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The Impact of Perfluorooctanoic Acid (PFOA) on the Mussel *Mytilus galloprovincialis*: A Multi-Biomarker Evaluation

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Abstract: Perfluorooctanoic acid (PFOA) has been widely studied due to its environmental persistence and bioaccumulation potential, raising concerns about its effects on aquatic life. This research evaluates the impact of PFOA on the antioxidant defenses and stress response systems of the mussel *Mytilus galloprovincialis*. Mussels were exposed to three concentrations of PFOA (1, 10, and 100 $\mu g \cdot L^{-1}$) over 28 days. Several biomarkers, including glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LPO), total antioxidant capacity (TAC), vitellogenin (VTG), ubiquitin (UBI), and caspase-3 (CASP) were analyzed. The results suggest stress responses, particularly in animals exposed to higher concentrations, as shown by GST and SOD activities which increased according to PFOA concentrations. Additionally, oxidative stress markers such as MDA and CAT showed variable responses depending on the exposure concentration tested. This study underscores the need for further investigation into the effects of PFOA on mollusks but also the need to unveil gender-specific responses in aquatic organisms exposed to this contaminant. The concentrations of PFOA used in our research are lower than those examined in previous studies, providing crucial insights into the impacts of even minimal exposure levels. It highlights the potential of *M. galloprovincialis* as a bioindicator in environmental monitoring programs, providing crucial insights for environmental management and policymaking regarding regulating and monitoring PFOA in marine settings. Consequently, in a country where seafood consumption is the second largest in Europe, implementing environmental policies and regulatory measures to manage and monitor PFOA levels in marine environments is crucial.

Keywords: perfluorooctanoic acid (PFOA); *Mytilus galloprovincialis*; antioxidant defense; oxidative stress; endocrine disruptors; environmental monitoring

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1. Introduction

Perfluorinated organic compounds (PFCs) have emerged as significant environmental contaminants due to their stable carbon-fluorine bonds, which contribute to their persistence and potential for bioaccumulation in the environment, humans, and various organisms, including aquatic animals like fish and bivalves [1–3]. These compounds are distinguished by replacing all hydrogen atoms on the carbon chain with fluorine, resulting

in extreme stability and unique properties such as low surface energy, hydrophobicity, and lipophobicity [4–6]. Perfluorooctanesulfonic acid (PFOS) and Perfluorooctanoic acid (PFOA) were historically the most used PFCs until 2001, when PFOS production ceased, shifting the prevalence to PFOA [7]. Exposure to these compounds, particularly PFOS and PFOA, has been linked to a range of adverse health effects, including hepatic, immunological, reproductive, neurobehavioral, and hormonal disturbances, as well as genotoxic and carcinogenic potential [8]. PFCs are used in a wide range of products and industries, such as textile treatment, metal planting, firefighting and semiconductors [9]. There are two ways that PFCs might enter the environment: directly or indirectly. The incorrect disposal or the discharge of waste into the atmosphere from the industrial facilities. PFCs are furthermore released directly into surface water through the effluent of wastewater treatment plants. Indirectly by precursors such as fluorotelomers alcohols that was transformed in perfluoroalkyl carboxylic acids (PFCAs) and perfluoro sulphonic acids (PFSAs) by abiotic or biological mechanisms [10]. PFCs are released into the environment, becoming widespread in water [11], sediment [12], dust [13], and wildlife [14,15]. Consequently, human exposure occurs through various routes, predominantly diet and inhalation [16]. Various studies have reported that the concentrations of PFOA and PFOS in surface water can vary considerably. PFOA levels have been documented between 0.09 and 1270 $ng \cdot L^{-1}$, while PFOS levels have been observed to range from 0.06 to 144 $ng \cdot L^{-1}$ [17–22]. Recognizing dietary intake as a major exposure route, the European Commission proposed directive 2017/0332, setting drinking water limits at 0.4 $\mu g \cdot L^{-1}$ for PFOS and 4 $\mu g \cdot L^{-1}$ for PFOA [23]. Directive 2017/0332, later amended and implemented as Directive 2020/2184, addresses PFAS concentrations in drinking water, focusing only on these substances' total and sum concentrations. It sets a new group limit of 0.5 µg/L for PFAS, along with a limit of 0.1 µg/L for 16 specific individual PFAS [24]. The European Food Safety Authority (EFSA) further highlighted fish, meat, eggs, milk, dairy products, and drinking water as significant sources of exposure [23]. Drinking water, particularly in contaminated areas, remains a significant source of PFAS exposure [25]. Tolerable weekly intakes (TWI) of 13 $ng \cdot kg^{-1}$ bw for PFOS and 6 $ng \cdot kg^{-1}$ bw for PFOA have been established [23]. Despite multiple exposure sources, diet remains the predominant route for most populations [26]. In 2020, EFSA set a new TWI for PFOA, PFOS, perfluorononanoic acid (PFNA), and PFHxS at 4.4 $ng \cdot kg^{-1}$ bw per week, significantly lower than previous limits [26]. The European Union and the United States Environmental Protection Agency have also explored alternatives to PFOA and its compounds [26]. The presence of high PFOA concentrations near fluorochemical plants in Fuxin, China, as reported by Zhao et al. [7], is a significant cause for concern. For PFOA, milk, dairy products, drinking water, and fish are the main sources of chronic exposure. Associations between PFOA levels and kidney and testicular cancers have been observed [27,28]. In 2018, the International Agency for Research on Cancer classified PFOA as possibly carcinogenic (Group 2B). PFOA and PFOS are not metabolized but accumulate in the liver, kidneys, and blood [29,30]. Exposure to PFAS during pregnancy has been linked to adverse health outcomes, including increased risks of preeclampsia, preterm birth, and low birth weight [23]. PFAS exposure induces oxidative stress in aquatic organisms, affecting antioxidant enzymes activities and detoxification pathways [31–37]. Disruption of nuclear receptors, fatty acid oxidation, and mitochondrial permeability contribute to oxidative stress [38]. According to some studies [39,40], this exposure to PFOA can have a negative effect on biological pathways related to detoxification, lipid metabolism, and xenobiotic metabolism. In a study performed on water flea (*Daphnia carinata*), the 48-h lethal concentration 50 (LC50) values for acute toxicity were 78.2 $mg \cdot L^{-1}$ (with a confidence interval of 54.9–105) for PFOA and 8.8 $mg \cdot L^{-1}$ (with a confidence interval of 6.4–11.6) for PFOS [41]. Sentinel organisms, like bivalves, play a crucial role in monitoring environmental pollution and assessing ecosystem health, especially in coastal marine environments [42]. They accumulate pollutants in their tissues by filtering large volumes of water for feeding. These organisms are highly sensitive to pollutants and provide early warning signs of environmental stress [43]. Mussels, in particular, are extensively used as sentinel organisms

to assess contamination levels in marine environments, as their efficient filtration abilities allow them to accumulate both organic and inorganic pollutants, making them effective bioindicators in aquatic ecosystems, and also they have a broad distribution [44]. By examining their responses to contaminants, we can infer the potential impacts on the broader ecosystem. The cellular antioxidant defence system protects cells from oxidative stress by activating specific enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). These enzymes are vital in mitigating oxidative damage and are frequently used as biomarkers to detect oxidative stress caused by exposure to environmental contaminants. Exposure to PFOA notably impacts the activity levels of these key antioxidant enzymes [45–48]. Specifically, the activities of SOD and CAT are reduced, whereas GPx activity significantly increases in response to PFOA exposure, indicating a shift in the oxidative balance within cells [42]. In certain studies, the activity levels of SOD, CAT, and GPx showed notable changes, which are crucial markers of oxidative stress. Specifically, CAT and SOD activities decreased, while GPx activity increased significantly in response to PFOA exposure [42,49]. Malondialdehyde (MDA), a byproduct of lipid peroxidation, is often used to measure the level of oxidative damage in cells. According to some studies, an increase in MDA levels suggest increased oxidative stress in *M. edulis* following exposure to PFOA [38,50–52]. Exposure to PFOA notably impacts the activity levels of these key antioxidant enzymes [45–48]. Specifically, the activities of SOD and CAT are reduced, whereas GPx activity significantly increases in response to PFOA exposure, indicating a shift in the oxidative balance within cells [42]. Previous studies have also shown that exposure to per- and polyfluoroalkyl substances (PFASs) triggers oxidative stress in various aquatic species [32,38,53]. In the hepatocytes of *Oreochromis niloticus*, the activities of SOD, CAT, and glutathione reductase (GR) increased following exposure to both PFOS and PFOA [33]. Additionally, increased MDA concentration was observed in *Pimephales promelas* after PFOS exposure, demonstrating these substances' oxidative impact [34,35].

Although PFOA has been globally phased out due to its persistence and toxicity, rising concentrations have been observed in drinking water (with a mean of 3.55 $\mu g \cdot L^{-1}$), seawater (ranging from 8.8 to 16.2 $ng \cdot L^{-1}$), and human blood (between 0.3 and 5.4 $\mu g \cdot L^{-1}$). This increase is attributed to the degradation of precursor compounds, terrestrial runoff, and atmospheric transport [54–57]. There have been a number of studies indicating the toxicity of exposure to PFAS in aquatic organisms, with various effects at cellular and molecular level, particularly in fish [38,58]. However, there are few or no studies on the toxicity of PFOA in other aquatic organisms, particularly in bivalves such as mussels. There is therefore an urgent need to assess the toxicity of PFOA in these organisms, which are also consumed by humans.

The current study aims to elucidate the biochemical mechanisms by which PFOA affects *Mytilus galloprovincialis*. The individuals were subjected to different PFOA concentrations (0, 1, 10, and 100 $\mu g \cdot L^{-1}$) over 28 days. GST, SOD, CAT, LPO, and TAC were used to assess antioxidant responses. Ubiquitin (UBI) and caspase (CASP) were also evaluated, which are involved in damaged protein degradation and apoptosis. Vitellogenin-like protein (VTG), a biomarker of endocrine disruption, was also measured. It is anticipated that the results of this study can help to understand the environmental impact of PFOA, particularly in marine species where these compounds can accumulate and pose risks to state exposure to various ecosystems

2. Materials and Methods

2.1. PFOA Stock Solution

For the preparation of the stock solution of PFOA (CAS No 335-67-1, Sigma-Aldrich, Shanghai, China), 0.1 g of the compound was dissolved in 100 mL of methanol (99.8% *v*/*v*; Honeywell, Seelze, Germany).

2.2. Experimental Trial

The experimental setup used to expose mussels to PFOA was as described previously [54–56,59]. *M. galloprovincialis* (Lamarck, 1819) were gathered manually at Guincho coast (Cascais, Lisbon, Portugal) in February 2023. Mussels of similar sizes were collected to avoid variations in bioaccumulation and biomarker responses caused by differences in size or weight. The specimens were transported to the laboratory facilities at NOVA School of Science and Technology in a thermal container, to maintain the temperature constant and avoid additional stress. The mussels were then acclimatized for five days in a 50 L aquarium using seawater from Guincho, equipped with filtration, recirculation, and aeration systems. They were maintained under controlled conditions with a pH of 8.1 ± 0.2, temperature at 20.0 ± 1.0 °C, and salinity of 33 ± 1 g·L^{-1}. The photoperiod was set to 12 h of light and 12 h of darkness, ensuring dissolved oxygen levels remained above 6 mg·L^{-1} throughout the acclimatization process.

Mussels (0.735 ± 0.209 g) were randomly distributed among four aquariums, each with 8 individuals. Each tank was properly separated to avoid mixing or any risk of cross-contamination and the exposure assay was carried out under static conditions. Moreover, water quality parameters (temperature, pH, and salinity) were checked daily in each aquarium.

The appropriate volume of PFOA was taken from the stock solution (Section 2.1) and added to each 8-L aquarium to achieve the concentrations required for the exposure assay (1, 10 and 100 µg·L^{-1}), and these conditions were renewed every 48 h. For controls, an additional aquarium with filtered seawater was used. The methanol concentration in each aquarium was estimated to be <0.01% according to the dilution factor.

The realistic criterion of 1000 ng·L^{-1} for the studied concentrations is based on documented levels of PFOA ranging from 0.09 to 1270 ng·L^{-1} and PFOS from 0.06 to 144 ng·L^{-1}, according to diverse studies [60], however, these concentrations can vary considerably. Recognizing dietary intake as a major exposure route, the European Commission pro-posed directive 2017/0332, setting drinking water limits at 0.4 µg·L^{-1} for PFOS and 4 µg·L^{-1} for PFOA. Higher concentrations to realistic concentrations were also tested to better understand their effects on the animals. In addition, a control aquarium was also used with ≤0.01% methanol in seawater. The animals were fed daily with 1.5 mg/L of *Chlorella* sp. (Shine superfood, Portugal) previously dissolved in seawater.

Ethics

This work complied with the relevant policies and standards regarding animal experimentation and welfare (e.g., Directive 2010/63/EU) and ARRIVE guidelines. The collection of organisms and experimentation was authorized by the national authorities (DGRM and ICNF) and the faculty's ethics committee. The researchers who carried out the experiments on the mussels are certified at level C by the European Federation for Laboratory Animal Science (FELASA) and have extensive experience in animal experimentation.

2.3. Sample Treatment

At the end of the experiment, the animals were weighed using an analytical balance (Sartorius, Germany) and measured with a micrometer. Subsequently, the whole body of each mussel (9 males and 23 females) was separated from the shell using a scalpel and tweezers, collected, and stored in microtubes at −45 °C. Gender identification was based on the visual observation of gonads, with males showing a white-cream coloration and females exhibiting an orange-pink coloration. The specimens were then processed using a Tissue Master 125 tissue homogenizer (Kennesaw, GA, USA) in a 3.0 mL phosphate-buffered saline (PBS) solution [61]. This PBS solution consisted of NaCl (140 mM; Panreac, Barcelona, Spain), Na_2HPO_4 (10 mM; Sigma-Aldrich, St. Louis, MO, USA), KCl (3 mM; Merck, Darmstadt, Germany), and KH_2PO_4 (2 mM; Sigma-Aldrich, Steinheim, Germany), adjusted to a pH of 7.3 ± 0.2. Following homogenization, the samples were centrifuged at 15,000× *g* for 10 min at 4 °C using a VWR CT 15RE centrifuge (Tokyo, Japan). The resulting

supernatant was then transferred to 1.5 mL microtubes (in duplicate) and stored at −45 °C until biomarker analysis could be conducted. Samples were thawed as needed for each assay, and all procedures were carried out on ice to maintain sample integrity.

2.4. Total Protein

The Bradford method was employed to ascertain the total protein content [62]. Calibration standards were prepared using Bovine Serum Albumin (BSA; Nzytech, Lisboa, Portugal) to establish a calibration curve ranging from 0 to 4 mg·mL^{-1}. Subsequently, 20 µL of each standard or sample was combined with 180 µL of Bradford Reagent in a 96-well microplate (Greiner Bio-One, GmbH, Kremsmünster, Austria). The absorbance of each well was measured at 595 nm using a Synergy HTX Multi-Mode Reader microplate reader (BioTek, Winooski, VT, USA). The total protein concentration in the samples was determined from the calibration curve, and the results were expressed in mg·mL^{-1}. The cytosolic protein concentration (PROT) was calculated for normalization purposes.

2.5. Biomarkers Analyses

2.5.1. Glutathione S-Transferase (GST) Activity

The protocol initially described by W. H. Habig et al. [63] was adapted and optimized for 96-well microplates [49]. To determine the specific activity of GST, a molar extinction coefficient of 5.3 mM^{-1}·cm^{-1} for 1-chloro-2,4-dinitrobenzene (CDNB) was employed. In each microplate well, 20 µL of the sample was combined with 180 µL of the substrate solution, which consisted of 19.6 mL of PBS buffer, 200 µL of reduced L-Glutathione (200 mM; GSH; Sigma-Aldrich, St. Louis, MO, USA), and 200 µL of 100 mM CDNB (Sigma-Aldrich, St. Louis, MO, USA). The absorbance at 340 nm was measured every minute for six minutes using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). GST activity results were normalized to cytosolic protein content and expressed as nmol·min^{-1}·mg^{-1} cytosolic protein.

2.5.2. Superoxide Dismutase (SOD) Activity

To assess SOD activity, the Nitroblue Tetrazolium (NBT) method, as described previously [64], was adapted for use with a 96-well microplate following the protocol described by Copeto et al. [59]. In each well, 200 µL of potassium phosphate buffer (50 mM; pH 8.0) was combined with 10 µL of EDTA (3 mM; Riedel-Haën, Seelze, Germany), xanthine (3 mM; Sigma-Aldrich, Shanghai, China), NBT (0.75 mM; Sigma-Aldrich, Steinheim, Germany), and the same volume of the respective sample. The reaction was initiated by adding 10 µL of xanthine oxidase (XOD; Sigma-Aldrich, Steinheim, Germany). Absorbance was monitored at 560 nm every two minutes for 20 min using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). SOD activity results were normalized to the total protein content and expressed as U·mg^{-1} cytosolic protein.

2.5.3. Catalase (CAT) Activity

CAT activity was determined following a method previously described [65], with modifications for a 96-well microplate [59]. In each well, 20 µL of the sample, 30 µL of methanol (Honeywell, Sellze, Germany), and 100 µL of potassium phosphate buffer (100 mM; pH 7.0; Sigma) were combined. The reaction was initiated by adding 20 µL of hydrogen peroxide (0.035 M; Sigma-Aldrich, Steinheim, Germany) to each well. The microplate was then placed on an orbital shaker (Optic Ivymen System, JP Selecta, Barcelona, Spain) and incubated in the dark at room temperature for 20 min. Subsequently, 30 µL of KOH (10 M; ChemLab, Zedelgem, Belgium) and 30 µL of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (32.4 mM in 0.5 M HCl; purpald; Aldrich, Steinheim, Germany) was added, followed by another incubation step under the same conditions for 10 min. Finally, 10 µL of potassium periodate (65.2 mM in 0.5 M KOH; Sigma-Aldrich) was introduced to each well and incubated for 5 min in the dark at room temperature. Absorbance readings were taken at 540 nm using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). A

calibration curve was established using formaldehyde (AppliChem, Darmstadt, Germany) standards ranging from 0 to 150 µM. CAT activity results were normalized to total protein content and expressed as nmol·min^{-1}·mg^{-1} cytosolic protein.

2.5.4. Lipid Peroxidation (LPO)

LPO was evaluated using the thiobarbituric acid assay, as previously described [59,66]. A calibration curve was constructed using malondialdehyde (MDA; Merck, Germany) concentrations ranging from 0 to 0.1 µM in Mili-Q ultrapure water. In 1.5 mL microtubes, 5 µL of the sample or standard was mixed with 45 µL of PBS buffer, 12.5 µL of sodium dodecyl sulfate (8.1% *w*/*v*; SDS; Sigma-Aldrich, Germany), 93.5 µL of trichloroacetic acid (20% *w*/*v*; TCA; Panreac, Barcelona, Spain), 93.5 µL of thiobarbituric acid (1% *w*/*v*; TBA; Sigma-Aldrich, Germany), and 50.5 µL of ultrapure water. Following a brief centrifugation at 3000× *g* for 30 s, the microtubes caps were punctured and then heated in a dry bath (Thermobloc Digital, Labnet, Dusseldorf, Germany) at 100 °C for 10 min. After cooling on ice, 62.5 µL of ultrapure water was added to each microtube, followed by another centrifugation at 3000× *g* for 30 s. Subsequently, 150 µL of each sample was transferred to the microplate wells, and the absorbance was read at 530 nm using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). The LPO results were expressed relative to the total protein content, being the [MDA] expressed as nmol·mg^{-1} cytosolic protein.

2.5.5. Total Antioxidant Capacity (TAC)

The total antioxidant capacity was determined following the protocol described by Kambayashi et al. [67]. Calibration curves were prepared using standards of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Aldrich, Russian Federation) diluted in a potassium phosphate buffer, which included potassium phosphate monobasic (5 mM; pH 7.4; Sigma), glucose (5.55 mM), and sodium chloride (154 mM; NaCl; Panreac), with concentrations ranging from 0 to 0.330 mM. Subsequently, 10 µL of the sample or standard, 10 µL of myoglobin (90 µM; Sigma, USA), and 150 µL of 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (600 µM; ABTS; Alfa Aesar, Karlsruhe, Germany) were dispensed into the microplate wells (Greiner Bio-one, GmbH, Kremsmünster, Austria). The reaction was initiated by adding 40 µL of hydrogen peroxide (500 µM; Sigma-Aldrich) to each well. After incubating for 5 min at room temperature, absorbance readings were taken using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). The TAC values were then standardized based on the total cytosolic protein content and expressed as µmol·mg^{-1} cytosolic protein.

2.5.6. Caspase-3 (CASP-3)

Caspase-3 levels were measured using an indirect ELISA [68]. To create a calibration curve, standards of caspase-3 (recombinant human active cc119; Merck, Rahway, NJ, USA) were prepared in PBS buffer at varying concentrations from 0 to 5 µg.mL^{-1}. Each microplate well (Greiner Bio-one, Microlon 600 High Binding, Frickenhausen, Germany) received 50 µL of sample or standard and was incubated at 4 °C for 24 h. The wells were washed with a 0.05% PBS-Tween solution (Panreac, Spain). A blocking step followed, involving 100 µL of 1% BSA in PBS (Nzytech, Lisboa, Portugal) added to each well and incubated at room temperature for 90 min. After three more washes with PBS-Tween, 50 µL of the primary antibody (anti-caspase 3 ab13847, Abcam, Amsterdam, the Netherlands) diluted to 1.5 µg·mL^{-1} in 1% BSA was added, and the wells were incubated again at 4 °C for another 24 h. Following three washes, 50 µL of the secondary antibody (anti-mouse IgG Fc specific-alkaline phosphatase, Sigma, State of Israel) diluted to 1.0 µg·mL^{-1} in PBS with 1% BSA was added and incubated at 37 °C for 90 min. After a final wash, 50 µL of substrate solution consisting of 157 mg Trizma hydrochloride (Tris-HCl; Sigma, USA), 58 mg NaCl (Panreac), 50 µL MgCl2 (5 mM; Fluka, BioChemika, Buchs, Switzerland), and 10 mg 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP; Sigma-Aldrich, Gillingham, UK) in 10 mL distilled water, pH 9, was added to each well. The reaction was stopped by adding

50 µL of 3M NaOH (Panreac, Spain) after 15 min at room temperature. Absorbance at 405 nm was recorded using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). Results were normalized to the total protein content and results are expressed as $\mu g \cdot mg^{-1}$ cytosolic protein.

2.5.7. Total Ubiquitin (UBI)

Total ubiquitin levels were measured via an indirect ELISA, following the protocol previously described [68]. Ubiquitin standards from UBPBio (Dallas, TX, USA) created a calibration curve with concentrations ranging from 0 to 0.8 $\mu g \cdot mL^{-1}$. The volume of sample or standard in each well was 50 µL. The primary ubiquitin antibody (P4D1; Sc-8017; Santa Cruz Biotechnology, Dallas, TX, USA) was diluted to 1.5 $\mu g \cdot mL^{-1}$ in 1% BSA (w/v) and dispensed into each microplate well. The results were adjusted for total cytosolic protein content and expressed as $\mu g \cdot mg^{-1}$.

2.5.8. Vitellogenin (VTG)

The vitellogenin levels were extracted and measured by following the Ueda (1970) method [69], adapted for a 96-well microplate. Briefly, 20 µL of either sample or standard was added to each well along with 140 µL of distilled water and 35 µL of vanadate-molybdate reagent (Sigma-Aldrich, St. Louis, USA). A calibration curve was set using potassium dihydrogen phosphate (inorganic phosphate) concentrations ranging from 0 to 30 $\mu g.mL^{-1}$. Absorbance readings were taken with a Synergy HTX microplate reader at 470 nm. The results were expressed in µg of inorganic phosphate per mg of protein.

2.6. Statistical Analysis

Statistical analyses were conducted using Prism 9 (GraphPad Software, version 9.5.1). The choice of test was determined based on whether the data met parametric assumptions. For data satisfying these assumptions, a one-way ANOVA followed by Dunnett's multiple comparison test was performed. In cases where parametric assumptions were not met, the Kruskal–Wallis test, also followed by Dunnett's multiple comparison test, was used. Additionally, the non-parametric Spearman's rank correlation was employed to assess the strength and direction of linear relationships between pairs of variables.

3. Results

3.1. Mortality Rate

From the original 32 mussels that initiated the experience all survived at the end of the exposure period.

3.2. Biomarkers of Oxidative Stress

3.2.1. Glutathione S-Transferase (GST)

Figure 1A presents GST activity (mean ± standard deviation) results and shows an increasing activity trend in response to the various concentrations of PFOA. At a concentration of 1 $\mu g \cdot L^{-1}$, there is a slight rise in GST activity compared to the control, although this difference is not statistically significant. At 10 and 100 $\mu g \cdot L^{-1}$, GST activity increases significantly compared to control.

3.2.2. Superoxide Dismutase (SOD)

Figure 1B presents SOD activity, expressed as mean ± standard deviation, and reveals an upward trend in activity across varying concentrations tested over time. The tested concentrations significantly differ from the control. At 1 $\mu g \cdot L^{-1}$, SOD activity significantly increases compared to the control. Although SOD activity is further elevated at 10 $\mu g \cdot L^{-1}$, it is not significantly different from the 1 $\mu g \cdot L^{-1}$ level, but are significant different from the control. The 100 $\mu g \cdot L^{-1}$ concentration shows similar SOD activity to the 10 $\mu g \cdot L^{-1}$ level, with a significant increase compared to controls.

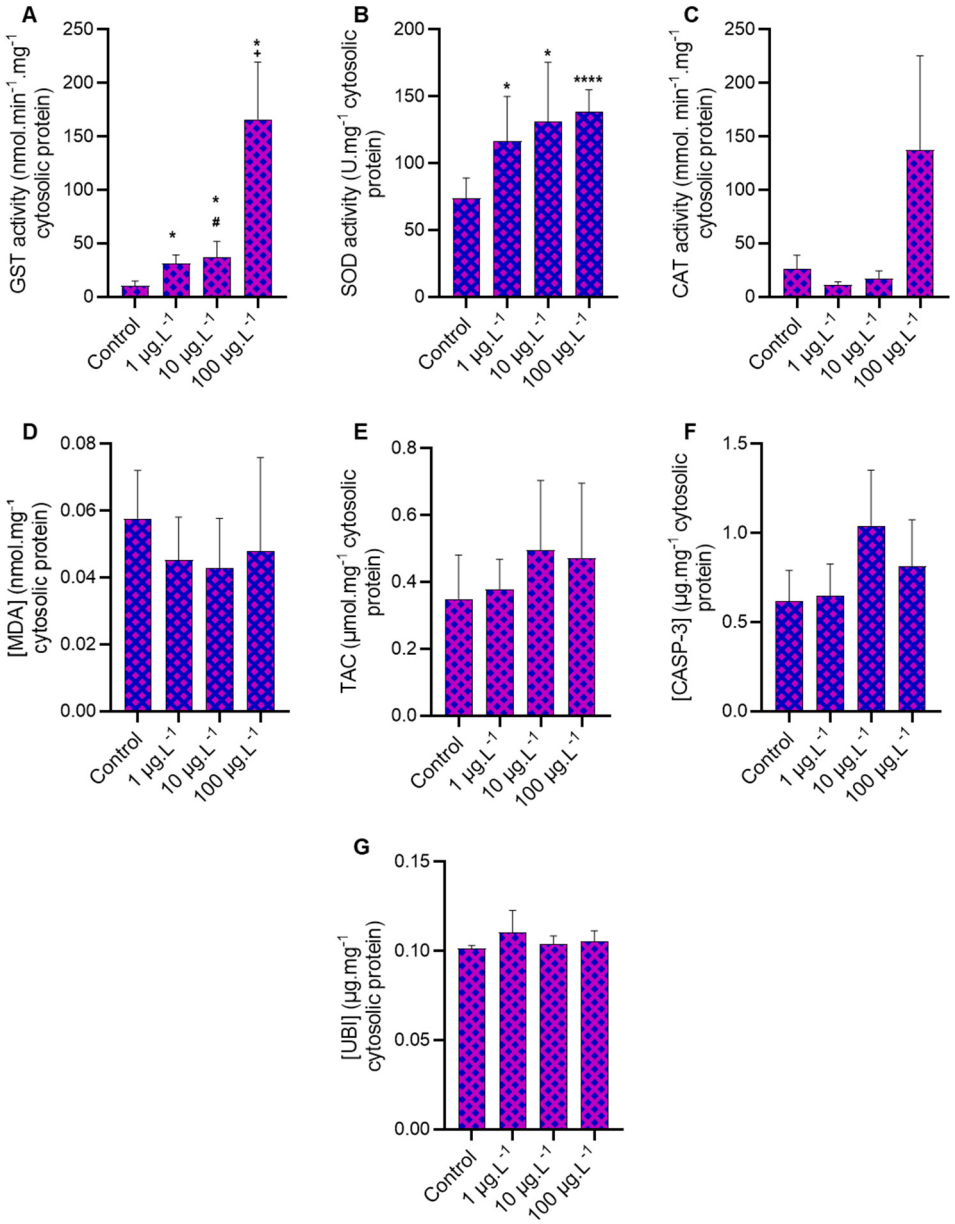

Figure 1. (**A**) GST, (**B**) SOD and (**C**) CAT activity, (**D**) LPO, (**E**) TAC, (**F**) CASP and (**G**) UBI levels in mussels exposed to different concentrations (0, 1, 10 and 100 µg·L^{-1}) of PFOA. All data are presented as mean ± s.d. *—$p < 0.05$ and ****—$p < 0.0001$, compared with control. #—$p < 0.05$, compared with 1 µg·L^{-1}, +—$p < 0.05$, compared with 10 µg·L^{-1}.

3.2.3. Catalase (CAT)

Figure 1C displays CAT activity results (mean ± standard deviation) for mussels exposed to varying concentrations of PFOA. The control group and lower concentrations (1 µg·L^{-1} and 10 µg·L^{-1}) exhibit relatively consistent and low levels of CAT activity, with only minor variations that are statistically non-significant when compared with the control group. At the 100 µg·L^{-1} exposure concentration, a marked increase but not statistically significant in CAT activity was observed, suggesting an antioxidant response at this higher concentration.

3.2.4. Lipid Peroxidation (LPO)

LPO is shown in Figure 1D, measured as the malondialdehyde (MDA) concentration, shows great variability and no discernible trend can be observed. Data suggests that the tested concentrations of the compound do not induce significant changes at this level.

3.2.5. Total Antioxidant Capacity (TAC)

Figure 1E shows a slight increase in total antioxidant capacity (TAC) according to the PFOA concentrations tested although no statistical differences were detected.

3.2.6. Caspase (CASP)

Figure 1F depicts caspase-3 concentrations across to the different PFOA concentrations, with higher average concentrations being determined in animals exposed to 10 $\mu g \cdot L^{-1}$ and at 100 $\mu g \cdot L^{-1}$ of PFOA, but no significant changes were detected.

3.2.7. Ubiquitin (UBI)

Figure 1G represents the ubiquitin (UBI) concentrations, which remained constant across the different PFOA concentrations tested. No significant changes were detected among the concentrations tested or in comparison to the control group.

3.2.8. Vitellogenin (VTG)

Figure 2 shows VTG values (mean ± standard deviation) for animals exposed to varying concentrations of PFOA. Although an increase in the VTG levels of the exposed females was observed according to the PFOA concentrations tested, this increase was not statistically significant. The VTG levels determined in the males remained similar for the PFOA concentrations tested.

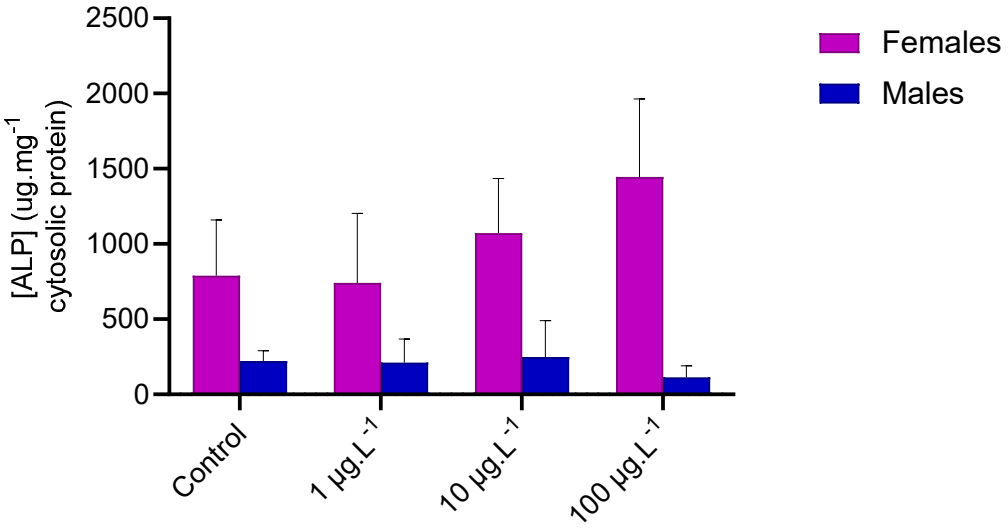

Figure 2. Total alkali-labile phosphate (ALP) concentration, in mussels exposed to different concentrations (0, 1, 10 and 100 $\mu g \cdot L^{-1}$) of PFOA. All data are presented as mean ± s.d.

3.3. Correlation Analyses

The results of correlation analysis (Spearman) are presented in Table 1.

Table 1. Spearman correlation matrix.

	GST	SOD	CAT	LPO	TAC	UBI	CASP	VTG
GST	1.00							
SOD	0.66 $p = 0.003$	1.00						
CAT	0.22 $p = 0.359$	0.06 $p = 0.780$	1.00					
LPO	−0.29 $p = 0.234$	0.04 $p = 0.856$	0.00 $p = 0.990$	1.00				
TAC	0.33 $p = 0.182$	0.48 $p = 0.020$	0.10 $p = 0.658$	0.09 $p = 0.695$	1.00			
UBI	0.26 $p = 0.308$	0.46 $p = 0.019$	−0.13 $p = 0.571$	−0.02 $p = 0.917$	0.64 $p = 0.003$	1.00		
CASP-3	0.22 $p = 0.367$	0.64 $p < 0.0001$	0.15 $p = 0.470$	−0.01 $p = 0.980$	0.82 $p < 0.0001$	0.58 $p = 0.004$	1.00	
VTG	−0.14 $p = 0.540$	0.26 $p = 0.172$	0.31 $p = 0.119$	0.31 $p = 0.113$	0.38 $p = 0.058$	0.14 $p = 0.501$	0.44 $p = 0.019$	1.00

The correlation matrix presented in Table 1 highlights significant relationships among several biochemical parameters related to oxidative stress and apoptotic activity, including GST, SOD, CAT, LPO, TAC, UBI, CASP-3, and VTG. A moderate positive correlation was observed between GST and SOD (r = 0.66, $p = 0.003$), indicating a potential link between glutathione transferase and superoxide dismutase activities. SOD also exhibited significant positive correlations with UBI (r = 0.46, $p = 0.019$) and CASP-3 (r = 0.64, $p < 0.0001$), suggesting that higher SOD activity is associated with moderate increased ubiquitin and caspase-3 levels. Furthermore, TAC showed a strong positive correlation with CASP-3 (r = 0.82, $p < 0.0001$), suggesting a close association between total antioxidant capacity and caspase-3 concentration, which may reflect an interaction between antioxidant defense mechanisms and apoptosis. UBI also showed a moderate significant correlation with TAC (r = 0.64, $p = 0.003$) and CASP-3 (r = 0.58, $p = 0.004$), further reinforcing an eventual connection between ubiquitin levels, antioxidant capacity, and apoptotic processes. In contrast, CAT and LPO did not show significant correlations with the other parameters, indicating that their roles may be more independent or complex within these interactions.

4. Discussion

It is well-known that PFASs induce oxidative stress [38]. Several studies indicate that PFAS exposure induces oxidative stress in aquatic organisms, affecting antioxidant enzyme activity and detoxification pathways [31–37]. The criteria of selecting 1 $\mu g \cdot L^{-1}$ for the lowest tested concentration was based on various studies that documented levels of PFOA in surface waters ranging from 0.09 to 1270 $ng \cdot L^{-1}$ [11,17–22]. This range reflects different contamination levels due to various factors such as proximity to industrial sources, urban runoff, and wastewater discharge. Higher concentrations were also tested to better understand their effects on the animals. Recent studies have shown the effects of different PFASs on oxidative stress biomarkers in marine organisms, specially mussels [70]. Glutathione S-transferase (GST) is a key enzyme in phase II of biotransformation, facilitating detoxification by catalyzing glutathione conjugating with toxic substances. This makes harmful compounds less reactive and easier to eliminate, indirectly contributing to antioxidant defenses by influencing glutathione availability [71]. In our study, GST activity in mussels showed significant changes after 28 days of exposure to PFOA, with enzyme levels rising notably in comparison to controls. Similarly, varying responses in GST activity were recorded by different studies and species following exposure to PFAS. For instance, in the Australian rainbowfish (*Melanotaenia fluviatilis*), GST activity increased

in the gills at the two highest concentrations of PFOA tested (1 and 10 mg·L^{-1}) during a 24-day exposure period. However, a decrease in activity was observed in the liver at the highest concentration [72]. Increased GST activity was also noted in the planarian *Dugesia japonica* after 10 days of exposure to PFOA across a range of concentrations (0.5, 5, 10, 20 mg·L^{-1}) [73] and in the digestive gland of the Mediterranean mussel *(M. galloprovincialis*) exposed to PFOS for 21 days (2–10 mg·L^{-1}) [74]. Inhibition of GST activity was also observed in the hepatocytes of Nile tilapia (*Oreochromis niloticus*) exposed for 24 h to PFOS or PFOA, particularly at the highest concentrations tested (1, 5, 15, and 30 mg·L^{-1}) [33]. These observations underscore the species-specific responses of GST activity to PFAS exposure, highlighting the intricate biochemical mechanisms in the detoxification and antioxidant defense systems of aquatic organisms, such as mussels. Elevated GST activity may protect against oxidative stress when antioxidant levels are diminished, as seen in previous studies where mussels transplanted to harbor areas exhibited increased GST levels to counteract oxidative damage [75]. Moreover, GST is a key enzyme in the biotransformation process, and its enhanced activity in mussels exposed to higher concentrations of contaminants, such as PFOA, suggests an adaptive detoxification response [71]. This enhanced GST activity could be linked to the organism's effort to mitigate the accumulation of harmful substances by facilitating their biotransformation and excretion. Of the antioxidant enzymes analyzed, SOD is considered the first line of defense that converts superoxide anion (O^{2-}) into hydrogen peroxide (H_2O_2), which is subsequently scavenged by CAT and/or GSH-Px [76]. Our findings suggest that after 28 days of exposure, SOD activity in mussels exposed to PFOA increased significantly compared to the controls. In a previous study, SOD activity determined in the green mussel (*Perna viridis*) exposed to a mixture of perfluorinated compounds including PFOS, PFOA, perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) for 7 days [77], increased at lower concentrations (0–100 µg·L^{-1}) but declined at the highest concentrations (100–10,000 µg·L^{-1}). Similarly, in the freshwater mollusk (*Unio ravoisieri*) exposed to PFOS at concentrations between 2 and 10 mg·L^{-1} for 7 days, SOD activity increased in those treated with 2 and 6 mg·L^{-1} but decreased in those exposed to 10 mg·L^{-1} [32]. An increase followed by a decrease in SOD activity was also observed in the amphipod (*Gammarus insensitives*) exposed to PFOS at concentrations of 1 mg·L^{-1}, 1.6 mg·L^{-1}, and 3.1 mg·L^{-1} over 4 days [78]. Conversely, in the zebrafish (*Danio rerio*), SOD activity in the liver increased after a 96-h exposure to 200 µg·L^{-1} of PFOS, while no changes were observed in the gills [79]. In goldfish (*Carassius auratus*), no changes in SOD activity were detected in the liver after exposure to PFOA concentrations of 1.21 and 12.1 µmol.L^{-1} over 4 days [80]. Other studies have shown a decrease in SOD activities following PFAS exposure, such as in the Chinese mitten crab (*Eriocheir sinensis*) exposed to high concentrations of PFOS (up to 10 mg·L^{-1}) for 21 days [81] and in the freshwater cladoceran (*Daphnia magna*) exposed to concentrations of 0.008–5 mg·L^{-1} of PFOS or PFNA, particularly at 0.2 mg·L^{-1}, over 7 days [82]. Since SOD serves as a primary defense against oxygen toxicity by catalyzing the conversion of superoxide anions into oxygen and hydrogen peroxide, an increase in SOD levels in the group exposed to the highest concentration, this suggests that this enzyme plays a crucial role in protecting mussels from exposure-related oxidative stress. This protective response has been also observed in mussels exposed to environmental stressors, where elevated SOD activities in the gills acted as a biomarker for oxidative stress [83]. Catalase (CAT), an enzyme within peroxisomes, is crucial for converting hydrogen peroxide (H_2O_2) into water and molecular oxygen. This study revealed that CAT activity in mussels significantly increased after 28 days of exposure to PFOA when compared to controls. Previous research has shown varied effects on CAT activity due to exposure to perfluorinated compounds. For example, CAT activity decreased in the liver of medaka fish (*Oryzias latipes*) after being exposed to PFOA at concentrations of 10, 50, and 100 mg·L^{-1} for 7 days [84] and in water fleas (*Daphnia magna*) exposed to PFOS or PFNA at a concentration of 0.04 mg·L^{-1} [82]. In contrast, increases in CAT activity were noted in both the digestive gland and gills of the freshwater mussel (*Unio ravoisieri*) exposed to PFOS ranging from 2–10 mg·L^{-1} [32]. The

Australian rainbowfish (*Melanotaenia fluviatilis*) showed increased CAT activity in the gills when exposed to 0.1 mg·L^{-1} of PFOA over 24 days. However, a decrease was observed in the liver at the highest concentrations tested [72]. Similarly, green mussels (*Perna viridis*) exhibited increased CAT activity after 7 days of exposure to PFOS, PFOA, PFNA, and PFDA up to a concentration of 100 µg·L^{-1} [77]. Furthermore, a notable increase in CAT activity was recorded in the hepatocytes of Nile tilapia (*Oreochromis niloticus*) exposed for 24 h to the highest concentrations of PFOS or PFOA tested (1, 5, 15, and 30 mg·L^{-1}) [33]. Conversely, in the liver of rice fish, CAT activity was inhibited following exposure to PFOA, particularly at a concentration of 50 mg·L^{-1} [84]. These findings suggest that the perfluorinated compound-induced response in CAT activity is sensitive and varies across species, likely due to differing interactions within their antioxidant enzymatic defense systems.

In this study, the changes observed in VTG concentrations are not statistically significant, even though there was an increase in vitellogenin levels, namely in females exposed to 100 µg·L^{-1}. As referred by other studies, estrogenic effects were only observed at PFOA concentrations equal or higher than 500 µg·L^{-1}, suggesting that 100 µg·L^{-1} is unlikely to have an estrogenic effect in mussels. In effect, previous research, such as that carried out by Kim et al. [85], identified PFOA as an estrogenic substance effective in inducing VTG in male common carp (*Cyprinus carpio*) at concentrations ranging from 500 to 50,000 µg·L^{-1}.

In the ALP method, the presence of baseline VTG levels is common, and an estrogenic effect is only considered when these levels are higher than 100 µg·P·mg^{-1} protein [86]. The use of the indirect-ALP method as a biomarker of VTG is not consensual in the scientific community, and some constraints have been pointed out. For example, a proteomic study by Sánchez-Marin [87] showed that the ALP method is not providing reliable information about Vtg levels. Thus, the methodology used may not be specific enough to distinguish between different phosphate sources, resulting in values that reflect a combination of factors, including natural reproductive biology and possible interference from other phosphorylated proteins. This lack of specificity can result in measurements that reflect multiple sources of phosphate, which are not necessarily related to vitellogenesis [88].

Additionally, PFOA has been shown to activate estrogen-responsive genes significantly, as evidenced by studies conducted by Wei et al. [89] in *Gobiocypris rarus*. However, Wei et al. [90] found PFOA to be a weaker estrogen inducer than 17β-estradiol (EE2), although its estrogen-like effects have been documented in various fish species. Molecular analysis in rainbow trout exposed to 200–1800 mg·L^{-1} of PFOA indicated estrogenic gene activations strongly correlated with those triggered by EE2 exposure [40]. PFOA also enhanced total aromatase activity, crucial for EE2 synthesis and regulation of internal levels [91]. Kang et al. (2019) reported that the gene expression patterns of VTG and choriogenin in Japanese medaka exposed to 10 mg·L^{-1} of PFOA for 21 days mirrored those in fish exposed to estradiol (E2), leading to adverse reproductive outcomes, including reduced fecundity [92]. Moreover, PFOA exposure was found to disrupt the thyroid T3/T4 ratio in the Australian rainbowfish (*Melanotaenia fluviatilis*), marking the first observation of its impact on circulating thyroid hormone ratios in fish. This disruption has implications for thyroid hormone homeostasis, an important indicator typically used to detect thyroid disturbances in laboratory and field studies [93–95].

In our study, the PFOA concentrations tested did not resulted in clear LPO or caspase-3 and ubiquitin changes following exposure, suggesting that these concentrations are not sufficient to trigger significant changes or there are defense mechanisms acting to protect cells.

However, it should be emphasized that due to the current lack of studies on the effects of PFOA on mussels, it is only possible to make comparisons with effects on other species, such as fish and few bivalve species. Regarding VTG, the discussion is made with fish species exposed to PFOA due to the lack of data in bivalves.

On the other hand, it should be noted that in most of these studies the concentrations of PFOA tested were higher (in the mg·L^{-1} range) than those tested in the present study. Therefore, up to 100 µg·L^{-1}, the detoxification and antioxidant mechanisms appear to

respond to protect the organism effectively. This also highlights the urgent need for further research in other aquatic animals, as bivalves.

Furthermore, our results, supported by the correlation analyses suggest a network of interconnected biochemical pathways where oxidative stress markers and ubiquitin are closely linked to apoptotic processes. In effect, the strong correlations observed between SOD, TAC, and CASP-3, along with their associations with ubiquitin, underscore the significance of these pathways at the cellular and molecular level to protect animals. However, the absence of significant correlations involving CAT and LPO suggests that further investigation is necessary to understand their roles within this context fully.

5. Conclusions

This study provides compelling evidence of the impact of PFOA on the antioxidant defense and stress response systems in the mussel *M. galloprovincialis*. The differential responses of oxidative stress markers, such as superoxide dismutase (SOD) and catalase (CAT), across the various PFOA concentrations tested, underscore the complexity of PFOA-induced oxidative stress. These findings highlight the importance of assessing the ecological risks of PFOA and similar contaminants in the marine biota.

It is also important to note that the concentrations used in this study are much lower than those tested by previous studies. The results suggest that up to 100 $\mu g \cdot L^{-1}$, the detoxification and antioxidant mechanisms can effectively protect the organism.

Moreover, the absence of observed mortality suggests that the mussels were able to manage their exposure to this level of contaminants successfully.

This research offers valuable insights for environmental management and policymaking, particularly in regulating and monitoring PFOA in marine ecosystems, especially in regions with high seafood consumption. Implementing stringent environmental policies to control PFOA levels in marine environments is crucial for mitigating potential risks to aquatic life and human health. Focusing on monitoring contaminant concentrations is essential for effective policy control. However, to achieve comprehensive contamination management, it must be complemented by preventive measures, remediation strategies, and health protection initiatives. By deepening our understanding of the biochemical effects of PFOA on marine organisms, this study contributes to the broader effort to address the challenges posed by persistent environmental contaminants.

Future research should continue to investigate the mechanisms underlying these responses and extend the scope to include other perfluorinated compounds and additional aquatic species. Such efforts are essential for developing comprehensive strategies for environmental protection and public health.

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