

Article

Evidence of Endocrine Disruption and Oxidative Stress in *Mytilus galloprovincialis* Exposed to 17 α -Ethinylestradiol

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Abstract

The presence of endocrine-disrupting compounds (EDCs) in aquatic environments has raised significant concerns, particularly regarding their impact on marine biota. Among these compounds, 17 α -ethinylestradiol (EE2), a synthetic estrogen widely used in oral contraceptives, is highly persistent and biologically active at very low concentrations. This study evaluated the effects of EE2 exposure on oxidative stress responses and endocrine disruption in *Mytilus galloprovincialis*, exposed for 28 days to three EE2 concentrations (10, 30, and 300 ng·L⁻¹). Biomarkers of oxidative stress, including the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST), as well as Lipid Peroxidation (MDA levels), total ubiquitin (UBI) and the endocrine disruption marker, vitellogenin-like protein (VTG) were assessed. Results showed significant increase in GST and a decrease in CAT activities followed by an elevation at 300 ng·L⁻¹, slightly higher than control values. Overall, results suggest an enhanced oxidative challenge. No significant changes were detected in MDA and UBI levels. VTG-like protein levels increased according to the EE2 concentrations tested, suggesting an effect on the mussel's endocrine system. These results show the activation of detoxification and antioxidant defense mechanisms in exposed mussels as a response to mitigate oxidative stress damage. Furthermore, it highlights the importance of using biomarkers in pollution monitoring studies and environmental risk assessment.

Keywords: 17 α -ethinylestradiol; *Mytilus galloprovincialis*; oxidative stress; antioxidant enzymes; vitellogenin; endocrine disruption



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1. Introduction

Environmental pollution, particularly from domestic and industrial effluents, presents a significant challenge. Aquatic ecosystems are especially vulnerable, as these pollutants ultimately reach the ocean [1]. Marine ecosystems are at high risk from industrial, agricultural, and domestic pollutants [2]. Therefore, researching the impacts of these pollutants is essential for ecosystem protection. Coastal areas host diverse habitats, including estuaries, biogenic reefs, rocky areas, sandy beaches, salt marshes, mudflats, and seagrass beds [3]. The protection of these areas is critical, as they are threatened by human activities such as overfishing, maritime transport, dredging, and chemical discharges [4].

Characterized by well-oxygenated environments, these coastal regions are preferred habitats for bivalves, making them important for ecotoxicological studies focused on food safety and pollutant impact assessment [5].

Mussels (*Mytilus* spp.) are commonly used as biological model organisms in research [6] due to their wide geographical distribution, sedentary nature, and high tolerance to diverse environmental conditions. Furthermore, they are well known for accumulating significant amounts of pollutants [7,8]. Their high filtration capacity and well-characterized physiology, morphology, and biochemistry make them effective “carriers” of contaminants through trophic chains, making them ideal organisms for toxicology and bioavailability studies [9]. Endocrine-disrupting compounds (EDCs) represent a significant threat due to their ability to disrupt the endocrine system, affecting hormone synthesis and enzyme activities [10,11]. Among EDCs, steroid hormones are commonly detected in wastewater. Natural estrogens, including estrone (E1), 17 β -estradiol (E2), and estriol (E3), mainly originate from human and animal excretion, whereas the synthetic estrogen 17 α -ethinylestradiol (EE2), extensively used in oral contraceptives, is also frequently reported. These compounds interfere with cellular apoptotic processes, influence the epigenetic regulation of specific target cells, and disrupt the cell cycle [12]. Despite their harmful effects, EDCs persist in the environment and are discharged through industrial, urban, and agricultural effluents. These compounds infiltrate soil and eventually contaminate groundwater and drinking water, posing severe risks to human development and reproduction [11,13].

EE2 is highly estrogenic and exhibits high resistance to degradation, making it a moderately persistent environmental contaminant. In fact, EE2 was reported to degrade in seawater between 5 and 10 days by photolysis and microbial activity [14,15]. However, the scientific literature reveals considerable variability, with results indicating that the degradation period for EE2 ranges from two to more than 40 days [16]. In wastewater treatment plants, EE2 is often incompletely removed (removal rates from 0.77% to 95%), leading to its widespread detection in aquatic environments. Concentrations ranging from 18 to 83 ng·L⁻¹ have been reported in coastal habitats worldwide (e.g., Douro River estuary in Portugal, the Venice Lagoon in Italy, and the Gulf of Gdańsk in the Baltic Sea) [17], with wastewater effluents being a significant source of contamination [16]. In Portugal, Ribeiro et al. reported concentrations as high as 83.1 ng·L⁻¹ in the Douro River estuary [18].

EE2 shows toxicity at concentrations as low as 0.1 ng·L⁻¹ and is recognized by the EU as an emerging aquatic pollutant [19]. EE2 bioaccumulates in trophic chains, affecting various marine organisms, with aquatic invertebrates being especially vulnerable due to prolonged exposure from early life stages [20].

Exposure to EE2 negatively affects bivalves by causing the overproduction of reactive oxygen species (ROS), activating antioxidant enzymes (e.g., SOD, CAT), which work to reduce oxidative stress and prevent cell death due to membrane disintegration [21].

EE2 can disrupt lysosomal membrane stability and cause gonadal damage in *M. galloprovincialis*. Additionally, it is known to induce vitellogenin gene expression in *Saccostrea glomerata* and affects metabolic pathways linked to anaerobic metabolism, osmotic

stress, and reproduction in both *Crassostrea virginica* and *Ruditapes philippinarum* [22–24]. In seahorses (*Hippocampus guttulatus*), EE2 exposure reduced egg viability, delayed sexual differentiation and maturation, decreased testosterone synthesis, and caused gestational disturbances [25]. In fish, EE2 inhibited sexual development in juvenile male western mosquitofish (*Gambusia affinis*), reduced DNA repair gene transcription in male zebrafish (*Danio rerio*), and increased vitellogenin levels in juvenile rainbow trout (*Oncorhynchus mykiss*) [20]. However, it must be highlighted that the toxicity thresholds for EE2 vary significantly across taxa, ranging from low $\text{ng}\cdot\text{L}^{-1}$ concentrations in teleost fish to $\mu\text{g}\cdot\text{L}^{-1}$ levels in many studies using bivalves [20]. This difference in sensitivity can be attributed to the fact that in fish, EE2 acts as a high-affinity agonist for nuclear estrogen receptors, while in bivalves, a vertebrate-like nuclear ER is lacking, and their estrogenic signaling likely works via modulation of the endogenous steroid metabolism [26,27].

EE2 can also reduce fertility rates and induce feminization in males, leading to the production and release of female-associated hormones or proteins like vitellogenin (VTG). VTG is a phosphoprotein synthesized in the liver of female ovoviviparous organisms during yolk formation (vitellogenesis) in response to the hormone estradiol (E2); however, VTG is also produced in males following exposure to EDCs like EE2 [28]. Thus, biomarkers such as vitellogenin (VTG); total ubiquitin, signaling target proteins for proteolytic degradation [29]; antioxidant enzymes and lipid peroxidation (LPO), indicators of oxidative stress; or glutathione S-transferase (GST), a detoxifying enzyme, are commonly used to measure biological responses to contaminants [30]. Regarding biomarkers' baselines for the mussel whole-tissues, the values reported in the literature are highly variable, ranging from nmol to $\mu\text{mol}\cdot\text{min}\cdot\text{mg}^{-1}$, depending on size, maturation stage, season, temperature, diet and geographical distribution, and assay methodologies [27,31–33].

Therefore, the main aim of this study was to evaluate the effects of EE2 on *M. galloprovincialis* exposed to different concentrations (0 , $10\text{ ng}\cdot\text{L}^{-1}$, $30\text{ ng}\cdot\text{L}^{-1}$, and $300\text{ ng}\cdot\text{L}^{-1}$) of this contaminant over 28 days. This was achieved by analyzing several stress biomarkers, including oxidative stress biomarkers (GST, CAT, SOD, and LPO), total ubiquitin, and vitellogenin levels. The novelty of the present study lies in providing a biomarker-based assessment of EE2 exposure in this species, combining VTG-like protein, antioxidant and detoxification enzymes, lipid peroxidation, and ubiquitin responses under environmentally relevant exposure conditions. This study explores the proteolytic degradation of damaged proteins (UBI levels), a pathway that is less frequently studied in bivalve ecotoxicology. Thus, this study improves the understanding of how EE2 affects multiple biological pathways in a sentinel marine mussel. Additionally, this study aims to contribute to a better understanding of the risks posed by exposure to EE2 in aquatic biota.

2. Materials and Methods

2.1. EE2 Stock Solution

To prepare the EE2 stock solution, 5 mg of EE2 (CAS No. 57-63-6, Sigma-Aldrich, St. Louis, MO, USA) was weighed and dissolved in 100 mL of methanol (99.8% V/V, Honeywell, Seelze, Germany). The required volume was added to each aquarium to achieve the target concentrations of 10, 30, and $300\text{ ng}\cdot\text{L}^{-1}$. Accounting for the dilution factor, the methanol concentration in each aquarium was determined to be $\leq 0.001\%$. An aquarium containing filtered seawater served as the control (containing the same percentage of solvent as the test aquaria). Additionally, another aquarium containing only filtered seawater was used as an additional control (free of solvent).

2.2. Experimental Trial

M. galloprovincialis (Lamarck, 1819) were manually collected from Guincho Beach (Cascais, Lisbon, Portugal) in March 2024. Mussels ($n = 32$) with an average length of 2.9 ± 0.3 cm and an average width of 1.4 ± 0.2 cm were collected to minimize variations in bioaccumulation and biomarker responses that could result from differences in size or weight. The specimens were transported to the NOVA School of Science and Technology Laboratory in a thermal box to maintain a constant temperature and minimize additional stress. The experimental setup used to expose the animals to EE2 was described previously [34,35]. Briefly, upon arrival, the mussels were acclimated for 7 days in a 50 L saltwater aquarium filled with seawater from Guincho Beach. The aquarium was equipped with filtration, recirculation, and aeration systems, and maintained under controlled conditions, including pH (8.1 ± 0.1), temperature (17.2 ± 0.1 °C), salinity (33 ± 1), and a 12 h light/dark photoperiod.

Mussels were randomly distributed into 8 experimental tanks of 8 L volume ($n = 2$ tanks per treatment), with 4 individuals per tank (total $n = 8$ animals per treatment). Each tank was considered a primary experimental unit (accounting for potential tank effects). The two tanks were independent replicates, and mussels within each tank (a total of 8 per treatment) were processed and analyzed individually, thus obtaining the variance inherent to the population. The weight and size of the bivalves are presented in the Supplementary Material (Table S1). The seawater in test tanks and target EE2 concentrations (0, 10, 30 and $300 \text{ ng}\cdot\text{L}^{-1}$) were renewed every 48 h. Water sampling was performed at the beginning (0 h after spiking), 24 h and 48 h later (before water renewing). Parameters, like pH, temperature, and salinity, were checked daily. Considering the dilution factor, the methanol concentration in each aquarium was estimated to remain below 0.001%. Another two tanks with seawater (containing the same amount of solvent) were used as the control. A tank with seawater was used as an additional control to assess solvent effect.

The concentrations of 10 and $30 \text{ ng}\cdot\text{L}^{-1}$ were chosen based on realistic environmental levels of EE2 [23], and the highest non-realistic concentration ($300 \text{ ng}\cdot\text{L}^{-1}$) was selected for comparison purposes and a better comprehension of the effects of EE2 in mussels.

The mussels were fed daily with $1.5 \text{ mg}\cdot\text{L}^{-1}$ of dried algae (*Chlorella* sp., Shine, Portugal). The feeding regimen was followed to mimic wild conditions, where mussels are simultaneously feeding and being exposed to contaminants.

Ethics

The animal trials followed the national and international policies (e.g., the 3 Rs principle of animal welfare) and the EU directive (2010/63/EU), even though it does not apply to invertebrates. The mussel sampling was authorized by the competent authorities (ref. PT2024OPCM000674901) and by the faculty ethical committee. Additionally, the researchers have a certification (level C) from the FELASA (European Federation for Laboratory Animal Science) to conduct experiments with animals.

2.3. Sample Treatment

At the end of the experimental period, the mussels were weighed in an analytical balance (Sartorius, Göttingen, Germany) and measured with a vernier caliper. The mussels were then opened, and gender was putatively identified based on gonad observation (white or cream for males and orange or reddish for females) [36]. Then, the edible tissue from each specimen (16 males and 16 females) was carefully removed with the aid of scissors and tweezers.

Then, each sample was individually homogenized using a Tissue Master 125 (Kennesaw, GA, USA) in 3 mL of phosphate-buffered saline (PBS; 140 mM NaCl, Panreac,

Barcelona, Spain; 10 mM Na₂HPO₄, Sigma-Aldrich, St. Louis, MO, USA; 3 mM KCl, Merck, Darmstadt, Germany; 2 mM KH₂PO₄, Sigma-Aldrich, Steinheim, Germany). The resulting lysate was transferred to 1.5 mL microtubes and centrifuged at 15,000 × g for 10 min at 4 °C using a VWR centrifuge (Hitachi Koki Co., Ltd., Tokyo, Japan). The pellet from each sample was discarded, and the supernatant was transferred to new 1.5 mL microtubes (in duplicate) and stored at −45 °C for subsequent biomarker analysis. Samples were thawed as required for each assay, with all procedures performed on ice to preserve sample integrity.

2.4. Total Protein Content

The total protein content of each sample was measured using the Bradford method [37]. The Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 100 mL of ethanol (Honeywell, Seelze, Germany) and 100 mL of phosphoric acid (H₃PO₄; Sigma-Aldrich, Darmstadt, Germany) and then diluting the solution to a final volume of 1 L with distilled water. A stock solution of Bovine Serum Albumin (BSA; Nzytech, Lisboa, Portugal) was used to prepare standards with concentrations ranging from 0 to 4 mg/mL of BSA in PBS buffer (pH 7.2 ± 0.1) to generate a calibration curve. In a 96-well microplate (Greiner Bio-One GmbH, Kremsmünster, Austria), 20 µL of each standard and sample were added, followed by 180 µL of Bradford reagent to bring the total volume in each well to 200 µL. The absorbance was measured at 595 nm using a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA). The LOD was calculated as 0.06 mg.mL^{−1} and LOQ as 0.18 mg.mL^{−1}. The total cytosolic protein was determined as mg.L^{−1} for biomarker normalization.

2.5. Biomarker Analysis

2.5.1. Glutathione-S-Transferase (GST) Activity

The protocol originally described by Habig et al. [38] was adapted and optimized for use with 96-well microplates [35]. GST activity was determined based on the conjugation reaction between the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and reduced L-glutathione (GSH), forming the GS-DNB complex, which interacts with GST in the sample. To prepare the substrate solution, 19.6 mL of PBS buffer (pH 7.2 ± 0.1) was mixed with 200 µL of 200 mM GSH (Sigma-Aldrich, St. Louis, MO, USA) and 200 µL of 100 mM CDBN (Sigma-Aldrich, St. Louis, MO, USA). In a 96-well microplate, 20 µL of each sample or blank (PBS or H₂O) was added in triplicate, and the volume in each well was adjusted to 200 µL with the substrate solution. Absorbance was measured at 340 nm using a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA) every minute for a period of 6 min. The specific activity of GST was calculated using the molar extinction coefficient of CDBN (5.3 mM^{−1} cm^{−1}). GST activity results were normalized to the cytosolic protein content and expressed as nmol.min^{−1}.mg^{−1} total protein.

2.5.2. Superoxide Dismutase (SOD) Activity

This assay was based on the indirect nitroblue tetrazolium (NBT) method previously described by Zhou et al. [39] and adapted and optimized for 96-well microplates [40]. In a 96-well microplate, each well received 200 µL of 50 mM potassium phosphate buffer (pH 8.0), 10 µL of 3 mM EDTA (Riedel-Haën, Seelze, Germany), 10 µL of 3 mM xanthine (Sigma-Aldrich, Shanghai, China), 10 µL of 0.75 mM NBT (Sigma-Aldrich, Steinheim, Germany), and 10 µL of the sample. The reaction was initiated by adding 10 µL of XOD (Sigma-Aldrich, Steinheim, Germany), and absorbance was measured every 2 min over 20 min using a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA). Results were normalized to cytosolic protein content and expressed as mU.mg^{−1} total protein.

2.5.3. Catalase (CAT) Activity

This assay was conducted using a method previously described by Johansson et al. [40], though adapted and optimized for 96-well microplates [34]. A calibration curve was prepared using formaldehyde standards (0–75 μM) in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1% BSA. Next, 50 μL of the sample, 30 μL of methanol (Honeywell, Seelze, Germany), and 70 μL of potassium phosphate buffer (100 mM; pH 7.0; Sigma, Darmstadt, Germany) were combined in each well. The reaction was initiated by adding 20 μL of 0.035 M hydrogen peroxide (Sigma-Aldrich, Steinheim, Germany) to each well, followed by a 20 min incubation at room temperature on an orbital shaker (Optic Ivymen System, JP Selecta, Barcelona, Spain) in the dark. Next, 30 μL of 10 M potassium hydroxide (KOH; ChemLab, Zedelgem, Belgium) and 30 μL of purpald (32.4 mM in 0.5 M HCl; purpald; Aldrich, Steinheim, Germany) were added, followed by an additional 10 min incubation under the same conditions. Finally, 10 μL of potassium periodate (65.2 mM in 0.5 M KOH; Sigma-Aldrich, Darmstadt, Germany) was added, and the mixture was incubated for 5 min in the dark. Absorbance was measured at 540 nm using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). Results were normalized to cytosolic protein content, and CAT activity was expressed as $\text{nmol} \cdot \text{mg}^{-1}$ total protein.

2.5.4. Lipid Peroxidation (LPO)

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA), a byproduct of LPO, using the Thiobarbituric Acid Reactive Substances (TBARS) assay [41]. A calibration curve was prepared with malondialdehyde (MDA; Merck, Germany) standards (0–0.1 μM) in ultrapure Milli-Q water. In 1.5 mL microtubes, 5 μL of each sample or standard were added to 295 μL of a solution consisting of 2.25 mL of PBS buffer (pH 7.2 ± 0.1), 0.625 mL of 8.1% (*w/v*) sodium dodecyl sulphate (SDS; Sigma-Aldrich, Germany), 4.675 mL of trichloroacetic acid (20% (*w/v*); TCA; Panreac, Spain), 4.675 mL of thiobarbituric acid (1% *w/v*; TBA; Sigma-Aldrich, Germany), and 2.525 mL of ultrapure Milli-Q water. The mixture was centrifuged at 3000 rpm for 1 min, followed by incubation in a dry bath (Digital Thermoblock, Labnet) at 100 °C for 10 min. After cooling on ice, 62.5 μL of ultrapure Milli-Q water was added to each microtube, and 150 μL of each sample or standard was transferred to a microplate well. Absorbance was measured at 530 nm using a Synergy HTX microplate reader. Results were normalized to cytosolic protein content, with MDA concentrations expressed as $\text{nmol} \cdot \text{mg}^{-1}$ total protein.

2.5.5. Total Ubiquitin (UBI)

Ubiquitin quantification was conducted using the ELISA (Enzyme-Linked Immunosorbent Assay) method [42], optimized for UBI. A calibration curve was generated using ubiquitin standards (UBPBIO-Ubiquitin-Proteasome, Biotechnologies, Dallas, TX, USA) with concentrations ranging from 0 to 0.8 $\mu\text{g} \cdot \text{mL}^{-1}$. For each well, 50 μL of either sample or standard was added to the microplate, followed by overnight incubation at 4 °C. After incubation, the microplate was washed twice with a 0.05% Tween-20 solution prepared in PBS buffer (pH 7.2 ± 0.1). Subsequently, 100 μL of a blocking solution containing 1% BSA in PBS was added to each well, and the plate was incubated at 37 °C for 90 min. The plate was then washed three times with the Tween-20 solution. A primary ubiquitin antibody solution (P4D1; Sc-8017; Santa Cruz Biotechnology, Dallas, TX, USA) was prepared by diluting it in PBS buffer (pH 7.2 ± 0.1) containing 1% BSA to a final concentration of 1 $\mu\text{g} \cdot \text{mL}^{-1}$. Then, 50 μL of the primary antibody was added to each well, and the plate was incubated overnight at 4 °C. Following this incubation, the microplate was washed three times, and 50 μL of secondary antibody (anti-mouse IgG Fc specific-alkaline phosphatase, Sigma-Aldrich, Steinheim am Albuch, Germany) diluted in PBS buffer (pH 7.2 ± 0.1) con-

taining 1% BSA to a final concentration of $1 \mu\text{g} \cdot \text{mL}^{-1}$ was added to each well. The plate was incubated at 37°C for 90 min. Subsequently, the microplate was rewashed, and $50 \mu\text{L}$ of substrate solution (containing 157 mg of Tris-HCl [Sigma, St. Louis, MO, USA], 58 mg of NaCl [Panreac, Barcelona, Spain], $50 \mu\text{L}$ of 5 mM MgCl_2 [Fluka, BioChemika, Buchs, Switzerland], and 10 mg of disodium 4-nitrophenyl phosphate hexahydrate [pNPP; Sigma-Aldrich, Gillingham, UK] prepared in 10 mL of distilled water at pH 9) was added to each well. The plate was incubated at room temperature for approximately 15 min. Finally, a stop solution (3 M NaOH; Panreac, Barcelona, Spain) was added, and absorbance was measured at 405 nm using a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA). Results were adjusted to cytosolic protein content and expressed as $\text{nmol} \cdot \text{mg}^{-1}$ total protein.

2.5.6. Vitellogenin (VTG)

The quantity of vitellogenin (VTG) was determined by measuring the amount of inorganic phosphate, using an indirect method described by Atkinson et al. [43], adapted to 96-well microplates. Standards with varying concentrations of inorganic phosphate (0 to $50 \mu\text{M}$; Sigma-Aldrich, St. Louis, MO, USA) were prepared to generate a calibration curve. The procedure began with extracting free phosphates from the sample using tertbutyl methyl ether (Sigma-Aldrich, St. Louis, MO, USA). For this, 1 mL of the sample was mixed with 1 mL of the organic solvent and agitated for 30 min. The aqueous phase was then discarded. In the next step, $800 \mu\text{L}$ of the organic phase was combined with $200 \mu\text{L}$ of 1 M NaOH and incubated at 50°C for 1 h. For quantification, $25 \mu\text{L}$ of aqueous phase was transferred to a well of a microplate (Synergy HTX, BioTek, Winooski, VT, USA), in triplicates with $35 \mu\text{L}$ of vanadate molybdate reagent (Sigma-Aldrich, USA) diluted 1 for 2 and $140 \mu\text{L}$ of MilliQ water. Absorbance was measured at 410 nm using a microplate reader. Results were normalized to cytosolic protein content and expressed as $\mu\text{g ALP} \cdot \text{mg}^{-1}$ total protein.

2.6. Quantification of EE2 in Seawater

2.6.1. Sample Preparation and Derivatization

Extraction was carried out using a volume of 10 mL of water sample (from the experimental aquaria) and placed into a 15 mL glass conical test tube and spiked with internal standard (Ethinylestradiol ($20,21\text{-}^{13}\text{C}_2$, 99%) (EE2D2) from Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) to the final concentration ($0.050 \mu\text{g L}^{-1}$). The derivatization involved the addition of: $600 \mu\text{L}$ of potassium carbonate 5% (K_2CO_3 , (99.0% purity) from Panreac Química SA, Barcelona, Spain), $10 \mu\text{L}$ of pyridine (Sigma-Aldrich $\geq 99.5\%$ purity), and $200 \mu\text{L}$ of acetic anhydride was obtained from Sigma-Aldrich LiChropur ($\geq 99\%$ purity; Buchs, Switzerland). The mixture was vortexed and allowed to react for 5 min at room temperature. After derivatization, the analytes were extracted by the dispersive liquid–liquid microextraction (DLLME) technique, in which EE2 was extracted, then a quick injection of $70 \mu\text{L}$ chloroform (CHCl_3 from Supelco EMSURE, Darmstadt, Germany) (extraction solvent) was added, resulting in a cloudy solution. This solution was vigorously shaken by hand. Finally, the conical tubes were centrifuged at 2000 rpm and 4°C for 10 min. Dispersive particles were collected at the bottom of the centrifuge tube by a glass Pasteur pipette with a latex bulb. The resulting organic phase was transferred to a micro-insert of $100 \mu\text{L}$ and injected into gas chromatography–mass spectrometry (GC–MS) for analysis.

2.6.2. Gas Chromatography–Mass Spectrometry (GC/MS) Analysis

The gas chromatograph Shimadzu GCMS-QP2010 Gas Chromatograph–Mass Spectrometer equipped with an autoinjector AOC-5000 (Shimadzu Corporation, Kyoto, Japan) was used to out the chromatographic analyses. Injections of $1 \mu\text{L}$ were made in the splitless mode with a 2.0 min purge-off time, and the injector temperature was set at 275°C . Helium

(99.9999%), at a constant flow rate of 2 mL min^{-1} was used as the carrier gas. Separation was performed using a fused-silica capillary column Zebtron ZB-5MS W/Guardian (Phenomenex, Torrance, CA, USA) coated with 5% polysilarylene and 95% of polydimethylsiloxane ($30 \text{ m} \times 0.25 \text{ mm ID}$, $0.25 \text{ }\mu\text{m}$ film thickness) with 10 m of Guardian capillary column but not with a stationary phase. The analysis was done following the oven program temperature: initial temperature $80 \text{ }^{\circ}\text{C}$ (held for 2 min), increased by $20 \text{ }^{\circ}\text{C min}^{-1}$ to $220 \text{ }^{\circ}\text{C}$ (held for 17 min), next increased by $20 \text{ }^{\circ}\text{C min}^{-1}$ to $250 \text{ }^{\circ}\text{C}$ and held at this temperature for 7 min, and increased again by $15 \text{ }^{\circ}\text{C min}^{-1}$ to $305 \text{ }^{\circ}\text{C}$, and held at this temperature for 5 min. The transfer line was set at $275 \text{ }^{\circ}\text{C}$, and the ion source was set at $200 \text{ }^{\circ}\text{C}$ with an electron impact ionization of 70 eV. The MS system was routinely set in selective ion monitoring (SIM) mode, and each analyte was quantified based on peak area using one target and two qualifier ion(s). For EE2 SIM, ions m/z set to 296 for quantification and 338; 213 for qualifiers; in the case of EE2d2 SIM, ions m/z set to 298 for quantification and 340; 252 for qualifiers; with a retention time of 28.90 min and 28.88 min, respectively.

2.6.3. Calibration Curves and Quantification

Calibration curves and respective linearity parameters were obtained through matrix-matched calibration in a water sample from the experimental aquaria. Appropriate volumes of the standard solution containing EE2 and EE2D2 were added to blank samples (free of EE2 residues), spiked at six concentration levels (ranging from 10 to $500 \text{ ng}\cdot\text{L}^{-1}$). Calibration curves were constructed by plotting the EE2/EE2D2 ratio obtained against the concentration of the analyte. The limit of quantification (LOQ) was set at $10 \text{ ng}\cdot\text{L}^{-1}$, corresponding to the lowest calibration standard. The calibration curve shows a good linearity across the entire range ($r^2 \geq 0.999$).

Recovery tests were made by spiking samples with EE2 standard before extraction and derivatization. The experimental recovery was obtained from the difference between two measurements (the sample and the spiked samples). These samples were spiked with the target compounds at two concentration levels ($250 \text{ ng}\cdot\text{L}^{-1}$ and $50 \text{ ng}\cdot\text{L}^{-1}$), recovery following the addition of $250 \text{ ng}\cdot\text{L}^{-1}$ was 99.4%, and recovery following the addition of $50 \text{ ng}\cdot\text{L}^{-1}$ was 103.6%.

2.7. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 9 software (version 9.5.1). Before further testing, outliers were removed using Iglewicz and Hoaglin's robust test for multiple outliers (available at contchart.com). The Shapiro–Wilk test was employed to assess normality, and the Brown–Forsythe test was employed to verify the homogeneity of variances. Following this, ANOVA was performed, and Tukey's HSD test was subsequently applied to identify significant differences ($p < 0.05$) between the control and exposure concentrations, as well as among the different exposure groups. Where assumptions were violated, the corresponding non-parametric or robust alternatives (Welch's ANOVA test and Dunnett T3, respectively) were used. To simplify the graphical analysis, box plots represent half of the value and the values between the first and third quartiles, the whiskers represent the maximum and minimum values, and the horizontal line represents the median.

3. Results

3.1. Mortality Rate

During the exposure period, the mortality rate for each group was 0% in control, 0% in $10 \text{ ng}\cdot\text{mL}^{-1}$, 12.5% (one mussel dead) in $30 \text{ ng}\cdot\text{mL}^{-1}$ and 0% in $300 \text{ ng}\cdot\text{mL}^{-1}$ (Supplementary material, Table S2).

3.2. Biomarkers Analysis

Detailed statistical results for each biomarker are provided in the Supplementary Material (Tables S3–S9).

Solvent-based and solvent-free controls were also compared, and no significant differences were found ($p > 0.05$); therefore, the controls displayed in biomarker figures represent the solvent-based controls.

3.2.1. Glutathione S-Transferase (GST)

GST activity (Figure 1) shows a significant increase in the EE2-exposed groups, with the highest activity being observed at 300 ng·L⁻¹. Statistical analyses ($p < 0.05$) indicate an EE2-induced effect between controls and animals exposed to 10 and 300 ng·L⁻¹.

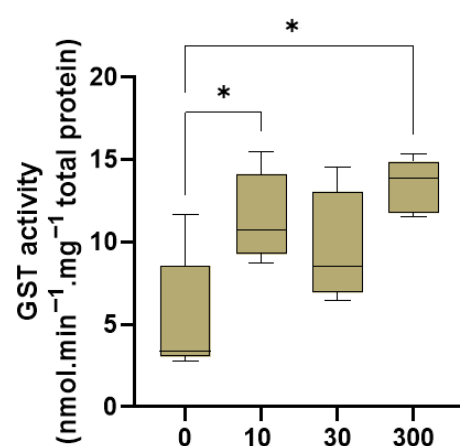


Figure 1. GST activity in mussels exposed to the different EE2 concentrations (0, 10, 30 and 300 ng·L⁻¹). Significant differences if * ($p < 0.05$). Boxes represent half of the value and the values between the first and third quartiles; the whiskers represent the maximum and minimum values. The line is the median value.

3.2.2. Superoxide Dismutase (SOD)

The results of SOD activity (Figure 2) show an increase in response to EE2 exposure compared to the control group. The group exposed to 300 ng·L⁻¹ exhibited the highest SOD activity, suggesting a dose-dependent response. However, no significant differences ($p > 0.05$) were found between controls and exposed animals.

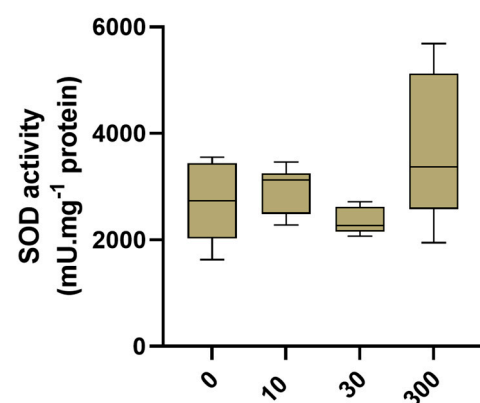


Figure 2. SOD activity in mussels exposed to the different EE2 concentrations (0, 10, 30 and 300 ng·L⁻¹). Boxes represent half of the value and the values between the first and third quartiles; the whiskers represent the maximum and minimum values. The line is the median value.

3.2.3. Catalase (CAT)

CAT activity (Figure 3) shows a significant decrease from the control to animals exposed to 10 and 30 ng·L⁻¹ ($p < 0.05$). However, at 300 ng·L⁻¹ no differences were detected compared to the control.

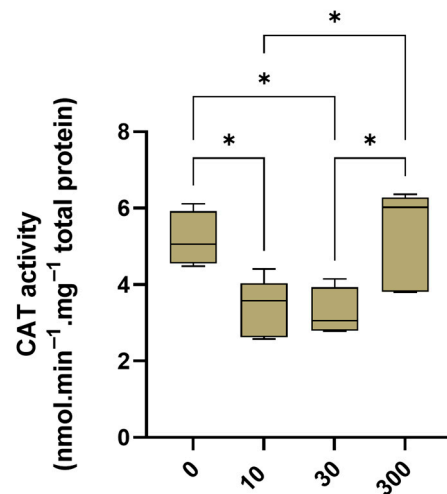


Figure 3. CAT activity in mussels exposed to the different EE2 concentrations (0, 10, 30, and 300 ng·L⁻¹). Significant differences if * ($p < 0.05$). Boxes represent half of the value and the values between the first and third quartiles; the whiskers represent the maximum and minimum values. The line is the median value.

3.2.4. Lipid Peroxidation (LPO)

The LPO (measured as MDA levels) is presented in Figure 4, showing an increase in MDA levels according to the tested EE2 concentrations, with the highest values being observed in the group exposed to 10 ng·L⁻¹. However, no significant differences ($p > 0.05$) were observed between the control and the exposed animals.

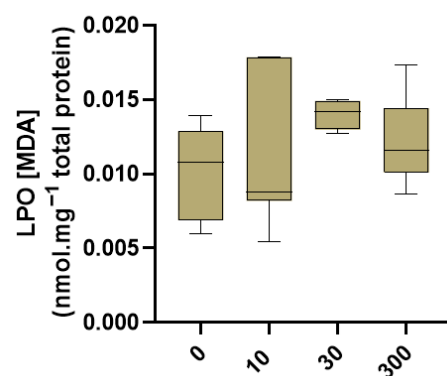


Figure 4. MDA levels in mussels exposed to the different EE2 concentrations (0, 10, 30, and 300 ng·L⁻¹). Boxes represent half of the value and the values between the first and third quartiles; the whiskers represent the maximum and minimum values. The line is the median value. LOD: 0.0016 μ M; LOQ: 0.005 μ M.

3.2.5. Ubiquitin (UBI)

The ubiquitin results (Figure 5) showed that the highest levels of UBI were found in mussels exposed to 300 ng·L⁻¹, revealing a highly variable response, and the lowest observed in animals exposed to 10 ng·L⁻¹. However, no significant changes ($p > 0.05$) were observed between controls and the exposed groups or among treatments.

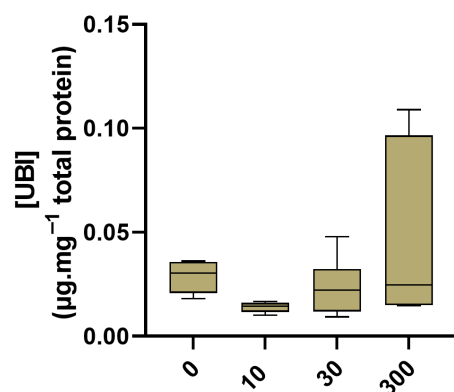


Figure 5. UBI levels in mussels exposed to the different EE2 concentrations (0, 10, 30, and 300 ng·L⁻¹). Boxes represent half of the value and the values between the first and third quartiles; the whiskers represent the maximum and minimum values. The line is the median value. LOD: 0.023 µg.mL⁻¹; 0.07 µg.mL⁻¹.

3.2.6. Vitellogenin (VTG)

Vitellogenin (VTG) levels are shown in Figure 6, showing an increase in a dose-dependent manner following EE2 exposure. These results demonstrate a clear estrogenic response, with VTG induction being most pronounced at the highest EE2 concentration tested (300 ng·L⁻¹).

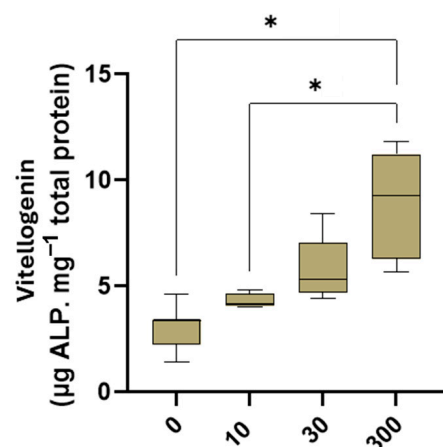


Figure 6. VTG levels in mussels exposed to the different EE2 concentrations (0, 10, 30, and 300 ng·L⁻¹). Significant differences if * ($p < 0.05$). Boxes represent half of the value and the values between the first and third quartiles; the whiskers represent the maximum and minimum values. The line is the median value. LOD: 0.98 µg.L⁻¹; LOQ: 3.25 µg.L⁻¹.

3.3. EE2 Determination of Seawater Physicochemical Parameters in Seawater

Table 1 presents the nominal and the real concentrations of EE2 measured in aquaria seawater at exposure times of 0, 24 and 48 h. No EE2 was detected in control tanks.

Calibration curves were obtained by matrix-matched calibration using seawater from the experimental aquaria, with six concentration levels ranging from 10 to 500 ng·L⁻¹. Good linearity was observed across the full calibration range ($r^2 \geq 0.999$). The limit of quantification (LOQ) was 10 ng·L⁻¹, corresponding to the lowest calibration level. Recovery assays performed at 50 and 250 ng·L⁻¹ yielded recoveries of 103.6% and 99.4%, respectively.

Results show that over 48 h, the real EE2 levels measured in the aquaria seawater remained constant and within the expected range, except for the nominal concentration of 30 ng·L⁻¹ where a slight decrease was observed. The 0 h sample for the nominal 30 ng·L⁻¹ treatment was not quantified because the sample was lost during handling prior to GC-MS

analysis. Nonetheless, statistics revealed that no significant changes ($p < 0.05$) were detected among sampling periods, and EE2 concentrations remained constant over time.

Table 1. EE2 nominal and measured concentrations ($\text{ng}\cdot\text{L}^{-1}$) measured in seawater samples after 0, 24 and 48 h.

Sample Time	0 h	24 h	48 h
EE2 (Nominal Concentration)	Measured Concentration		
0	n.d.	-	-
10	11.6 ± 0.8	9.9 ± 0.5	10.8 ± 1.4
30	-	23.6 ± 2.6	25.4 ± 3.9
300	303.1 ± 49.1	294.2 ± 26.7	305.8 ± 8.4

LOD: $3.3 \text{ ng}\cdot\text{L}^{-1}$, LOQ: $10 \text{ ng}\cdot\text{L}^{-1}$; n.d. (not detected).

Regarding the physicochemical parameters, they remained stable during the experiment in all experiment tanks (Supplementary Material, Table S10).

4. Discussion

Studying the effects of xenobiotics on marine biota is crucial for understanding their impact on aquatic ecosystems. These environments often experience stress conditions that lead to cellular and molecular alterations, potentially causing oxidative stress and subsequent cellular damage.

Mortality is a widely used endpoint to assess the severity of a xenobiotic's adverse effects. In the present study, no significant mortality was observed after 28 days of exposure to the different EE2 concentrations. This indicates that the tested concentrations did not reach the threshold to cause death in these mussels. Only one death was recorded in animals exposed to $30 \text{ ng}\cdot\text{L}^{-1}$.

Regarding experimental conditions, EE2 concentrations measured in seawater remained stable over each 48h period, confirming the compound's high persistence in aquatic environments.

Previous studies support these findings, showing that these concentrations do not cause mortality in this species, even when combined with other compounds such as sodium lauryl sulfate [21] or salicylic acid [44]. Additionally, the lack of mortality at the tested concentrations is consistent with findings from other authors, including Islam et al. [45], who reported no mortality at concentrations up to $1.25 \times 10^6 \text{ ng}\cdot\text{L}^{-1}$ of EE2 in the *Oyster Saccostrea glomerata*. Similarly, Leonard et al. [46] observed no mortality in *Lampsilis fasciola* mussels exposed to $1000 \text{ ng}\cdot\text{L}^{-1}$ of EE2, and Lopes et al. [47] registered no mortality in *M. galloprovincialis* exposed to up to $625 \text{ ng}\cdot\text{L}^{-1}$ of EE2.

In contrast, fish show higher sensitivity. For example, studies conducted on different species like zebrafish (*Danio rerio*) [48] and rare minnow (*Gobiocypris rarus*) [49] exposed to EE2 showed different results. For rare minnows, exposure to $4 \text{ ng}\cdot\text{L}^{-1}$ for 21 days showed no mortality but led to reproductive failure and severe gonadal alterations. While in zebrafish, exposure to $10 \text{ ng}\cdot\text{L}^{-1}$ resulted in >60% mortality over 90 days.

The resilience of bivalves, found in the present study, may be linked to the fact that bivalves have some additional protective mechanisms against exposure to contaminants, such as reducing their filtration rate, decreasing the metabolic rate, or keeping their shells closed for extended periods, decreasing the uptake of contaminants [21]. However, shell closure is metabolically costly and can trigger cellular stress and protein induction (such as UBI elevation) due to nutrient cessation [21,50]. Alternatively, EE2 in the $\text{ng}\cdot\text{L}^{-1}$ range typically induces chronic, sublethal effects, such as reproductive impairment and tissue damage, rather than immediate death [47]. In the present study, exposure to EE2 elicited a response of the antioxidant defense system, particularly CAT. Other observed changes in-

cluded increased GST activities, related to cellular detoxification mechanisms, and elevated VTG levels, a biomarker of endocrine disruption.

The response patterns of some biomarkers analyzed were non-monotonic; specifically, CAT and GST showed a biphasic trend. Regarding GST, significant induction occurred at both the lowest ($10 \text{ ng}\cdot\text{L}^{-1}$) and highest ($300 \text{ ng}\cdot\text{L}^{-1}$) EE2 concentrations, but little change was observed at $30 \text{ ng}\cdot\text{L}^{-1}$. SOD activity increased mainly at the highest concentration. Such non-linear concentration–response behavior is not uncommon for endocrine-active contaminants, which frequently elicit biphasic responses [51]. Although non-statistically significant, the increased SOD activity suggests that EE2 can cause oxidative stress, leading to an upregulation of the antioxidant defense system.

CAT activity decreased in animals exposed to 10 and $30 \text{ ng}\cdot\text{L}^{-1}$ EE2, followed by an increase in animals exposed to $300 \text{ ng}\cdot\text{L}^{-1}$ when compared to the lower concentrations; however, this increase remained near the control levels. One possible explanation is that the low concentration of EE2 generates mild reactive oxygen species (ROS) production or triggers signaling (e.g., via Nrf2 or estrogen-receptor related pathways) that modulate CAT expression. Similar activation of antioxidant enzymes in bivalves at sub-toxic pollutant levels has been documented; for example, chronic EE2 exposure in mussels was reported to elevate overall antioxidant enzyme activities compared to controls [44].

Conversely, Lopes et al. [21] observed the inhibition of CAT and SOD activities in mussels at the highest exposure concentrations of EE2 ($125 \text{ ng}\cdot\text{L}^{-1}$), when mixed with a surfactant, attributing this to overwhelming stress levels.

In this study, SOD activity remained near control levels in animals exposed to 10 and $30 \text{ ng}\cdot\text{L}^{-1}$ but increased at $300 \text{ ng}\cdot\text{L}^{-1}$, although non-significantly, indicating that only the highest EE2 concentration caused stress to trigger a more pronounced SOD response.

Similarly, Cunha et al. [44] found elevated SOD activities in *M. galloprovincialis* exposed to EE2 (at $\sim 100 \text{ ng}\cdot\text{L}^{-1}$ for 28 days), suggesting an activation of the antioxidant defenses following exposure. In the present study, the absence of a significant SOD change at 10 and $30 \text{ ng}\cdot\text{L}^{-1}$ could be related to the lower concentrations tested compared to those tested by Cunha et al.

Animals exposed to 10 and $300 \text{ ng}\cdot\text{L}^{-1}$ also showed a significant increase in GST activities. GST is involved in detoxification, conjugating toxic compounds with glutathione (GSH) for elimination. Thus, the increased GST activity suggests that EE2 induced detoxification mechanisms to eliminate the contaminant and avoid cellular damage. This is a common response linked to the chemical removal prior to the onset of damage [52]. In addition, the lack of a significant increase in MDA levels suggests that the antioxidant system and the detoxification mechanisms successfully prevented lipid peroxidation.

Our findings are in line with other studies reporting increased GST activity in bivalves exposed to pharmaceuticals or estrogenic compounds. For instance, Cunha et al. [44] found that mussels exposed to $125 \text{ ng}\cdot\text{L}^{-1}$ EE2 alone had significantly higher GST activity than controls. Interestingly, they noted that when EE2 was combined with salicylic acid ($0.5 \text{ mg}\cdot\text{L}^{-1}$), GST induction was suppressed, suggesting an antagonistic interaction; however, under single-compound exposure, GST remained active as a primary detoxification route [44].

Together, the observed activities of CAT, SOD, and GST demonstrate that mussels employ a multi-faceted antioxidant strategy to deal with EE2 exposure, agreeing with the broader literature stating that pollutants like EE2 elicit integrated antioxidant and biotransformation defenses in bivalves [44].

Despite the activation of some oxidative stress defenses, indicators of cellular damage, like UBI and MDA levels, showed no statistically significant changes across EE2 treatments. These findings suggest that the antioxidant and detoxification responses were largely

successful in mitigating oxidative injury over the 28-day exposure. Our findings align with previous studies showing that moderate, sub-lethal EE2 exposure can trigger antioxidant defenses without causing immediate oxidative damage. Cunha et al. [44] also reported that mussels chronically exposed to EE2 exhibited elevated antioxidant enzyme activities (SOD, GPx, and GST) but showed no significant increase in lipid peroxidation or protein carbonylation compared to controls. Furthermore, the same authors demonstrated that marked lipid and protein oxidative damage was only observed when EE2 was combined with an additional stressor (salicylic acid), highlighting that EE2 alone, at environmentally relevant levels, may not cause evident cellular injury. This aligns with our observation that single-compound exposure to EE2 did not elicit MDA or UBI changes, despite the induction of some antioxidant enzymes. It appears that *M. galloprovincialis* was capable of effectively counteracting the ROS produced by exposure to EE2, thereby preventing cell damage.

On the other hand, Canesi et al. [53] found a significant MDA rise in mussel digestive gland tissue upon exposure to a low-dose mixture of EDCs (including EE2), leading to oxidative damage, which coincided with a depression of catalase transcription.

Mechanistically, the lack of elevated MDA and UBI levels in EE2-exposed mussels may reflect an effective adaptive or compensatory cellular response. The induced antioxidants (CAT, SOD, and GST) and possibly other antioxidant enzymes or compounds (e.g., glutathione peroxidase) likely neutralized the ROS, preventing lipid and protein damage.

Additionally, the relatively stable UBI levels across treatments, except for mussels exposed to $300 \text{ ng}\cdot\text{L}^{-1}$ where results were highly variable, suggest that the proteasome system was not overwhelmed in mussels exposed to EE2, and damaged proteins were likely degraded efficiently, keeping ubiquitin-protein conjugate levels steady.

It is also worth noting that *M. galloprovincialis* is considered a robust species that frequently endures fluctuations in oxygen levels, pollutants, and other stressors in coastal environments [54–56]. They possess constitutively high antioxidant baseline levels, as an adaptation to intertidal oxidative challenges [57], which likely contribute to the resilience observed in the present study. However, it is possible that with a more prolonged exposure period, the cell's antioxidant mechanisms might lose efficiency.

Several studies have shown a strong correlation between exposure to EE2 and VTG expression [58,59]. For instance, Ciocan et al. [59] exposed *M. edulis* to 5 and $50 \text{ ng}\cdot\text{L}^{-1}$ of EE2 for 10 days and found a significant increase in VTG and ER2 mRNA expression at the early stage of gametogenesis; however, no significant changes were found in mature mussels. In our study, Vitellogenin-like (VTG-like) protein levels increased according to the EE2 exposure concentrations, confirming the estrogenic mode of action of this contaminant. It is important to note that the present study utilized waterborne exposure. However, in natural environments, bivalves are also exposed to EE2 through dietary uptake of contaminated suspended particles. Given the lipophilic nature of EE2, dietary ingestion may lead to higher internal doses and potentially more severe endocrine disruption than waterborne exposure alone [60].

While fish are typically more sensitive and show VTG induction at much lower concentrations, our results show that mussels also respond to xenoestrogens by synthesizing vitellogenin or its analogs. Notably, even the lowest tested concentration ($10 \text{ ng}\cdot\text{L}^{-1}$) led to an increase in VTG-like proteins, suggesting that *M. galloprovincialis* is sensitive to EE2 at environmentally relevant concentrations. These results agree with field and laboratory studies; for instance, *M. galloprovincialis* collected from polluted canals in Venice was found to have significantly elevated VTG-like protein compared to those from reference sites, suggesting exposure to environmental xenoestrogens [61]. Additionally, Quinn et al. [62]

exposed zebra mussels (*Dreissena polymorpha*) to municipal effluent, known to contain a mixture of xenoestrogens, and observed a clear increase in VTG (measured as ALP).

However, work by Morthorst et al. [63] raised concerns about false positives in VTG determination when using the ALP method, as it can be influenced by other phosphorylated proteins. They found no estrogen-induced increase in freshwater mussels (*Unio tumidus*) exposed to 17 β -estradiol for seven weeks. Fernandez-González et al. [64] exposed *M. galloprovincialis* to 100 ng·L⁻¹ EE2 and found no evidence of VTG induction in the gonads at the transcriptional level and stated that VTG could not be an adequate biomarker for xenoestrogenicity in marine mussels. This suggests that a cautious interpretation of our findings is necessary; while the elevation in VTG-like proteins was evident, the eventual non-specific interference in the assay performed must be considered.

Studies in fish (e.g., male rainbow trout or zebrafish) exposed to EE2 concentrations ranging from 10 to 100 ng·L⁻¹ caused the induction of vitellogenin [62]. In contrast, mussels exhibit only modest, though still biologically relevant, VTG responses at comparable concentrations. This reduced sensitivity may be attributed to a lower affinity of molluscan estrogen receptors for EE2, variations in endocrine signaling pathways, or the presence of limiting co-factors involved in vitellogenin synthesis. In fact, the distinct sensitivity thresholds observed between mussels and fish are related to different endocrine systems [65]. In fish, EE2 acts as a potent agonist of the nuclear estrogen receptor, leading to rapid transcriptional changes such as the induction of vitellogenin [26,66]. Whereas in mussels, the typical vertebrate estrogen receptor is missing or unresponsive to estrogens [65]. Instead, estrogenic signaling in these invertebrates may occur via modulation of endogenous steroid metabolism [65].

Other studies also indicate that longer periods of exposure (3 to 4 months) to low EE2 concentrations (tens of ng·L⁻¹ or less) have led to significant VTG elevation [61,62].

The biological implications of VTG-like protein induction in mussels are significant, as it can indicate endocrine disruption, with concomitant impairment of their reproductive capacity. For example, Blaise et al. [67] observed that caged freshwater mussels downstream of a sewage effluent had skewed sex ratios with a higher proportion of females, suggesting that chronic estrogenic exposure might impair male viability or alter sex determination.

5. Conclusions

This study demonstrates that, after 28 days of exposure, *M. galloprovincialis* exhibited significant biochemical alterations, specifically in GST and CAT activities. These changes reflect a coordinated response to detoxify EE2 and mitigate oxidative stress through diverse antioxidant mechanisms. Notably, MDA and UBI levels showed no significant changes across all tested concentrations, suggesting that the antioxidant defenses effectively prevented lipid peroxidation and protein damage.

However, the observed increase in VTG-like proteins correlated with EE2 concentrations, suggesting potential endocrine disruption. These findings reinforce the use of *M. galloprovincialis* as a sentinel species for monitoring xenobiotics, including EDCs, in coastal environments. Our results indicate that even at low ng·L⁻¹ levels, EE2 can trigger an estrogenic response, which carries significant implications for both the organism's health and population-level dynamics, particularly during sensitive developmental or reproductive windows.

Given the contradictory results in the current literature regarding EE2 exposure and VTG induction, further research is crucial. The various studies on EE2 exposure and VTG induction appear to show contradictory results, and further research is needed to clarify this issue. Thus, future studies should utilize: PCR (qPCR) to determine mRNA expression levels of vitellogenin genes; ER binding assays; mass spectrometry (LC-MS/MS) for precise

quantification of VTG-like protein isoforms; and enzyme-linked immunosorbent assays (ELISA) for bivalve-specific protein sequences detection.

Furthermore, ALP assay results must be interpreted with caution due to the risk of false positives; integrating complementary tools (e.g., proteomics, transcriptomics) will be essential to validate these pathways.

Future research should also explore the co-exposure of EE2 with other environmental stressors (e.g., rising sea temperatures, hypoxia, changing salinity, or mixture with other contaminants) to better understand their ecological relevance.

Finally, since mussels are a food source in the human diet, the presence of these contaminants poses a potential risk to human health, which warrants further investigation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jmse14090829/s1>, Supplementary Table S1. Size and weight of *Mytilus galloprovincialis* across the different exposure concentrations. Supplementary Table S2. Mortality rate registered by treatment and tank during the experiment period. Supplementary Table S3. Biomarker responses in *Mytilus galloprovincialis* exposed to the different EE2 concentrations. Supplementary Table S4. *p*-values for Glutathione-S-Transferase (GST) Activity. Supplementary Table S5. *p*-values for Superoxide Dismutase (SOD) Activity. Supplementary Table S6. *p*-values for Catalase Activity. Supplementary Table S7. *p*-values for Lipid Peroxidation. Supplementary Table S8. *p*-values for Total Ubiquitin. Supplementary Table S9. *p*-values for Vitellogenin. Supplementary Table S10. physicochemical parameters during the experiment period (average).

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