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Article

# Antioxidant Responses Induced by PFAS Exposure in Freshwater Fish in the Veneto Region

Elisabetta Piva <sup>1‡</sup>, Sophia Schumann <sup>1‡</sup>, Serena Dotteschini <sup>1</sup>, Ginevra Brocca <sup>2</sup>, Giuseppe Radaelli <sup>2</sup>, Andrea Marion <sup>2</sup>, Paola Irato <sup>1</sup>, Daniela Bertotto <sup>3\*</sup> and Gianfranco Santovito <sup>1\*</sup>

<sup>1</sup> Department of Biology, University of Padova, Italy.; elisabetta.piva.2@studenti.unipd.it (E.P.), sophia.schumann@studenti.unipd.it (S.S.), serena.dotteschini@studenti.unipd.it (S.D.), paola.irato@unipd.it (P.I.)

<sup>2</sup> Department of Comparative Biomedicine and Food Science, University of Padova, Italy; ginevra.brocca@unipd.it (G.B.), giuseppe.radaelli@unipd.it (G.R.)

<sup>3</sup> Department of Industrial Engineering, University of Padova, Italy.; andrea.marion@unipd.it (A.M.),

<sup>‡</sup> Equally contributed as first author

\* Correspondence: daniela.bertotto@unipd.it (D.B.), gianfranco.santovito@unipd.it (G.F.)

**Abstract:** In recent decades, the interest in PFAS has grown exponentially around the world, due to the toxic effects induced by these chemical compounds in humans, as well as in other animals and in plants. However, current knowledge related to the antistress responses that organisms can express when exposed to these substances is still insufficient and therefore it requires further investigation. The present study focuses on antioxidant responses in *Squalius cephalus* and *Padogobius bonelli*, exposed to significant levels of PFAS in an area of the Veneto region subjected to a recent relevant pollution case. These two ubiquitous freshwater species were sampled in three rivers characterized by different concentrations of PFAS. Several biomarkers of oxidative stress have been evaluated and the results suggest that PFAS chronic exposure induces some physiological responses in the target species, at both cellular and tissue scales. The risk of oxidative stress seems to be kept under control by the antioxidant system by means of gene activation at the mitochondrial level. Moreover, the histological analysis suggests an interesting protective mechanism against damage to the protein component based on lipid vacuolization.

**Keywords:** antioxidant enzymes; freshwater fish; oxidative stress; PFAS; Veneto region

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## 1. Introduction

In recent years, the scientific community is focusing on “emerging contaminants”, i.e. chemicals that are not commonly monitored in the environment but have the potential to enter it and cause known or suspected adverse ecological and human health effect. Within this group, the per- and polyfluorinated compounds (PFAS) stand out as major players.

PFAS are a heterogeneous group of highly fluorinated aliphatic substances, similar to the non-fluorinated analogs from which they derived, except that in their molecular structure fluorine atoms replace all hydrogen substituents. At least one “perfluoroalkyl moiety” -  $C_nF_{2n+1}$  - [1], therefore characterizes PFAS. Since the 1940-50s, PFAS have been widely produced and manufactured all over the world [2]. Initially considered non-toxic and inert, these chemicals were subsequently associated with many adverse health effects, and concern about their toxicity increased exponentially.

Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) have been among the first to be detected in human blood and in the environment, and are considered some of the most widespread PFAS, as shown in several studies [3].

Many of these synthetic chemical compounds are known to be both hydro- and lipophobic, as well as chemically and thermally stable [1]. They have also dielectric properties, low surface energy, low friction properties [3], and a surfactant nature [4].

PFAS are extremely resistant to hydrolysis, photolysis, biodegradation and metabolism [5]. They are characterized also by high mobility and persistence, as they can travel great distances dispersing in surface and ground water bodies, as well as in air in the form of dust particulates [6]. Their chemical and physical characteristics support their intensive use in many industrial and commercial applications such as firefighting foams, water and oil repellents, surfactant agents, lubricants, and coating agents [2].

Since the early 2000s, PFAS began to be detected in the environment, even far away from where they were originally used or manufactured. They have been traced as far as the Arctic and Antarctica [7]. PFAS have been detected in several different matrices, from aquatic ecosystems (surface and ground waters) to sediments, flora and fauna [8]. In 2011, the Italian Ministry of Environment, Land and Sea commissioned the control of PFAS contamination in the Italian water streams to the Institute of Water Research of the National Research Centre (IRSA-CNR) [9]. The results of these analyses were communicated in 2013 and it turned out that in some provinces of the Veneto region drinking water samples exhibited extremely high PFAS concentrations. Twelve types of PFAS were identified, e.g. perfluorobutanoic acid (PFBA), perfluorobutanesulfonic acid (PFBS), and specially PFOA and PFOS [10].

Modern eco-physiological studies are focusing on if and how organisms can respond to environmental chemical stressors. The measurement of appropriate biomarkers is therefore crucial to determine the interaction between chemicals and biological systems. Toxicants, like PFAS, act at the molecular level causing an alteration in reactive oxygen species (ROS) production followed by a cellular redox imbalance. This can subsequently cause DNA damage, enzyme activity modifications, and a subsequent cascade of oxidative damage of the entire organism [11-12].

To protect cells from these damages, organisms have evolved various antioxidant multi-enzymatic systems [13-17]. Some of these enzymes can be used as good molecular biomarkers for contaminant-mediated oxidative stress and may represent also good markers of physiological response in organisms chronically exposed to xenobiotics [18].

The aim of the present research was first of all to identify potential effects and physiological responses induced by chronic exposure to PFAS in freshwater fish. A second goal was to study if the extent of these effects correlate to different environmental PFAS levels. For this purpose, two ubiquitous species in the Veneto region were studied: *Squalius cephalus* (European chub) and *Padogobius bonelli* (Padanian goby). These two species are particularly interesting because they occupy two different environmental niches and have different feeding habits. Therefore they can be affected differently by PFAS and can express species-specific responses.

The approach to this study was truly multidisciplinary, integrating biochemistry, molecular biology, and histology at locations of a fluvial system with mapped pollution levels. The expression of two genes, *gpx4* and *sod2*, were analyzed in fish sampled in three different aquatic sites to evaluate the antioxidant response in mitochondria, one of the main endogenous sources of ROS and the targets of PFAS toxicity, where the encoded enzymes are specifically expressed. Two biochemical assays were used to evaluate lipid peroxidation and total antioxidant capacity. The liver was chosen as the primary site of PFAS accumulation.

The results suggest that PFAS chronic exposure induces some physiological responses in the target species, at both cellular and tissue scales. The risk of oxidative stress seems to be kept under control by the antioxidant system by means of gene activation at the mitochondrial level. Furthermore, the histological analysis suggests an additional interesting protective mechanism against damage to the protein component based on lipid vacuolization.

## 2. Materials and Methods

### 2.1. Sampling sites

The present research is based on a fieldwork activity that took place on March 2021. The three sampling sites were located in three different water streams in the province of Vicenza (Italy), characterized by different levels of PFAS contamination (monitoring data were provided by the Regional Agency for Environmental Prevention and Protection of Veneto, ARPAV, in 2020).

The first location, referred to as Site 1, is Torrente Leogra in Torrebelticino (45.71779193 N; 11.30627616 E). PFAS concentrations for this site were always below the detection threshold, so it was initially considered as a control site. However, subsequent analyses aimed at verifying the presence of PFAS in fish residing in this environment have shown that even the control specimens have accumulated these contaminants in their tissues, albeit in very low concentrations (Schumann et al., Biomonitoring the effect of accumulating perfluorochemicals on physiological responses in freshwater fish from different ecological niches, submitted).

The second site, referred to as Site 2, is Roggia Moneghina in Grumolo delle Abbadesse (45.51163342 N, 11.65604081 E). This site was characterized by a low level of PFAS contaminations, mostly represented by PFOS (95.5 ng/L).

Finally, the third site is the Retrone river in Altavilla Vicentina (45.51350834 N, 11.5045197 E), characterized by a very high level of PFAS pollution (PFOA: 582.6 ng/L; PFOS: 95.5 ng/L).

Given this evidence on pollutant levels, these three sites are referred to in this work as 'Very low polluted Site', 'Low polluted Site' and 'Highly polluted Site', respectively.

## 2.2. Samples preparation

Ten specimens of each species, *S. cephalus*, and *P. bonelli*, were sampled from each of the three sites by electrofishing (authorized 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). For each species, the animals were of similar size: *S. cephalus* 19.99 cm ( $\pm 5.14$ ) and *P. bonelli* 6.81 cm ( $\pm 0.65$ ). After being caught, fish were immediately anesthetized and then euthanized with an overdose of essential clove oil, prepared in water-soluble form by dilution with ethyl alcohol (concentration 0.007 ml/L). All specimens were weighed and measured for total body length and dissected. Liver was removed, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analyzed. Liver samples of 6 individuals for each species and site were fixed also with 4% paraformaldehyde for histological analysis.

## 2.3. Primers Design, Total RNA extraction, *sod2*, and *gpx4* cDNA synthesis.

Amino acid and nucleotide sequences of *sod2* and *gpx4* from teleost fish obtained from the NCBI database were aligned by MUSCLE to identify conserved domains for primer design. At first, the species that are phylogenetically closer to *S. cephalus* and *P. bonelli* were identified. A similarity between *Periophthalmus magnuspinnatus* (mudskipper) and *P. bonelli* was identified, as these two species belong to the same family, namely Gobiidae. In the same way, a similarity between *Pimephales promelas* (Fathead minnow) and *S. cephalus* was identified, since both belong to the same family namely Leuciscidae.

The identities between the coding regions of the sequences were validated by a BLAST comparison (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The multiple alignments of the sequences with ClustalW2 led to the identification of the most conserved regions. Primers were designed in the coding regions and the primer sequences were analyzed with the IDT Oligo Analyzer tool (<https://eu.idtdna.com/pages/tools/oligoanalyzer>). Primer sets are shown in Table S1.

Total RNA was purified from liver tissues of both species using PRIMEZOL™ reagent (Canvax, Córdoba, Spain) according to the manufacturer's protocol. Further purification was performed with 8M LiCl [19] to remove glucidic contaminants and the quantification was performed using the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA); RNA integrity was assessed by running an aliquot of

RNA (1000 ng/ $\mu$ L) on a denaturing gel stained [20]. The cDNA synthesis was performed using a Biotechrabbit™ cDNA Synthesis Kit (Berlin, Germany) at 50°C for 1 h + 99°C for 5 minutes, from 1  $\mu$ g of total RNA in a 20  $\mu$ L reaction mixture, containing 2  $\mu$ L of dNTP Mix (10 mM each), 0,5  $\mu$ L of RNase Inhibitor, 40 U/  $\mu$ L, 0,5  $\mu$ L of Oligo (dT) 12-18 (10  $\mu$ L), 4  $\mu$ L of 5x Reverse Transcriptase Buffer, 1  $\mu$ L of RNA Template, 1  $\mu$ L of RevertUPTM II Reverse Transcriptase and PCR Grade Water up to 20  $\mu$ L. PCR reactions were performed with 50 ng of cDNA and GRS Taq DNA polymerase (Grisp, Porto, Portugal). The PCR program was the following: 95°C for 5 minutes and 40 x (95° for 30 s, Ta for 30 s, 72°C for 30 s), final elongation at 72°C for 5 minutes.

#### 2.4. qRT-PCR analysis

To evaluate the expression of mitochondrial *sod2* and *gpx4* mRNAs, real-time qRT-PCR analysis was performed. cDNAs for both target genes were amplified with the specific primers reported in Table S1. To control for variation in the efficiency of cDNA synthesis and PCR amplification reactions, the housekeeping gene *gapdh* was used as a control and amplified with species-specific primers (Table S1). qRT-PCR amplifications were carried out using the qPCRBIO SyGreen Mix Separate-ROX kit (PCR Biosystems, Wayne, PA, USA) and the following program: 95°C for 2 minutes, 40 x (95°C for 20 seconds and 60°C for 60 seconds) and then the dissociation stage 95°C for 15 s, 60°C for 1 minute, 95° for 15 s and 60°C for 15 s.

#### 2.5. Lipid peroxidation (MDA) assay

The Lipid Peroxidation assay was performed only in *S. cephalus* liver (8 per site) using the abcam® ab118970 Lipid Peroxidation (MDA) Assay Kit (Colorimetric/Fluorometric) (Waltham, MA, USA). This kit provides a convenient tool for the sensitive detection of malondialdehyde (MDA). In the lipid peroxidation assay, the MDA in the samples reacts with thiobarbituric acid (TBA) to generate an MDA-TBA adduct. The MDA-TBA adduct can be easily quantified colorimetrically (OD = 532 nm) or fluorometrically (Ex/Em = 532/553 nm). Each sample was at first weighed (the weight was kept between a range of 5-10 mg) then washed with cold phosphate-buffered saline (PBS). 150  $\mu$ L of ultrapure water and 3  $\mu$ L of BHT were added to each sample. Tissue samples were then vortexed and homogenized with pestles sitting on ice and briefly centrifuged, after adding 163  $\mu$ L of 2N perchloric acid. At the end of this process, 110  $\mu$ L of supernatant was collected and then 330  $\mu$ L of TBA was added to both samples and standards. After incubation at 95°C for 60 minutes, the samples were kept on ice for 10 minutes. The samples' optical density (OD) was finally read on a microplate reader TECAN Infinite® F200PRO (Männedorf, Switzerland) at 532 nm. Further calculations were performed following the manufacturer's protocol. Data were normalized against total protein concentration.

#### 2.6. Total Antioxidant Capacity (TAC) assay

The Total Antioxidant Capacity (TAC) assay was performed in *S. cephalus* and *P. bonelli* liver using abcam® TAC 65329 kit (Waltham, MA, USA). The kit for the TAC assay can measure the combination of both small antioxidant molecules and antioxidant enzymes, also being able to discriminate the former with the use of the Protein Mask present in the kit. For the analysis, 9 liver tissues of *P. bonelli* (3 for each site) and 12 liver tissues of *S. cephalus* (4 for each site) were analyzed. To perform the assay, 10-15 mg of liver were washed in cold PBS and then resuspended in 1 mL of cold PBS. The samples were homogenized using pestles sitting on ice and then the homogenates were incubated for 10 minutes on ice. The samples were finally centrifuged for 5 minutes at 4°C at top speed to remove insoluble materials. After this step, the supernatant was collected and transferred to new tubes kept on ice. For TAC analysis, 100  $\mu$ L of supernatant were mixed with 100  $\mu$ L of Cu<sup>2+</sup> working solution. For small molecules analysis, 100  $\mu$ L of supernatant (previously diluted 1:1 with Protein Mask) were mixed with 100  $\mu$ L of Cu<sup>2+</sup> working solution. The Protein Mask prevents the reduction of Cu<sup>2+</sup> by proteins thus allowing the analysis of

only small antioxidant molecules. The reduced  $\text{Cu}^+$  ion is chelated with a colorimetric probe giving an absorbance peak around 570 nm, proportional to the total antioxidant capacity. The samples' OD was read on a microplate reader TECAN In-finite® F200PRO (Männedorf, Switzerland). The enzymatic antioxidant capacity was calculated by difference between TAC and small molecule antioxidant capacity. Data were normalized against total protein concentration.

### 2.7. Estimation of the Total Amount of Proteins

The total amount of proteins (TP) in the cellular extracts was assessed by the Folin phenol reagent method [21], using growing concentrations of bovine serum albumin as standard

### 2.8. Hepatosomatic index (HSI)

Total body weights (g) and lengths (cm) of all specimens were collected together with the weight of each liver. From these measurements, the hepatosomatic index (HSI = weight of liver x 100/total fish weight) was determined.

### 2.9. Histology

Six samples per species and site were evaluated according to morphological shifts in the liver tissue of *P. bonelli* as in *S. cephalus*. Liver samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) till they were dehydrated in alcohol. The paraffin-embedded serial sections (5- $\mu\text{m}$ ) were cut with a Leica CM1850 microtome (Leica Biosystems, Deer Park, IL, USA) and stained with hematoxylin and eosin (H&E staining). The grade of fatty degeneration in the liver tissue was determined using a ranking system to describe the status of degeneration (severe, moderate, low, no degradation) which was defined before observation. Samples were analyzed randomly without looking at the labeling to exclude an observation bias.

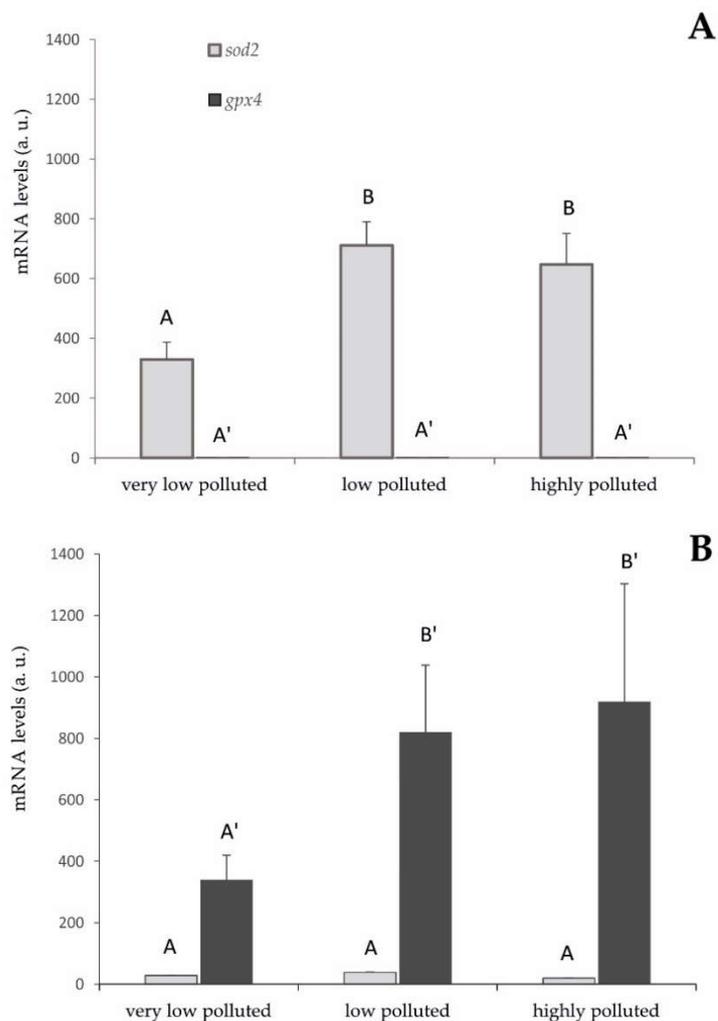
### 2.10. Statistical analysis

All data were expressed as the average of the analyzed samples  $\pm$  standard deviation (SD). Statistical analyses were performed with the PRIMER statistical program. One-way ANOVA was followed by the Student-Newman-Keuls test to assess significant differences ( $p < 0.05$ ). The semi-quantitative comparison of the different histological sections was performed using Spearman rank correlation to describe the monotonic relation between pollution and grade of steatosis.

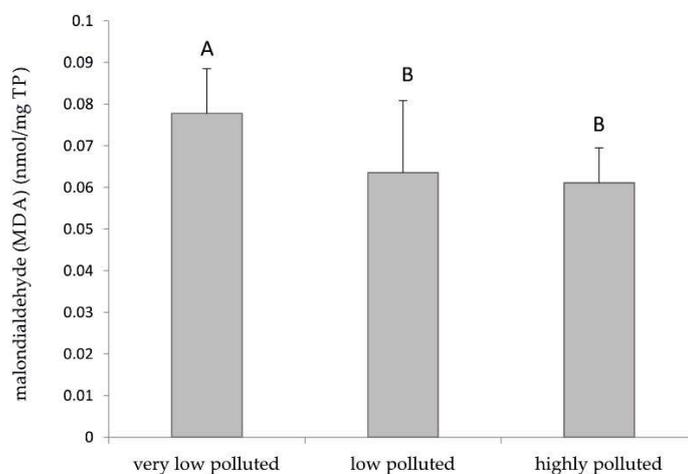
## 3. Results

### 3.1. qRT-PCR

The mRNA expression levels of *sod2* and *gpx4* in liver tissues have been analysed in both species and the results are reported in Figure 1.



**Figure 1.** *gpx4* and *sod2* mRNA levels in (A) *S. cephalus* and (B) *P. bonelli* liver. Values (arbitrary units) are indicated as mean  $\pm$  SD. Transcription levels were normalized to the *gapdh* housekeeping gene. Different letters correspond to significant statistical differences ( $p < 0.05$ ) among different sites (Student-Newman-Keuls test). Five specimens per site.



**Figure 2.** Malondialdehyde mean concentrations in *S. cephalus* liver. Values (arbitrary units) are indicated as mean  $\pm$  SD. Different letters correspond to significant statistical differences ( $p < 0.05$ ) among different sites (Student-Newman-Keuls test). Eight specimens per site.

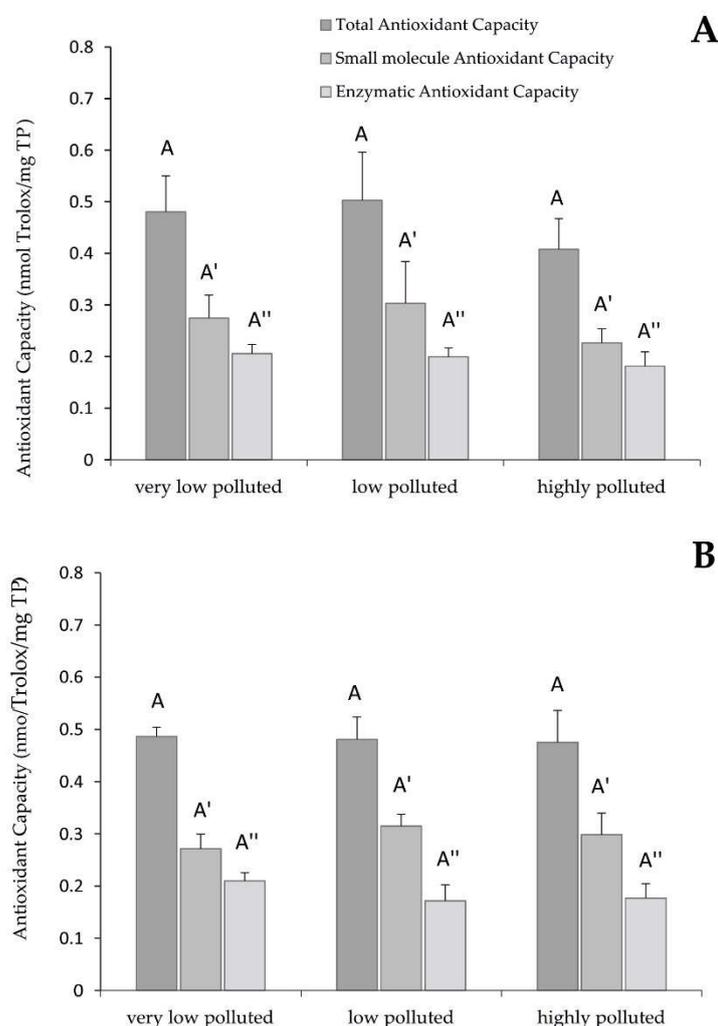
In *S. cephalus*, *sod2* was more expressed than *gpx4* (Figure 1A). The differences are between 600 and 1400 times ( $p < 0.001$ ). The expression of this gene is statistically higher in the fish sampled from the low and highly polluted sites with respect to those inhabiting the very low polluted river ( $p < 0.05$ ).

A diametrically opposite situation is found in *P. bonelli*, where *gpx4* is more expressed than *sod2* (Figure 1B). The differences are between 10 and 40 times ( $p < 0.001$ ).

Similarly to the previous situation, the expression of this gene is statistically higher in the fish sampled from the low and highly polluted sites with respect to those living in the very low polluted river ( $p < 0.05$ ).

### 3.2. Lipid peroxidation (MDA) assay

The lipid peroxidation levels in the liver of fish sampled in both low and highly polluted sites are significantly lower ( $p < 0.05$ ) than those measured in fish from the least polluted site, although the difference is only about 20% (18% for the low polluted site, 21% for the highly polluted one) (Figure 2).



**Figure 3.** Total antioxidant capacity (sum of small molecules antioxidant capacity and enzymatic antioxidant capacity) of (A) *S. cephalus* and (B) *P. bonelli* liver. Values (nanomoles of Trolox per milligrams of total proteins) are indicated as mean  $\pm$  SD. Different letters correspond to significant statistical differences ( $p < 0.005$ ) among different sites (ANOVA test). *P. bonelli*: three specimens per site. *S. cephalus*: four specimens per site.

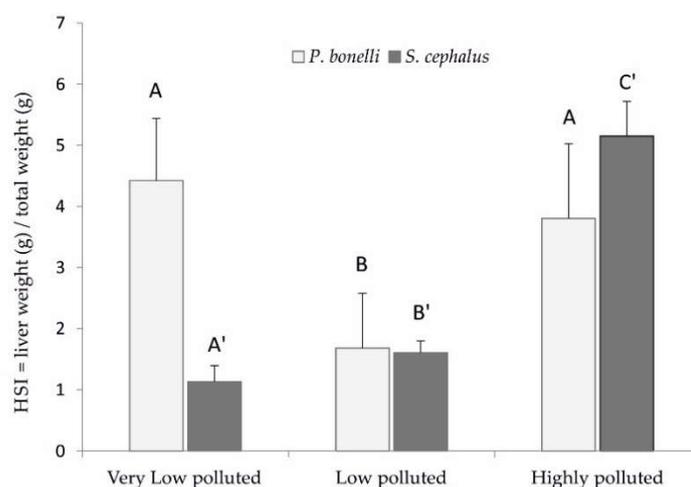
### 3.3. Total Antioxidant Capacity (TAC) assay

Figure 3A reports the results related to the Total Antioxidant Capacity in the liver of *S. cephalus*. It is possible to notice that there are no statistically significant differences among the specimens sampled in the three sites.

Even in the liver of *P. bonelli*, the antioxidant capacity does not vary with respect to the different degree of pollution by PFAS, despite the levels being slightly lower (but not statistically) in the fish sampled in the highly polluted site (Figure 3B).

### 3.4. Hepatosomatic index (HSI)

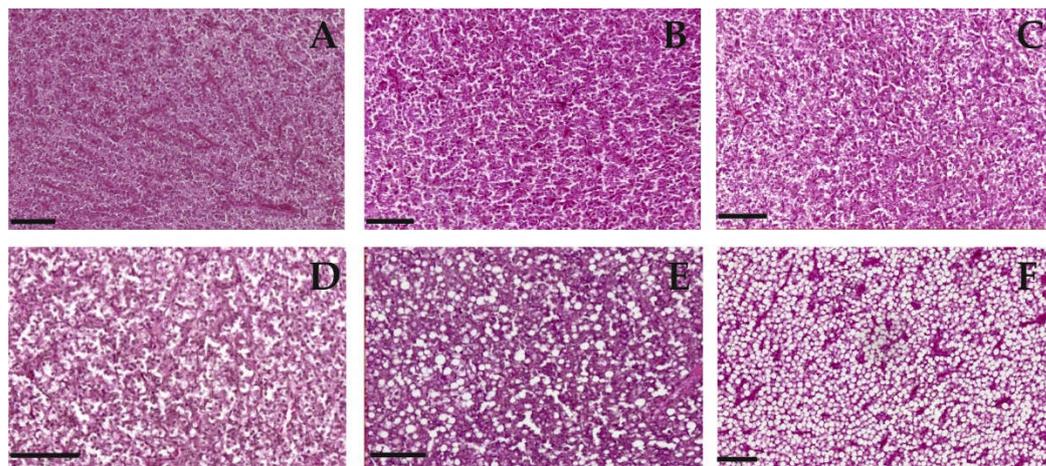
Figure 4 shows the hepatosomatic index (HSI) values calculated on *P. bonelli* and *S. cephalus*. In *P. bonelli*, the only statistically significant difference is related to the specimens sampled in the low polluted site, with values that are less than half that of the other fish analysed ( $p < 0.05$ ). Concerning the HSI calculated on *S. cephalus*, there are statistically significant differences for all three experimental groups, with the lowest values in the fish sampled at the very low polluted site and the highest in the specimens from the highly polluted site ( $p < 0.05$ ). In particular, the latter express values that are at least three times higher than other sampled fish.



**Figure 4. Hepatosomatic index in *P. bonelli* and *S. cephalus*.** Values (grams of liver weight per grams of total body weight) are indicated as mean  $\pm$  SD. The three means were statistically compared with each other. Different letters correspond to significant statistical differences ( $p < 0.05$ ) among different sites (Student-Newman-Keuls test). *P. bonelli* specimens: site 1 n=9, site 2 and 3 n=10. *S. cephalus* specimens: site 1 n=8, site 2 n=9, site 3 n=6.

### 3.5. Histopathological diagnosis of the liver

*P. bonelli* showed a proportional increase of hepatocyte cytoplasmic vacuolization with raising PFOS concentration found in the organism (4.64  $\mu\text{g}/\text{kg}$  in the very low polluted, 10.78  $\mu\text{g}/\text{kg}$  in the low and 112.41  $\mu\text{g}/\text{kg}$  in the highly polluted site). Two out of four analyzed fish from the low polluted site showed severe microvesicular steatosis. In contrast, all five analyzed samples from the highly polluted site shown severe macrovesicular steatosis, characterized by single fat filled vacuole. The level of steatosis highly correlated with the level of PFOS accumulation in *P. bonelli* ( $\rho = 0.721$ ), especially in specimens from the highly polluted area, which showed high-level macrovesicular steatosis in the liver. In *S. cephalus*, there was a very limited steatosis and unrelated to the accumulated amount of PFOS ( $\rho = -0.315$ ).



**Figure 5.** Lipid vacuolization in liver of (A-C) *S. cephalus* and (D-E) *P. bonelli* caught in the three different rivers with a (A,D) very low, (B,E) low and (C,F) high concentration of PFAS. Increasing vacuolization was assessed only in *P. bonelli* liver, and it is proportional to increasing hepatic PFAS concentration (from 139.25  $\mu\text{g}/\text{kg}$  per whole body weight). Scale bars indicate 50  $\mu\text{m}$ . Six specimens per site.

#### 4. Discussion

In the last decades, the Veneto provinces of Vicenza, Verona, and Padova have been affected by one of the most extensive PFAS contamination in the world [10]. Most of the research efforts on the physiological effects of PFAS have been focused on human health. Many studies have shown that the exposure of the human population to these pollutants could lead to several pathologies. Since PFAS can interact with many molecular components of the cell, they can also interfere with different metabolic pathways [22-23] leading to an increase in ROS production and pathologies such as immunotoxicity, neurotoxicity, and alterations of the reproductive system [24]. It has been shown that PFAS can also cause endocrine disruption and alteration of lipid metabolism [3,25-26].

By contrast, very little is known about the physiological effects that PFAS could produce on non-human organisms. Even less is known to date about the interactive effects of different compounds of PFAS when present simultaneously [27]. This study aims at verifying the physiological effects and responses induced by PFAS in freshwater fish.

From the gene expression analysis, it is possible to verify the activation of the antioxidant gene expressions in the liver that is related to the exposure to PFAS and the accumulation of these pollutants inside the tissues. In *S. cephalus* the PFAS induce an increase of *sod2* expression. In particular, going from a very low level of environmental pollution to a slightly higher level, the mRNA expression for this gene doubled. This higher level of expression remains almost unchanged even in the specimens sampled in the highly polluted site by PFAS.

These results are compatible with an increase in superoxide radical formation at the mitochondrial level since superoxide dismutase is the specific scavenger of this ROS and the very specific cellular localization of SOD2 [28]. However, they also indicate that liver cells do not seem to respond proportionally to the concentration of PFAS, although it should promote a greater risk of oxidative stress. It is possible to hypothesize that the measured expression levels of SOD2 are already protective against this risk. In fact, these animals have been chronically exposed to PFAS, and therefore had time to balance the antioxidant response through negative feedback control, which is characteristic of the expression of proteins that were part of the antioxidant system [29]. To verify these hypotheses, it should be useful to evaluate the effect of acute stress, for example through *in vivo* experiments under controlled laboratory conditions.

La limitatissima espressione della GPX4 sembra inspiegabile, visto che questo enzima dovrebbe essere coinvolto nell'eliminazione dell'acqua ossigenata prodotta dalla SOD2. A hypothesis is that other components of the antioxidant defence system may be

activated in specimens of *S. cephalus* after PFAS exposure, such as *Prdx3* and *Prdx5*, two isoforms of peroxiredoxins specifically expressed in mitochondria. Peroxiredoxins are enzymes that act in a specific way by reducing hydrogen peroxide and other peroxides. The upregulation of mitochondrial peroxiredoxins could compensate the limited expression of GPX4. In order to verify this hypothesis, it will be essential to evaluate the gene expression of these antioxidant enzymes in the liver.

A diametrically opposite situation was found in *P. bonelli*, where the presence of PFAS pollution, induced an increase of *gpx4* expression. The mRNA levels of this gene triple as PFAS concentration increases going from a very low level of pollution to a slightly higher level. This higher level of expression remains almost unchanged even in the specimens sampled in the highly polluted site. The obtained results are in line with a possible increase in the formation of peroxides at the mitochondrial level, as GPX4 is specifically expressed in mitochondria and provides a specific scavenging action against this type of ROS [30]. It remains to clarify whether the formation of hydrogen peroxide occurs by di-electronic reduction of molecular oxygen or by monoelectronic reduction of the superoxide radical. However, limited levels of *sod2* induction seems to exclude the second hypothesis.

As a response to greater environmental presence of PFAS and higher hepatic accumulation of these substances, liver cells do not seem more responsive. Similar to what has been proposed for *S. cephalus*, we can hypothesize that the expression levels of *gpx4* are already protected against the risk of oxidative stress. However, also in specimens of *P. bonelli* living in the very high polluted river, the upregulation of mitochondrial peroxiredoxins could explain the limited expression of GPX4.

As for the biochemical analyses, the data of lipid peroxidation do not correlate with the levels of PFAS found in the three rivers. In particular, an increase in lipid peroxidation associated to an increase in the accumulation of PFAS and therefore of a supposed raising in ROS formation could have been expected. Instead, our results indicate a little decrease of this parameter as the level of environmental pollution increases. This evidence can be explained by the increase in the expression of antioxidant enzymes previously discussed and the different timing of exposure. During the acute phase of exposure to PFAS, there could have been a likely increase in ROS formation. This may have led to an increase in lipid peroxidation and consequently to activation of genes coding antioxidant proteins, especially at the level of mitochondria, to counteract the damage of cell membranes. However, this study shows that, in the chronic exposure of fish from the contaminated sites, the maintenance of high levels of the antioxidant response may favours a prompt elimination of ROS produced by PFAS, also leading to a lower-than-normal level of lipid peroxidation.

Regarding the liver TAC, which is the result of post-transcriptional expression of antioxidant proteins and molecules, the absence of statistically significant differences among the experimental groups could be mainly due to a lack of activation of the antioxidant defenses at the cytoplasmic level. The target of PFAS toxicity are mitochondria and, the expression of antioxidant enzymes is proven. However, the amount of antioxidant protein biosynthesized by these organelles is lower than the one produced at the cytoplasmic level, where many more antioxidant enzymes and isoforms are expressed, possibly covering the increase in antioxidant activity in the mitochondria.

The histological analyses show a differential response of the two species towards PFAS exposure. Specifically, it is possible to notice a positive correlation between lipid vacuolization (steatosis) in *P. bonelli* but not in *S. cephalus*.

A positive correlation between hepatocytes vacuolization and PFAS accumulation in liver was shown also in eels exposed to PFOA [31], zebrafish exposed to PFOS [30] and rats exposed to PFOA and PFOS [32]. The increase in vacuolation could be considered a defense mechanism against the toxic effects of PFAS. In fact, it is known that these compounds show their toxicity against cytoplasmic proteins which are purely hydrophilic molecules. By increasing the amount of fat inside the cell, the hydrophilicity of the intracellular environment is reduced, making the interaction between PFAS and the protein

component less probable. The accumulation of lipids in *P. bonelli* could lead to a reduction of the specific weight of the tissue and therefore to a reduction of HSI, as actually occurs in the specimens of this species exposed to the low polluted environment. However, it seems that this defense mechanism is insufficient when there is an increase in the accumulation of PFAS in the organ, as it occurs in specimens exposed to the high polluted environment (Schumann et al., Biomonitoring the effect of accumulating perfluorochemicals on physiological responses in freshwater fish from different ecological niches, submitted). In this case, they also require a proliferation of hepatocytes to implement the detoxifying action, which it brings the HSI back to increase.

On the other hand, steatosis does not seem to be a defense mechanism that characterizes *S. cephalus*, probably because this species is able to accumulate much more PFAS in its own tissues and in particular in the liver (Schumann et al., Biomonitoring the effect of accumulating perfluorochemicals on physiological responses in freshwater fish from different ecological niches, submitted). The need of liver mass increase becomes more dominant, with a consequent increase in HSI, as verified in specimens from low and especially high polluted sites. Hepatic hyperplasia and hypertrophy, together with steatosis, is a consequence of the accumulation of PFAS in this organ well known in mammals [33-34] but also found in zebrafish chronically exposed to PFAS [35-36].

This study also shows that vacuolization is species-dependent and might correlate, in case of PFAS pollution, with the habits of the species. Decreased vacuolization can result from a reduction of the cytoplasmic glycogen and/or lipids caused by insufficient energy intake or use of resources due to glucocorticoid-induced glycogenolysis [32]. It is, therefore, presumable to hypothesize that this species, notoriously potamodromous, responded to xenobiotic stress through greater use of energy resources. On the contrary in *P. bonelli*, a sedentary species, the PFAS stress seems to have induced the phenomenon of steatosis. This phenomenon is commonly associated with overnutrition or chronic toxicity. It has already been observed in other studies, for example in zebrafish fry chronically exposed to PFOS [32,37].

## 5. Conclusions

This study provided new data on the effects of chronic exposure to PFAS on two freshwater fish species. The multidisciplinary approach has led to interesting results on the different sensitivity of the two species and on the different physiological responses that they can elicit when their surrounding environment changes. Results also highlighted that stress responses are very species-dependent, and this is something that will certainly prompt future studies.

The results obtained in this work could also provide useful information on the impact of PFAS within the trophic chains and consequently within products intended for food consumption. This issue is now more relevant than ever since, on September 20, 2021, after a legal battle with the Veneto Region, the results of food analysis sampled from the so-called "red zone" were published. These analyses show that PFAS contamination does not only concern drinking water but also foods of various kinds. This is of particular concern since recent research has identified the consumption of food, particularly fish and shellfish, as one of the main roads of exposure to PFAS [38].

From the present work it also emerges that field studies are more complex to interpret since they don't allow definite conclusions about the causality of the observed effects. For example, it must be taken into account that per- and poly-fluoroalkyl may not be the only pollutants present in the three sampled sites, although this is an assumption on which the present study is based. In this respect, the obtained data help formulating hypotheses and indicating approaches that will likely promote further investigation in the near future. In light of the growing knowledge about this topic, new urgent actions appear unavoidable to reduce the potential damage [39]. It is now apparent that PFAS pollution routes fast, so that it has reached the Venice lagoon already, thus impacting the lagoon organisms and consequently the regional economy.

Most of the studies that focus on PFAS-induced effects in fish always use very high concentrations of single PFAS compounds. However, as the present study highlights possible toxic responses at low PFAS concentrations, it appears extremely interesting to address future studies using similar concentrations.

New laboratory experiments, carried out under controlled conditions, will certainly be necessary to establish the correlation between physiological changes, histological damages, and exposure to a specific pollutant or class of pollutants. Even if studies on the toxicity of single compounds are certainly necessary to gain basic toxicological information, the real exposures to which humans and other animals are subjected are characterized by a coexistence of different chemical compounds. For this reason, focusing on the toxicity of mixed chemicals could be a promising approach to this topic that may likely improve future health and environmental risk assessment [40].

**Supplementary Materials:** Table S1: Primer pairs used for qRT-PCR. Amplicon sizes and annealing temperatures (Ta) are also indicated.

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