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Revealing hidden risks: *in vitro* analysis of PFAS hazards in *Mytilus galloprovincialis* gills and digestive gland

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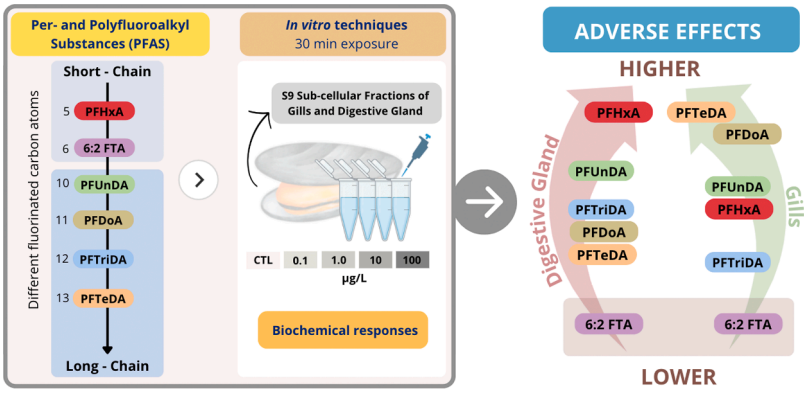
HIGHLIGHTS

- Of the PFAS investigated, 6:2 FTA was identified as the least toxic for both organs.
- Digestive gland was more sensitive to PFHxA, with reduced antioxidant defenses.
- Gills showed high oxidative stress from PFDoA and PFTeDA exposure.
- Anti-cholinesterase capacity observed in gills exposed to PFHxA and PFTriDA.
- *In vitro* assays can offer a reliable tool to assess PFAS adverse effects in mussels.

GRAPHICAL ABSTRACT



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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals known for their persistence and bioaccumulation, leading to widespread environmental contamination. Despite their recognised environmental risks, particularly to aquatic wildlife, including marine invertebrates, detailed impact studies are limited. PFAS can be categorised according to the length of the compound chain, with short-chain PFAS announced as a safer alternative to the more commonly used long-chain PFAS. However, recent evidence suggests that also short-chain PFAS pose significant environmental risks. The present study evaluated the adverse effects of six PFAS compounds—two short-chain (PFHxA, 6:2 FTA) and four long-chain (PFUnDA, PFDoA, PFTriDA, PFTeDA)— on the digestive gland and gills of mussels, *Mytilus galloprovincialis*, using *in vitro* assays. The results showed organ-specific responses: the digestive gland was more sensitive to PFHxA, with increased catalase activity and decreased total antioxidant capacity, and cellular damage was observed only at higher concentrations of PFTriDA. Gills were more affected by PFDoA and PFTeDA, with inhibited antioxidant enzyme activity and increased oxidative stress. PFHxA and PFTriDA also showed inhibition of acetylcholinesterase activity. 6:2 FTA

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had the lowest effects for both organs, while PFHxA was the most harmful. These findings underscore the need for thorough risk assessments of PFAS, considering both chain length and organ-specific effects.

1. Introduction

Globally, aquatic foods from aquaculture and fisheries represent approximately 17 % of the world's animal protein consumption, providing sustainable livelihoods for 10–12 % of the global population [1]. Nonetheless, aquaculture and shellfish harvesting in coastal and estuarine areas are constantly threatened by the presence of human-generated chemicals from different sources, being industrial waste identified as the main cause of marine environmental contamination [2,3]. Among them are per- and polyfluoroalkyl substances (PFAS), which are a group of chemicals characterized by their unique physicochemical properties (including oil and water repellence, temperature and chemical resistance, and surfactant properties) being used in a wide range of applications such as firefighting foams, non-stick metal coatings for kitchenware, paper food packaging, textiles for furniture and outdoor clothing [4]. These contaminants encompass an estimated 5000 to 10,000 chemicals, the majority of which exhibit significant resistance to environmental degradation under natural conditions, leading to their designation as "forever chemicals" [5]. Because of their unique properties, widespread production, and long-term persistence, PFAS have a wide environmental distribution, already found in coastal areas and estuaries including aquaculture and shellfish harvesting areas along the Atlantic and Mediterranean coasts [6–9]. PFAS and their transformation products were also identified in aquatic wildlife (namely fish and bivalves) and humans, demonstrating their potential for bioaccumulation and biomagnification [10–12]. Due to their bioaccumulation potential and associated risks for human health, PFAS have received increasing global public attention and have been classified as persistent organic pollutants of emerging concern [13]. These substances have been associated with numerous health issues, including impaired immune functions, chronic autoimmune disorders, hepatotoxicity, cancer, decreased fertility, and abnormal development [14,15]. Considering that dietary intake is one of the major routes of human PFAS exposure [16,17], the European Food Safety Authority (EFSA) has set tolerable weekly intakes (4.4 ng per kg of body weight per week) for perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), recommending the monitoring of PFAS of different chain lengths [16]. As a countermeasure of specific PFAS restrictions [18,19], the industry strives to generate alternative PFAS molecules and for most of the substitutes the information on their hazardous properties, environmental fate, and toxic effects is still very limited or non-existent [17]. There is, therefore, an urgent need to characterize the toxicity profiles of "emerging PFAS" congeners and obtain knowledge about putative adverse effects on endogenous resources such as bivalves, both under their natural environmental habitats but also subjected to lab-controlled conditions [5,20,21]. Newly emerging short-chain PFAS are significantly more persistent and mobile in aquatic environments, leading to increased potential risks for both human health and the environment [20,22]. This was recently emphasized in the Zürich Statement on PFAS, where scientists and policymakers point to the necessity of obtaining a mechanistic understanding of their toxicity for establishing a sound assessment and management of PFAS [23].

Given their persistence and widespread distribution, it is also important to note that PFAS concentrations in aquatic systems vary significantly across different environments, reflecting the global scale of contamination. Among the world's oceans, the highest levels have been detected in the Indian Ocean (41.1 ng/L), followed by the North Pacific (12.8 ng/L), the North Atlantic (4.0 ng/L), the South Atlantic (2.6 ng/L), the Arctic (1.1 ng/L), the South Pacific (0.5 ng/L), and the Southern Oceans (<0.1 ng/L) [7]. In surface waters from four river basins in Portugal (Ave, Leça, Antuã, and Cértima), the compounds detected at the highest concentrations were PFOS (22.6 ng/L) during the dry season and perfluorobutanoic acid (PFBA) (22.6 ng/L) during the wet season [24]. Llorca et al. [25] also quantified PFAS concentrations from surface water in Spain and the PFAS with the highest concentrations detected was PFOS (264 ng/L). In Melbourne (Australia), the average concentration of the sum of 33 PFAS (ΣPFAS) compounds in surface waters was measured at 78.6 ng/L [26]: the PFAS compounds with the highest median concentrations included PFBA (11.3 ng/L), perfluorohexanoic acid (PFHxA) (9.2 ng/L), PFOA (8.3 ng/L), PFOS (8.0 ng/L), perfluoropentanoic acid (PFPeA) (7.5 ng/L), perfluoroheptanoic acid (PFHpA) (3.2 ng/L), and perfluorohexanesulfonic acid (PFHxS) (2.9 ng/L). Furthermore, Bonato et al. [27] reported that PFAS concentrations of 3.4 μg/L had already been found in the surface water of the Veneto region (Italy). In the same study, concentrations of 7.9 μg/L were also detected in spring water and in industrially contaminated areas in the province of Vicenza concentrations peaked at up to 700 μg/L. In the open Western Mediterranean Sea waters, the ΣPFAS ranged from 246 to 515 pg/L for surface water samples [28]. According with Aminot et al. [6] the ΣPFAS concentrations (ng/L) found in European aquaculture areas were in Adriatic Sea (NW Greece): 0.1; Aveiro lagoon (Portugal): 0.1 – 0.4; Butrint lagoon (Albania): 0.3 – 0.5; Goro lagoon, northern Adriatic Sea (Italy): 0.1 – 1.0, Bergen fjord (Norway), salmon farm: 0.2 – 0.7 and bivalve farm: 0.3 – 2.0; Ebro River delta (Spain): 0.8 – 1.6; south coast (United Kingdom (UK)): 3.9 – 8.4; Northern Tyrrhenian Sea (Italy): 25.3 – 29.8. PFOS was the dominant compound in the Exe Estuary (UK) and was also present at notable levels in the Goro Lagoon (Italy), the Aveiro Lagoon (Portugal), and in fish farming facilities in the Norwegian fjord. In seawater, Hung et al. [29] in sea areas of Korea, found PFAS concentrations ranging from 1.74 (Gwangyang bay) to 5.09 (Masan Bay) ng/L. Moreover, the regulation of PFAS in drinking water has become a growing concern globally. The European Union has established a recommendation that the ΣPFAS be less than 500 ng/L, or the sum of 20 select PFAS be less than 100 ng/L [30].

Furthermore, the bioaccumulation of PFAS in aquatic organisms is another critical concern, particularly as these compounds tend to accumulate at different food chain levels, including bivalves and fish. In what concerns bivalves, concentrations of PFOS were already found in *Mytilus galloprovincialis* in ten Portuguese estuaries and the concentrations ranged between 62.96 to 79.80 ng/g wet weight (WW) (Neiva), with a reported maximum concentration of 125.9 ng/g WW [31]. In addition, in clams collected in the Washington, DC metropolitan area, the ΣPFAS ranged from 4 to 23 μg/Kg, with PFOA being the dominant one (3–20 μg/Kg) [32]. In Charleston South Carolina (SC), in whole fish, the mean of ΣPFAS (ng/g WW) varied from 12.7 to 33.0 ng/g WW [33]. In the River Po (Italy), fish specimens were collected and, after analysis, the predominant PFAS was PFOS, present in all samples in higher concentrations (reaching a maximum of 126.4 ng/g and 114.4 ng/g in bleak and channel catfish, respectively) [34].

Regarding the impacts of PFAS on wildlife, studies have shown that exposure to PFAS can impair the development and reproduction of aquatic organisms. For example, Wang et al. [35] observed a reduction in body size and changes in sex ratios in adult zebrafish following five months of chronic PFAS exposure at 50 μg/L. Additionally, the larvae of *M. galloprovincialis* were found to be particularly sensitive to PFOS and PFOA, with exposure to concentrations ranging from 0.01 to 1000 μg/L over 48 h leading to delayed shell development, even at the lowest concentration of 0.01 μg/L [36]. PFAS exposure has been associated with immunotoxicity in aquatic organisms, leading to compromised immune function and increased susceptibility to diseases and infections. For instance, Espinosa-Ruiz et al. [37] demonstrated that exposure of

European sea bass (*Dicentrarchus labrax*) to PFOS (4.83 μg/Kg diet) significantly altered immune responses, including increased peroxidase activity in skin mucus and heightened phagocytic capacity of leukocytes, while also decreasing the bactericidal activity of skin mucus. Furthermore, some PFAS have been found to exhibit genotoxic effects in aquatic organisms, causing significant DNA damage such as strand breaks, chromosomal aberrations, and increased apoptosis. This was observed in the marine mussel (*Perna viridis*) exposed to PFOS, PFOA, PFNA, and PFDA (0.1, 1, 10, 100 and 1000 μg/L) for 7 days [38]. These authors identified PFOS as the most genotoxic, with the lowest EC_{50} value of 33 μg/L, compared to 594 μg/L for PFOA, 195 μg/L for PFNA, and 78 μg/L for PFDA. The study demonstrated that these effects intensified with higher concentrations and longer exposure durations. Additionally, research by Bernardini et al. [39] has assessed the effects of C6O4 (perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy)acetic acid), a new alternative to traditional PFOA, on the clam species *Ruditapes philippinarum*. Clams were exposed to environmental concentrations of C6O4 (0.1 μg/L and 1 μg/L) for 7 and 21 days. The results showed that, although chemical analyses indicate C6O4 accumulates in clam tissues at about five times lower levels than PFOA, transcriptomic analyses revealed numerous changes in gene expression. Many of these altered pathways, including immune response, apoptosis regulation, nervous system development, lipid metabolism, and cellular membrane functions, were similar in clams exposed to both C6O4 and PFOA.

In light of the above-mentioned and considering the lack of information regarding PFAS impacts towards marine organisms, the present study expands the investigation to six PFAS: perfluorohexanoic acid (PFHxA, $C_5F_{11}COOH$), (Perfluorohexyl)acetic acid (6:2 FTA, $C_6F_{13}CH_2COOH$), perfluoroundecanoid acid (PFUnDA, $C_{10}F_{21}$ COOH) Perfluorododecanoic acid (PFDoA, $C_{11}F_{23}COOH$), perfluorotridecanoic acid (PFTriDA, $C_{12}F_{25}COOH$), and perfluorotetradecanoic acid (PFTeDA, $C_{13}F_{27}COOH$). Based on the length of the PFAS fluorinated carbon chain ($C_n F_{2n+1}$), these can be identified as "long-chain" or "short-chain" PFAS. The term "long-chain PFAS" refers to a carbon chain length equal to or greater than C8 for perfluorocarboxylic acids (PFCAs), including PFOA, and carbon chain lengths equal to or greater than C6 for perfluoroalkanesulfonic acids (PFSA), including PFOS and PFHxS [40,41]. In contrast, the term "short-chain PFAS" refers to perfluorosulfonic acids (PFSA) with $C < 6$ and perfluorocarboxylic acids (PFCAs) with $C < 7$ [40,41]. Long-chain PFAS tend to bind strongly to particles and have a high potential for bioaccumulation, whereas short-chain ones are more commonly found in the aqueous phase of the environment [42]. Considering the knowledge gap on the impacts of PFAS in bivalves, the present study assessed, for the first time, the toxic effects of these six PFAS (PFHxA, 6:2 FTA, PFUnDA, PFDoA, PFTriDA and PFTeDA) in *Mytilus galloprovincialis* gills and digestive gland after *in vitro* exposure. *In vitro* assays allow the screening of large numbers of samples and the testing of multiple contaminants simultaneously, which facilitates the identification of potential hazards more rapidly. By studying the effects of PFAS on isolated tissues, *in vitro* experiments provide valuable mechanistic insights into the underlying toxicity pathways, identifying early warning signals of potentially hazardous substances. This understanding enhances the existing knowledge of how PFAS interact with biological systems and elicit adverse effects.

2. Materials and methods

2.1. Chemicals

Perfluorohexanoic acid (PFHxA, CAS number 307–24–4), (Perfluorohexyl)acetic acid (6:2 FTA, CAS number 53826–12–3), Perfluoroundecanoid acid (PFUnDA, CAS number 2058–94–8), Perfluorododecanoic acid (PFDoA, CAS number 307–55–1), Perfluorotridecanoic acid (PFTriDA, CAS number 72629–94–8), Perfluorotetradecanoic acid (PFTeDA, CAS number 376–06–7) were purchased from LGC Standards (Table 1). Primary stock solution (200 mg/L) was prepared by dissolving each PFAS in methanol (MeOH), except for the 6:2 FTA which was dissolved in Dimethyl sulfoxide (DMSO). Each stock solution was diluted 10 times with distilled water to obtain an intermediate solution (20 mg/L). This solution was serially diluted to obtain the following concentrations: 1000, 100, 10 and 1 μg/L, to achieve the selected concentrations in the reaction mixture as detailed in Section 2.2. The concentrations tested (0.1; 1.0; 10.0 and 100.0 μg/L) were based on the values recommended by European Union for drinking water (100 – 500 ng/L) [30], concentrations found in the environment [17,27] and also in concentrations that have caused effects on marine invertebrates subjected to laboratory exposures [36,38,39].

2.2. In vitro assay

Specimens of *Mytilus galloprovincialis* were collected in the Mira Channel (Ria de Aveiro coastal lagoon, Portugal), and only PFOS concentrations (between 0.1 and 0.4 ng/L) were detected near the coast of Aveiro [6].

After sampling, mussels were immediately transported to the laboratory where they were kept under controlled temperature (17 ± 1 ºC) and salinity (30 ± 1), over two weeks for their depuration and acclimation to laboratory conditions. Three days after arrival at the lab and during the rest of the depuration and acclimation period, organisms were fed with Oceanlife® solution (composed of 1 spoon of Oceanlife Coral Food®, 1 mL of Oceanlife Phyto Marine®, and 4 mL of deionized water for 100 L), administered at a concentration of 50 μL/L every other daily.

After this period, organisms were dissected and the gills (G) and digestive gland (DG) of 40 individuals were removed, resulting in 4 pools, each made of tissues from 10 different individuals: 4 pools of 10 G and 4 pools of 10 DG were used for the entire assay. The potential impacts of PFAS on specific tissues were assessed by performing *in vitro* experiments, exposing S9 fractions for each organ, according to OECD guidelines [43] with some modifications (centrifugation speed and exposure time). Each pool was homogenized in phosphate buffer (K_2HPO_4, 0.1 M pH 8.2) (1:2 p/v ratio) using a Potter Elvehjem homogenizer. The homogenates were then centrifuged at 10,000 *g* for 20 min at 4 °C. The resulting supernatants (S9 sub-cellular fractions) were collected, aliquoted and stored at −80 °C until required or immediately used. For each PFAS, four sets of G- and DG-S9 sub-cellular fractions underwent a 30-min exposure to every test concentration (0.1; 1.0; 10.0 and 100.0 μg/L). *In vitro* experiments were accompanied by a control (CTL) treatment for each tissue, where no contaminant was added. The preparation of reaction mixtures and the dilution of S9

Table 1
Overview of Per- and Polyfluoroalkyl Substances (PFAS) Tested and Corresponding Chemical Specifications. For each PFAS compound, the following information is shown short name, Chemical Abstracts Service (CAS) number, molecular formula, and number of fully fluorinated carbon atoms in structure.

Compound	Short name	CAS	Molecular formula	No. fluorinated carbons
Perfluorohexanoic acid	PFHxA	307–24–4	$C_5F_{11}COOH$	5
(Perfluorohexyl)acetic acid	6:2 FTA	53826–12–3	$C_6F_{13}CH_2COOH$	6
Perfluoroundecanoid acid	PFUnDA	2058–94–8	$C_{10}F_{21}COOH$	10
Perfluorododecanoic acid	PFDoA	307–55–1	$C_{11}F_{23}COOH$	11
Perfluorotridecanoic acid	PFTriDA	72629–94–8	$C_{12}F_{25}COOH$	12
Perfluorotetradecanoic acid	PFTeDA	376–06–7	$C_{13}F_{27}COOH$	13

sub-cellular fractions to the working protein concentration followed the guidelines of OECD No. 319B [43], except for the total antioxidant capacity and lipid peroxidation tests, for which 0.5 g of fresh weight tissue (FW) was used. To achieve a standardized working protein concentration of 1 mg/mL for *in vitro* exposure, the S9 sub-cellular fraction was diluted with phosphate buffer (K_2HPO_4, 0.1 M pH 8). This concentration was selected to prevent enzyme saturation and, consequently, to guarantee precise measurements of enzyme kinetics [43]. Once the reaction mixtures were prepared, each set was spiked (1:10 v/v) with the working stock solutions of the selected chemicals (refer to Section 2.1) and then incubated for 30 min, shielded from light, at 25 ± 2 °C on an orbital shaker at 1200 *g*. Following *in vitro* exposure, biochemical measurements were conducted as described in Section 2.3.

2.3. Biochemical parameters

To evaluate the biochemical alterations triggered in mussels following exposure to six PFAS (PFHxA, 6:2 FTA, PFUnDA, PFDoA, PFTriDA and PFTeDA), a battery of biochemical parameters was employed as biomarkers. These parameters encompassed: i) measuring antioxidant enzyme activities, specifically superoxide dismutase (SOD), catalase (CAT) and total antioxidant capacity (TAC); ii) quantifying detoxification enzyme activities, such as carboxylesterases (CbEs) and glutathione S-transferases (GSTs); iii) assessing cellular damage, measuring levels of lipid peroxidation (LPO) and iv) evaluating neurotransmission, by measuring the activity of the enzyme acetylcholinesterase (AChE). The quantification of protein (PROT) content to a standardized working protein concentration of 1 mg/mL for *in vitro* assays was conducted using the Biuret method outlined by Robinson and Hogden [44]. The absorbance was read at 540 nm. Bovine serum albumin standards (ranging from 0 to 40 mg/mL), a universally acknowledged PROT benchmark, were employed to construct a calibration curve. The results were expressed as mg per mL and achieved a final concentration of 1 mg/mL through a series of dilutions.

2.3.1. Antioxidant defenses

The evaluation of superoxide dismutase (SOD) activity followed the protocol outlined by Magnani et al. [45]. The sample was blended with Tris-EDTA buffer (composed of Tris base and ethylenediaminetetraacetic acid (EDTA) at pH 8.2). The reaction started with the addition of pyrogallol solution (dissolved in 0.01 mmol/L HCl). After a 1-min incubation period, the absorbance was measured again at 420 nm. Results were denoted in units (U) per mg of PROT, where one unit of enzyme activity indicates the inhibition of 50 % of pyrogallol autoxidation.

The assessment of CAT activity followed the procedure delineated by Johansson and Borg [46]. In this assay, CAT facilitated the decomposition of hydrogen peroxide (H_2O_2) into formaldehyde in the presence of methanol. The formaldehyde generated was measured using calibration standards spanning from 0 to 150 μmol/L. After stirring for 5 min, CAT activity was monitored at 540 nm, and the outcomes were expressed in units (U) per mg of PROT, where U represents the quantity of enzyme leading to the formation of 1 nmol of formaldehyde per min.

The TAC content was assessed following the method outlined by Benzie and Strain [47], with adaptations by Hagger et al. [48]. TAC quantifies an organism's capability to eliminate or neutralize free radicals or reactive oxygen species (ROS). The FRAP (ferric reducing antioxidant power) assay employed in this study is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by the antioxidants present in the sample. Aqueous solutions of known concentrations of $FeSO_4{*}7H_2O$ were measured using calibration standards ranging from 0 to 1000 μmol/L. This reduction leads to the formation of a blue-coloured complex known as the ferrous tripyridyltriazine complex, which can be quantified spectrophotometrically at 593 nm after 10 min of incubation. The results were expressed as μmol per g of FW.

2.3.2. Detoxification enzyme activities

The activity of CbEs was determined following the original procedure by Hosokawa and Satoh [49], subsequently refined by Solé et al. [50]. This method relies on the enzyme's capability to hydrolyze ester substrates, liberating p-nitrophenol in the process. The commercial colorimetric substrate, p-nitrophenyl butyrate (pNPB), was used because of its ability to produce p-nitrophenol after enzymatic hydrolysis by CbEs. As CbEs act on these substrates, p-nitrophenol is released, resulting in a yellow coloration that intensifies over time. The rate of p-nitrophenol formation, indicative of CbEs activity, was monitored at 405 nm over a 5-min period at 15-s intervals. For activity quantification, an extinction coefficient (ε) of 18 mM^{-1} cm^{-1} was applied, and the results were expressed in nmol per min per mg of PROT.

The activity of GSTs followed the protocol initially outlined by Habig et al. [51] with modifications by Carregosa et al. [52]. This method hinges on the role of GSTs in facilitating the conjugation of glutathione (GSH) with electrophilic centres on diverse substrates, such as 1-chloro-2,4-dinitrobenzene (CDNB). Upon interaction with CDNB, GSH forms a thioether compound, and the subsequent increase in thioether concentration serves as an indicator of GSTs activity. The kinetic reaction of thioether formation was tracked at 340 nm over a 5-min duration at 15-s intervals. Thioether formation was quantified utilizing an extinction coefficient (ε) of 9.6 $mM^{-1}cm^{-1}$, and results were expressed as nmol per min per mg of PROT.

2.3.3. Cellular damage

The determination of LPO levels followed the method initially described by Ohkawa et al. [53], with adaptations by Carregosa et al. [52]. This technique determines the quantity of malondialdehyde (MDA), a byproduct of lipid peroxidation, by subjecting it to reaction with thiobarbituric acid (TBA) under elevated temperature conditions, resulting in the formation of a pink-red chromogen. The chromogen was subsequently measured spectrophotometrically, with absorbance readings taken at 535 nm. The concentration of MDA in the sample was determined using an extinction coefficient (ε) of 156 mM^{-1} cm^{-1}, and results were expressed in nmol of MDA equivalents formed per g of FW.

2.3.4. Neurotransmission

The measurement of AChE activity followed the methodology initially outlined by Ellman et al. [54], with adjustments made by Mennillo et al. [55]. This method relies on the enzymatic hydrolysis of acetylthiocholine iodide (ATChI) by AChE, resulting in the production of thiocholine. Thiocholine subsequently reacts with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to form a yellow-colored product, 5-thio-2-nitrobenzoate, which can be quantified spectrophotometrically at 412 nm. The kinetic reaction was monitored over a 5-min period at intervals of 15 s. For quantification purposes, an extinction coefficient (ε) of 13.6 mM^{-1} cm^{-1} was utilized, representing the molar absorptivity of the 5-thio-2-nitrobenzoate product. Results were expressed in nmol per min per mg of PROT.

2.4. Data analysis

2.4.1. Statistical analyses

Results for all biochemical parameters and replicates (N = 4, corresponding to 10 mussels per pool, per tissue and per PFAS concentration) were analyzed using permutational multivariate analysis of variance (PERMANOVA) with the PERMANOVA+ add-on in PRIMER v6 [56]. Paired t-tests were used to determine statistical significance, with p-values less than 0.05 considered significant. The null hypothesis tested was that exposure to A (PFHxA), B (6:2 FTA), C (PFUnDA), D (PFDoA), E (PFTriDA), or F (PFTeDA) had no toxic effects on DG or G mussels, with no significant differences between the tissues examined. Significant differences between PFAS at the same concentration are marked by different letters in each Figure. Additionally, significant differences between the control (CTL) and each PFAS at a given concentration are

identified with *p*-values provided in supplementary material (SM) tables.

2.4.2. Multivariate analyses

For the cluster analysis, the biochemical results were imported into PERMANOVA + add-on in PRIMER v6. A similarity matrix was constructed using the Bray-Curtis similarity method. Cluster analysis was performed using hierarchical clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). This approach allowed the visualization of similarities and dissimilarities between the tested PFAS

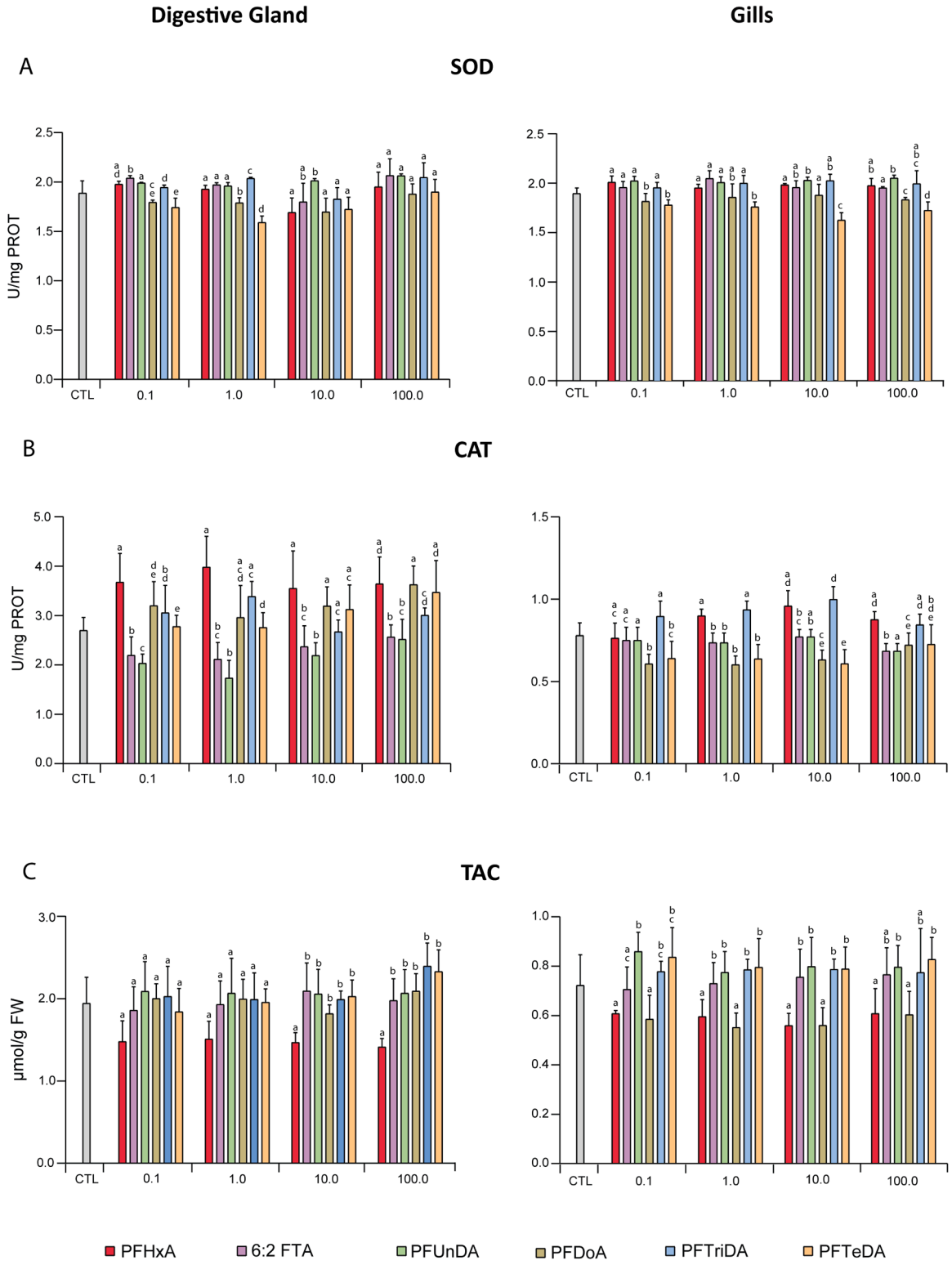

Fig. 1. A: Superoxide dismutase activity (SOD); B: Catalase activity (CAT); C: Total Antioxidant Capacity (TAC) of S9 *Mytilus galloprovincialis* digestive gland and gills exposed to different treatments (CTL; PFHxA; 6:2 FTA; PFUnDA; PFDoA; PFTriDA and PFTeDA) at different concentrations (0.1; 1.0; 10 and 100 µg/L). Results are reported as mean + SD. Significant differences ($p < 0.05$) among different PFAS at the same concentration are indicated by different letters. N = 4, corresponding to 10 mussels *per* pool *per* tissue.

under different concentrations.

An ordination analysis by Principal Coordinates (PCO) was performed using all the biochemical results obtained for the different treatments and PFAS that were first normalized. The Euclidean distances between the centroids, representing the average coordinates of all treatment points (0.1; 1.0; 10.0; 100.0 μg/L of A: PFHxA, B: 6:2 FTA, C: PFUnDA, D: PFDoA, E: PFTriDA, or F: PFTeDA), were then calculated for both tissues (DG and G). These distances were visualized by PCO analysis. In the PCO graph, biochemical parameters with a Pearson correlation ($\rho = 0$) were represented as overlapping vectors. This analysis identified the treatments with the most significant effects on each biochemical parameter and enabled the grouping of treatments based on their similarity distances.

3. Results

3.1. Biochemical parameters

3.1.1. Antioxidant defenses

At each exposure concentration and comparing PFAS, the activity of SOD was reduced in the DG exposed to PFDoA and PFTeDA. A similar trend was observed in the G (Fig. 1A). Among concentrations, for each PFAS, in the DG, significant differences were mainly found for PFUnDA, with significantly higher activity at the highest concentration compared to the remaining ones (Tabel 1 SM). For the G, PFTeDA was the PFAS that varied the most among concentrations, with inhibited activity compared to CTL (Table 1SM).

Regardless of the concentration tested, in the DG, CAT activity was significantly higher in the presence of PFHxA while lower activity was observed for 6:2 FTA and PFUnDA. In the G, except for the highest concentration, lower activity was found for PFDoA and PFTeDA, while the highest was recorded for PFTriDA (Fig. 1B). For each PFAS, in the DG most differences to the CTL were observed for PFHxA and PFUnDA, which induced higher and lower activity levels, respectively (Table 1SM). In G, the activity increases in PFHxA and PFTriDA at 1.0 and 10.0 μg/L compared to the CTL. An opposite response was recorded for PFTeDA (Table 1SM).

In the DG, at each tested concentration, lower TAC levels were observed with PFHxA exposure. In the G, the TAC content was reduced for PFHxA and PFDoA regardless of the concentration (Fig. 1C). Among

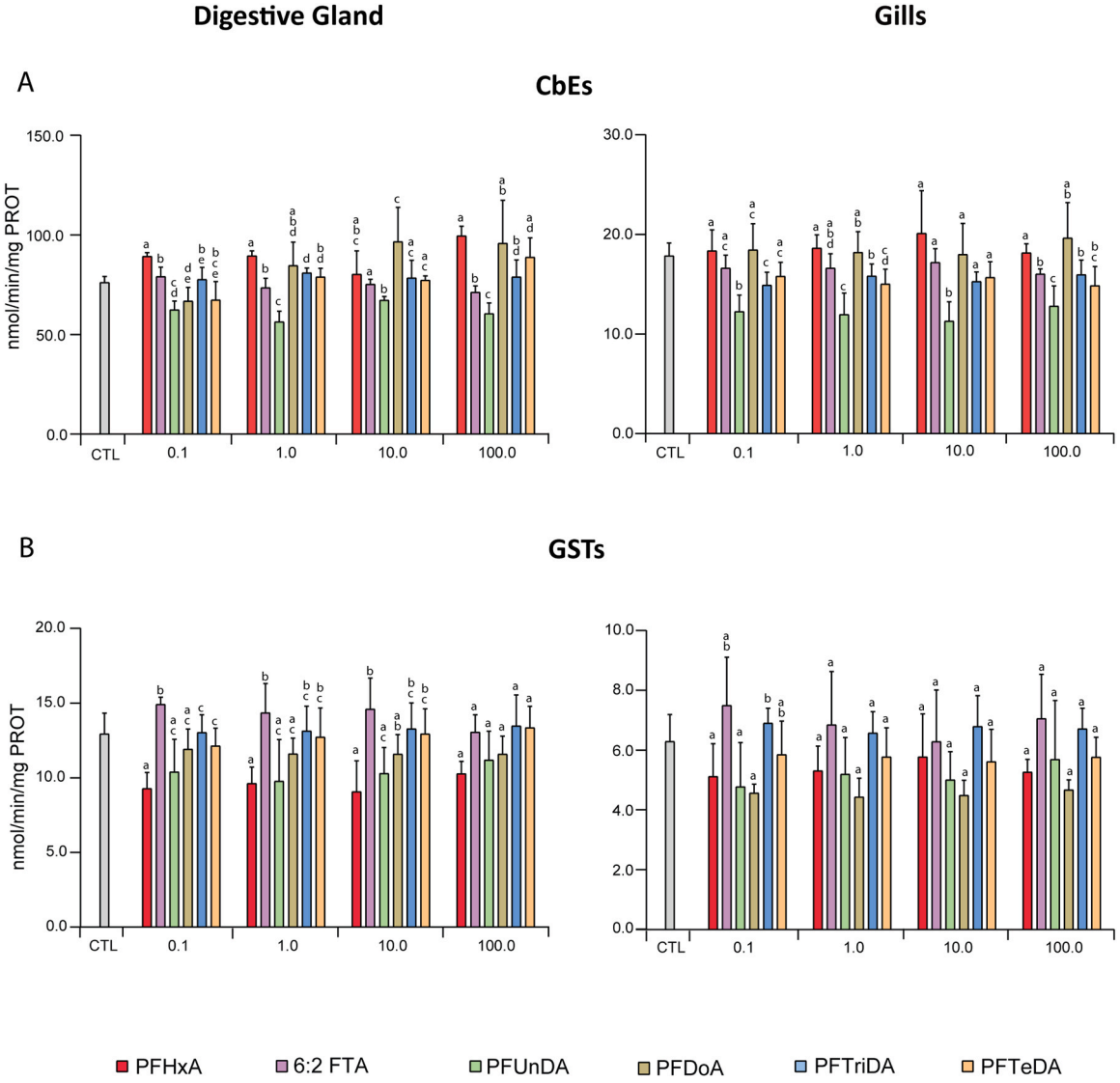

Fig. 2. A: Carboxilesterases activity (CbEs); B: Glutathione S-transferases activity (GSTs) of S9 *Mytilus galloprovincialis* digestive gland and gills exposed to different treatments (CTL; PFHxA; 6:2 FTA; PFUnDA; PFDoA; PFTriDA and PFTeDA) at different concentrations (0.1; 1.0; 10 and 100 μg/L). Results are reported as mean + SD. Significant differences ($p < 0.05$) among different PFAS at the same concentration are indicated by different letters. N = 4, corresponding to 10 mussels *per* pool *per* tissue.

concentrations, few differences were detected, with PFHxA leading to lower TAC content compared to the CTL, both for DG and G (Table 1SM).

3.1.2. Detoxification enzyme activities

Regardless of the concentration tested, the activity of CbEs was reduced in mussels exposed to PFUnDA in both DG and G compared to the remaining PFAS (Fig. 2A). Among concentrations, in the DG, higher activity was recorded for PFHxA compared to CTL. In the G, differences were observed for PFUnDA, with lower activity compared to uncontaminated G (Table 1SM).

In the DG, when comparing PFAS, at each concentration, the lowest GSTs activities were observed with PFHxA, PFUnDA and PFDoA. A similar pattern was seen in the G for PFDoA (Fig. 2B). Among concentrations, in the DG, differences were observed for PFHxA, with lower activity compared to the CTL. In the G, no significant differences were noticed regardless of the PFAS (Table 1SM).

3.1.3. Cellular damage

In the DG and G, levels of LPO were similar among PFAS, regardless of the concentration tested, except for PFTriDA and PFTeDA in the DG, which showed the highest LPO levels at the highest concentration (Fig. 3). Among concentrations, in the DG only at the highest concentration of PFTriDA significantly higher LPO levels were observed compared to the CTL, while for the G, no significant differences were noticed regardless of the PFAS (Table 1 SM).

3.1.4. Neurotransmission

In the DG, when comparing PFAS, differences in AChE activity were mostly noted for PFDoA (0.1, 10.0 and 100.0 μg/L) and PFTriDA (0.1, 1.0 and 100.0 μg/L). In the G, the lowest AChE values were observed in mussels exposed to PFHxA (1.0 and 10.0 μg/L) and PFTriDA (0.1, 1.0 and 100.0 μg/L) (Fig. 4). Comparing concentrations, in the DG, the activity of AChE was maintained among most of the concentrations, regardless of the PFAS. In the G, the activity of AChE was significantly inhibited in the presence of PFHxA (1.0, 10.0 e 100.0 μg/L) and PFTriDA (0.1, 1.0 and 100.0 μg/L) compared to the CTL (Table 1 SM).

3.2. Multivariate analyses

3.2.1. Digestive gland vs gills

The first principal component (PCO1) of the PCO graph (Fig. 5) accounted for 82 % of the total variation and separated the two organs on opposite sides: DG on the negative side and G on the positive side. The second principal component (PCO2) explained 9.2 % of the total variation, and in the case of DG separated the PFTriDA, 6:2 FTA, PFunDA and CTL treatments on the positive axis from the PFHxA, PFDoA and PFTeDA treatments on the negative axis. Regarding the G, it separated the PFTriDA, PFHxA, PFunDA, 6:2 FTA treatments on the positive axis from the CTL, PFDoA and PFTeDA treatments on the negative axis. The parameters GSTs ($p = -0.951$), CbEs ($p = -0.985$), CAT ($p = -0.963$), TAC ($p = -0.977$) and LPO ($p = -0.994$) showed a strong correlation with the negative side of PCO1. The remaining parameters, SOD ($p = 0.885$) and AChE ($p = -0.558$), revealed a strong correlation with the positive and negative sides, respectively, of PCO2.

3.2.2. Digestive gland

Cluster analysis (Fig. 6A) showed a first division (groups A and B) that distinguished PFAS $A_{0.1;1.0;10;100}$ (group A) from the remaining treatments (group B). Group B is divided into two subgroups (B1 and B2), separating treatments $D_{0.1;1.0;10;100}$, F_{100}, $E_{0.1;1.0;100}$ (B1) from the others treatments ($F_{0.1;1.0;10}$, CTL, E_{10}, $B_{0.1;1.0;10;100}$, $C_{0.1;1.0;10;100}$). Cluster B1 was further subdivided, isolating the treatment $D_{0.1;1.0;10;100}$ (B11) from the others (B12). Additionally, cluster B2 separated the treatment $C_{0.1;1.0;10;100}$ (B22) from the remaining treatments (B21).

In Fig. 6B, the PCO graph showed 38.4 % of the total variation for PCO1. The PCO1 axis separated the PFAS $C_{0.1;1.0;10;100}$, $B_{0.1;1.0;10;100}$ and E_{100} on the positive side from the other treatments on the negative side ($A_{0.1;1.0;10;100}$, $F_{0.1;1.0;10;100}$, $D_{0.1;1.0;10;100}$ and $E_{0.1;1.0;10}$). Concerning PCO2, which explained 24.8 %, PFAS $A_{0.1;1.0;10;100}$, $F_{0.1;1.0;10}$, $D_{0.1;1.0}$, $C_{0.1;1.0;10;100}$, $B_{1.0}$ were isolated on the positive axis from the remaining treatments ($D_{10;100}$, $E_{0.1;1.0;10;100}$, CTL, $B_{0.1;100}$) on the negative axis. The parameters SOD ($p = 0.433$), CAT ($p = -0.879$), CbEs ($p = -0.810$) and TAC ($p = 0.721$) showed a strong correlation with PCO1, while the remaining parameters GSTs ($p = -0.556$), AChE ($p = 0.673$) and LPO ($p = -0.782$) showed a greater correlation with PCO2.

3.2.3. Gills

Cluster analysis (Fig. 6C) subdivided the biochemical results into two groups (A and B), separating the treatments $D_{0.1;1.0;10;100}$ and $F_{0.1;1.0;10;100}$ (cluster B) from the remaining ones (A). Group A was subdivided into A1 and A2, isolating the treatment $C_{0.1;1.0;10;100}$ (A2) from the other ones (A1). The group A1 was subdivided comprised two

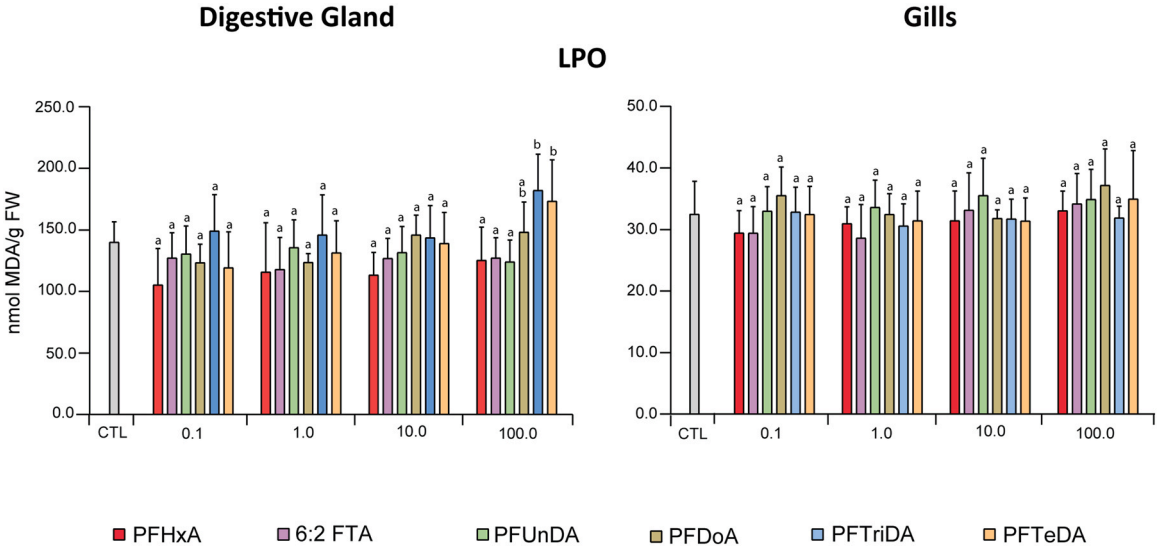

Fig. 3. Lipid peroxidation (LPO) levels of S9 *Mytilus galloprovincialis* digestive gland and gills exposed to different treatments (CTL; PFHxA; 6:2 FTA; PFUnDA; PFDoA; PFTriDA and PFTeDA) at different concentrations (0.1; 1.0; 10 and 100 μg/L). Results are reported as mean + SD. Significant differences ($p < 0.05$) among different PFAS at the same concentration are indicated by different letters. N = 4, corresponding to 10 10 mussels *per* pool *per* tissue.

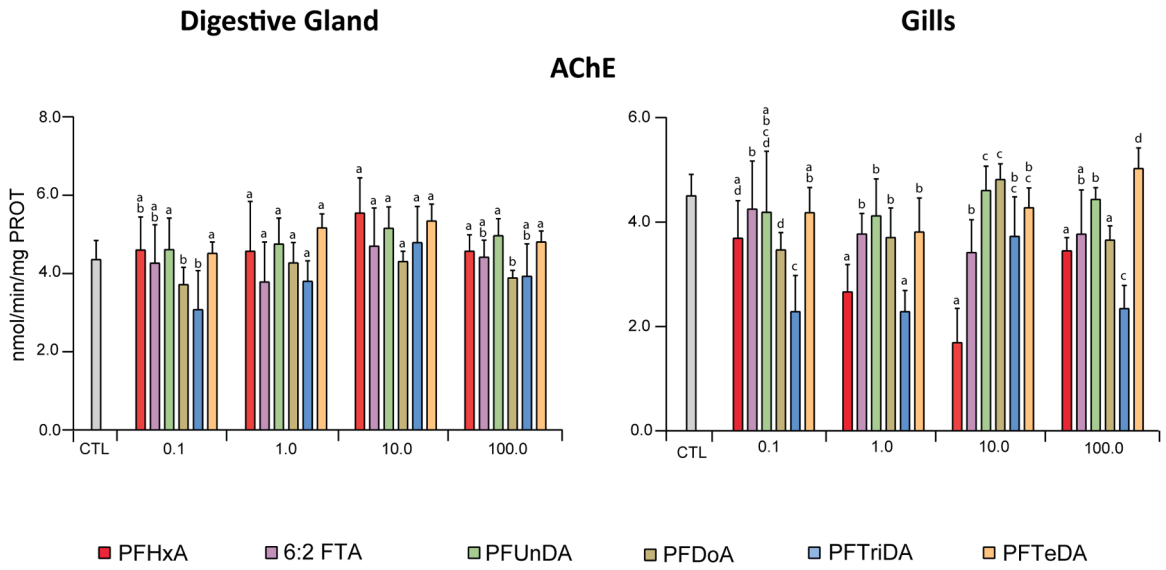

Fig. 4. Acetylcholinesterase (AChE) activity of S9 *Mytilus galloprovincialis* digestive gland and gills exposed to different treatments (CTL; PFHxA; 6:2 FTA; PFUnDA; PFDoA; PFTriDA and PFTeDA) at different concentrations (0.1; 1.0; 10 and 100 μg/L). Results are reported as mean + SD. Significant differences ($p < 0.05$) among different PFAS at the same concentration are indicated by different letters. N = 4, corresponding to 10 10 mussels *per* pool *per* tissue.

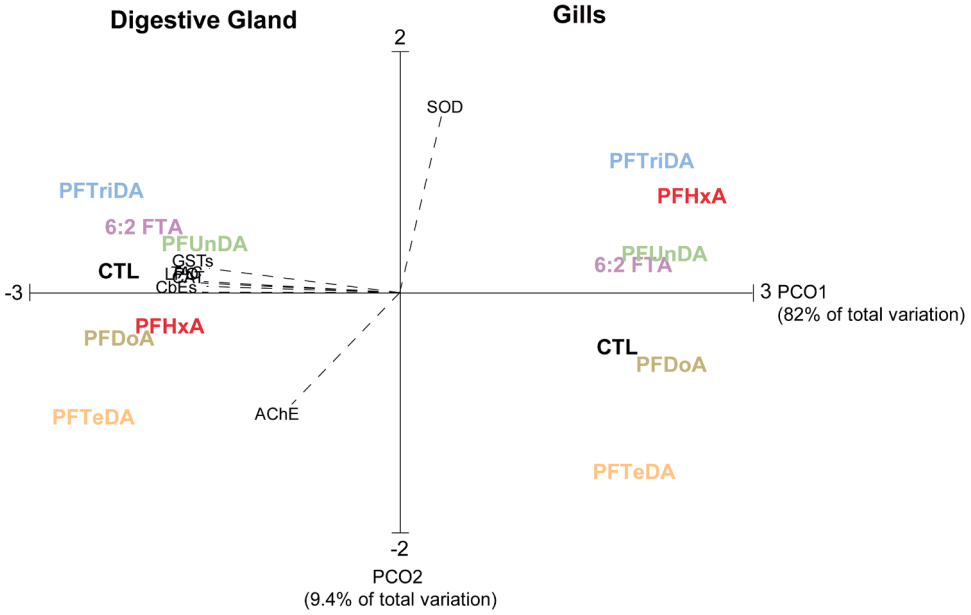

Fig. 5. Centroids ordination diagram (PCO) based on the overall biochemical parameters tested for each organ (digestive gland and gills) for each treatment (CTL; PFHxA; 6:2 FTA; PFUnDA; PFDoA; PFTriDA and PFTeDA).

other clusters: A11 ($A_{0.1;1.0;10;100}$) and A12 (CTL, $B_{0.1;1.0;10;100}$, $E_{0.1;1.0;100}$).

In the PCO graph shown in Fig. 6D, the PCO1 axis explained 38.5 % of the total variation. This axis isolated treatments $F_{0.1;1.0;10;100}$ and $D_{0.1;1.0;10;100}$ on the positive axis from the others on the negative axis. The PCO2 revealed a total variance of 31.2 % and separated treatments $C_{0.1;1.0;10;100}$, $E_{0.1;1.0;10;100}$, $B_{0.1;1.0;10;100}$, $F_{0.1;1.0;10;100}$ (positive side) from treatments $A_{0.1;1.0;10;100}$, $D_{0.1;1.0;10;100}$ and CTL (negative side). The axis PCO1 demonstrated a strong correlation with the parameters SOD (p = 0.842), CAT (p = 0.922), GSTs (p = 0.208), LPO (p = - 0.226) and AChE (p = - 0.687), while the remaining parameters TAC (p = 0.877) and CbEs (p = - 0.954) showed a greater correlation with PCO2.

4. Discussion

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals that have been extensively used in industry and consumer products worldwide since the 1950s [57]. Due to their persistence and ability to bioaccumulate, they have become widespread environmental contaminants [10,11,58]. The environmental release and fate of PFAS are complex, with varying degrees of mobility and persistence depending on their specific chemical structures [59]. Broadly, PFAS can be divided into two main groups, depending on the number of fluorinated carbons: short-chain (C < 6 for perfluorosulfonic acids (PFSAs) and C < 7 for perfluorocarboxylic acids (PFCAs)) and long-chain PFAS (C ≥ 8 for perfluorocarboxylic acids (PFCAs), and C ≥ 8 for perfluoroalkanesulfonic acids (PFSA)) [41]. Short-chain or emerging PFAS, were introduced to replace or supplement traditional PFAS in response

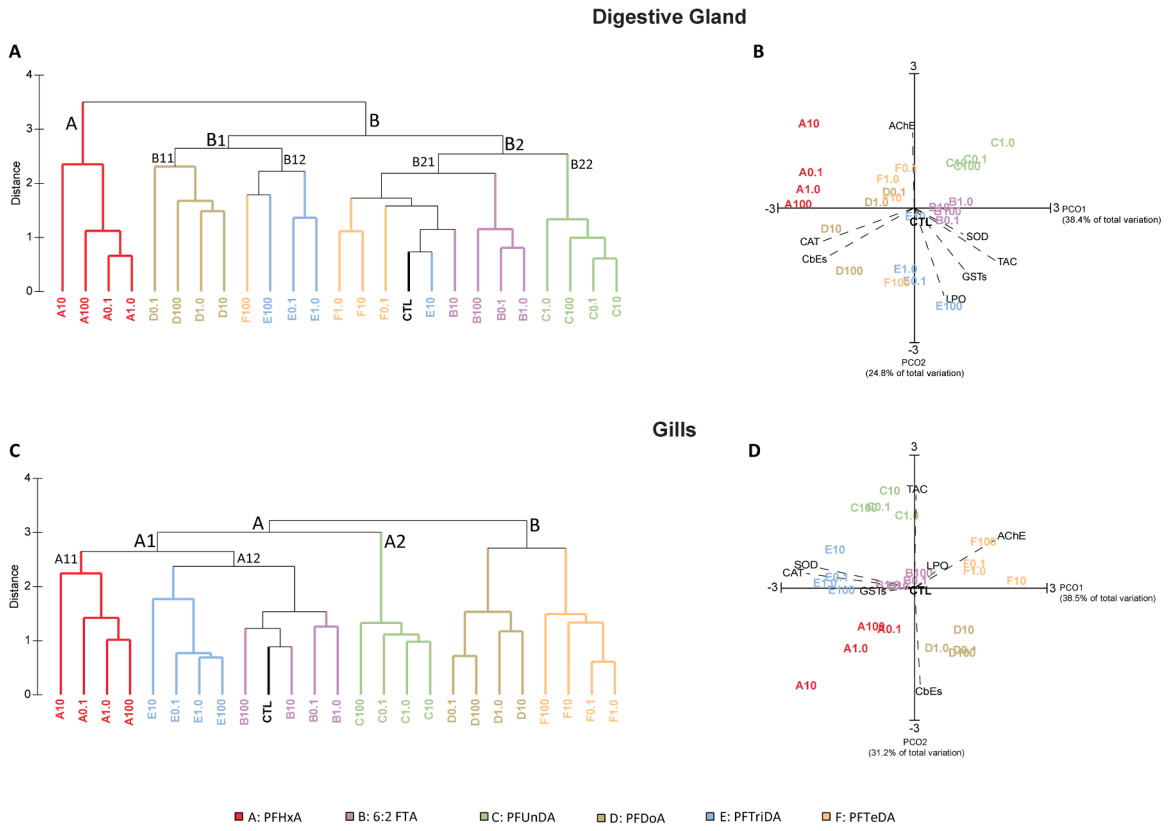

Fig. 6. A: Cluster analysis for S9 *Mytilus galloprovincialis* digestive gland among tested PFAS at different concentrations; B: Centroids ordination diagram (PCO) measured in S9 *Mytilus galloprovincialis* digestive gland unexposed (CTL) and exposed to different PFAS (A: PFHxA; B: 6:2 FTA; C: PFUnDA; D: PFDoA; E: PFTriDA and F: PFTeDA) at different concentrations (0.1; 1.0; 10 and 100 μg/L). C: Cluster analysis for S9 of S9 *Mytilus galloprovincialis* gills among tested PFAS at different concentrations; D: Centroids ordination diagram (PCO) measured in S9 *Mytilus galloprovincialis* gills unexposed (CTL) and exposed to different PFAS (A: PFHxA; B: 6:2 FTA; C: PFUnDA; D: PFDoA; E: PFTriDA and F: PFTeDA) at different concentrations (0.1; 1.0; 10 and 100 μg/L). For PCO, Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0$): SOD = Superoxide dismutase activity; CAT= Catalase activity; TAC = Total Antioxidant Capacity; CbEs = Carboxilesterases activity; GSTs = Glutathione S-transferases activity; LPO = Lipid peroxidation levels and AChE = Acetylcholinesterase activity.

to increasing regulatory pressures. Long-chain or traditional PFAS, are well-documented for their bioaccumulative properties [60]. In contrast, the emerging short-chain PFAS have been promoted by the chemical industry as safer and more environmentally sustainable, with a purportedly lower potential for human accumulation [61]. However, short-chain PFAS are equally persistent as their long-chain counterparts, possessing different yet equally concerning properties, and have already become widespread in the environment [24–26,29,34,62]. As a result, there is growing concern among experts about whether these emerging substitutes are truly safer alternatives to the older PFAS compounds [63]. Given the limited understanding of PFAS impacts on bivalves, this study provides the first assessment of the toxic effects of six PFAS compounds (Short - chain: PFHxA, 6:2 FTA; Long - chain: PFUnDA, PFDoA, PFTriDA, and PFTeDA) on the digestive gland (DG) and gills (G) of *Mytilus galloprovincialis* following *in vitro* exposure. This evaluation is crucial for elucidating the impact of fluorinated carbon chain lengths on environmental impacts, as well as for formulating comprehensive risk assessments and management strategies for PFAS contamination.

In vitro techniques serve as rapid, cost-effective, and reliable screening tools for evaluating the toxicity of various chemicals with high precision and reproducibility [64,65]. These methods enhance our understanding of the cellular and molecular mechanisms underlying responses to environmental pollutants across different tissues, thereby supporting more extensive *in vivo* experiments involving whole organisms or mesocosms [66]. The examination of tissue-specific responses using the S9 sub-cellular fraction is a sensitive approach that aids in detecting tissue damage and the direct effects of chemicals, making it a widely accepted biomarker [67–69]. In the present study, an S9-based *in vitro* assay was employed to explore potential interactions between PFAS and mussels' antioxidant defenses, biotransformation processes, cellular damage and acetylcholinesterase (AChE) activity. The antioxidant system was assessed by measuring total antioxidant capacity (TAC) and the activity of specific enzymes, namely superoxide dismutase (SOD) and catalase (CAT). These enzymatic and non-enzymatic scavengers are essential in mitigating oxidative stress by neutralizing reactive oxygen species (ROS) and thereby preventing cellular damage [70]. Among them, the enzyme SOD plays a key role as the first line of defense against oxidative stress by catalyzing the conversion of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2). This initial reaction is crucial because superoxide radicals are highly reactive and can cause significant cellular damage if not properly managed. Subsequently, the enzyme CAT contributes to the antioxidant defense by breaking down H_2O_2 into water and oxygen. This subsequent step is important because, while H_2O_2 is less reactive than O_2^-, it can still be toxic at elevated concentrations and may lead to the formation of hydroxyl radicals, which are even more damaging [70–72]. The TAC integrates the effects of both enzymatic and non-enzymatic antioxidants, providing a comprehensive measure of the organism's overall antioxidant defense system [73]. Moreover, another defense mechanism involves an organism's capacity to detoxify pollutants by converting them into more water-soluble and less toxic forms, which facilitates their excretion from the cells [50,70]. In the present study, the biotransformation enzymes examined were carboxylesterases (CbEs) and glutathione S-transferases (GSTs), which play a pivotal role in phase I and phase II detoxification processes, respectively. However, when these enzymes are ineffective or insufficiently active, cellular damage can occur due to lipid peroxidation (LPO), which corresponds to

the oxidative degradation of lipids within cell membranes, initiated by ROS. This process generates lipid radicals and secondary products, which can compromise membrane integrity and disrupt cellular functions [74,75]. Additionally, the present study evaluated the degradation rate of acetylthiocholine in the presence of PFAS by measuring the enzyme AChE activity. As a key member of the cholinesterase (ChE) family, AChE hydrolyses acetylthiocholine iodide (ATCI) into choline and acetate [76]. This enzymatic reaction occurs in the postsynaptic membrane and is crucial for terminating synaptic transmission by degrading acetylcholine, thereby preventing excessive stimulation of neurons. A decrease in AChE activity can lead to the accumulation of acetylcholine, resulting in prolonged synaptic signalling and potential adverse biological effects [77].

The present findings regarding the biochemical responses of the two organs revealed, through Principal Coordinates (PCO) analysis, a distinction between the two organs; DG treatments (negative PCO1 axis) and G treatments (positive PCO1 axis). Such separation highlights the dissimilarities in the biochemical profiles between the two tissues and indicates a differential response of these organs to pollutants. The G of mussels serve as the primary respiratory organ, allowing for the exchange of gases, such as oxygen and carbon dioxide, between the animal and its aquatic environment [78]. The large surface area and high perfusion rate of the gill epithelium facilitate the entry of various substances, including potential pollutants, making the G sensitive to environmental changes. In addition to respiration, this organ also plays a vital role in the filtering and feeding process. Mussels use their G to filter large volumes of water, capturing suspended particles, including microorganisms, as a source of food [79,80]. The DG, also known as the hepatopancreas, is responsible for the digestion and absorption of these filtered particles, as well as the production of enzymes and other biochemical compounds necessary for the mussel's metabolism [78]. According to the PCO, the basal values of CbEs, CAT, TAC, and LPO were found to be higher in the DG because this organ is actively involved in metabolic processes, including detoxification, digestion, and storage, which require a higher level of enzymatic and antioxidant activities to manage the oxidative stress and metabolic demands associated with these functions [81]. Giannessi et al. [68] and Cruz et al. [67] also observed different basal values for the two organs (DG and G) in *in vitro* tests with S9 fractions of *M. galloprovincialis*, with the predomination of biochemical parameters being more evident for the DG.

Concerning PFAS exposure to DG, cluster analysis distinguished PFHxA from the other compounds. Based on the biochemical responses, this distinction was evidenced in the PCO by the induction of CAT activity and a reduction of TAC. A decrease in TAC, coupled with an increase in CAT activity, suggests that while the mussels are struggling to cope with oxidative stress, CAT activity was enhanced to counteract the excess H_2O_2 generated by PFHxA exposure. However, the overall antioxidant defenses, including other enzymes and non-enzymatic antioxidants, may be depleted or insufficient to fully mitigate oxidative damage. This imbalance increases the risk of cellular damage, such as lipid peroxidation, protein oxidation, and DNA damage. The findings of the study conducted by Jiao et al. [82] indicate that the antioxidant enzymes, specifically SOD and CAT, were inhibited and that there was a reduction in TAC in the organs (liver, spleen and kidney) of *Carassius auratus* following 30-day exposure to varying toxic doses: 0; 5; 15; 45 and 135 mg/L of PFHxA. The contrast between these findings highlights the impact of exposure duration and concentration on the antioxidant responses of different species. In the case of the present study, mussels were exposed to lower concentrations of PFHxA for only 30 min (*in vitro*), and there was a specific upregulation of CAT activity, indicating an acute, immediate response to oxidative stress. However, prolonged *in vivo* exposure to significantly higher concentrations of PFHxA in *C. auratus* resulted in a more severe outcome, with the fish failing to effectively activate their antioxidant defenses, leading to a widespread inhibition of key enzymes like SOD and CAT. This suggests that the severity of oxidative stress and the organism's ability to respond are strongly influenced by both the concentration and duration of PFHxA exposure. Nevertheless, studies conducted by Sanches et al. [69] revealed an increase in SOD activity in *M. galloprovincialis* DG and G after a 30 min exposure (*in vitro*) to the ionic liquid 1-butyl-3-methylimidazolium chloride, corroborating the idea that effects are not only dependent on exposure duration but also on the contaminant. In addition, the present findings revealed an inhibition of GSTs activity after exposure to PFHxA, indicating that as this enzyme also contributes to the neutralization of ROS through the conjugation of glutathione (GSH) with reactive compounds, its inhibition may be associated with a reduction in the cell's antioxidant capacity. Similarly, Cruz et al. [67] found no changes in the GSTs activity after *in vitro* exposure of *M. galloprovincialis* DG cell fractions to the pharmaceuticals sodium lauryl sulphate, salicylic acid and caffeine. In contrast, PFUnDA caused a different response in the DG of mussels, resulting in the inhibition of both CAT and CbEs activities. This result highlights a notable difference in how PFAS with varying chain lengths impact enzymatic functions. The observed inhibition by PFUnDA (long–chain) can be attributed to its unique chemical structure compared to PFHxA (short–chain) [41,83]. PFUnDA, which has a longer carbon chain, likely exerts a more significant steric effect on the active sites of CAT and CbEs. The extended fluorinated chain may induce conformational changes in the enzymes or interfere with their interaction with natural substrates, leading to stronger inhibition. Studies conducted by Giannessi et al. [68] also observed that short-term exposure of *M. galloprovincialis* DG to different fluoroquinolone antibiotics (ciprofloxacin, enrofloxacin and danofloxacin) results in distinct responses specifically in SOD activity. In longer chain fluorocarbons, it was found that at the highest concentration tested (100 μg/L) of PFTriDA, despite enhanced antioxidant capacity, this compound caused cellular damage, shown by higher LPO levels. This result indicates that although PFTriDA can augment the overall antioxidant capacity (shown by higher TAC content), this activation is insufficient to neutralize the ROS that are responsible for the occurrence of LPO. Other studies have demonstrated that, despite the activation of antioxidant enzymes, these are insufficient to prevent cellular damage in mussels [84–86].

Regarding the exposure of G to PFAS, the cluster analysis revealed a discernible differentiation between all the PFAS evaluated, indicating notable differences in the characteristics of the compounds. Following the initial cluster separation, two distinct groups were identified, which isolated the compounds PFDoA and PFTeDA. Subsequent PCO analysis further highlighted the distinctiveness of these two PFAS compounds in comparison to the others. The analysis demonstrated that PFDoA and PFTeDA exhibited biochemical parameters that contributed to this separation. Specifically, these compounds caused a marked inhibition of the antioxidant enzymes SOD and CAT, suggesting that the presence of free radicals associated with exposure to these PFAS may have led to the deactivation of enzymatic activity. This indicates that the toxicity caused by PFDoA and PFTeDA likely exceeded the antioxidant capacity of the mussel G tissues due to elevated levels of oxidative stress generated by these contaminants. Corroborating our findings Giannessi et al. [68] also found an inhibition of SOD activity in cell fractions from G of *M. galloprovincialis* exposed to different fluoroquinolone antibiotics after an *in vitro* exposure (30 min). Furthermore, the compound PFTeDA (the PFAS tested with the highest number of fluorinated carbon atoms) exhibited an increase in AChE activity, indicating that following short-term exposure to this PFAS, did not affect acetylcholine-related neural functions in mussels. These findings are consistent with observations by Cruz et al. [67], who found an increase in AChE activity in G of *M. galloprovincialis* after short-term exposure to different drugs. However, exposure to PFHxA and PFTriDA resulted in a clear inhibition of this enzyme, revealing their anti-cholinesterase capacity. In short-term *in vitro* exposure studies with ionic liquids in *M. galloprovincialis*, Sanches et al. [69] also observed a decrease in AChE activity in G, suggesting that impacts may occur more rapidly, depending on the contaminant. Therefore, the present findings, along

with existing literature, highlight that inhibition of AChE may depend on the specific contaminant as well as the duration of exposure, with some contaminants requiring longer exposure before inducing an inhibitory effect. PFUnDA stood out from the other compounds on the negative axis of PCO1 due to its ability to inhibit the activity of CbEs. This inhibition can be attributed to the chemical structure of PFUnDA, which may have a high affinity for the enzyme's active site. This is likely due to specific interactions, such as hydrogen bonds or hydrophobic interactions, leading to more effective inhibition compared to other compounds that lack such favorable interactions. The study by Solé and Sanchez-Hernandez [87] has already demonstrated the inhibition of CbEs activity in aquatic organisms exposed to pharmaceuticals and personal care products, contaminants considered to be emerging contaminants such as PFAS. In the present study, despite the biochemical consequences, exposure to PFAS did not result in discernible cellular damage in the G of the mussels. This observation lends support to the hypothesis that mussels may possess alternative defense mechanisms, not measured in this study, that contributed to the regulation of ROS balance and safeguard against oxidative stress.

Nevertheless, it was observed that for both organs (DG and G), the 6:2 FTA compound showed the closest biochemical values to CTL, indicating that this emerging short-chain PFAS induced less toxicity in the organisms. These findings are consistent with the literature, as Hoke et al. [88] have demonstrated that the 6:2 fluorotelomer sulfonic acid (6:2 FTSA), from the same family of compounds as the 6:2 FTA, is not classified as an aquatic risk under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) or European Classification, Labelling and Packaging (CLP) regulations and exhibits low bioconcentration and biomagnification factors.

5. Conclusion

This study addresses the knowledge gap regarding per- and polyfluoroalkyl substances (PFAS) toxicity in bivalves by evaluating six PFAS compounds (PFHxA, 6:2 FTA, PFUnDA, PFDoA, PFTriDA, and PFTeDA) on the digestive gland (DG) and gills (G) of mussels, *Mytilus galloprovincialis*, through short-term *in vitro* exposure. The results reveal organ-specific responses: the DG was more sensitive to PFHxA, showing increased catalase (CAT) activity but reduced total antioxidant capacity (TAC), indicating insufficient antioxidant defenses. However cellular damage in the DG was only observed at higher concentrations of PFTriDA (100 μg/L). In contrast, the G were more affected by PFDoA and PFTeDA, exhibiting inhibited antioxidant enzymes and high oxidative stress, while PFHxA and PFTriDA also showed acetylcholinesterase (AChE) inhibition. Notably, 6:2 FTA was the least toxic, suggesting emerging short-chain PFAS like 6:2 FTA may be considered safer but still require further scrutiny. Conversely, PFHxA, despite being a short-chain PFAS, demonstrated significant toxicity, emphasizing the need for thorough risk assessments of all PFAS types. Overall, this study highlights the importance of considering both PFAS chain length and organ-specific responses in toxicity evaluations and supports the need for comprehensive risk assessments and management strategies.

Environmental implications

Per- and polyfluoroalkyl substances (PFAS) are known as "forever chemicals" due to their persistence, long half-lives and potential human toxicity. Their widespread presence in aquatic systems poses significant risks to wildlife and potentially to humans. This study aims to assess the *in vitro* toxic effects of six PFAS on the digestive gland and gills of mussels, *Mytilus galloprovincialis*, highlighting the importance of biomarkers to provide rapid and effective information on the potential impact of PFAS on marine organisms. Overall, this study reveals the need of considering PFAS chain length and organ-specific responses in toxicity evaluations.

CRediT authorship contribution statement

Marta Cunha: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alessandro Nardi:** Writing – review & editing, Validation, Supervision, Conceptualization. **Rosa Freitas:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Amadeu M.V.M. Soares:** Resources, Funding acquisition. **Ana M. Gil:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.136823.

Data availability

Data will be made available on request.

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