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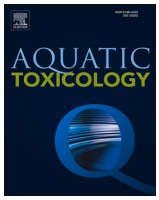

Chronic PFAS exposure induces the activation of selenium-dependent glutathione peroxidases and catalase as antioxidant defences in the European chub (*Squalius cephalus)* (Linnaeus, 1758) kidney

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A R T I C L E I N F O

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A B S T R A C T

The Veneto Region (Italy) experienced one of the heaviest contaminations by per- and polyfluoroalkyl substances (PFAS), pollutants of emerging concern due to their environmental persistence and bioaccumulation potential in animal tissues. Hence, there is a need to study their impact on freshwater fish inhabiting contaminated rivers, particularly at the level of the antioxidant system, since PFAS are known to cause an imbalance in reactive oxygen species (ROS) production, thereby increasing the risk of oxidative stress. This study examines the physiological responses triggered by chronic exposure to three distinct environmental concentrations of PFAS in the European chub (*Squalius cephalus*). The sites were classified as "control" (with a PFAS concentration < 5 ng/L), "low polluted" (5.64 ng/L) and "highly polluted" (582.6 ng/L). Biochemical and molecular analyses were performed on the kidney, one of the main organs for xenobiotic bioaccumulation. The catalase (CAT) and selenium-dependent glutathione peroxidases (Se-GPXs) expression was quantified at both active protein and mRNA transcript levels. Results confirm the activation of antioxidant defences against the risk of PFAS-induced oxidative stress. There is a differential induction in the biosynthesis of enzymes inside specific intracellular compartments: CAT in peroxisomes in the "low polluted" site and Se-GPXs in the cytoplasm in the "highly polluted" one. The gene *gpx1* was the only isoform whose mRNA level corresponded to that of the active protein, suggesting the highest contribution to the biosynthesis of Se-GPXs at high PFAS concentrations. Conversely, *gpx4* increased its transcription level in the "low polluted" site, which didn't match with an increase in protein content, leading us to hypothesise an involvement of specific cytoplasmic mRNA-protein complexes, called stress granules, acting in the temporary silencing of *gpx4*.

GLOSSARY

ANOVA: one-way analysis of variance
ARPAV: Regional Agency for Environmental Prevention and Protection of Veneto
BSA: bovine serum albumin
cat: catalase gene
CAT: catalase protein
CNR: Italian National Research Council
DTT: 1,4-Dithiothreitol

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EDTA: ethylenediaminetetraacetic acid
G3BP1: GTPase Activating Binding Protein 1
gpx1: glutathione peroxidase 1 gene
GPX1: glutathione peroxidase 1 protein
gpx2: glutathione peroxidase 2 gene
GPX2: glutathione peroxidase 2 protein
gpx3: glutathione peroxidase 3 gene
GPX3: glutathione peroxidase 3 protein
gpx4: glutathione peroxidase 4 gene
GPX4: glutathione peroxidase 4 protein
GPXs: glutathione peroxidases
GSH: reduced glutathione
GSSG: oxidised glutathione
H_2O: water
H_2O_2: hydrogen peroxide
HSD: honestly significant difference
LOQ: limit of quantification
$NADP^+$: oxidised nicotinamide adenine dinucleotide phosphate
NADPH: nicotinamide adenine dinucleotide phosphate
O_2: molecular oxygen
PABP: poly(A)-binding protein
PFAS: per- and polyfluoroalkyl substances
PFCs: polyfluorocarbons
PFOA: perfluorooctanoic acid
PFOS: perfluorooctanesulfonic acid
PRDX3: peroxiredoxin 3 protein
prdx4: peroxiredoxin 4 gene
PRDX5: peroxiredoxin 5 protein
PRDXs: peroxiredoxins qRT-PCR: quantitative real-time PCR
ROS: reactive oxygen species
Se: selenium
SGs: stress granules
TEM: transmission electron microscopy
TIA-1: T-Cell-Restricted Intracellular Antigen-1

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of fluorinated organic compounds with distinct and unique chemical and physical properties, enabling their use in various industrial processes (Buck et al., 2021). They have a hydrophobic aliphatic tail composed of 4–16 carbons and are distinguished from polyfluorocarbons (PFCs) by the absence of aromatic groups. All or only some hydrogen atoms are replaced by fluorine atoms on the skeletal carbon structure, according to the C_nF_{2n+1} formula (Buck et al., 2011).

Since the 1950s, their production has increased steadily, and their use in various sectors has led to uncontrolled release into the environment, particularly in freshwater systems. The main contaminated sites correspond to the most industrialised areas. However, thanks to the strong fluorocarbon bond, PFAS are very stable and persistent in the environment (Glüge et al., 2020). For this reason, they are also detected in remote areas of the planet (Russell et al., 2008). PFAS have garnered scientific attention since the early 2000s, when they were first detected in human blood, prompting the initiation of studies aimed at understanding their potential toxic effects on the health of organisms (Travis, 2024). The Veneto region is one of the most notable cases of PFAS contamination. In 2013, a study by the CNR Water Research Institute revealed a high presence of PFAS in aquifers, particularly perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) (Polesello et al., 2013). The subsequent monitoring activities of the Regional Agency for Environmental Prevention of Veneto (ARPAV) revealed that the contamination affected a total area of 190 km^2, encompassing the provinces of Verona, Padua, and Vicenza (Pitter et al., 2020).

As confirmed by other studies (Liu et al., 2007; Panaretakis et al., 2001; Wielsøe et al., 2015; Yao and Zhong, 2005), PFAS can enter cells, leading to an increase in the production of reactive oxygen species (ROS), which are toxic intermediates in the metabolism of molecular oxygen (O_2). About 3 % of the O_2 in cells is improperly converted into ROS (Santovito et al., 2005). Over 90 % of the latter are generated in mitochondria, representing the main intracellular compartments for ROS production (Filho, 2007). ROS regulate multiple biological processes at physiological concentrations, including cell proliferation, inflammatory response, cell signalling and stress defence (Bakiu et al., 2024; Finkel, 2011; Shields et al., 2021). However, high concentrations of ROS can cause an imbalance in the redox equilibrium, resulting in a state of oxidative stress, which can damage various macromolecules, including DNA, lipids, and proteins (Liguori et al., 2018). For this reason, ROS concentrations are maintained within a homeostatic range, thanks to the action of multiple antioxidant molecules that detoxify the cells from them. Oxidative stress represents the breakdown of the balance between the formation and elimination of ROS. Hydrogen peroxide (H_2O_2) represents the second intermediate of ROS chain formation, deriving from the dielectronic reduction of O_2. Compared to other ROS, H_2O_2 has a non-radical nature and represents the least hazardous form; nevertheless, the Haber-Weiss and Fenton reactions can lead to the production of hydroxyl radicals, the most dangerous ROS for an organism's health (Das et al., 2015). Therefore, organisms have evolved multiple antioxidant enzymatic and non-enzymatic defences to limit the risk of oxidative stress. Among the firsts, glutathione peroxidases (GPXs), peroxiredoxins (PRDXs) and catalase (CAT) collaborate to counteract H_2O_2, reducing it to water (H_2O) and O_2 in different cellular compartments.

Previous studies have shown that PFAS contamination in the rivers of the Veneto region leads to a significant increase in oxidative stress in particular fish species, including the European chub *Squalius cephalus* (Linnaeus, 1758). Our previous results (Piva et al., 2022) suggested that the liver can cope with this stress condition by activating its mitochondrial-level antioxidant defences. Nevertheless, further analyses of the same specimens revealed high stress levels in both muscle and blood tissues (Schumann et al., 2024), underscoring the need to investigate additional organs and anti-stress components. The kidney was found to be affected by PFAS-induced stress, particularly in the endoplasmic reticulum, due to the downregulation of the peroxiredoxin 4 (*prdx4*) gene (Pacchini et al., 2025). Indeed, the antioxidant enzyme encoded by this gene specifically protects this cellular compartment from oxidative stress by acting as a scavenger of H_2O_2 (Tavender and Bulleid, 2010). It remains to be verified whether the other parts of the cell also suffer from oxidative stress or whether the antioxidant system is active in other cell organelles.

The kidney was identified as one of the primary target tissues in fish for PFAS bioaccumulation. In two bioconcentration studies on *Oncorhynchus mykiss*, PFAS accumulated significantly in blood, kidney and liver (Martin et al., 2003; Goeritz et al., 2013). Considering the high affinity between PFAS and blood serum proteins, it is reasonable that highly perfused organs, such as the liver and kidney, could be more affected by PFAS bioaccumulation (Xiong and Li, 2024).

The kidney is also the primary organ for xenobiotic elimination, detoxifying the organism from toxic molecules using multiple systems (Inui et al., 2000). PFAS can be eliminated at the renal level via glomerular filtration, a passive diffusion process, or through two other active pathways: tubular secretion and reabsorption. This process is mediated by PFAS renal carriers, which are members of two transporter families: the solute carrier (SLC) and ATP-binding cassette transporter (ABC) families. The former do not require ATP hydrolysis; on the contrary, the latter are ATP-dependent transporters and principally work as efflux transporters (Niu et al., 2023). It is conceivable that greater PFAS bioaccumulation requires higher kidney activity to eliminate these substances, thereby increasing metabolic energy demand. This entails the inherent risk of increased ROS production by the mitochondrial electron transport chain, leading to the activation of the antioxidant system to counteract oxidative stress. Therefore, this work aims to

evaluate the efficiency of the kidney of *S. cephalus* in antioxidant defences, to mitigate the risk of oxidative stress following chronic environmental stress by PFAS in freshwater streams of the province of Vicenza (Veneto, Italy). In particular, this work analysed the induction of selenium-dependent glutathione peroxidases (Se-GPXs) and CAT enzymes at the level of active proteins. The mRNA accumulation for CAT and four Se-GPX isoforms was also measured to investigate the involvement of different cellular compartments.

2. Materials and methods

2.1. Study area

The study was conducted in the Veneto Region (northeastern Italy), an area known since 2013 for large-scale PFAS pollution caused by long-term industrial emissions in the provinces of Vicenza, Verona, and Padua, primarily attributed to the Rimar-Miteni (RM) industrial facility. The plant was located in Trissino (Vicenza) and discharged wastewater into the Agno-Fratta-Gorzone river system, resulting in the widespread dispersion of PFAS downstream through a complex hydrological network (Polesello et al., 2013). The RM plant ceased its operations and was declared bankrupt in November 2018. From that moment, the Veneto Region launched extensive environmental monitoring programs to assess PFAS levels in surface and groundwater.

Fish samples were collected from three rivers in Vicenza province with different levels of documented PFAS contamination. These sites were selected based on historical PFAS monitoring data on surface waters provided by ARPAV (Table S1). The absence of other environmental contaminants was also verified (metals, pesticides, and volatile organic compounds were below the detection limits), enabling PFAS to be identified as the primary potential pollutant.

A map of the study area is provided in Figure S1, along with the locations of sampling sites and the former RM plant. Leogra Torrent, in Torrebelvicino (VI) (45.71779193 N; 11.30627616 E), was located in the upper Brenta River basin, upstream of any significant urban or industrial activity. It presented a PFAS concentration lower than the detection threshold (< 5 ng/L) and was therefore selected as a "control site". The Roggia Moneghina River, located in Grumolo delle Abbadesse (VI) (45.5116 N; 11.6560 E), was positioned along the mid-course of the Bacchiglione River, in an area moderately affected by historical PFAS sources, and was therefore chosen as a "low polluted" site. The Retrone River, located in Altavilla Vicentina (VI) (45.51350834 N, 11.5045197 E), was part of the Fratta-Gorzone River system, historically known to receive industrial discharges from the former RM plant. Given its high contamination level, it was selected as a "highly polluted" site.

2.2. Sampling activity

Ten specimens of *S. cephalus* were collected from each site, with a comparable size range (19.99 ± 5.14 cm) and weight (42.36 ± 21.07 g), using the electrofishing technique (authorised by decree of the director of the Agri-environment, Planning and Management of Fish and Wildlife Hunting of the Veneto Region, n. 336 of 14 December 2020). Electrofishing was carried out in a single pass using a backpack electrofisher (Smith-Root LR-24 Backpack Electrofisher) throughout the wadeable habitat (< 1 m depth), and one net collected the fish. The sampling activity was conducted in March 2021 by a single team and on a single day, ensuring minimal environmental variation between the three sites (temperature within a 0.5-degree range; pH approximately 8.0–8.1). Although the number of specimens analysed is limited, it is adequate to obtain significant results in studies of stress physiology, as demonstrated by other research (Solé et al., 2021; Altwaijry et al., 2023; Atli et al., 2024). After the sampling, individuals were immediately euthanised with an overdose of clove oil (0.070 mL/L), a widely accepted and effective method for minimising stress and pain in teleost species, particularly under field conditions (Keene et al., 1998; Ross and Ross, 2008). The procedure complied with the general principles of the Directive 2010/63/EU and AVMA guidelines for the euthanasia of animals (2020). Fish were rapidly dissected to extract organs (including the kidney), which were promptly frozen with liquid nitrogen and stored at −80 °C until processing.

2.3. Cell-free extract preparation

To obtain a cell-free extract for biochemical analyses, kidney samples were homogenised in a buffer solution containing Tris–HCl 20 mM (pH 7.6), EDTA 1 mM, DTT 1 mM, sucrose 0.5 mM, and KCl 0.15 mM. This process involved mechanically crushing the tissue in ice using a high-speed mixer-homogeniser (Polytron PT 3000, Kinematica AG, Luzern, Switzerland). After centrifugation at 13,000 × *g* for 1 h at 4 °C, the cell-free extracts were immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. CAT activity assay

Aebi's method (Aebi, 1984) was employed to quantify CAT activity. The principle is based on the rapid enzymatic reduction of H_2O_2 to H_2O, resulting in a decrease in absorbance at 240 nm, as measured using a UV-1800 UV–Vis Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). This allowed the quantification of CAT levels as active protein, expressed as CAT units per milligram of protein (U CAT/mg proteins). A CAT unit is the enzyme quantity that reduces 1 μmol of H_2O_2 per minute.

2.5. Se-GPX activity assay

The Se-GPX activity was quantified using the spectrophotometric method described by Livingstone et al. (Livingstone et al., 1992), which employed H_2O_2 as the substrate. Se-GPXs reduce H_2O_2 employing reduced glutathione (GSH), which is converted to its oxidised form (GSSG). GSH is subsequently regenerated by glutathione reductase through the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. The consumption of NADPH was measured by the decrease in its absorbance at 340 nm using a UV-1800 Spectrophotometer to estimate the Se-GPX content as active proteins, expressed as Se-GPX units per milligram of protein (U Se-GPX/mg protein). The unit of Se-GPX corresponds to an enzyme quantity capable of catalysing the oxidation of 1 μmol of NADPH per minute.

2.6. Total protein quantification

CAT and Se-GPXs values were normalised to the total protein content of each sample. The total amount of protein in the cell-free extract was quantified through the Folin-Ciocalteu phenol reagent method of Lowry et al. (Lowry et al., 1951). Increasing concentrations of bovine serum albumin (BSA) were used as standards for the calibration curve.

2.7. Total RNA extraction, quantification, integrity assessment and cDNA synthesis

According to the TripleXtractor protocol (Grisp, Porto, Portugal), the guanidine thiocyanate-phenol-chloroform method was performed to extract the total RNA content by precipitation from kidney samples. This was subsequently quantified with the NanoDropTM 2000/2000c spectrophotometer (Thermo Scientific, Wilmington, USA). The RNA purity was assessed by calculating the 260/280 and 260/230 absorbance ratios. The first was used to verify the absence of protein and DNA contamination, and the second was used to verify the absence of lipids and phenol contamination. Electrophoresis in a 1 % agarose denaturing gel was performed to verify the RNA integrity and absence of genomic contamination. Samples were then diluted to 1000 ng/L and reverse transcribed using the cDNA Synthesis Kit (Biotechrabbit, Berlin, Germany).

2.8. Primer design and validation by qualitative PCR

Primers used for amplifying and quantifying *gpx4* were previously designed by Piva et al. (2022) on the characterised sequence of *S. cephalus*. Conversely, primers for the quantification of *gpx1, gpx2, gpx3* and *cat* were designed using cDNA sequences of phylogenetically related species within the Cypriniformes group, collected from the NCBI database: the fathead minnow (*Pimephales promelas*) and the grass carp (*Ctenopharyngodon idella*). Primers for quantitative real-time PCR (qRT-PCR) (listed in Table S2) were designed using the Primer3 v 4.1.0 program (https://primer3.ut.ee/) to select 100–200 nucleotides (nt) of the coding region. Primers were subsequently analysed *in silico,* through the IDT OligoAnalyzer™ tool (https://eu.idtdna.com/calc/analyzer) and the Beacon Designer™ program (http://premierbiosoft.com/qOligo/Oligo.jsp?PID=1). A multiple alignment among the Cypriniformes cDNA sequences was performed with the EMBL-EBI T-Coffee program (https://www.ebi.ac.uk/jdispatcher/msa/tcoffee?stype=protein), to verify that primers were designed in the highly conserved gene regions (identical nt > 90 %). A qualitative PCR was performed to verify the amplification efficiency of primers, followed by 1.5 % agarose gel electrophoresis to visualise the specific amplification products. Table S3 contains the list of tested primers for *gpx2* amplification trials. Given the impossibility of amplifying with any of the tested primer pairs, qRT-PCR was not performed for this gene.

2.9. Quantification of mRNA expression by qRT-PCR

The qRT-PCR analyses allowed the evaluation of transcription levels of the five studied genes: *gpx1, gpx3, gpx4,* and *cat*. Each sample was analysed in triplicate. The amplification thermal profile included 95 °C for 2 min, 38 cycles at 95 °C for 20 s and 60 °C for 1 min (amplification plot), and finally 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s (dissociation curve, to verify the absence of genomic contamination). To evaluate primer efficiencies, standard curves were established using increasing concentrations of a cDNA pool, thereby excluding individual-specific responses. According to the comparative Ct method (Livak and Schmittgen, 2001), only primers with an amplification efficiency close to 1 were used to ensure a comparable amplification efficiency. Each sample was normalised on the transcription level of *gapdh*, the housekeeping gene. Gene expression was quantified through the formula $2^{-\Delta\Delta Ct}$ from the Pfaffl mathematical model (Pfaffl, 2001).

2.10. Statistical analysis

Results are presented in mean values ($n = 10$) ± Standard Deviations. Statistical analysis was performed using Prism GraphPad software (Version 10.3.1, Boston, MA, USA). For each group, a Shapiro-Wilk normality test was performed to verify the normality of the dataset. Given the normality condition in all groups, parametric tests were performed. A one-way analysis of variance (ANOVA) test was conducted to verify statistically significant differences among the sampling sites, followed by Tukey's HSD test to determine the significance of these differences. Statistical significance was considered at a p-value cutoff of 0.05. To reflect the magnitude of significance, results are reported and graphically indicated at three levels: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***), as recommended in statistical reporting guidelines (Altman et al., 1983).

3. Results

3.1. Enzymatic tissue activity

CAT activity is statistically higher (64 %; $p = 0.00033$) in specimens sampled in the "low polluted" site compared to those from the "control" one (Fig. 1a). CAT activity measured in kidney samples from the "highly polluted" site is not statistically different from the controls ($p = 0.46$).

The graph in Fig. 1b shows the Se-GPX activity in the kidney of fish from the "highly polluted" site, which is statistically higher (61 %; $p = 0.0024$) than that measured in specimens from the "control" site. Se-GPX activity in kidney samples from the "low polluted" site is not statistically different with respect to controls ($p = 0.51$).

3.2. gpx1, gpx2, gpx3, gpx4, and cat mRNA levels

Fig. 2 reports the transcript levels of *cat, gpx1, gpx3*, and *gpx4* in kidney tissues. Regarding *gpx2*, no amplification of the target mRNA was detected, suggesting an absence or extremely low transcription levels of this gene in the kidney tissue.

No statistically significant differences in the *cat* (CTRL vs low: $p =$

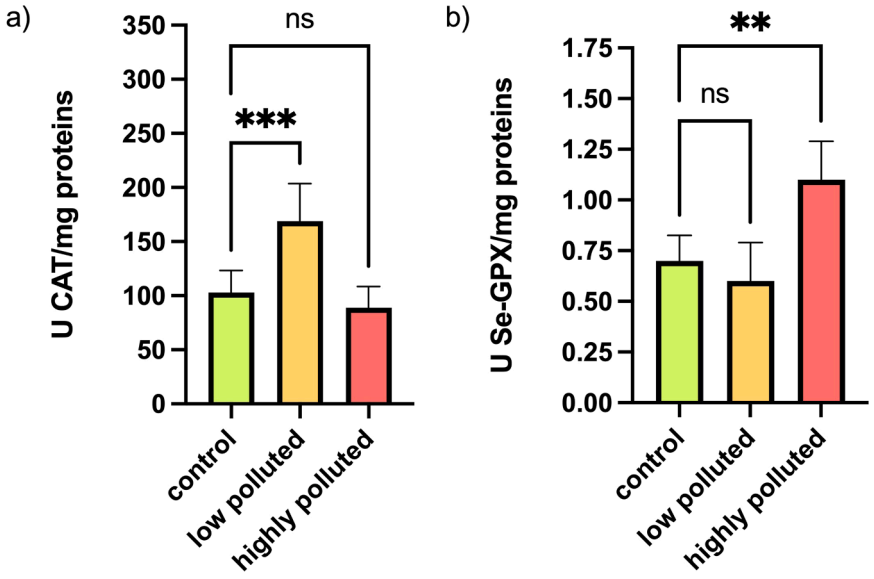

Fig. 1. Enzymatic tissue activity of (a) CAT and (b) Se-GPX proteins. Different colours represent the three sites at different PFAS concentrations: the "control" site in green, the "low polluted" site in yellow, and the "highly polluted" site in red. Asterisks indicate statistically significant differences against the "control" site (ns: no significant differences; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

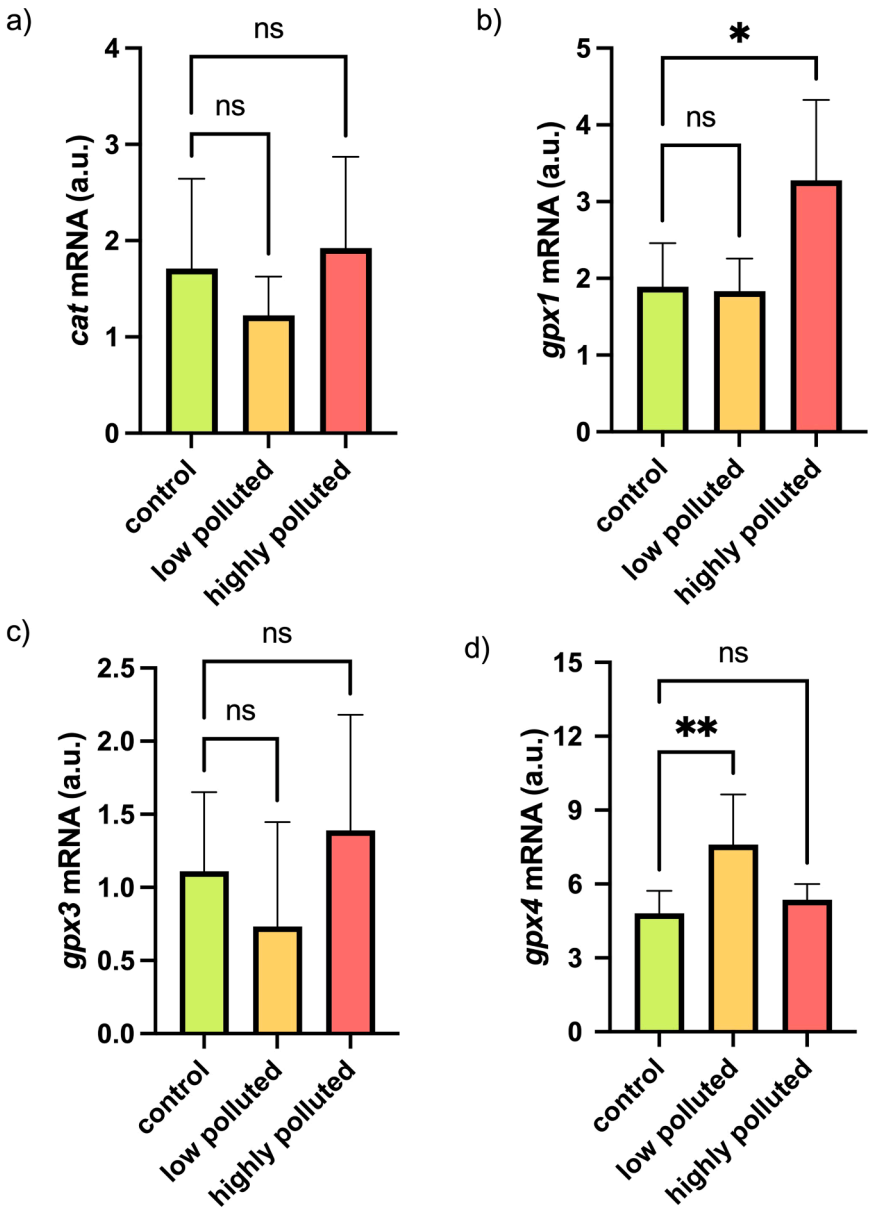

Fig. 2. mRNA expression levels of (a) cat, (b) gpx1, (c) gpx3 and (d) gpx4. Different colours represent the three sites at different PFAS concentrations: the "control" site in green, the "low polluted" site in yellow, and the "highly polluted" site in red. Asterisks indicate statistically significant differences against the "control" site (ns: no significant difference; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

0.50; CTRL vs high: $p = 0.86$) and *gpx3* (CTRL vs low: $p = 0.65$; CTRL vs high: $p = 0.80$) gene expression levels were detected among the specimens from the three sampling sites (Fig. 2a and 2c).

The graph in Fig. 2b shows *gpx1* mRNA level in the kidney of fish from the "highly polluted" site that is statistically higher (73.3 %; $p = 0.015$) than that measured in specimens from the "control" site. The *gpx1* mRNA level from the "low polluted" site is not statistically different with respect to controls ($p = 0.99$).

The *gpx4* mRNA levels were statistically higher in the kidney tissue of specimens sampled in the "low polluted" site (58 %; $p = 0.0038$) compared to the "control" one (Fig. 2d). No statistically significant differences in the *gpx4* expression levels were detected between the specimens from the "highly polluted" site and the "control" one ($p = 0.72$).

4. Discussion

Multiple factors can increase the rate of ROS formation, including exogenous stressful conditions, such as exposure to environmental contaminants and xenobiotics (Lushchak, 2016; Pollard et al., 2024). Among these, PFAS are known to lead to an imbalance in the cellular redox equilibrium, which in turn increases the risk of oxidative stress (Wielsøe et al., 2015).

In the "low polluted" site, where PFAS concentrations are low, it is plausible that the generation of ROS, particularly H_2O_2, is moderate and therefore requires a lower antioxidant activation by the cells. From the results of enzymatic activity, it appears that such activation only involves CAT. In fact, it is evident that there was a statistically significant increase in CAT in the kidney from the "low polluted" site compared to the control one. This suggests that CAT plays a preventive action against oxidative stress at low PFAS concentrations, representing a first line of defence against increased H_2O_2 (Nitta et al., 2020), and is involved in the primary stress response. Therefore, at these low PFAS concentrations, the generation of ROS, particularly H_2O_2, is moderate enough to stimulate CAT activity without overwhelming its capacity, allowing efficient detoxification.

On the other hand, exposure to higher PFAS concentrations does not cause a further increase in CAT activity, which remains stable at control values. It is conceivable that high PFAS concentrations, present in the "highly polluted" site, could increase ROS production, including H_2O_2. Previous studies showed that high levels of H_2O_2 can inhibit CAT activity (Lardinois et al., 1996), as confirmed by our results in the *S. cephalus* kidney. One hypothesis is that CAT is being inhibited due to excessive ROS accumulation, which can alter the enzyme's structure or function (Lardinois et al., 1996). However, if this were to happen, we would see a decrease in CAT activity below control levels, but this does not occur. Another possibility is that CAT, under those environmental conditions, is reaching its maximum antioxidant capacity. Although catalase is one of the key enzymes responsible for H_2O_2 detoxification, its high efficiency is limited to peroxisomes, where this enzyme is located primarily (Nitta et al., 2020). The PFAS-induced increase in the rate of H_2O_2 formation could involve other intracellular sites, such as the cytoplasm or mitochondria, where CAT is absent (Ighodaro and Akinloye, 2018). However, the absence of CAT elevation at high pollution levels does not appear to be a significant factor in the risk of oxidative stress. This is likely because Se-GPX activity increases simultaneously, providing a second line of defence against PFAS-induced oxidative stress in this organ. This suggests a shift in antioxidant strategy, where the system relies less on CAT and more on Se-GPXs to reduce H_2O_2 and other peroxides, especially when ROS production exceeds CAT's detoxification ability. Therefore, a phasic response of the scavenger system against H_2O_2 is conceivable. At low PFAS exposure, this response involves activation of CAT, while at higher concentrations of these xenobiotics, Se-GPXs likely play a predominant role. The differential induction of biosynthesis of these enzymes confirms that antioxidant defences work complementarily: some are activated when the others are weakened or inhibited. Godin and Ganrett (Godin and Garnett, 1992) also found that CAT and GPX activities were inversely correlated, even in various mammalian species, suggesting that low GPX activity is compensated by high CAT activity. Benedetti et al. (Benedetti et al., 2007) demonstrated that the exposure to cadmium of the Antarctic fish *Trematomus bernacchii* induces an increase in GPX activity, while CAT activity remains unaltered.

The gene expression results have provided interesting indications of the mechanisms underlying these variations in enzyme activity, although no direct statistical correlation was observed. In fish sampled from the "low polluted" site, the *cat* transcription shows an unexpected trend unrelated to enzyme activity, as a high biosynthesis of the active protein at low concentrations of PFAS does not correspond to an increase in *cat* mRNA level. Similarly, there was an increase in *gpx4* transcription without a corresponding rise in Se-GPX activity. The mRNA-protein mismatch of certain antioxidant enzymes has also been observed in other studies examining the physiological stress responses of fish to environmental stressors (Chatzidimitriou et al., 2020; Sattin et al., 2015).

This discrepancy is not directly attributable to the different half-lives of the respective molecules (Lavut and Raveh, 2012), which led to the hypothesis of post-transcriptional regulation involving stress granules (SGs) (Drago et al., 2022). SGs are membrane-free foci, 0.1–2 μm in diameter, that form in the cytoplasm of eukaryotic cells (Hirose et al., 2023). They contain complexes of silenced mRNA blocked in the early phase of translation, ribosomal subunit 40S, eukaryotic initiation factors (eIF4E, eIF4G, eIF4A, eIF4B, eIF3, eIF2), some non-translating messenger ribonucleoproteins (mRNPs) and different mRNA-binding proteins, acting as translational repressors (Kedersha et al., 2002; Protter and Parker, 2016). Oxidative stress induced by various stress factors, such as thermal shock, hyperosmolarity, viral infection and UV irradiation, can trigger the formation of SGs (Anderson and Kedersha, 2009; Chen and Liu, 2017). Their assembly begins with the phosphorylation of the eukaryotic translation initiation factor 2 subunit 1 (eIF2α) (Mokas et al., 2009), which enables the selection of mRNAs to be stored and protected from degradation. SGs exhibit selective mRNA recruitment rather than random sequestration. This selectivity is influenced by cis-acting sequence elements, RNA-binding proteins (RBPs), codon usage bias, poly(A) tail length, and even the subcellular localisation of the transcripts (Khong et al., 2017). This implies that mRNAs coding for different antioxidant enzymes can be differentially regulated post-transcriptionally, depending on the stress context.

In fact, this regulatory system aims to efficiently respond to changing environmental conditions by reinitiating the translation of mRNA that is already present inside SGs (Piva et al., 2024). This follows the cell's need to rapidly implement the biosynthesis of proteins targeted to counteract a stress peak (Waris et al., 2014), thereby improving cell viability during the response (Treeck and Parker, 2019). The fact that the analysed organisms are exposed to chronic stress would justify the presence of SGs (Ratti et al., 2020). To verify it, it would be appropriate to evaluate primarily the gene expression of some proteins involved in their assembly, which could include T-cell-restricted intracellular antigen 1 (TIA-1), TIA-1-related protein (TIAR), Ras-GTPase-activating protein SH3 domain-binding proteins 1 and 2 (G3BP1/2), and tristetraprolin (TTP) (Anderson and Kedersha, 2008). These nucleation proteins participate in SG formation by directly binding target mRNAs and self-associating to promote SG growth (Mitchell and Parker, 2014). Data from the literature show that high levels of TIA-1 mRNA expression frequently correspond to low levels of antioxidant enzyme biosynthesis, supporting the hypothesis of post-transcriptional control operated by SGs in the case of oxidative stress risk, not only in fish (Nicorelli et al., 2018; Piva et al., 2024), but also in other aquatic animals (Drago et al., 2021).

Therefore, the higher CAT activity measured in fish on the "low polluted" site would not result from the increased transcription of the *cat* gene but rather from the translation of its messengers being previously transcribed and stored inside SGs. Instead, the increase in *gpx4* mRNA could represent a preventive response against the increased risk of oxidative stress in the mitochondria due to exposure to PFAS. The translation could take place at a later stage, should the need arise to activate antioxidant defences in this cellular compartment, rather than immediately investing metabolic energy in the biosynthesis of enzymes. For that reason, the lack of correspondence between gene expression and enzymatic activity for CAT and Se-GPX may reflect enzyme-specific regulation by SGs, depending on how cells prioritise their response to chronic PFAS exposure. This hypothesis is supported by growing evidence in the literature, which shows that stress granules differentially sort transcripts related to oxidative stress, enabling the selective regulation of their translation during cellular stress (Aulas et al., 2017; Sanders et al., 2020).

Regarding the fish sampled in the "highly polluted" site, results show a statistically significant increase only in the expression of *gpx1*. These data suggest that high concentrations of PFAS result in the development of peroxides at the cytoplasmic level, activating the GPX1 protein, which operates especially in this cellular compartment. Additionally, Kim et al. (Kim et al., 2020) reported a significant upregulation of *gpx1* transcription in zebrafish embryos only under the highest dose of PFAS treatment. This result correlates well with the Se-GPX activity data, as the increase in the gene transcript corresponds to the rise in the active protein content. Since *gpx1* is the only one among the investigated genes actively transcribed in fish from the "highly polluted" site, we can deduce that this isoform contributes more to preventing oxidative stress caused by peroxides. However, based on what has been previously said about the post-transcriptional regulation carried out by SGs, we cannot exclude the possibility that the other GPX isoforms may also contribute to Se-GPX activity.

Among these proteins, we can certainly exclude GPX2, given the impossibility of detecting its mRNA in the kidney. This lack of amplification likely reflects the tissue-specific expression pattern of *gpx2*, which is predominantly transcribed in the intestinal epithelium rather than in kidney tissue. This result is consistent with the evidence in the literature, which indicates a predominant expression and activity of the GI-GPX

protein (gastrointestinal glutathione peroxidase) encoded by *gpx2* gene (Chu et al., 1997) in the intestinal epithelium inside enterocytes (Florian et al., 2001), where it plays a critical role in neutralising lipid hydroperoxides and protecting against oxidative stress associated with digestion (Brigelius-Flohé and Maiorino, 2013).

Consequently, we can exclude the involvement of *gpx2* in the antioxidant response in the chub kidney, and the absence of its expression does not reflect a technical failure but rather its tissue-specific regulation. The same consideration also applies to GPX3. In fact, it is an extracellular enzyme (Brigelius-Flohé and Maiorino, 2013), whereas PFAS alter the cellular redox balance with an increase in the concentration of ROS, mainly at the intracellular level. Therefore, it is plausible that *gpx3* transcription is not activated, thereby saving metabolic energy to favour the transcription of more useful isoforms that detoxify the cell from peroxides formed at the intracellular level.

Concerning the expression of *gpx4*, the lack of increased mRNA levels is interesting, as this enzyme is specifically expressed in mitochondria (Pei et al., 2023), a target of PFAS toxicity (Hagenaars et al., 2013). These data suggest that the fish kidney is not subject to mitochondrial stress in the "highly polluted" site despite the higher levels of PFAS. However, it is known that in these intracellular compartments, other enzymes can intervene as a protective system, such as H_2O_2 scavengers. These include PRDXs, a group of antioxidant enzymes widely distributed among eukaryotic organisms (Al-Asadi et al., 2019; Bakiu and Santovito, 2015; Hewitt and Degnan, 2023; Tolomeo et al., 2016). In particular, isoforms 3 and 5 are expressed in the mitochondria, where they represent an essential defence against chemical and physical stress (Ježek et al., 2024). This redundancy of mitochondrial isoforms appears to be functional, as PRDX5 seems to act at a basal level and in low-stress situations, while PRDX3 is activated when stress increases (Drago et al., 2022; Tolomeo et al., 2019). Preliminary data obtained by analysing the mRNA expression of these two PRDXs in the same fish considered in the present study seem to confirm this trend, with both isoforms compensating for the absence of GPX4 protein induction (unpublished results).

It is essential to acknowledge that this study is based on certain key assumptions; therefore, it is crucial to consider its limitations.

PFAS water concentrations used for site classification were not directly measured at the time of specimen collection. Instead, they were acquired from official reports provided by ARPAV, a certified regional authority, which constantly publishes data on systemic environmental monitoring. However, despite these data being considered reliable, as obtained through standardised and accredited analytical protocols, the lack of in situ validation may introduce uncertainty.

A direct correlation between environmental PFAS concentrations and their bioaccumulation in the kidney was assumed, due to the scarce quantity of material, which made it impossible to measure PFAS inside the tissue. This may create ambiguity, but other experimental data support this hypothesis. The same analysis of other organs (liver, gills, intestine, and skeletal muscle) revealed a correspondence between PFAS levels in the water and those found in the liver and skeletal muscle (Schumann et al., 2024). Considering the common role in xenobiotic detoxification, it is plausible that the kidney could also serve as an accumulation site, reflecting the liver pattern. In addition, it is reasonable to assume that PFAS accumulation in this organ may equal or even surpass that in muscle tissue, as documented in other freshwater fish species (Goeritz et al., 2013; Surma et al., 2021). A correlation between PFAS in the water and bioaccumulation levels in the kidney was found in samples collected during another sampling campaign on *S. cephalus* (April 2024) (unpublished results).

The presence of other environmental contaminants, such as heavy metals, pesticides, and volatile organic compounds, was not directly investigated, as these substances were reported to be below the detection limits according to ARPAV monitoring data. Although this reduces the likelihood of perturbative effects, the possibility of undetected synergistic or additive interactions cannot be excluded entirely. Considering the inherent complexity of the environmental experimental setup, eliminating all variables presents a significant challenge. The minimal presence of other contaminants below the detection limit, combined with common physical conditions, represents a good starting point. To isolate PFAS-specific effects, future studies under controlled laboratory conditions with defined concentrations of individual compounds or mixtures are recommended. The present study aimed to evaluate the impact of chronic stress on the chub kidney. Considering the long half-life of this species and its high mobility, replicating the same experiments of prolonged exposure in the laboratory could mask other significant intrinsic limitations.

The lack of *gpx2* gene expression potentially represents a critical point in the experiment. Nevertheless, its predominant expression in the intestinal epithelium is well known; therefore, it is conceivable that it would not be found in the kidney tissue. The study aimed to obtain an overall picture of all Se-GPXs, testing also *gpx2* expression and verifying its absence and lack of influence on the kidney. Consequently, after testing multiple primers and not revealing expression, we concluded that its absence was due to undetectable mRNA levels in kidney tissue. Therefore, *gpx2* is considered not to be influential and is excluded from the molecular analysis. While this is consistent with the hypothesis, it may nonetheless limit the completeness of the GPX family profile in the investigated organ, and further investigation may be required.

5. Conclusions

In conclusion, this study has provided valuable insights into the antioxidant response mechanisms in the kidney of *Squalius cephalus* exposed to varying concentrations of PFAS in the aquatic environment. The results suggest a biphasic response of the antioxidant system, with catalase activation at low PFAS concentrations and a greater involvement of selenium-glutathione peroxidases at higher concentrations.

The observed discrepancy between gene expression and enzymatic activity indicates the presence of complex post-transcriptional regulatory mechanisms, potentially mediated by SGs. This highlights the importance of considering multiple levels of regulation when studying oxidative stress responses. Transmission electron microscopy (TEM) analysis can identify SGs inside cells by visualising anomalous electron-dense, membrane-less aggregates in the cytoplasm near ribosomal components. To investigate the molecular composition of these structures, the TEM technique can be combined with immunolabeling, aiming to confirm the specificity of the proteins within cells, using antibodies tagged with SG markers (G3BP1, TIA-1, PABP).

The absence of gpx2 expression and the lack of activation of gpx3 and gpx4 under high-pollution conditions underscore the tissue- and subcellular-specificity of antioxidant responses. This suggests that other systems, such as mitochondrial peroxiredoxins, may play a complementary role in protecting against PFAS-induced oxidative stress.

Despite the study's limitations, such as the lack of direct PFAS measurements in tissues and the potential presence of other contaminants, these findings provide a solid foundation for future research. Future studies could benefit from more detailed analyses of PFAS bioaccumulation in kidney tissues and experiments under controlled conditions to isolate the specific effects of PFAS.

Ultimately, this research significantly contributes to our understanding of the complex antioxidant responses in fish exposed to emerging environmental contaminants, such as PFAS, highlighting the importance of an integrated approach that considers both molecular mechanisms and physiological responses.

CRediT authorship contribution statement

Sara Pacchini: Writing – original draft, Validation, Supervision, Formal analysis, Data curation. **Giacomo Vanzan:** Writing – original draft, Formal analysis, Data curation. **Elisabetta Piva:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Sophia Schumann:** Writing – review & editing, Methodology, Investigation,

Conceptualization. **Martina Cortese:** Writing – review & editing, Investigation. **Laura Drago:** Writing – review & editing, Methodology, Investigation. **Shaghayegh Kholdihaghighi:** Writing – review & editing, Investigation. **Chiara Fogliano:** Writing – review & editing, Investigation. **Daniela Bertotto:** Writing – review & editing, Methodology, Investigation. **Andrea Bottacin-Busolin:** Writing – review & editing, Investigation. **Paola Irato:** Writing – review & editing, Resources, Methodology, Investigation. **Andrea Marion:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition. **Gianfranco Santovito:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Andrea Marion reports financial support was provided by European Commission. If there are other authors, they 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107524.

Data availability

Data will be made available on request.

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