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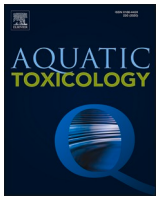

Metabolic and oxidative status alterations induced in *Ruditapes philippinarum* exposed chronically to estrogen 17α-ethinylestradiol under a warming scenario

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ABSTRACT

The presence of pharmaceuticals in the aquatic environment is an ongoing concern. However, the information regarding their effects under different climate change scenarios is still scarce. 17α-ethinylestradiol (EE2) is widely present in different aquatic systems showing negative impacts on aquatic organisms even when present at trace concentrations (≈1 ng/L). Nevertheless, its impact on bivalves is poorly understood, especially considering the influence of climate change factors. This study aimed to assess the toxicological impacts of EE2 under current and predicted warming scenarios, in the edible clam *Ruditapes philippinarum*. For this, clams were exposed for 28 days to different EE2 concentrations (5, 25, 125, 625 ng/L), under two temperatures (17 °C (control) and 21 °C). Drug concentrations, bioconcentration factors and biochemical parameters, related to oxidative stress and energy metabolism, were evaluated. Results showed that under actual and predicted temperature scenarios EE2 concentrations led to a disturbance in redox homeostasis of the clams, characterized by an increase in oxidized glutathione in contaminated organisms compared to control ones. Nevertheless, clams were capable to cope with the stressful conditions, activating their defence mechanisms (especially at the highest exposure concentration and in particular at increased temperature), and no oxidative damage occured. Although limited effects were observed, the present findings indicate that under both temperatures contaminated clams altered their biochemical performance, which can impair their sensitivity and protection capacity to respond to other environmental changes and/or affect their capacity to grow and reproduce. The results presented here highlight the need for further research on this thematic, considering that climate change is an ongoing problem, and the levels of some pharmaceutical drugs will continue to increase in marine/estuarine environments.

1. Introduction

The presence of pharmaceutical drugs in aquatic systems and their potential persistence in these environments has become an emerging concern (Gaw et al., 2014). Although the reported concentrations are usually low, ranging from few ng/L up to μg/L (Álvarez-Muñoz et al., 2015; Radović et al., 2015; Rocha et al., 2016), the bioactivity and the capacity of these compounds to be effective at low concentrations might exert effects on non-target organisms once in the aquatic environment (Li, 2014). Additionally, the presence of mixtures of pharmaceuticals in those environments may be of special concern due to additive or synergistic effects that may possess on aquatic fauna (Almeida et al., 2018a, 2018b). Therefore, an increasing number of studies has addressed the potential impact of pharmaceutical pollution on aquatic biota from different aquatic systems (for review: Almeida et al., 2020; Ebele et al., 2017; Gaw et al., 2014; Vieira et al., 2021), including studies addressing the impact of these pollutants in marine organisms (for a review: Gaw et al., 2014). Several studies demonstrated that bivalves are excellent model organisms to assess the toxicity of several environmental stressors, including pharmaceuticals (Almeida et al., 2017; Andrew

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et al., 2008; MEneff et al., 2014). Their ecologic and socio-economic value combined with their sedentary lifestyle, well-characterized life--cycles, wide distribution, filter feeding capacity, sedentary behaviour and bioaccumulation potential make bivalves good bioindicators in ecotoxicology and monitoring programs. The uptake of pharmaceutical compounds in wild bivalves has been shown (McEneff et al., 2014; Rocha et al., 2016), and several reports have evaluated the biological effects of such contaminants on these organisms under laboratory-controlled conditions (Almeida et al., 2017; Andrew et al., 2008; Brew et al., 2020; Freitas et al., 2019).

Among pharmaceuticals, hormones are a relevant class due to their abundant use in human and veterinary medicine but also due to their impacts on non-target organisms' health (Li, 2014). These compounds can be classified as Endocrine Disrupting Chemicals (EDCs) due to their capability to alter the functioning of the endocrine system by mimicking endogenous hormones, leading to adverse health effects in non-target organisms (Oliveira et al., 2016). 17α-ethinylestradiol, also known as EE2, is a synthetic hormone produced from estradiol (E2), a natural hormone. It is the most used steroid estrogen in combined oral contraceptives showing a high estrogenic potency (Adeel et al., 2017). Besides its vast use in birth control pills, EE2 is also used in hormone replacement treatments and as a therapy for prostate cancer in men and breast cancer in postmenopausal women (Aris et al., 2014). A vast number of reports showed the presence of EE2 in the aquatic systems worldwide with concentrations in the ng/L range probably as a consequence of its wide spread use (Pojana et al., 2004; Ribeiro et al., 2009; Rocha et al., 2016; Zuo et al., 2006). EE2 concentrations of 1–3 ng/L were found in the Sado Estuary (Portugal) by Rocha et al. (2013). Similar concentrations of EE2 were also found in Mondego River (Portugal) by the same authors (Rocha et al., 2014), but also in coastal surface waters of the Baltic Sea as described by Beck et al. (2005). Higher concentrations of EE2 (20 – 100 ng/L) were found in different aquatic systems worldwide. Nevertheless, these high concentrations are commonly associated with very polluted aquatic environments and are not frequently found, thus representing extreme values (see for review: Almeida et al., 2020).

Besides its frequent occurrence in aquatic environments, the high chemical stability (high half-life time), low predicted non-effect concentrations reported for aquatic wildlife (0.1–0.5 ng/L) and its tendency to accumulate in aquatic biota (relatively high lipophilicity), make the presence of this pharmaceutical in aquatic systems, even at trace concentrations, a great concern (Adeel et al., 2017; Andrew et al., 2010; Aris et al., 2014). These facts lead to the inclusion of EE2 on the Watch List of contaminants of emerging concern (CECs) in surface waters in the European Union (Directive, 2013, 2008, 2000). Although the number of publications concerning the biological effects of EE2 on marine organisms has recently increased, most of the studies focus on vertebrate species (Meina et al., 2013; Robinson et al., 2007). In particular, information regarding the effect of this synthetic hormone on marine invertebrates, such as bivalves, is still limited (for review: Almeida et al., 2020). The reported effects on bivalves focus on the reproductive behavior and function (Andrew et al., 2010; Brew et al., 2020; Capolupo et al., 2018; Smolarz et al., 2017), with scarce information regarding other physiological and biochemical effects that may be induced by the presence of this drug. However, it has been demonstrated that a large number of endocrine disruptors, such as estrogens, are reactive chemicals and capable of acting at different sites within an organism, triggering multiple mechanisms of toxicity leading to responses not only linked to endocrine effects (Canesi et al., 2008; Souza et al., 2013). An overproduction of reactive oxygen species (ROS) is one general type of toxicity commonly observed in organism subjected to general stressors, typically associated with increase of basal metabolic rates. The scavenging of ROS overproduction is assured by the antioxidant system (enzymatic and non-enzymatic). A lack of response by this system leads to a redox imbalance towards oxidative pathways. This imbalance can result in oxidative damage in biomolecules such as DNA, proteins and lipids (Regoli and Giuliani, 2014).

Low-lying coastal areas (e.g., estuaries) are among the most vulnerable marine ecosystems as they are, not only subject to pollution that may arise from industrialization and population increase, but also highly impacted by climate change related factors (IPCC, 2019, 2014). Consequently, organisms from those areas will be exposed to a combination of multiple stressors, resulting in a wide range of environmental and biological impacts. Alongside with the impacts caused by pollutants, organisms in coastal ecosystems are also subject to environmental changes, as those foreseen under climate change scenarios. These changes are likely to affect the sensitivity of organisms to pollutants as well as the behaviour of pollutants and, consequently, its toxicity (Almeida et al., 2018b; Costa et al., 2020; Freitas et al., 2015; Serra--Compte et al., 2018). Different studies have shown that climate change associated factors, namely warming, are responsible for physiological and biochemical impairments in marine species (Matozzo et al., 2013; Velez et al., 2017). Bivalves are susceptible to increase in temperature, with several studies reporting modifications on metabolic rate, mitochondrial function, oxidative status and gene expression in bivalves exposed to thermal stress (Cherkasov et al., 2006; Coppola et al., 2018; Velez et al., 2017). Furthermore, studies have demonstrated that these environmental changes may modulate responses of bivalves to contaminants, in a chemical- and species-dependent manner (Costa et al., 2020; Pirone et al., 2019; Serra-Compte et al., 2018). Nevertheless, there is still a need for additional information to fully understand the toxic impacts that drugs may pose to this group of organisms, especially considering predicted climate change scenarios.

Based on the lack of information regarding the toxicological impacts of EE2 on marine bivalves, in the present study the Manila clam, *Ruditapes philippinarum,* was selected as a bioindicator species. Clams were exposed for 28 days to increasing EE2 concentrations, under actual seawater temperature (17 °C) and a predicted warming scenario (+4 °C), representing the worst-case projected scenario according to the Intergovernmental Panel on Climate Change (IPCC, 2019). Different biochemical markers were measured, to assess oxidative stress and metabolic capacity alterations in clams, caused by the presence of EE2 under two different temperature scenarios.

2. Materials and methods

2.1. Sampling and test organism

The clam *Ruditapes philippinarum* was selected to evaluate the toxicological impacts of EE2 under actual and predicted warming scenarios. Specimens were collected at the Ria de Aveiro (Northwest Atlantic coast of Portugal) in September 2019 and transported to the laboratory with seawater from the sampling site. In order to minimize the effect of body weight on biochemical responses, 180 individuals with similar size were selected (length: 3.81 ± 0.42 cm; width: 3.06 ± 0.51 cm) for laboratory experiments.

After sampling, to allow clams to depurate and acclimate to laboratory conditions, organisms were kept for 10 days in artificial seawater (salinity 30 ± 1, Tropic Marin® SEA SALT from Tropic Marine Center), under continuous aeration, with constant temperature (17 ± 1 °C) and a photoperiod of 12:12 h (light/dark). Clams were fed with Algamac Protein Plus. A stock solution of food was prepared every week in an aqueous solution (0.66 g/L) and 150,000 cells/animal/day was added to the exposure medium every 2–3 days. Seawater was renewed every 2–3 days.

2.2. Experimental conditions

After depuration, organisms were subjected to a chronic toxicity test (28 days) where five concentrations of EE2 (purity ≥98%, MW=296.40 g/mol) (0 (control), 5, 25, 125 and 625 ng/L) at two different temperatures (17±1 °C (control) and 21±1 °C) were tested, making a total of 10 treatments. A stock solution of EE2 (1 mg/L) was prepared using water

as a solvent. The lowest concentrations of EE2 used were selected according to concentrations of EE2 found in different aquatic systems (Ribeiro et al., 2009; Rocha et al., 2016, 2014), whereas the highest concentration was selected to represent EE2 levels found at highly polluted areas but also to simulate a discharge of EE2 into surface waters (Brossa et al., 2005; Montagner and Jardim, 2011). EE2 blanks (containers with the same medium conditions but without clams) were prepared to assess possible drug concentration loss during the experiment (two containers per treatment). Taking into consideration the water temperature of the sampling area during September (16–19 °C) (IPMA, 2019), a temperature of 17 °C was chosen as control temperature. The temperature of 21 °C was chosen to simulate potential warming conditions under a worst case climate change scenario, based on the predicted increase of seawater temperature from 1.0 to 4.0 °C (IPCC, 2019). To maintain the temperature levels, containers were placed in different climatic rooms. Before starting the experimental period, temperature of 21 °C was reached gradually (+2 °C every 2–3 days) to avoid any additional stress to organisms.

The experimental setup consisted of six individuals per container and three containers per treatment (six individuals/replicate, three replicates/treatment, making a total of eighteen individuals/treatment). Containers with 3 L of artificial seawater (salinity 30 ± 1) were used for each replicate and submitted to continuous aeration and to a photoperiod of 12:12 h (light:dark).

During the exposure period, the water from each aquarium was completely renewed once a week, both in exposure aquaria and blanks, after which EE2 concentrations were re-established in order to maintain constant the concentrations during the experiment. From the exposure medium (contaminated aquaria with clams), water samples were collected from each aquarium immediately after spiking (identified as Beginning) to ensure nominal chemical concentration. In blanks (contaminated aquaria without clams), water samples were collected from each aquarium immediately after spiking and immediately before water renewal (identified as Ending) to evaluate possible EE2 losses between weekly water renewals. Water samples were stored at −20 °C for further evaluation. Due to the limited quantity of water collected from each aquarium (2 mL), water samples from the exposure medium were pooled; i.e., samples from the same sampling time and from replicates of the same treatment were combined and one value was obtained for each treatment ($n = 1$, corresponding to one sampling week). Water samples from blanks (pooled replicates) per treatment were analyzed and mean values with standard deviation values were obtained for each treatment ($n = 3$, corresponding to three sampling weeks).

During the experiment, clams were fed with Algamac Protein Plus (150,000 cells/animal/day), every 2–3 days. Mortality was checked daily. At the end of the exposure period, clams were frozen individually in liquid nitrogen and stored at −80 °C for further evaluation. Two individuals per replicate (six per treatment) where used for biochemical analysis and quantification of EE2 in soft tissues. Both analyses were performed in the same individuals.

2.3. Quantification of 17α-ethinylestradiol concentrations in water and clams' soft tissues

Concentrations of EE2 were measured in water and soft tissues by using a high-performance liquid chromatography-fluorimetric (HPLC-FL) detection method. Water samples were analyzed following the detection method described by Patrolecco et al. (2013) with slightly modifications. Water samples were filtered and extracted with solid phase extraction (Oasis HLB 6cc 150 mg solid-phase extraction cartridges, Waters), followed by HPLC analysis. Soft tissues samples were analyzed by using the method described by Bai and Acharya (2019), with slightly modifications. Tissue samples (3 g, corresponding to the pool of the 6 organisms per treatment) were mixed with Na_2EDTA solution and then added with acetonitrile and methanol. After adding anhydrous Na_2SO4 and NaCl, the mixture was centrifuged at 9240 rpm for 10 min. The supernatant obtained was evaporated with a stream of nitrogen. The residue was re-dissolved in 200 μL of mobile phase and injected into the HPLC. The chromatographic analyses were carried out using an HPLC system with a PerkinElmer Series 200 variable flow pump, coupled to a JASCO FP-1520 fluorescence detector (JASCO, Tokyo, Japan). The detection was performed using an excitation wavelength of 230 nm and an emission of 302 nm. The system was controlled by a PerkinElmer interface module (NCI 900 Network Chromatography Interface) and chromatograms were processed by a PerkinElmer TotalChrom Navigator software. An Haisil HL C18 5 μm 250 × 4.6 mm chromatographic column (Higgins Analytical, Inc.) was used. The analyses were performed at room temperature at a flow rate of 1.1 mL/min and an injection volume of 100 μL was used. The mobile phase consisted of acetonitrile/water (45/55%, v/v) acidified at pH 3.6 with glacial acetic acid. The recovery was >73 % for water samples and >64 % for soft tissues. The detection limit (LOD) calculated as a signal-to-noise ratio of 3:1, was 1 ng/L for water samples and 10.0 ng/g DW (dry weight) for soft tissues.

2.4. Biochemical parameters evaluation

Prior to the evaluation of biochemical parameters, the whole soft tissue of clams was pulverized and divided in 0.5 g fresh weight (FW) aliquots (two individuals per replicate and six individuals per treatment). For each parameter assessment, supernatants were obtained by extraction with a specific buffer for each analysis (1:2, w/v). For extraction procedure, samples were homogenized with TissueLyser II (Frequency: 20 per second (s); time: 1.30 min) and centrifuged for 20 min, at 10 000 *g* (3 000 *g* for ETS) at 4 °C. Supernatants collected after the extraction were used to determine electron transport system activity (ETS) activity, glycogen (GLY) content, total protein (PROT) content, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, glutathione-*S*-transferases (GSTs) activity, lipid peroxidation (LPO) and oxidized (GSSG) glutathione content. To determine the activity of GR, GPx, GSTs enzymes and the content of GLY and PROT, supernatants extracted with a potassium phosphate buffer (50 mM K_2HPO4, 50 mM KH_2PO4, 1 mM ethylenediamine tetraacetic acid disodium salt hydrate (EDTA), 1 % (v/v) Triton X-100, 1 mM dithiothreitol (DTT), pH 7.0) was used. For LPO assessment, supernatants were obtained using 20 % (w/v) trichloroacetic (TCA). For ETS evaluation, a homogenizing buffer (0.1 M Tris-HCl, 15 % (w/v) polyvinylpyrrolidone (PVP), 153 mM magnesium sulfate (Mg SO_4), and 0.2 % (v/v) Triton-X 100, pH 8.5) was used for the extraction. GSSG was measured in a supernatant extracted using a potassium phosphate buffer (0.1 M K_2HPO4, 0.1 M KH_2PO4, 5 mM EDTA, 0.1 % (v/v) Triton X-10, pH 7.5) containing 0.6 % (v/v) sulfosalicylic acid.

Two technical replicates of each sample were performed for all biochemical parameters. A microplate reader (Synergy™HT, Biotek Instruments, Inc.) was used to evaluate all biochemical biomarkers.

2.4.1. Energy – related parameters

ETS activity was determined based on King and Packard (1975) with modifications by De Coen and Janssen (1997). The absorbance was measured at 490 nm, for 10 min with intervals of 25 s. The amount of formazan formed in each well was calculated using the extinction coefficient (Ɛ) 15 900 M^{-1} cm^{-1} and results were expressed in nmol per min per g FW.

The GLY content was evaluated spectrophotometrically at 492 nm, using the sulfuric acid method, as described by Dubois et al. (1956), with minor adaptations (Carregosa et al., 2014). Glucose standards (0–10 mg/mL) were used for a calibration curve. The results were expressed in mg per g of FW.

The PROT content was determined by the biuret method (Robinson and Hodgen, 1940), using bovine serum albumin (BSA) as standard (0–40 mg/mL). Absorbance was measured at 540 nm. Results were presented as mg per g of FW.

2.4.2. Enzymatic antioxidant and biotransformation defenses

Glutathione reductase activity was determined spectrophotometrically, according to Carlberg and Mannervik (1985), with few modifications described by Carregosa et al. (2014). The assay was performed following the oxidation of NADPH at a wavelength of 340 nm. Enzymatic activity was expressed as U per g of FW (U = μmol NADPH oxidized/min), using the molar extinction coefficient of 6 220 M^{-1} cm^{-1}.

The activity of GPx was quantified following the methodology described by Paglia and Valentine (1967), with minor modifications described by Carregosa et al. (2014). The activity was measured indirectly, assessing NADPH oxidation by the action of GR. The absorbance was measured at 340 nm, for 5 min in 10 s intervals. GPx activity was presented as U per g of FW (U = μmol NADPH oxidized/min) using the molar extinction coefficient of 6 220 M^{-1} cm^{-1}.

The activity of GSTs was determined following the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB), as described by Habig et al. (1974), with minor alterations (Carregosa et al., 2014). Absorbance was read at 340 nm and the results expressed as U per g of FW (U = μmol CDNB conjugated/min), using the molar extinction coefficient of 9600 M^{-1} cm^{-1}.

2.4.3. Indicators of cellular damage

Levels of LPO were evaluated spectrophotometrically at 535 nm, by the method described by Ohkawa et al. (1979) and modifications proposed by Carregosa et al. (2014), through the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Results were expressed as μmol MDA equivalents per g of FW, using the molar extinction coefficient of 15 600 M^{-1} cm^{-1}.

Concentrations of GSSG concentrations determined according to Rahman et al. (2007). Absorbance was measured at 412 nm and results were calculated using a calibration curve of GSSG standards (0–90 μmol/L) and expressed as nmol per g of FW.

2.5. Data analysis

Bioconcentration factor (BCF) was estimated by dividing the concentration of EE2 detected in clams' tissues by the EE2 concentration detected after spiking in the water from exposure aquaria (contaminated aquaria with animals).

Concentrations of EE2 in water samples and clam tissues as well all biochemical parameters were subjected to hypothesis testing performed by Permutational multivariate analysis of variance with one factor design (temperature x EE2 concentration) using PERMANOVA Add-on in Primer v6 (Anderson et al., 2008). A one-way hierarchical design was followed in this analysis. When significant differences were observed in the main test, pair wise comparisons were performed. *p*-values were obtained using Monte Carlo test (Anderson and Robinson, 2003). Pair wise test with *p*-values lower than 0.05 resulted in the rejection of the null hypothesis, being considered as significantly different ($p < 0.05$). Biochemical results were also separately submitted to a PERMANOVA analysis with a two factors design: EE2 concentration as factor 1 and temperature as factor 2, to test the interaction between the two factors on each parameter.

Statistical evaluation was performed for results of EE2 quantification in water samples and in biochemical parameters. The null hypotheses tested were: a) in blanks, for a given EE2 concentration and temperature level, no significant differences exist between sampling periods (significant differences are identified with # in Table 1); b) in blanks, for a given EE2 concentration and sampling periods, no significant differences exist between temperatures (significant differences are identified with an * for sampling period "Beginning" and with ** for sampling period "Ending" in Table 1); c) for a given biomarker and for each temperature level, no significant differences exist among the EE2 concentrations (in figures, significant differences among treatments are identified with lowercase letters for temperature 17 °C and uppercase letters for temperature 21 °C); d) for a given biomarker and for each EE2 concentration, no significant differences exist between temperature levels (in figures, significant differences between temperature levels are represented with an asterisk).

3. Results

3.1. 17α-ethinylestradriol concentrations in water and clams' tissues

Results revealed that EE2 concentrations quantified in the exposure medium collected after spiking were below the nominal concentrations in all experimental treatments (Table 1). In blanks, EE2 concentrations in water collected at different sampling times (Beginning vs Ending) showed, in general, significant differences at all concentrations regardless the temperature tested, except at concentration 125 ng/L at 17 °C and 625 ng/L at 21 °C (Table 1). Nevertheless, considering concentrations measured immediately after spiking (Beginning), losses of EE2 were lower than 20 % between water renewals. These results were considered acceptable to conduct the experiment and indicates relative stability of the compound during the exposure. Between temperatures and for each sampling period, in general, no significant differences were observed for each tested concentration, except at 25 ng/L for the Beginning and at 125 ng/L for the Ending sampling periods (Table 1).

Concentrations of EE2 detected in *R. philippinarum* tissues were below the limit of detection (10 ng/g dry weight) in all treatments,

Table 1
17α-ethynilestradiol concentrations (ng/L) in water samples collected from exposed medium and blanks, and in clams' whole soft tissues (ng/g DW (dry weight)). Results obtained for water samplesc collected from blanks are means with standard errors (n=3). For blanks, # indicates significant differences ($p<0.05$) between samples collected after (Beginning) and before (Ending) water changes; * indicates significant differences ($p<0.05$) between temperatures for samples collected after water exchange (Beginning); ** indicates significant differences ($p<0.05$) between temperatures for samples collected before water exchange (Ending).

		Water Samples (ng/L)				Tissue Samples (ng/g DW)	*BCF*
Temperature (°C)	EE2 concentration (ng/L)	*Exposure medium Beginning*	*Blanks Beginning*		*Ending*		
17	CTL (0)	<LOD	<LOD		<LOD	<LOD	–
	5	3.8	4.0 ± 0.2	#	3.5 ± 0.3	<LOD	–
	25	19.3	23.1 ± 2.1	#	18.5 ± 1.4	<LOD	–
	125	96.7	97.9 ± 8.9		100.9 ± 2.0	<LOD	–
	625	488.7	505.1 ± 1.7	#	487.0 ± 11.5	15.21	0.031
21	CTL (0)	<LOD	<LOD		<LOD	<LOD	–
	5	3.8	4.0 ± 0.1	#	3.6 ± 0.4	<LOD	–
	25	21.4	17.9 ± 0.6*	#	20.1 ± 0.8	<LOD	–
	125	102.3	100.4 ± 8.8	#	89.9 ± 2.6**	<LOD	–
	625	512.9	503.2 ± 37.2		485.8 ± 25.4	12.51	0.024

(Water samples - LOD: 1 ng/L; Tissue samples – LOD: 10 ng/g dry weight. Bioconcentration factor (BCF): mean concentration of EE2 measure in clams' tissue/mean concentration of EE2 measured in the water every week after spiking.

except in clams exposed to the highest concentration of EE2 (625 ng/L), with EE2 concentrations of 15.21 and 12.51 ng/g DW in clams maintained at 17 and 21 °C, respectively. At this exposure concentration, BCF was similar for both temperatures tested (Table 1).

3.2. Biochemical parameters evaluation

3.2.1. Energy – related parameters

ETS activity at 17 °C showed no significant differences among tested concentrations. At 21 °C, although the 5 and 125 ng/L concentrations increased, none of the tested concentrations were significantly different from the control. No significant differences were observed between temperatures, regardless of the EE2 concentration tested (Fig. 1A).

At 17 °C, the GLY content showed significant differences among clams exposed to the highest (625 ng/L) and the lowest (5 ng/L) EE2 concentrations, but no differences were found relative to control. At 21 °C, a significant increase in GLY levels was detected in clams exposed to the highest concentration of EE2 (625 ng/L), when comparing to control, 5 and 125 ng/L groups. No significant differences were observed between temperatures for each of the concentrations tested (Fig. 1B).

The PROT content at 17 and 21 °C was significantly higher in clams exposed to 625 ng/L compared with clams at 5 ng/L, but no significant differences were detected among EE2 concentrations and the control group. No significant differences were observed between tested temperatures for each EE2 concentration (Fig. 1C).

3.2.2. Enzymatic antioxidant and biotransformation defenses

The activity of GPx at 17 °C was significantly lower in organisms exposed to 125 ng/L compared with the ones exposed to 25 and 625 ng/L. At 21 °C a significant increase was observed in clams exposed to 25 and 625 ng/L compared to control. Significant differences between temperatures were observed at the highest exposure concentration, with higher values at 21 °C (Fig. 2A).

At a temperature of 17 °C GR significantly increased at the lowest concentration (5 ng/L) and a significant decrease in clams exposed to 125 ng/L was observed compared to the control. GR activity at 21 °C showed a significant increase between organisms exposed to the lowest concentration (5 ng/L) and control organisms. At each tested concentration, no significant differences were observed between temperatures (Fig. 2B).

Concerning GSTs activity, at 17 °C no significant differences were observed among concentrations. However, at 21 °C a significant increase in GSTs activity was observed at the highest exposure concentration compared to the control. Significant differences between temperatures were observed at the highest exposure concentration, with higher values at 21 °C (Fig. 2C).

3.2.3. Indicators of cellular damage

At a temperature of 17 °C, clams exposed to 625 ng/L showed significantly lower LPO levels compared with the control and the remaining concentrations. LPO levels at 21 °C showed significantly lower LPO levels in clams exposed to 25 ng/L EE2 compared to the control organisms. At each tested concentration, no significant differences were observed between temperatures (Fig. 3A).

At 17 °C the GSSG content increased significantly in clams exposed to 25, 125 and 625 ng/L EE2 compared to control group. At 21 °C similar results were observed, but in this case only clams exposed to 125 ng/L EE2 showed a significant increase compared with the control group. Significant differences between temperatures were observed at the concentration 25 ng/L EE2, with higher values at 17 °C (Fig. 3B).

The statistical analyses performed with all biomarkers showed only a significant interaction between EE2 concentration and temperature for GSTs ($p<0.05$).

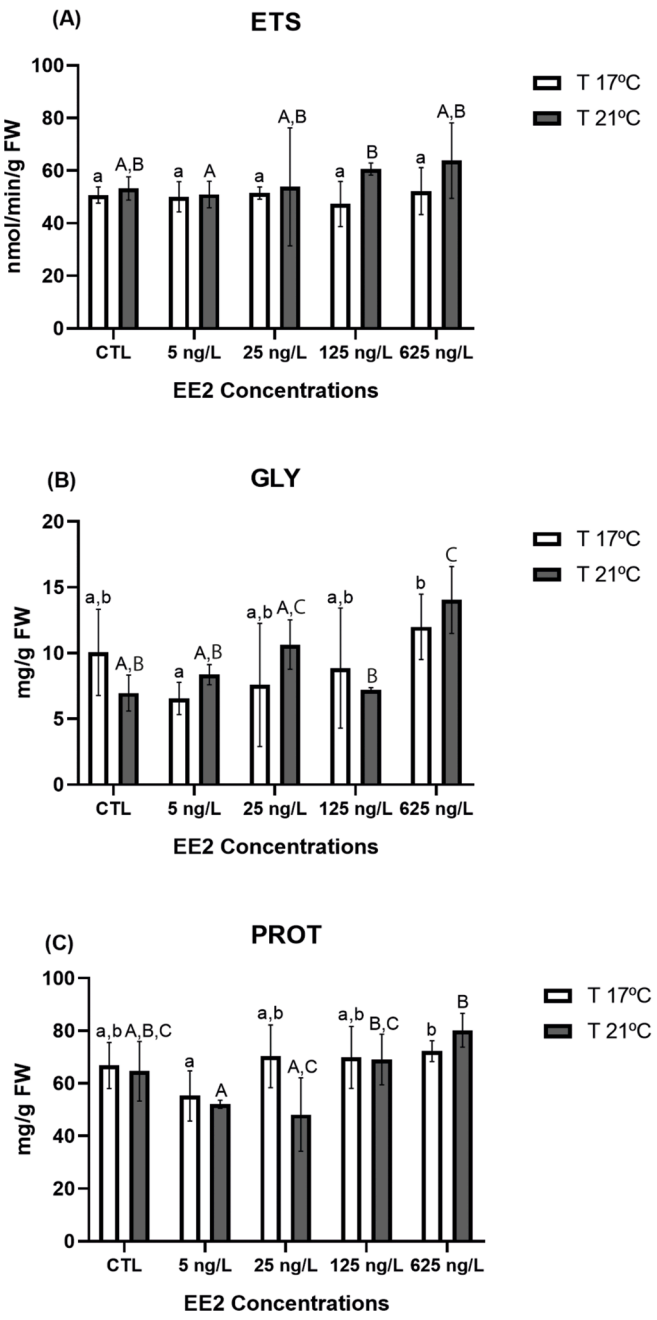

Fig. 1. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in *Ruditapes philippinarum* exposed to EE2 (17α-ethinylestradriol) (0 (control), 5, 25, 125 and 625 ng/L) at two different temperatures (17±1 °C (control) and 21±1 °C) for 28 days. Results are means with standard errors (n=6). Significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters (17 °C) or uppercase letters (21 °C). For each EE2 concentration, differences between temperatures are identified by an asterisk.

4. Discussion

In the present study, the pharmaceutical drug uptake, as well as the impacts induced in the clam *Ruditapes philippinarum* were assessed after a chronic exposure (28 days) to a range of EE2 concentrations. Exposure took place at two different temperatures to understand the influence of warming on the effects caused by EE2.

Concerning EE2 levels found in the exposure medium the present results showed that, immediately after contamination, the spiked

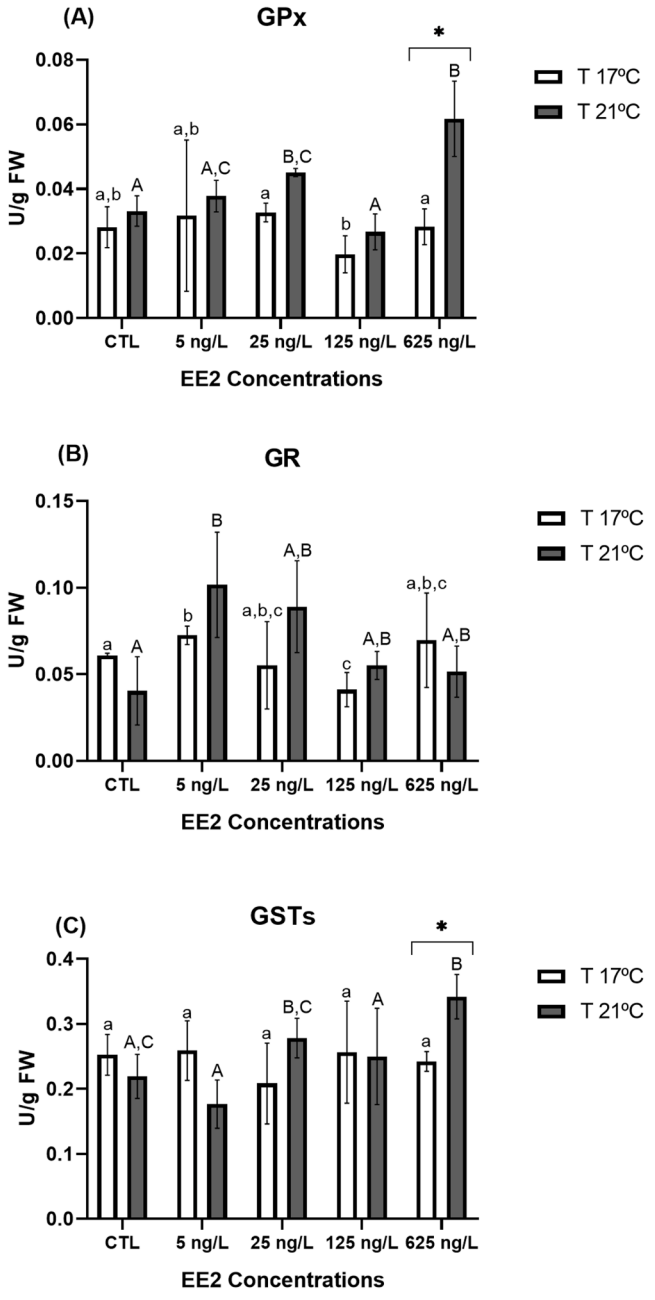

Fig. 2. A: Glutathione peroxidase (GPx) activity; B: Glutathione reductase (GR) activity; C: Glutathione S-transferase (GSTs) activity, in *Ruditapes philippinarum* exposed to EE2 (17α-ethinylestradriol) (0 (control), 5, 25, 125 and 625 ng/L) at two different temperatures (17±1 °C (control) and 21±1 °C) for 28 days. Results are means with standard errors (n=6). Significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters (17 °C) or uppercase letters (21 °C). For each EE2 concentration, differences between temperatures are identified by an asterisk.

concentrations were slightly below nominal concentrations. The low solubility of EE2 in water, due to its high hydrophobicity could justify the observed differences (Aris et al., 2014). Nevertheless, EE2 concentrations in blanks did not differ very much among sampling times (after spiking and before water renewal), which could be explained by its high chemical stability. Still information regarding chemical behaviour of EE2 in aquatic ecosystems is scarce (Aris et al., 2014).

Only a few studies adressed the presence of EE2 in wild bivalves from marine coastal systems, reporting concentrations in the ng/g range (Caban et al., 2016; Chiu et al., 2018). Studies concerning the uptake of EE2 in marine bivalves under laboratory-controlled conditions are even scarcer. Ricciardi et al. (2016) detected radiolabeled EE2 in the mussel, *Mytilus edulis,* exposed to ≈ 1 μg/L, for 38 days, at a concentration of 278.8 ng/g DW (dry weight). The oyster, *Crassostrea virginica,* exposed to similar concentrations of EE2 (1 μg/L) for 1, 5 and 10 days presented a tissue concentration of EE2 of 9.41 ng/DW, but only on the first day of exposure (Brew et al., 2020). Such results could reflect that clams may have a high capacity for detoxification and/or transformation of EE2. However, still limited information has been published with regard to the metabolic pathways of EE2 in invertebrates, including bivalves (Aris et al., 2014). In the present study, EE2 tissue concentrations were found to be below the limit of detection LOD (10 ng/g DW) at all tested concentrations excepted for the highest one. At this concentration clams presented a low BCF, highlighting potentially high EE2 detoxification or/and transformation capacity,corroborating the previously mentioned studies with oysters and mussels. Furthermore, similar results were obtained in clams exposed to different temperatures (17 and 21 °C), indicating that increased temperature may not influence bivalves' bioconcentration of EE2. Up to now, few studies have showed that bivalves exposed to pharmaceuticals under temperature challenges were not more susceptible to drug uptake (Almeida et al., 2021; Freitas et al., 2019). Nevertheless, the high LOD of the used analytical technique, considering the low concentrations of EE2 tested in the present study, does not allow us to draw any definite conclusions regarding the bioaccumulation of EE2 in the tissues of clams at control or at high temperatures, and further investigation is needed.

The exposure to contaminants, including pharmaceuticals, can impair the mitochondrial electron transport system (ETS) activity and energy reserves content, such as carbon hydrates, lipids and proteins. Glycogen (GLY) is the main energy reserve of bivalves being considered crucial to compensate for periods of food shortage, to mitigate short periods of stress or contribute to gametogenesis (Hummel et al., 1988). In the present study, at the control temperature (17 °C) no alterations on ETS activity and GLY levels were observed, regardless the EE2 concentration tested. These results may indicate that the stress induced by EE2 was not high enough to change metabolic capacity of the clams and alter energy reserves expenditure. Almeida et al. (2018a) found a similar pattern in the clam *R. philippinarum* exposed to carbamazepine and to cetirizine, highlighting that low concentrations tested were not enough to induce alterations in metabolism and GLY expenditure of clams. A similar response was observed under the warming scenario (21 °C). Associated with no changes on their metabolic capacity, clams were able to prevent the use of their energy reserves, maintaining or even increasing (at the highest EE2 concentration) the GLY content. Previously, Almeida et al. (2021) also showed no alteration on GLY content in clams exposed to carbamazepine and cetirizine under warming conditions. These findings may indicate the capacity of this species to preserve GLY content up to certain stress levels.

Under adverse conditions, such as drug exposure, protein content can increase as a consequence of protein biosynthesis stimulation, as reported by Cappello et al. (2017), in the mussel, *M. galloprovincialis,* exposed to the synthetic progesterone drospirenone (20, 200, 2000 and 10 000 ng/L), for 7 days. Nevertheless, the results presented here showed that neither EE2 exposure nor the exposure to the combination of both stressors (EE2 and higher temperatures) significantly impacted total protein content of clams highlighting once again that the stress level exerted may not have significantly altered the consumption levels of this energy reserve and, furthermore, the increase of GPx and GSTs activity observed at 21 °C and 625 ng/L of EE2 resulted from increased capacity of each enzyme and not from increased enzymes production.

In terms of defences, antioxidant system is responsible for the scavenging of reactive oxygen species (ROS), allowing the maintenance of the intracellular redox balance (Regoli and Giuliani, 2014). Enzymatic antioxidant mechanisms correspond to the organisms first line of defence against ROS production. Among antioxidant enzymes, glutathione peroxidase (GPx) catalyzes the elimination of hydroperoxides

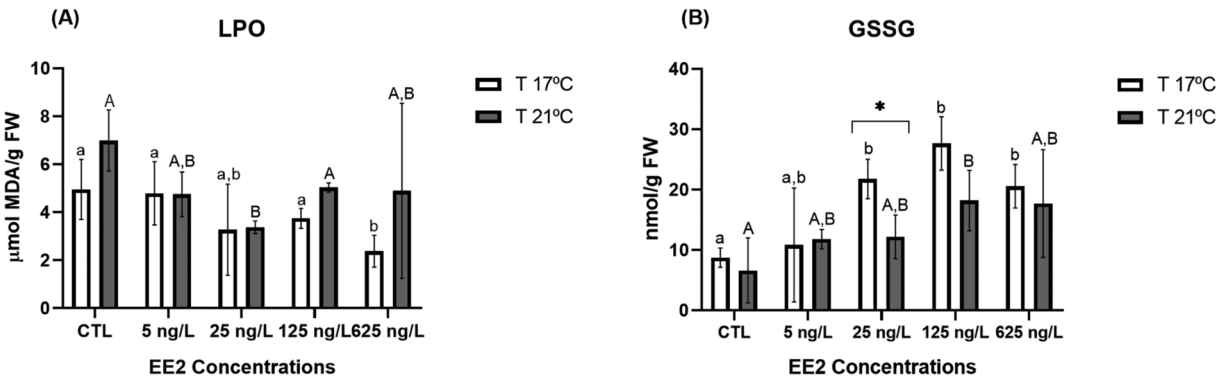

Fig. 3. A: Lipid peroxidation (LPO) levels; B: Oxidized glutathione levels, in *Ruditapes philippinarum* exposed to EE2 (17α-ethinylestradriol) (0 (control), 5, 25, 125 and 625 ng/L) at two different temperatures (17±1 °C (control) and 21±1 °C) for 28 days. Results are means with standard errors (n=6). Significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters (17 °C) or uppercase letters (21 °C). For each EE2 concentration, differences between temperatures are identified by an asterisk.

and its activity is directly dependent on reduced glutathione levels. At control temperature (17 °C), GPx activity was not affected by the presence of EE2, which may indicate that ROS produced as a consequence of the presence of EE2 in clams were not enough to stimulate this enzymatic antioxidant defence. Similar results were obtained by Maranho et al. (2014), showing no alterations on GPx activity in the polychaete *Hediste diversicolor* exposed to EE2 (0.01, 0.1, 1, 10 and 100 ng/g). Nevertheless, our results showed that under a warming scenario, GPx activity was enhanced in clams exposed to the highest concentration of EE2 (625 ng/L), even though at environmental relevant concentrations of EE2 (5 ng/L) no changes were observed. Assessing temperature (25 and 30 °C) effects in mussel *Mytilus coruscus,* Hu et al. (2015) showed an increase in GPx activity, after 3, 7 and 14 days of exposure at the highest temperature, corroborating the impact of thermal stress on GPx. Since at CTL conditions organisms exposed to both temperatures presented similar GPx activity, our findings may indicate that temperature rise enhanced the sensitivity of clams to pollutants, leading to enzyme activation in the presence of EE2, especially noticed at the highest tested concentration. The activity of GPx is correlated with glutathione reductase (GR) activity, which is responsible for the reduction of GSSG to GSH to reestablish the intracellular levels of the reduced form (GSH). Nevertheless, no clear pattern was observed in terms of GR responses as a function of EE2 concentrations or temperature. In fact, although clams tended to present higher GR activity at warming conditions and lower EE2 concentrations, this response was not effective at the highest exposure concentrations, with clams showing limited GR activity. Similarly, Maranho et al. (2014) reported an increase in GR activity in *H. diversicolor* exposed to low concentrations of EE2 (sediments spiked with 0.1 ng/g EE2), supporting the responsiveness of GR activity at low EE2 concentrations. Activation of antioxidant defences, namely an increase in GR enzymatic activity, under a warming scenario was reported by Park et al. (2008) in the clam, *Laternula elliptica*, supporting the increase reported here in clams exposed to the combination of low concentrations of EE2 and increased temperature.

Biotransformation pathways can play a significant role in ROS production, due to the catalytic activity of intermediate enzymes (Regoli and Giuliani, 2014). Glutathione-*S*-transferases (GSTs) are a family of biotransformation enzymes, belonging to phase II drug-metabolism, responsible for the formation of thioesters conjugates of several xenobiotics in order to improve their excretion rate by the organism. GSTs are also involved in the deactivation of endogenous secondary metabolites formed in the organism, such as hydro-peroxides (Regoli and Giuliani, 2014). The present results showed no significant alterations of GSTs at temperature of 17 °C, regardless the concentration tested, suggesting that GSTs may not be significantly involved in EE2 biotransformation. Previous studies revealed that different invertebrate species can reveal different responses to EE2 exposure, as reported by Souza et al. (2013) when two copepod species (*Eudiaptomus gracilis* and *Mesocyclops luckarti*) were exposed to concentrations of 10 ng/L of EE2, for 14 days, and only *E. gracilis* presented a decrease in GSTs activity. Nevertheless, clams exposed to non-environmentally relevant concentrations of EE2 (625 ng/L) in the warming scenario presented significantly higher GSTs activity, with a significant interaction between these two factors. Thus, our results indicate that GSTs are more responsive to EE2 presence when subjected to warming conditions. Similarly, Freitas et al. (2019) detected an increment in GSTs activity in *M. galloprovincialis* exposed to drugs under increased temperature.

Several studies have assessed the impact of steroid estrogens on lipid metabolism. Canesi et al. (2008) showed a decrease on lysosome membrane stability followed by an increase in LPO levels, on mussel *M. galloprovincialis* exposed to a mixture of EDCs, including EE2. Our findings revealed that at control temperature clams tended to decrease their LPO levels along the increasing exposure gradient, but significantly lower values were only found at the highest concentration (625 ng/L). Nevertheless, under a warming scenario, clams exposed to the highest EE2 concentration maintained LPO levels. The occurrence of oxidative damage in cellular membranes is conditioned by the global efficacy of the antioxidant defence mechanisms. The present results showed the neutralization of ROS by GSH, resulting in the increase of GSSG, which could explain the decrease in MDA content observed with EE2 exposure, regardless of the absence of an antioxidant enzymes activation. At a warming scenario, the increased activity reported for GSTs and GPx could support the lack of overall alterations observed on LPO levels under stressful scenarios, which is in accordance to results reported by other authors under similar conditions (Matozzo et al., 2013; Velez et al., 2017)

Reduced (GSH) and oxidized (GSSG) forms are the main non-enzymatic antioxidant mechanism of most living beings. The present study revealed that the GSSG content increased along the increasing exposure gradient, especially noticed at control temperature. Higher levels of oxidized glutathione could result from direct oxidation of GSH, supporting a redox imbalance as a consequence of drug presence. Similar results were reported by Almeida et al. (2015), in the clam *R. philippinarum* exposed chronically (28 days) to a range of concentrations of the pharmaceutical drug carbamazepine. In this study, a similar pattern was observed in clams exposed to EE2 at both temperatures. Alterations in GSSG and/or GSH levels, following stressors exposure, can be directly correlated with disturbances in enzymes from the glutathione cycle, namely GR and GPx, leading to a disturbance of redox balance, as demonstrated by Correia et al. (2016). The authors detected an increase on GSH/GSSG followed by an GR increase in clams exposed to a combination of concentrations of the pharmaceutical drug paracetamol under different salinities. Such changes are frequently considered as a widespread toxicological response, regardless of the stressor tested.

5. Conclusions

The present study provides news insights on the toxic impacts of 17α-ethinylestradiol under two different temperature scenarios, in the marine clam *R. philippinarum* through evaluation of energy-related and oxidative stress biomarkers. Our findings suggest disturbances in some antioxidant endpoints at the highest, non-environmentally relevant concentrations of EE2. Although a disruption of redox status may be present, clams seemed capable to cope with ROS overproduction. Environmental relevant concentrations of EE2 revealed a small impact in the overall redox status, compared to the highest ones. Furthermore, the present study showed that the combination of warming scenarios with EE2 may impact clams' antioxidant system, probably due to an increase in the organisms' sensitivity towards stressors presence, mainly when the pharmaceutical is present at high concentrations (125 and 625 ng/L). Even though environmentally relevant concentrations of EE2, at both temperatures, did not seem to have major impact in the endpoints approached, our findings reinforce the importance of studies focusing on the combination of pharmaceutical drugs with abiotic factors resulting from the ongoing climate change.

CRediT authorship contribution statement

Mónica G. Silva: Methodology, Formal analysis, Writing – original draft. **Valdemar I. Esteves:** Resources. **Valentina Meucci:** Resources, Methodology. **Federica Battaglia:** Resources, Methodology. **Amadeu MVM. Soares:** Funding acquisition. **Carlo Pretti:** Resources, Methodology, Writing – original draft. **Rosa Freitas:** Conceptualization, Resources, Funding acquisition, Writing – review & editing.

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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