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Effects of the antineoplastic drug cyclophosphamide on the biochemical responses of the mussel *Mytilus galloprovincialis* under different temperatures☆

Vanessa Queirós [a], Ulisses M. Azeiteiro [a], Carlos Barata [b], Juan Luis Santos [c], Esteban Alonso [c], Amadeu M.V.M. Soares [a], Rosa Freitas [a,*]

[a] Biology Department & CESAM, University of Aveiro, 3810-193, Aveiro, Portugal
[b] Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research, IDAEA-CSIC, Jordi Girona 18, 08034, Barcelona, Spain
[c] Departamento de Química Analítica, Escuela Politécnica Superior, Universidad de Sevilla, Spain

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ABSTRACT

Cyclophosphamide (CP) is an antineoplastic drug widely used in chemotherapy treatments with high consumption rates and that has been detected in the aquatic environment. After being released into the aquatic environment, CP may cause adverse effects on aquatic organisms since antineoplastics are well-known cytotoxic, genotoxic, mutagenic and teratogenic drugs. Moreover, predicted environmental changes, such as the temperature rising, may alter the impacts caused by CP on organisms. Thus, the present study aimed to assess the effects caused by CP chronic exposure in the mussel *Mytilus galloprovincialis*, under actual and predicted warming scenarios. Organisms were exposed for 28 days to different concentrations of CP (10, 100, 500 and 1000 ng/L) at control (17 ± 1.0 °C) and increased (21 ± 1.0 °C) temperatures. Biochemical responses related to metabolic capacity, energy reserves, oxidative stress and neurotoxicity were assessed. The results showed that the organisms were able to maintain their metabolic capacity under all exposure conditions. However, their antioxidant defense mechanisms were activated mostly at higher CP concentrations being able to prevent cellular damage, even under the warming scenario. Overall, the present findings suggest that temperature rise may not alter the impacts of CP towards *M. galloprovincialis*.

1. Introduction

Antineoplastic drugs (also known as anticancer, cytostatic or cytotoxic drugs) are an important class of pharmaceuticals used in the treatment of neoplastic diseases (e.g.: chemotherapy treatments). These drugs belong to a major class of pharmaceuticals identified as "antineoplastic and immunomodulating agents", class L of the Anatomical Therapeutic Chemical (ATC) classification, included in the sub-class of the antineoplastic agents (L01) (WHOCC, 2020). Considering that cancer is identified by the World Health Organization (WHO) as the second leading cause of death worldwide and an increase of 50% in the number of cancer cases is expected by 2040 in comparison to the number of cases in 2018 (Wild et al., 2020), it is also expected an increase in the consumption of these drugs and the development of new ones in the near future. In Europe, different countries have reported consumption values of antineoplastic drugs up to tonnes per year (Besse et al., 2012; Cristóvão et al., 2020; Franquet-Griell et al., 2017b). As an example, in the United Kingdom the consumption per capita of cyclophosphamide (CP), one of the most consumed antineoplastic in Europe, was reported to be of about 21 μg/inhab/day in 2009 (Rowney et al., 2009) and of 40 μg/inhab/day in 2014 (Booker et al., 2014).

After consumption, antineoplastic drugs are excreted through the urinary and/or digestive systems as metabolites or parent compounds and are discharged into the hospital and household wastewaters (Ioannou-Ttofa and Fatta-Kassinos, 2020; Isidori et al., 2016). Ultimately, these drugs reach the wastewater treatment plants (WWTPs) where conventional (mostly) treatments are applied to remove contaminants present in the waters. Nonetheless, inefficient removal rates of these drugs, which can be as low as 10%, have been reported for WWTPs by several authors (Buerge et al., 2006; Ioannou-Ttofa and Fatta-Kassinos,

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* Corresponding author.
E-mail address: rosafreitas@ua.pt (R. Freitas).

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2020; Martín et al., 2014; Negreira et al., 2014) and, consequently, these drugs can potentially reach different water compartments. Several studies have already reported the presence of these drugs in different water compartments (see for review Queirós et al., 2021), namely hospital effluents, WWTPs influents and effluents as well as freshwater ecosystems (Franquet-Griell et al., 2017a; Ghafuri et al., 2018; Gómez-Canela et al., 2014; Martín et al., 2011; Negreira et al., 2013a; Olalla et al., 2018). Predicted environmental concentrations (PECs) have also been calculated for several antineoplastics drugs (Cristóvão et al., 2020; Kümmerer et al., 2016; Martín et al., 2014; Rowney et al., 2009; Verlicchi et al., 2020). Since antineoplastic drugs are characterized as cytotoxic, genotoxic, mutagenic and teratogenic (Besse et al., 2012) when in the aquatic environment they may pose at risk the existent wildlife, with recent studies showing these effects in various aquatic species (Araújo et al., 2019; Fonseca et al., 2019; Kolarević et al., 2016; Russo et al., 2018).

Among antineoplastic drugs, CP is considered one of the most relevant pharmaceuticals due to its wide use in the treatment of various neoplastic diseases and high consumption levels worldwide (Gómez-Canela et al., 2020; Gouveia et al., 2019; Queirós et al., 2021). This drug was developed in the late 1950s and is currently used in the treatment of several neoplastic diseases, such as lymphomas and breast cancer, and also in the treatment of autoimmune diseases, being recognized also as an immunosuppressant drug (Emadi et al., 2009). Cyclophosphamide belongs to the oldest class of antineoplastic drugs, the alkylating agents (L01A group, ATC classification), being a nitrogen mustard analog (L01AA sub-group) (WHOCC, 2020). This drug mode of action (MoA) consists of DNA alkylation, forming DNA cross-links with consequent DNA synthesis inhibition and further cell death (Ponticelli et al., 2018; Zhang et al., 2006).

Although CP PECs for surface water are in the ng/L range, the measured concentrations (MEC) indicate an underestimation of PEC values, with the highest CP MEC in the literature being 687 μg/L (hospital wastewater effluent) (Hamon et al., 2018; Queirós et al., 2021). As for other pharmaceutical classes (Almeida et al., 2020a; Ebele et al., 2017; Mezzelani et al., 2018b), higher attention has been given to the presence of antineoplastic drugs, namely CP, in freshwater ecosystems (Bebianno and Garcia da Fonseca, 2020; Verlicchi et al., 2020) and, as far as we know, there is no information regarding CP concentrations measured in estuarine and marine ecosystems. However, the removal rates reported for CP in WWTPs range from 10% to 100% (Gómez-Canela et al., 2012; Isidori et al., 2016), which, along with CP physical-chemical characteristics (low biodegradation, low octanol/-water partition coefficient (log K_{ow} of 0.63) and a half-live in the order of years) (Buerge et al., 2006; Kosjek and Heath, 2011), are factors that enhance the probability of this drug to reach coastal zones. Under laboratory conditions, CP showed toxicity towards different marine species (Fernandes et al., 2020; Fonseca et al., 2019, 2018). In particular, the mussel species *Mytilus edulis* and *Mytilus galloprovincialis* have been used to study the effect of various antineoplastics drugs, including CP, by assessing responses related to neurotoxicity, cytotoxic, genotoxicity and oxidative stress, among others (Canty et al., 2009; Fernandes et al., 2020; Trombini et al., 2016). The use of bivalve species as bioindicators can be of great interest due to the fact that they have an important socio-economic status, are sensitive to a wide range of stressors, are sessile organisms, present a filter-feeding behavior and, thus, they may bioaccumulate several pollutants, including pharmaceuticals (Almeida et al., 2020a, 2020b; Burket et al., 2019). These characteristics make bivalve species suitable bioindicators to be used in ecotoxicological studies and assess several endpoints, such as physiological and biochemical responses (Costa et al., 2020b; Freitas et al., 2019; Hu et al., 2015; Khan et al., 2020; Strehse and Maser, 2020; Świacka et al., 2019; Zuykov et al., 2013).

Along with exposure to pollutants, organisms are nowadays exposed to climate changes such as temperature rise. In fact, by the end of the 21st century (2081–2100) and relative to the years 1986–2005, it is expected an increase of the global mean surface temperature, which is likely to be between 1 and 3.7 °C (depending on the scenario) (IPCC, 2019). Environmental changes, as those foreseen under climate change scenarios, may significantly affect bivalves performance, including their sensitivity towards pollutants and bioaccumulation patterns as already proved (Costa et al., 2020b; Franzellitti et al., 2020; Leite et al., 2020; Maulvault et al., 2018; Maynou et al., 2021; Pirone et al., 2019; Velez et al., 2017).

Taking this into account, the present study aimed to evaluate the impact of the antineoplastic drug CP after a chronic exposure (28 days), at relevant environmental concentrations (ng/L range), of the estuarine bivalve species *M. galloprovincialis*, under actual and predicted warming conditions. Bioaccumulation of CP and biochemical markers related to mussel's metabolic capacity, oxidative stress and neurotoxic status were evaluated at the end of the exposure period.

2. Material and methods

2.1. Chemicals

Cyclophosphamide monohydrate, 97% (Cytoxan) (CAS Number: 6055-19-2) was purchased from Acros Organics and used to prepare the stock solutions. A biological safety cabinet as well as appropriate protection (impervious chemotherapy protection gown, double powder-free latex gloves and safety goggles) were used for safe handling of the cytotoxic drug and solutions preparation. A CP stock solution (50 mg/L) was prepared in ultrapure Milli-Q water and used to prepare the spiking solutions.

2.2. Sampling procedures and experimental conditions

In the present study, the mussel *M. galloprovincialis* (Lamarck, 1819) was used as a model organism. Mussels were collected in June 2020 from Ria de Aveiro (NW Portugal) and were maintained in the laboratory for 15 days before the beginning of the experiment, for depuration and acclimation to the laboratory conditions. During this period, organisms were maintained at 17 ± 1 °C, 12 h light:12 h dark photoperiod and continuous aeration in artificial seawater (salinity 30 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Center). Seawater was renewed every two days and the mussels were not fed during the first week. Afterward, and during all the experiment, mussels were fed with Algamac protein plus (150.000 cells/animal/day) three times per week.

After acclimation, mussels with similar size (mean length: 5.9 ± 0.6 cm; mean width: 3.4 ± 0.3 cm) were selected and distributed into different glass aquaria (750 mL of medium), with 2 organisms per aquarium and 3 aquaria per treatment (total of 6 organisms per treatment) to test the impacts induced by chronic exposure (28 days) to CP under different temperature conditions. The following treatments were tested: Control (CTL, clean seawater with organisms with 0 ng/L of CP), 10, 100, 500 and 1000 ng/L of CP, at temperature 17 °C. The treatments control, 100 and 1000 ng/L of CP were also tested at 21 °C. The concentrations tested were selected based on previous studies (Fonseca et al., 2019, 2018) and relevant environmental levels. During the exposure period mussels were maintained at constant aeration, temperature (17 ± 1 and 21 ± 1 °C) and salinity (30 ± 1), with daily checking and readjustments if necessary. Seawater was renewed weekly, after which the CP concentrations and seawater parameters were re-established.

In order to quantify possible drug losses along the exposure period, during the first week of the experimental period, water samples from blanks (spiked seawater without organisms) were taken at the third day after spiking and before water renewal from the following treatments: at 17 °C, 500 ng/L and 1000 ng/L; at 21 °C, 1000 ng/L. For each treatment, 2 replicates were used.

At the end of the experiment, mortality was only recorded at 21 °C: 17% (corresponding to one mussel) at 100 and 1000 ng/L treatments.

From each treatment, the surviving mussels were sampled, immediately frozen in liquid nitrogen and maintained at −80 °C until further analysis. The whole soft tissues of each individual were homogenized separately in liquid nitrogen, divided into 5 aliquots with 0.5 g fresh weight (FW) each, and stored at −80 °C. Samples from all individuals were used for biochemical parameters determination.

After the biochemical analysis, the remaining tissues from 3 organisms per treatment, with exception of the CTL, were also used for CP quantification. Due to the lack of tissues from organisms from the treatment 1000 ng/L at 21 °C, for this treatment only 2 organisms were used for CP quantification.

2.3. Cyclophosphamide quantification on water and tissues

Concentrations of CP on water and soft tissues were quantified by the high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) detection method and as describe by Martín et al. (2011) and Martín et al. (2017), respectively (for more details see the supplementary material). The recovery, considering a standard, was 90% for water samples and 60% for tissues. The limits of detection (LOD) were 2 ng/L for water samples and 0.04 μg/kg dry weight (DW) for whole soft tissues. The limits of quantification (LOQ) were 5 ng/L for water samples and 0.14 μg/kg DW for whole soft tissues.

2.4. Biochemical parameters

The following biochemical parameters were determined in the present study: metabolic capacity (ETS, electron transport system activity); energy reserves content (PROT, protein content); oxidative stress related biomarkers that included antioxidant enzyme activities (CAT, catalase; GR, glutathione reductase; GPx, glutathione peroxidase), biotransformation enzymes of phase I (CbEs, carboxylesterases) and phase II (GSTs, glutathione S-transferases); redox status (GSH/GSSG, ratio between reduced (GSH) and oxidized (GSSG)) and cellular damage ((LPO, lipid peroxidation levels; PC, protein carbonylation levels). In addition, neurotoxicity (AChE, acetylcholinesterase activity) was also evaluated.

Subsamples used for biochemical analyses were subjected to extraction using the following buffers, specific to each biomarker, on a proportion of 1:2 (w/v): phosphate buffer (PROT, CAT, GR, GPx, GSTs, CbEs, LPO, PC and AChE), magnesium sulfate buffer (ETS) and KPE phosphate buffer (GSH/GSSG) (for further details see Andrade et al., 2018). After the addition of the specific buffers, samples were submitted to 90 s of high-speed shaking in a tissue-lyser (TissueLyser II, Qiagen) and then to 20 min of centrifugation at 10000 *g* or 3000 *g* (depending on the biomarker) at 4 °C. Afterward, supernatants were collected and immediately used or stored at −80 °C until further analysis. To determine each biochemical parameter, each sample replicate was read at least in duplicate in a microplate reader (BioTek Synergy HT).

The activity of ETS was measured by applying King and Packard (1975) method with De Coen and Janssen (1997) modifications. The absorbance was read at 490 nm, for 10 min with intervals of 25 s, followed by the calculation of the amount of formazan formed using the extinction coefficient $\varepsilon = 15.9\ mM^{-1}cm^{-1}$. Results were expressed in nmol per min per g of FW.

For total PROT content determination, the Biuret method was performed as described by Robinson and Hogden (1940) using, bovine serum albumin (BSA) as standard (0–40 mg/L). Absorbance was read at 540 nm and results were expressed in mg per g of FW.

The activity of CAT was quantified according to the Johansson and Borg (1988) method and with Carregosa et al. (2014) modifications. Formaldehyde standards (0–150 μmol/L) were used to obtain the standard curve. The absorbance was measured at 540 nm and the enzyme activity was expressed in U per g of FW, in which U represents the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde per min.

The activity of GR was quantified following the Carlberg and Mannervik (1985) method. The absorbance was measured at 340 nm and the extinction coefficient $\varepsilon = 6.22\ mM^{-1}cm^{-1}$ was used to calculate GR activity. The activity was expressed in U per g FW, in which U represents the amount of enzyme that caused the formation of 1.0 μmol of NADPH oxidized per min.

The activity of GPx was quantified according to the Paglia and Valentine (1967) protocol. The absorbance was measured at 340 nm, at each 10 s, for 5 min. The activity was calculated using the extinction coefficient $\varepsilon = 6.22\ mM^{-1}cm^{-1}$ and results were expressed as U per g of FW, in which U represents the number of enzymes that caused the formation of 1.0 μmol oxidized NADPH per min.

The activity of GSTs was quantified based on the Habig et al. (1974) method with modifications by Carregosa et al. (2014). Absorbance was measured at 340 nm and the activity was calculated using the extinction coefficient $\varepsilon = 9.6\ mM^{-1}cm^{-1}$. The activity was expressed in U per g of FW, in which U represents the amount of enzyme that catalysis the formation of 1 nmol of dinitrophenyl thioether per min.

The activity of CbEs was quantified following Hosokawa and Satoh (2001) method with adaptations carried out by Solé et al. (2018). The activity was measured using the commercial colorimetric substrate p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB). Absorbance was measured at 405 nm for 5 min with 15 s intervals between readings and the extinction coefficient $\varepsilon = 18\ mM^{-1}cm^{-1}$ was used to determine the activity. Results were expressed nmol per min per g of FW.

The concentration of GSH and GSSG was determined following the Rahman et al. (2006) method and used as standards (0–60 μmol/L) to obtain a calibration curve. Absorbance was measured at 412 nm and the ratio GSH/GSSG was determined taking into account the number of thiol equivalents (GSH/(2 * GSSG)).

Levels of LPO were estimated according to the method described by Ohkawa et al. (1979), through the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Absorbance was measured at 535 nm and the extinction coefficient $\varepsilon = 156\ mM^{-1}cm^{-1}$ was used to calculate LPO levels, expressed in μmol of MDA formed per g of FW.

Levels of PC were estimated using the method described by Mesquita et al. (2014) with Udenigwe et al. (2016) adaptations. Absorbance was measured at 450 nm and PC levels were estimated using the extinction coefficient $\varepsilon = 22.3\ mM^{-1}cm^{-1}$. The results were expressed in nmol per g of FW.

The AChE activity was determined following the method of Ellman et al. (1961), with Mennillo et al. (2017) modifications. AChE activity was measured at 412 nm for 5 min and calculated using the extinction coefficient $\varepsilon = 13.6\ mM^{-1}cm^{-1}$. Results were expressed in nmol per min per g of FW.

2.5. Statistical analysis

The effect of temperature and CP concentration on the measured responses was analysed using a two-way ANOVA. CP concentrations 10 and 500 ng/L were excluded from the analysis since were only performed at 17 °C. The effect of all tested CP concentrations used at 17 °C was further compared using one-way ANOVA. Data that did not meet ANOVA assumptions of normality and variance homoscedasticity were analysed by a Kruskal–Wallis test. A Tukey's post-hoc test or the non-parametric equivalent (Zar, 1996) was used to compare the different conditions. Statistical analysis was performed using the IBM SPSS Statistics software (version 26.0. Armonk, NY: IBM Corp).

3. Results

3.1. Cyclophosphamide quantification on water and tissues

Results of CP quantifications on water from blanks and tissue samples are presented in Table 1 and Table 2, respectively. The concentrations of CP in water collected on the 3rd day after spiking were slightly

Table 1
Concentrations of CP in the water (ng/L) on the 3rd day after contamination and before water renewal (day 7), corresponding to the first exposure week. The following conditions were analysed: 500 and 1000 ng/L at 17 °C and 1000 ng/L at 21 °C. Results are presented as mean ± standard deviation.

CP in the blanks (ng/L)			
Conditions		**3rd day**	**End of week**
17 °C	500 ng/L	471.60 ± 69.18	348.28 ± 80.67
	1000 ng/L	1048.40 ± 35.85	784.93 ± 281.93
21 °C	1000 ng/L	942.78 ± 70.50	688.01 ± 451.72

Table 2
Concentrations of CP in mussels' soft tissues exposed for 28 days to 10, 100, 500 and 1000 ng/L at 17 °C and 100 and 1000 ng/L at 21 °C. Results are presented as mean ± standard deviation (3 replicates per condition, with exception of condition 21 °C - CP 1000 ng/L, which only has 2 replicates due to lack of tissue).

CP concentration in mussels' soft tissues (ng/g Dry Weight)		
Conditions		
17 °C	10 ng/L	4.11 ± 5.62
	100 ng/L	26.23 ± 6.29
	500 ng/L	11.13 ± 16.56
	1000 ng/L	15.93 ± 8.19
21 °C	100 ng/L	21.00 ± 7.45
	1000 ng/L	14.75 ± 5.44

below (<6%) the nominal concentration in two conditions: 500 ng/L at 17 °C and 1000 ng/L at 21 °C. The drug losses between the 3rd day after spinking and the end of the week (before water renewal) ranged between 25% and 27% across all treatments, which shows the stability of CP in water along one week of experiment. Regarding concentrations of the drug on tissue samples, the results revealed that CP accumulated in mussel tissues. At both temperatures, the highest CP tissue concentration was found in mussels exposed to 100 ng/L. Although the statistical analysis was not performed with these data, due to the limited number of replicates, the results showed similar accumulation in tissues at the two temperatures.

3.2. Biochemical parameters

3.2.1. Metabolic capacity and protein content

Regarding the metabolic capacity (ETS), there were no significant differences among all tested treatments (Table 3, Fig. 1A).

A significant increase of the PROT content was observed in mussels exposed at 17 °C and 10 and 1000 ng/L of CP comparing to non-

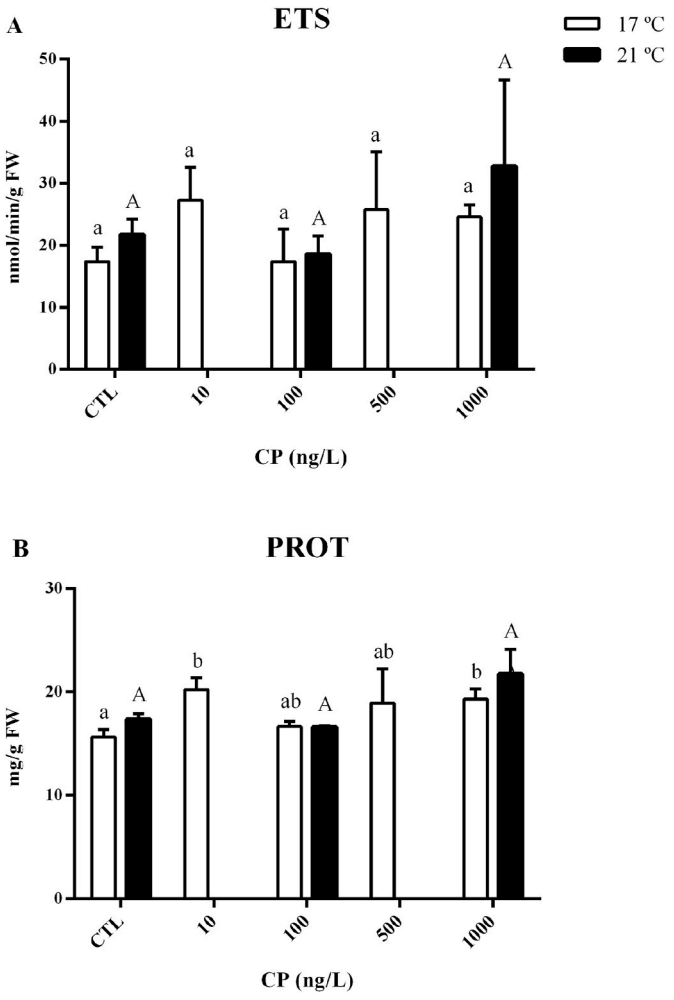

Fig. 1. A - Electron transport system activity (ETS) and B - Protein content (PROT), in *Mytilus galloprovincialis* exposed to cyclophosphamide (CP) concentrations (10, 100, 500 and 1000 ng/L) and two temperatures (17 and 21 °C). Results are expressed as means + standard deviation. Significant differences between mussels exposed to the same concentration and different temperatures are represented in the figures with an asterisk (*). Different letters indicate significant differences among concentrations for each temperature (lower case letters for 17 °C, upper case letters for 21 °C). Different letters and * are based on ANOVA followed by post-hoc Tukey's multiple comparison tests ($p < 0.05$).

Table 3
Two-way ANOVA results testing the effects of CP and temperature on biochemical parameters (Electron transport system activity - ETS; Protein content - PROT; Glutathione peroxidase - GPx; Glutathione S-transferases - GSTs; Carboxylesterases - CbEs; ratio between reduced (GSH) and oxidized (GSSG) - GSH/GSSG; Lipid peroxidation levels - LPO; Protein carbonylation levels - PC; Acetylcholinesterase activity - AChE) of *Mytilus galloprovincialis* mussels exposed for 28 days to 0 (CTL, control), 100 and 1000 ng/L and across the temperatures of 17 °C and 21 °C. Within 17 °C, all CP treatments (0 ng/L (CTL, control), 10, 100, 500 and 1000 ng/L) were also compared by one-way ANOVA. The *p-value* of the interaction between factors is presented and significant differences are identified by asterisks ($p < 0.05$*). Catalase (CAT) and glutathione reductase (GR) activities did not meet ANOVA assumptions, thus were analysed by non-parametric tests.

Conditions	**CP exposure**			**Temperature**			**Interaction**			**One-way ANOVA**		
	df	F	*p-value*	df	F	*p-value*	df	F	*p-value*	df	F	*p-value*
Biochemical parameters												
ETS	2, 12	4.81	0.029*	1, 12	2.28	0.16	2, 12	0.44	0.65	4, 10	2.21	0.14
PROT	2, 12	9.33	0.004*	1, 12	2.51	0.14	2, 12	0.73	0.50	4, 10	3.97	0.035*
GPx	2, 12	52.51	<0.001*	1, 12	29.00	<0.001*	2, 12	15.44	<0.001*	4, 10	17.5	<0.001*
GSTs	2, 12	2.85	0.097	1, 12	0.004	0.95	2, 12	5.63	0.019*	4, 10	8.65	0.003*
CbEs_pNPA	2, 11	7.46	0.009*	1, 11	0.008	0.93	2, 11	0.19	0.83	4, 10	3.81	0.039*
CbEs_pNPB	2, 11	20.93	<0.001*	1, 11	0.36	0.56	2, 11	0.49	0.62	4, 10	5.65	0.012*
GSH/GSSG	2, 12	24.11	<0.001*	1, 12	0.03	0.86	2, 12	8.45	0.005*	4, 10	5.14	0.016*
LPO	2, 12	2.018	0.18	1, 12	0.05	0.83	2, 12	0.98	0.40	4, 10	0.55	0.705
PC	2, 11	7.49	0.009*	1, 11	0.09	0.77	2, 11	0.64	0.548	4, 10	2.20	0.14
AChE	2, 12	54.84	<0.001*	1, 12	13.41	0.003*	2, 12	8.28	0.006*	4, 10	7.54	0.005*

contaminated mussels. At 21 °C, no significant differences were observed among concentrations. No significant differences were observed between temperatures, regardless of the concentration tested (Table 3, Fig. 1B).

3.2.2. Oxidative stress

At 17 °C, CAT and GR activities significantly increased in mussels exposed to 500 and 1000 ng/L comparing to non-contaminated ones, whereas at 21 °C only at 1000 ng/L a significant increase was verified

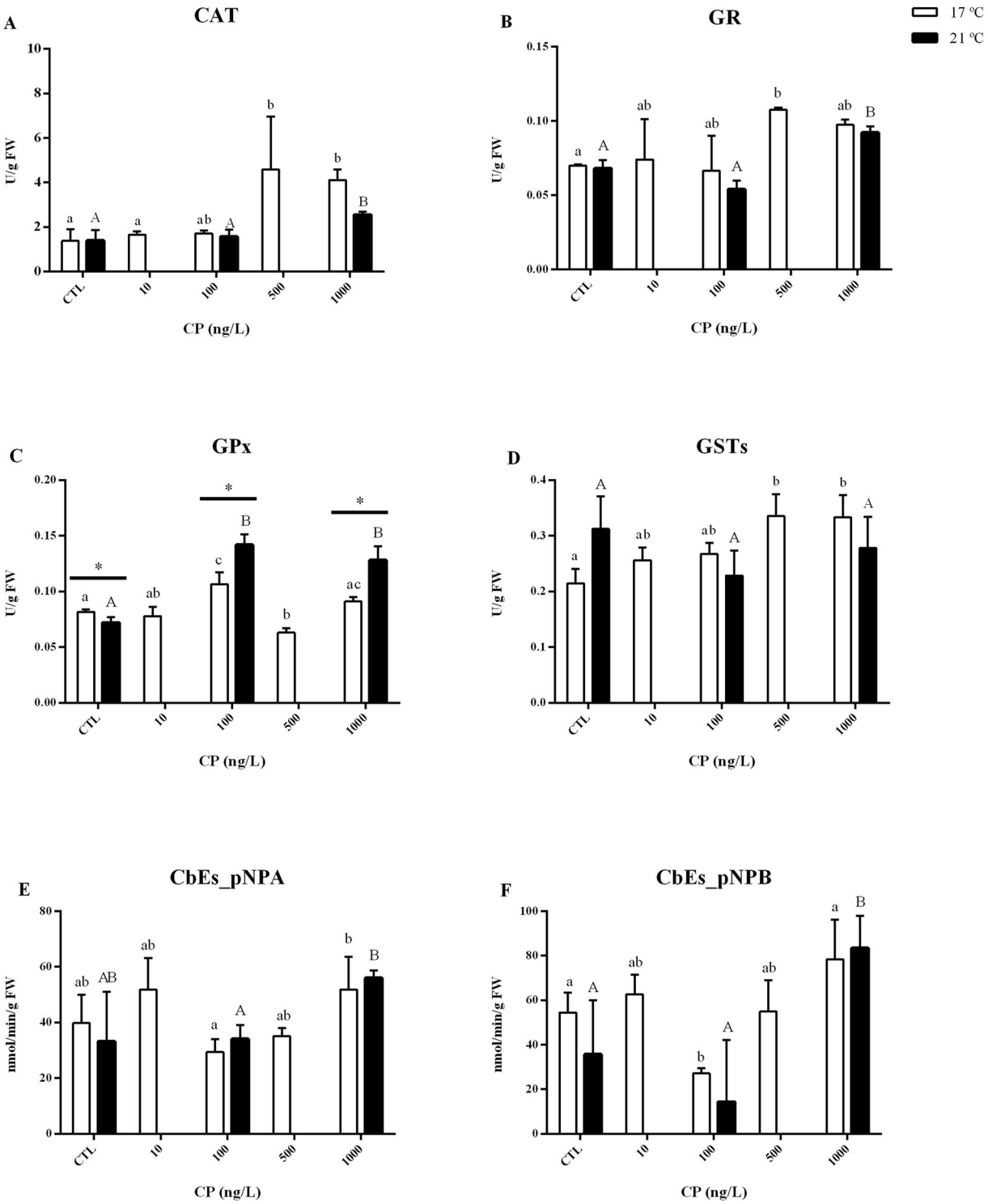

Fig. 2. A - Catalase activity (CAT), B - Glutathione reductase activity (GR), C - Glutathione peroxidase activity (GPx), D - Glutathione S-transferases activity (GSTs), E - Carboxylesterases activity with substrate p-nitrophenyl acetate (CbEs_pNPA), F - Carboxylesterases activity with substrate p-nitrophenyl butyrate (CbEs_pNPB), in *Mytilus galloprovincialis* exposed to cyclophosphamide (CP) concentrations (10, 100, 500 and 1000 ng/L) and two temperatures (17 and 21 °C). Results are expressed as means + standard deviation and as medians + interquartile range, in the case of non-parametric data. Significant differences between mussels exposed to the same concentration and different temperatures are represented in the figures with an asterisk (*). Different letters indicate significant differences among concentrations for each temperature (lower case letters for 17 °C, upper case letters for 21 °C). Different letters and * are based on parametric and non parametric ANOVA followed by post-hoc Tukey's or the non parametric equivalent multiple comparison tests ($p < 0.05$).

(Fig. 2A and B).

The activity of GPx at 17 °C significantly increased at 100 ng/L and decreased at 500 ng/L, whereas at 21 °C GPx activity increased significantly at 100 ng/L and 1000 ng/L in comparison to CTL mussels (Fig. 2C). A significant effect of the temperature *per se* and its interaction with CP was observed for GPx (Table 3). Higher temperature significantly increased GPx activity in contaminated mussels, although a contrary effect was observed in control organisms (Fig. 2C, Table 3).

At 17 °C the activity of GSTs increased significantly at 500 and 1000 ng/L in comparison to control mussels, while at 21 °C no significant differences were observed among concentrations (Fig. 2D). These differences among temperatures accounted for the significant interaction term (Table 3).

Regarding CbEs activity, both enzymes followed similar patterns. At the two temperatures tested (17 and 21 °C), there was a significant increase of activity between mussels exposed to CP 100 ng/L and CP 1000 ng/L (Fig. 2E and F). Furthermore, with the pNPB substrate, a significant activity decrease was observed between non-contaminated mussels and mussels from 100 ng/L CP treatment at 17 °C.

The ratio GSH/GSSG decreased at 500 ng/L and 1000 ng/L under 17 °C and only at 1000 ng/L in mussels maintained at 21 °C. Differences between temperatures were observed at 100 and 1000 ng/L, with significantly higher values at 21 °C for 100 ng/L exposed organisms and an opposite pattern at 1000 ng/L, resulting in a significant interaction between temperature and exposure concentrations (Fig. 3A, Table 3).

No significant differences were observed in terms of LPO levels among all treatments. A similar response was observed for PC levels, but in this case, at 21 °C, significantly higher values were observed at 1000 ng/L exposed organisms. For both LPO and PC levels no significant differences were observed between temperatures (Fig. 3B and C, Table 3).

3.2.3. Neurotoxicity

At the two temperatures tested, mussels exposed to the highest CP concentration (1000 ng/L) showed a significant increase of AChE activity, with significantly higher values at 21 °C exposed mussels (significant main and interaction terms in Table 3 and Fig. 4).

4. Discussion

In the present study, the effects induced in the mussel species *M. galloprovincialis* were evaluated after chronic exposure to CP, under an actual and predicted scenario of increasing water temperature (17 and 21 °C) to understand if temperature rise influences the impacts induced by CP. This topic is of utmost importance since the information available regarding CP effects in non-target organisms, such as estuarine bivalve species, is still scarce and, as far as we know, there is no data on the possible effects of this drug under climate change scenarios.

Regarding CP quantification on water samples, the results here presented suggest a low degradation rate of this drug along one-week exposure period (between water renewals). Negreira et al. (2013b) also reported CP stable behavior over time, under several conditions, such as different temperatures and solvents. In terms of CP quantification in mussel tissues, this drug was bioaccumulated by *M. galloprovincialis*, even when exposed to the lowest exposure concentration. Although there is a lack of research regarding bioaccumulation levels of antineoplastic drugs in bivalve species (this is the first study approaching this topic with these species, as far as we know), other studies evaluated the bioaccumulation potential of bivalves using other pharmaceutical classes (Almeida et al., 2015, 2020a,b; Burket et al., 2019; Moreno-González et al., 2016; Mezzelani et al., 2018a; Ricciardi et al., 2016; Serra-Compte et al., 2018). Nonetheless, the present results should be analysed with precaution due to the limited amount of samples used for CP quantification.

Previous studies already discussed the relationship between metabolic capacity and protein content in vertebrate and invertebrate aquatic

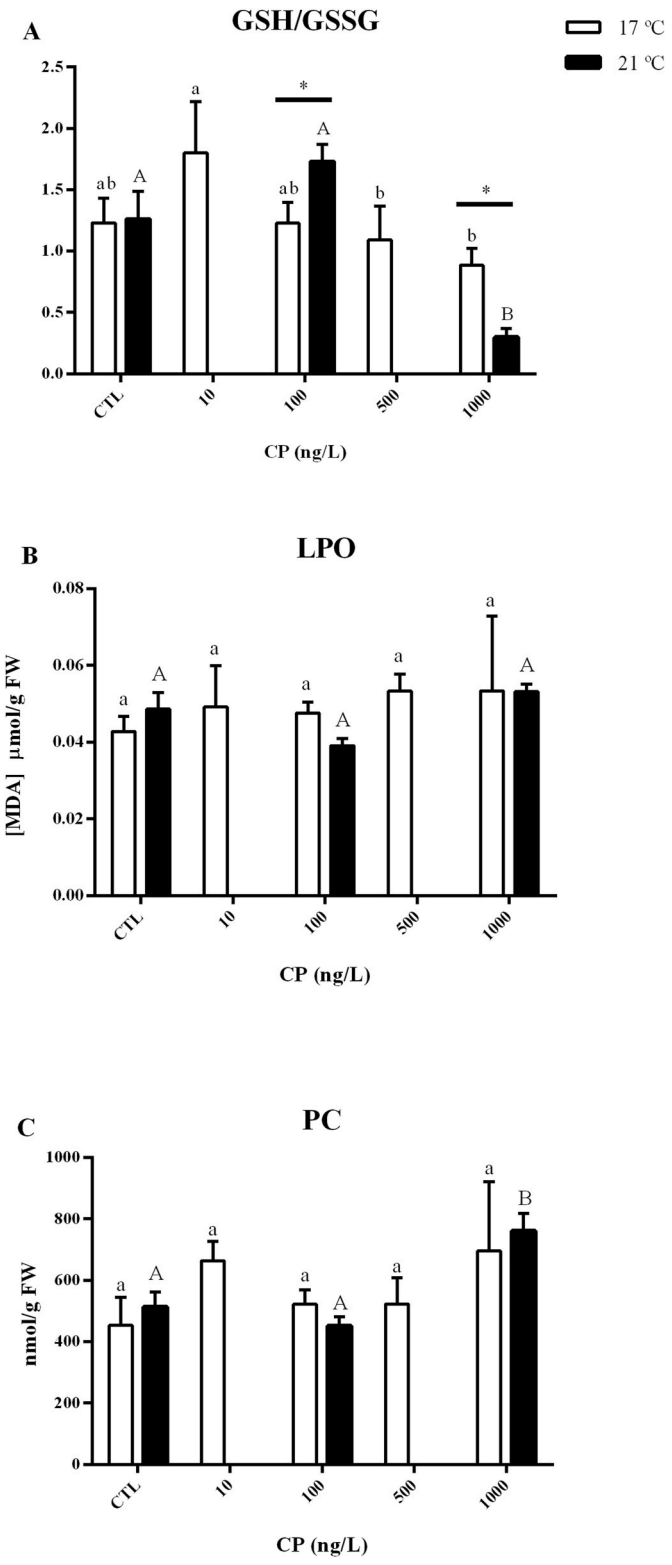

Fig. 3. A - Reduced and oxidized glutathione ratio (GSH/GSSG), B - Lipid peroxidation (LPO), C - Protein carbonylation (PC), in *Mytilus galloprovincialis* exposed to cyclophosphamide (CP) concentrations (10, 100, 500 and 1000 ng/L) and two temperatures (17 and 21 °C). Results are expressed as means + standard deviation. Significant differences between mussels exposed to the same concentration and different temperatures are represented in the figures with an asterisk (*). Different letters indicate significant differences among concentrations for each temperature (lower case letters for 17 °C, upper case letters for 21 °C). Different letters and * are based on ANOVA followed by post-hoc Tukey's multiple comparison tests ($p < 0.05$).

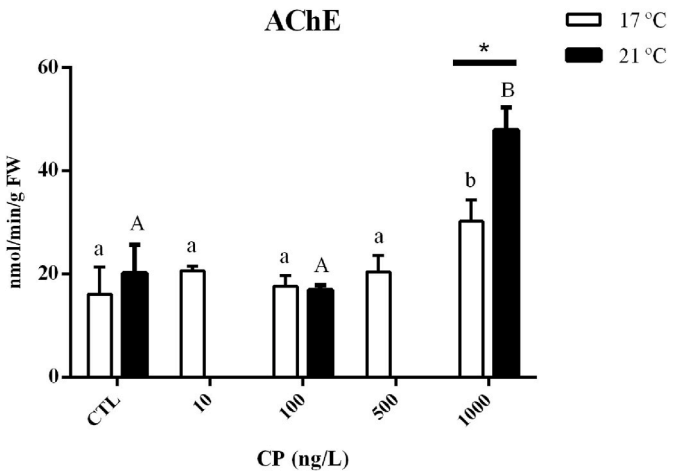

Fig. 4. Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* exposed to cyclophosphamide (CP) concentrations (10, 100, 500 and 1000 ng/L) and two temperatures (17 and 21 °C). Results are expressed as means + standard deviation. Significant differences between mussels exposed to the same concentration and different temperatures are represented in the figures with an asterisk (*). Different letters indicate significant differences among concentrations for each temperature (lower case letters for 17 °C, upper case letters for 21 °C). Different letters and * are based on ANOVA followed by post-hoc Tukey's multiple comparison tests ($p < 0.05$).

species exposed to environmental stresses (De Coen and Janssen, 1997; Smolders et al., 2003; Teixeira et al., 2017). The metabolic capacity of organisms can be assessed through the ETS activity, which is related to the energy consumption occurred in mitochondria (De Coen and Janssen, 1997). The present findings indicate that exposed organisms were able to maintain their metabolism similar to control levels regardless of the exposure concentration, at both of the tested temperatures. The maintenance of the metabolic capacity between the contaminated and non-contaminated mussels may indicate that the CP concentrations tested might not be stressful enough to generate any change in the organism's metabolic capacity. Furthermore, although mussels at 21 °C tended to present higher ETS activity, the absence of significant differences between the two temperatures can indicate the capacity of mussels to maintain their metabolic capacity even under temperature rise. Similar results were already observed by other authors when exposing bivalve species, including *M. galloprovincialis*, to different concentrations of different contaminants and to different temperatures (Costa et al., 2020b, 2020a; Leite et al., 2020). Accompanying the lack of metabolic activation, an increment of energy reserves was noticeable at 17 °C in mussels exposed to the lowest and the highest concentration of CP (10 and 1000 ng/L), with organisms showing increased PROT content, which may indicate that organisms were able to enhance the production of proteins under the exposure conditions and this behavior could be associated with the production of enzymes (such as antioxidant enzymes). At the intermediate concentrations (100 and 500 ng/L) mussels were not able to respond to the stress caused by these concentrations and, as a consequence, PROT content (and ultimately enzymes production) tended to be closer to control levels. At the highest exposure temperature (21 °C), although it is possible to observe a tendency to an increase in PROT content along with increasing concentrations, the present results suggest that the organisms were not able to increase the production of proteins, maintaining PROT content similar to those control levels.

Besides alteration on bivalve's metabolic capacity and energy reserves content, an increase in reactive oxygen species (ROS) production can occur as a consequence of exposure to stressful conditions, such as the presence of pollutants. The increase of ROS production can lead to organisms' responses in an attempt to avoid oxidative stress and cellular damages (e.g.: lipid peroxidation), including the activation of their antioxidant defenses, such as the antioxidant enzymes catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) (Regoli and Giuliani, 2014). These responses were already observed in bivalves, such as mussels (Faria et al., 2018; Gonzalez-Rey and Bebianno, 2014; Grbin et al., 2019; Morosetti et al., 2020; Regoli and Principato, 1995). CP is metabolized by cytochrome P450, producing acrolein as one of the metabolic products and main responsible for ROS increase in cells (e.g.: human and mouse) exposed to this drug (Jeelani et al., 2017; Korkmaz et al., 2007; Tripathi and Jena, 2009). Taking this into account, and since mussels also present cytochrome P450, CP may have the potential to cause a deleterious effect on mussels' oxidative status. For this reason, antioxidant defenses and cellular damage levels were evaluated in mussels exposed to this drug. The results obtained showed that CAT activity increased at the two highest concentrations at 17 °C and at the highest concentration at 21 °C. The activation of this antioxidant enzyme suggests an oxidative impairment under these conditions, a common behavior under certain oxidative stress levels (Costa et al., 2020b; Cruz et al., 2016; Regoli et al., 2011). A similar response was observed by Fonseca et al. (2018) when the polychaete *Hediste diversicolor* was exposed to 500 ng/L of CP, while a decrease of CAT activity to control levels was observed at 1000 ng/L. Other antineoplastic drugs, namely cisplatin (CDDP), have also caused, in *M. galloprovincialis*, an enhancement of CAT activity along with increasing concentrations (Trombini et al., 2016). Moreover, from the obtained results, temperature rise seemed not to influence the activity of CAT since similar enzyme activity was obtained at the two temperatures, which may be related to limited metabolic activation mentioned before, as a response to the increasing temperatures.

Along with CAT, the enzyme GPx can reduce hydrogen peroxide (H_2O_2) into H_20 as well as lipid hydroperoxides, through the oxidation of GSH to GSSG, and thus, contributing to ROS reduction in the cells. The activity of GPx at 17 °C increased at 100 ng/L followed by a decrease at 500 ng/L and, at the highest concentration (1000 ng/L), an increase to levels similar to the control ones. As in CAT, GPx activity is commonly higher at lower stress conditions, as a response to the increasing of oxidative stress (Regoli and Giuliani, 2014). However, the continuous increase of stress can lead to an inhibition of GPx activity (Regoli and Giuliani, 2014). This pattern was observed for the intermediate concentrations (100 and 500 ng/L). At the highest concentration, the organisms were able to maintain this antioxidant enzyme activity at control levels to cope and try to overcome the stress caused. These results are also in accordance with the results presented by Fernandes et al. (2020), where exposure to CP (1000 ng/L), lead to an increase of GPx activity in digestive glands of *M. galloprovincialis* at the 3rd and 7th day of exposure (with a total of 14 days of exposure). Similar results were also observed by Fonseca et al. (2017) when exposing polychaetes to CDDP, which resulted in higher total and selenium-dependent GPx values at higher concentrations (100 ng/L). Moreover, in the present study, GPx was significantly affected by the increasing temperature, showing higher activity levels at the highest temperature for both concentrations tested. The present findings suggest that, especially at 21 °C, GPx was activated to prevent cellular damage. The temperature was a significant factor in the induction of GPx activity, which was already described by other authors for different bivalve species (Andrade et al., 2019; Hu et al., 2015; Morosetti et al., 2020; Verlecar et al., 2007).

Glutathione reductase (GR) is an enzyme responsible for converting GSSG into GSH and thus fundamental to maintain an appropriate GSH/GSSG ratio, maintaining the redox balance. In the present study, GR activity at 17 °C increased at 500 ng/L and tended to control levels in the remaining treatments, a response that can be related to a tentative to compensate the overproduction of GSSG that failed at the highest concentration. In fact, at this concentration, GPx activity (GPx is responsible for converting GSH into GSSG) decreased, diminishing the oxidation of GSH into GSSG, which is confirmed by the GSH/GSSG ratio values similar to control levels. Furthermore, similar GSH/GSSG values obtained at CTL, 100, 500 and 100 ng/L exposed mussels at 17 °C

evidenced the role of GR and GPx on the maintenance of the GSH and GSSG balance. However, under warmer conditions, the GSH/GSSG ratio was significantly reduced at the highest concentration, which may result from higher GPx activity at this condition, leading to higher GSSG content and, thus, decreasing the ratio GSH/GSSG. To the best of our knowledge, the present study is the first to evaluate the effects of CP using GR activity and GSH/GSSG ratio as biomarkers in bivalve species. However, Aguirre-Martínez et al. (2015) also measured the GR activity after exposing clams to tamoxifen (antineoplastic drug) and, as in the present findings, also verified an increased activity within increasing concentrations.

Organisms exposed to xenobiotics can also activate biotransformation enzymes, such as glutathione S-transferases (GSTs), which are involved in cell detoxification processes by catalyzing conjugation reaction of lipid hydroperoxides with GSH producing products that can be excreted from the cell (Regoli and Giuliani, 2014). The GSTs have been reported to have an important role in CP detoxification from cells (e.g.: human and mice cells) (Conklin et al., 2015; Dirven et al., 1994; Pinto et al., 2009). The results obtained indicated that under control temperature GSTs were activated and involved in the detoxification of CP at higher concentrations. Fernandes et al. (2020) also verified increased levels of GSTs in mussel's digestive glands exposed to the same drug, reinforcing the role of this enzyme in the detoxification of CP in this mussel species. Aguirre-Martínez et al. (2015) also found increased levels of GSTs activity when exposing the clam *R. philippinarum* to tamoxifen. These results are also in line with other studies where GSTs proved to have a role in pharmaceuticals detoxification (Aguirre--Martínez et al., 2015; Cruz et al., 2016). Nevertheless, opposite results were found by other authors when exposing polychaetes to CP and CDDP (as well as mixtures of antineoplastic drugs), where GSTs activity was generally inhibited along with increasing concentrations (Fonseca et al., 2019, 2018, 2017). However, our results showed that GSTs were not activated at the highest temperature, which is also referred by other authors although with different contaminants, including pharmaceuticals (Almeida et al., 2021; Leite et al., 2020; Morosetti et al., 2020). Carboxylesterases (CbEs) are also important biotransformation enzymes, involved in the phase I enzyme system, contributing to the contaminants excretion (Ribalta et al., 2015). The present findings indicate a tendency to biotransformation enzyme activation at higher concentrations, regardless of the temperature.

The present study further demonstrated that non-contaminated mussels and contaminated mussel maintain their LPO levels, regardless of the concentration tested and the temperature. These findings reinforce that the mechanisms of defense activated at higher concentrations were enough to eliminate ROS and prevent cellular damage. Similarly, Fernandes et al. (2020), after the exposure of the same mussel species to CP (1000 ng/L), observed depletion of LPO values on the digestive glands after the 7th day of exposure, and, although this decrease was followed by an increase, the authors argued that by the 7th day the antioxidant defense system as well as the biotransformation enzymes, were able to counteract the ROS increase. Trombini et al. (2016) exposed *H. diversicolor* to CDDP (14 days) and verified an increase of LPO levels during the first week and then a decrease in gills and digestive gland, once again showing the efficiency of the antioxidant defense system. Nevertheless, the present study further revealed protein carbonylation (PC, a result of proteins oxidation by ROS) at the highest concentration tested (1000 ng/L) at 17 °C, which indicates certain oxidative damage at higher concentrations.

Finally, AChE is a key enzyme directly involved in the functioning of the neuromuscular system of several organisms (Aguirre-Martínez et al., 2016). This enzyme, which belongs to a major class of enzymes denominated as cholinesterases (ChEs), mode of action consists in the hydrolysis of the neurotransmitter acetylcholine into choline and acetate (Lionetto et al., 2013). This reaction occurs in the postsynaptic membrane and with it, the synaptic transmission ends. Inhibition of AChE activity has been widely used to determine neurotoxic effects on aquatic species exposed to pollutants, in particular, organophosphorus and carbamate pesticides but also other classes of pollutants (Coppola et al., 2020; De la Torre et al., 2002; Frasco et al., 2005; Lionetto et al., 2013). Contrarily, in the present study, an increase in AChE activity occurred in organisms exposed to the highest CP concentration at both temperatures. In fact, AChE increase can be related to cell apoptosis and consequent release of the enzyme as reported by Zhang et al. (2002). Furthermore, the temperature has been reported to be a factor in the increase of AChE activity (Costa et al., 2020b, 2020a; Pfeifer et al., 2005), which is in accordance with the present findings since the effect of the highest temperature, combined with the CP concentration, caused an enhancement of AChE activity comparing to CP acting alone (at 17 °C, control temperature). Fernandes et al. (2020) exposed the mussel *M. galloprovincialis* to CP (1000 ng/L) and verified that gills from exposed mussels presented higher levels of AChE activity on the 3rd day, comparing to the respective controls. This increase was accompanied by a decrease in the following days, being the activity similar to control levels. The author interpreted this pattern as maintenance of the AChE activity registered at the beginning of the experiment that then succeeded to lower activity levels. Other antineoplastic drugs, namely Cisplatin (CDDP), have also caused similar results to the present findings, namely increasing AChE activity along with concentrations, on *M. galloprovincialis* and *H. diversicolor* (14 days) (Fonseca et al., 2017; Trombini et al., 2016). On the contrary, in a study conducted by Fonseca et al. (2018), the species *H. diversicolor* was exposed for 14 days to the same concentrations of CP used in the present study, and no alterations were observed in the AChE activity, which may be due to a failure of docking between AChE and the drug, as argued by the authors.

5. Conclusion

Overall, in the present study, at lower CP concentrations (10 and 100 ng/L), mussels' antioxidant defenses were unchanged, regardless of the temperature tested. In fact, these defenses were generally activated only at higher concentrations (500 and 1000 ng/L). Furthermore, the decrease of GSH/GSSG ratio only at the highest concentration also indicates the redox balance loss at the referred concentrations. Regarding cellular damage, the present findings suggest that the antioxidant defense system was able to prevent lipid peroxidation (LPO) caused by CP exposure, regardless of the temperature. Moreover, although the interaction between concentration and temperature was verified in some biomarkers, in general, the results here presented showed that temperature did not enhance a significant response by mussels, maybe due to a decrease in organism's metabolism or due to an adaptative capacity exhibit by this species. However, future studies are of utmost importance to better understand the possible effects of antineoplastic chronic drug exposure on *M. galloprovincialis* as well as other bioindicator species, especially under climate change scenarios.

Author contribution

Vanessa Queirós; Biochemical analyses and data analyses, paper writing; Ulisses M. Azeiteiro: funding, Supervision, paper editing and revision; Carlos Barata: funding, Supervision, paper editing and revision; Juan Luis Santos: Chemical analyses and data analyses; Esteban Alonso: Chemical analyses and data analyses; Amadeu MVM. Soares: funding, Supervision, paper editing and revision; Rosa Freitas: funding, Supervision, paper editing and revision.

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.2021.117735.

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