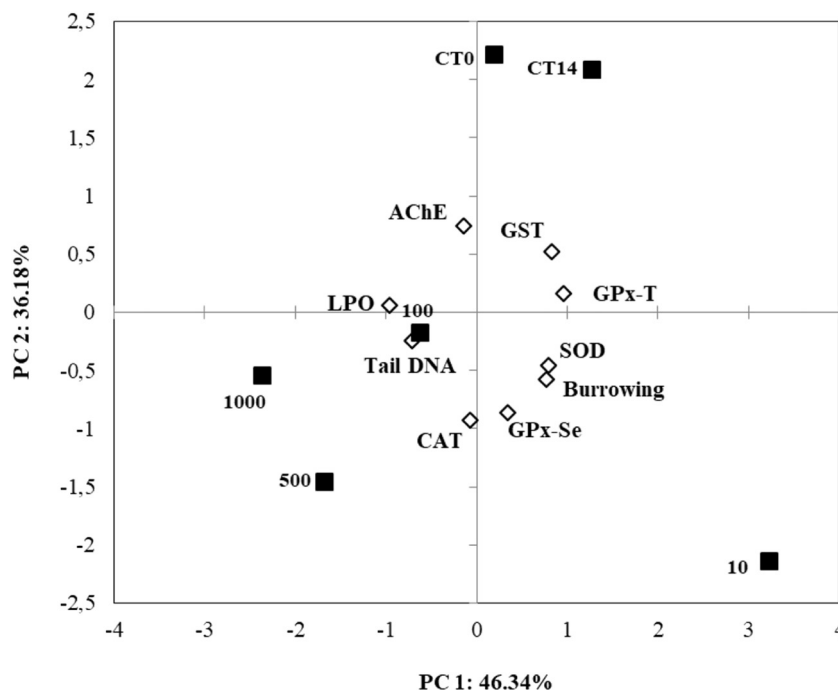


Fig. 7. Principal Component Analysis (PCA) integrating responses of antioxidant enzymes (SOD, CAT, Se-GPx, T-GPx, GST), AChE, DNA Tail, oxidative damage (LPO) and burrowing behavior of *N. diversicolor*, exposed to control conditions (CT0: day 0; CT14: day 14) and to different CP concentrations (10, 100, 500 and 1000 ng L⁻¹).

The administration of anticancer drugs relies on the enhancement of ROS levels to trigger cancer cells death through signaling pathways and cellular damage (Conklin, 2004; Gorrini et al., 2013). CP produces additional oxidative stress through the acrolein metabolite (Arumugam et al., 1999; Dumontet et al., 2001; Oboh and Ogunraku, 2010). In this sense, its cytotoxic MoA combined with the suppression of cellular defenses are effective to impair normal growing cells, including marine non-target species (Fonseca et al., 2017).

In the present study, although significant differences were not statistically detected in SOD activity when compared to controls ($p > 0.05$), data showed a slight induction of SOD activity at the two lowest concentrations (Fig. 4A), triggered as a potential consequence of polychaetes adaptation to overcome the stress from the drug (Sun and Zhou, 2008). At the two highest levels of CP, SOD activity was inhibited (Fig. 4A), as observed at 14-day of exposure to the anticancer drug CisPt, at 100 ng Pt L⁻¹ (Fonseca et al., 2017). In rats, the inhibition of SOD activity was also observed in animals treated with intraperitoneal injection, either with 5 or 100 mg CP kg⁻¹ (Patel and Block, 1985). Conversely, in the marine mussel *Mytilus galloprovincialis*, over the 2-week exposure to CisPt (100 ng Pt L⁻¹), a higher induction and detoxification of SOD activity was observed in the digestive gland compared to the gills, denoting the primary role of the former in recovery processes in this species (Trombini et al., 2016; Faria et al., 2009). Giving the predominant role of SOD as the first line of defense in ROS scavenging, by converting the highly reactive superoxide anion (O₂⁻) into hydrogen peroxide (H₂O₂), its depletion yields cell redox imbalance by O₂⁻ accumulation, thus elevating the oxidizing potential (Ben Ameer et al., 2012; Cozzari et al., 2015; Djordjevic et al., 2011).

Regarding the second line of defense against ROS, CAT activity acts as a catalyst of the SOD activity by-product H₂O₂ for its detoxification into water (Faria et al., 2009; Gonzalez-Rey and Bebianno, 2012). Even though it is expected a coping behavior among enzymes in the metabolic pathway, CAT activity responses depicted a non-monotonic profile over CP levels (Fig. 4B), might be attributed to other ROS detoxifying process, as stated by Aguirre-Martínez et al. (2013) and Gonzalez-Rey and Bebianno (2012). In the case of CP metabolism, the CYP450 activation for PAM and acrolein detoxification combined with the uncoupled catalytic cycle may be accountable to form extra amounts of oxyradicals that need to be neutralized (Dumontet et al., 2001; Harskamp et al., 2012; Oboh and Ogunraku, 2010). The burst of superoxide induced by CP that cannot be efficiently eliminated through SOD activity is promptly buffered by the GSH (Aquilano et al., 2014). The electrophilic acrolein produces cellular damage after binding to GSH and reduces their availability inside the cells, hence impairing the antioxidant glutathione dependent-system and also increasing free radical generation (Peña-Llopis et al., 2002; Singh et al., 2014).

The activity of Se-GPx is an important alternative to counteract harmful electrophiles produced during oxidative damage (Djordjevic et al., 2011) and is a key enzyme that detoxifies H₂O₂ and converts lipid hydroperoxydes to non-toxic alcohols (Charushila and Subodhini, 2015). The present results demonstrate that Se-GPx was unaffected at any of the CP levels to which worms were exposed to (Fig. 4C). In contrast, *N. diversicolor* exhibited a clear induction of that enzyme when specimens were exposed to CisPt, as an offset of CAT activity decrease (Fonseca et al., 2017), once both enzymes are associated to enzymatic hydrogen peroxide scavenging (Cozzari et al., 2015). As in the present study CAT activity was not inhibited. It may be hypothesized that the activation of Se-GPx was not particularly relevant to counteract severe oxidative challenge. Notwithstanding, T-GPx is also recognized as the most important enzymatic reductor agent of lipid peroxides and emerges as a compensatory response in cases where SOD activity is inhibited (Aquilano et al., 2014). In contrast, the excess of non-neutralized oxyradicals, such as superoxide, may lead to exhaustion or inhibition of the defense system culminating in T-GPx inactivation, as observed in worms exposed to 500 and 1000 ng L⁻¹ (Fig. 4D). Thus, once T-GPx activity decreased, more hydrogen peroxide is accumulated

in the cell to the point of causing oxidative damage and activation of inflammatory pathways (Yu et al., 2006). T-GPx activity inhibition also occurred in the digestive gland of the clam *Ruditapes philippinarum* exposed to the antiepileptic carbamazepine and the antibiotic novobiocin, as a reflection of pro-oxidant forces overcoming antioxidant defenses (Aguirre-Martínez et al., 2016).

GST is involved in the Phase II biotransformation metabolism by catalyzing the nucleophilic attack of GSH onto highly electrophilic compounds, thus denoting an important role in homeostasis as well as in detoxification and clearance of the drugs (Aguirre-Martínez et al., 2013; Faria et al., 2009; Hayes et al., 2005). Such enzyme can reduce lipid hydroperoxides together with GPx, and is related to protection against apoptosis (Prabhu et al., 2004). Several studies have pointed out its induction in aquatic invertebrates after pharmaceuticals exposure as an outcome of its activation and antioxidant role (Martin-Diaz et al., 2009; Buffet et al., 2014; Aguirre-Martínez et al., 2013; Maranhão et al., 2014; Aguirre-Martínez et al., 2016; Pires et al., 2016 a,b). The mismatch between our results and those reported may be related to different patterns of response associated to the drug's MoA and disparities across species regarding their defensive systems to metabolize xenobiotics (Cozzari et al., 2015; Faria et al., 2009; Sun and Zhou, 2008). No CP immunotoxicity was observed in the aquatic gastropod *Lymnaea stagnalis* even at therapeutic exposure levels, which was suggested to be related to the lack of appropriate converting enzymes of the pro-drug (Boisseaux et al., 2017). Contrarily, polychaetes *N. diversicolor* exhibited a sharp decline in the biotransformation system in a 14-day bioassay conducted with the electrophilic CisPt, which resembles the highly electrophilic binding to the drug (Fonseca et al., 2017). Nevertheless, Gonzalez-Rey and Bebianno (2012) observed a significant decrease of GST activity in mussels *Mytilus galloprovincialis* exposed to ibuprofen (250 ng L⁻¹), corroborating the data regarding the polychaete *Diopatra neapolitana*, in systems containing carbamazepine (3 µg L⁻¹) (Pires et al., 2016b).

Lipid peroxidation is initiated by the attack on fatty acids of biomolecules that possess sufficient reactivity to abstract a hydrogen atom from methylene carbon in the side chain (Halliwell and Chirico, 1993). Cellular lipids are the primary targets of generated ROS and subsequently LPO occurs, as in human cells exposed to hydroxyl-cyclophosphamide (Dumontet et al., 2001). Morphological changes were observed in type II-alveolar rats epithelial cells as a result of oxidative stress (e.g. decrease in SOD and glutathione reductase activities) and an enhancement of LPO occurs after 7 days of exposure to 150 mg CP kg⁻¹ body weight (Sulkowska et al., 1998). LPO is one of the main toxic effects caused by CP and responsible for several side effects during chemotherapy (Sulkowska et al., 1998), due to the production of the reactive aldehyde acrolein formed by β-elimination of aldophosphamide. Besides an important component produced by CP for cytotoxicity in cancer cells, this metabolite is also known as an overall secondary reactive species-product of LPO that triggers further chain reaction (Dumontet et al., 2001; Conklin, 2004; Singh et al., 2014). The increased levels of LPO by-products at the higher CP levels (500 and 1000 ng L⁻¹) (Fig. 6) may be a result of an inefficient biochemical antioxidant defense system (Gonzalez-Rey and Bebianno, 2012; Freitas et al., 2016; Pires et al., 2016a; Fonseca et al., 2017), depicted by the depletion of SOD, T-GPx and GST activities, as indicated in the first axis of the PCA (Fig. 7). Besides enzymes activity suppression, chemotherapy treatment produce additional ROS generation (Conklin, 2004), which may lead to DNA damage, mutations and apoptosis to normal growing cells, such as observed in marine non-target species exposed to cisplatin and methotrexate (Fonseca et al., 2017; Moreira et al., 2016).

CP has bifunctional and S_N1 molecular properties, with two reactive moieties towards bases of opposite DNA double strands (Anderson et al., 1995). The major alkylating agent following CP metabolism is PAM, which undergoes spontaneous hydrolysis to form reactive aziridium intermediate species, that binds to nucleophilic N7-position of guanine in DNA. Alkylation to oxygen atoms of DNA, especially at

the O6-position of guanine, are also generated at lower frequency than N-alkyl adducts, however its biological relevance is even greater considering the readily mispair of bases during DNA replication (Fu et al., 2012). The array of lesions caused by CP alkylation impair genome integrity by inducing mutagenesis, besides DNA replication and eventual responsive signaling for cell death if DNA repair mechanisms are not efficient (Deans and West, 2011; Fu et al., 2012). DNA damage caused by CP is widely described in vertebrates cells, in which administration by therapeutically treated-patients and rats exposed to this drug showed gene mutations, sister chromatid exchange, DNA adducts, DNA-DNA and DNA-protein crosslinks in somatic cells, especially by the ultimate crosslinking metabolite PAM (Crook et al., 1986; Codrington et al., 2004). Few studies were so far conducted to access genotoxicity responses to anticancer drugs considering *in vivo* uptake by marine organisms (Fonseca et al., 2017; Parrella et al., 2015). In the present study, results showed a significant increase in DNA damage in CP-treated animals compared to controls (Table 2). Animals exposed to CP, particularly to 1000 ng L⁻¹, had a significantly higher density of DNA in the tail compared to controls (CT14) ($p < 0.05$). The grade of DNA damage was similar among CP treatments, although the lowest and highest concentrations indicated more burden of mid damage.

DNA damage caused by anticancer drugs were addressed in other aquatic biological models, at levels ranging from low ng L⁻¹ to µg L⁻¹: the bivalve mollusc *Mytilus galloprovincialis*, exposed to cisplatin (Trombini et al., 2016); the microcrustaceans *Ceriodaphnia dubia* and *Daphnia magna*, exposed to 5-FU, capecitabine, cisplatin, doxorubicin, etoposide and imatinib (Parrella et al., 2014a, 2014b, 2015); and ZFL cell lines of the fish *Danio rerio* to the tyrosine kinase inhibitor imatinib (Novak et al., 2016). However, the 14-day exposure of *N. diversicolor* to the alkylating agent cisplatin (Fonseca et al., 2017) yielded no genotoxicity effect at a similar range of concentrations. Cytotoxic metabolites of CP are transported and diffused into cells interacting with DNA sites after prodrug enzymatic activation, certified with the increasing DNA damage over increasing CP levels, however, this needs to be confirmed with CP levels accumulated in the worms.

5. Conclusions

The present study highlights the cytotoxic effects of the antineoplastic drug CP on normal proliferative cells of polychaetes, by binding to DNA and interfering in antioxidant status. Chronic exposure of polychaetes *N. diversicolor* to CP levels of 500 and 1000 ng L⁻¹ in seawater led to an inhibition of the antioxidant enzymes activity, stimulating LPO by-products and DNA damage. The analysed biomarkers suggest that the prodrug CP is probably metabolically activated by *N. diversicolor* into PAM and acrolein, respectively responsible for the antineoplastic properties and toxic effects observed, nonetheless, this needs to be confirmed.

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