Biochemical aspects of susceptibility to stressors in two small cyprinids

***Squalius laietanus* and *Barbus meridionalis* from the NW Mediterranean**

Montse Solé¹, Silvia Lacorte² and Dolors Vinyoles³

¹Institute of Marine Sciences, ICM-CSIC. Passeig marítim de la Barceloneta 37-49
08003 Barcelona, Spain.

²Department of Environmental Chemistry, IDAEA-CSIC, c/Jordi Girona 18, 08034
Barcelona, Spain

³Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals (Vertebrats),
Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal, 643, E-08028
Barcelona, Spain

Abstract

Specimens of two endemic cyprinids, *Squalius laietanus* (Catalan chub) and *Barbus meridionalis* (Mediterranean barbel), were sampled from a reference site in a small stream of the Ripoll River (NW Mediterranean) outside of their reproductive season. Biomarkers involved in xenobiotic-mediated responses were individually contrasted in fish of both species and 17 perfluoroalkyl substances (PFASs) analysed in muscle to reveal bioaccumulation trends. The parameters were in muscle: cholinesterases, metabolic lactate dehydrogenase (LDH) and citrate synthase (CS); and in liver: cytochrome P450 dependent activities (EROD and BFCOD), carboxylesterase (CE), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX) and catalase (CAT). All markers are considered adaptive defence mechanism to face stress. Sensitivity to a model pesticide: dichlorvos was also contrasted *in vitro* in muscular acetylcholinesterase (AChE) and hepatic CE to reveal species sensitivity to neurotoxic chemicals. Enzymatic activities related to protective mechanisms such as butyrylcholinesterase (BuChE), CE and CAT were higher in chub whereas the antioxidant defences GR and GPX were higher in barbel. Aerobic CS was also higher in barbel while anaerobic LDH was so in chub. EROD activity did not differ between the two species but BFCOD activity was higher in barbel. Levels of PFAS were higher in barbel likely due to its benthic habitat. The *in vitro* tests revealed higher sensitivity to dichlorvos of muscular AChE in chub (lower IC₅₀) which was probably compensated by a higher catalytic efficiency of CE. All these former biochemical particularities are discussed in terms of fish ecological performance in front of anthropogenic stressors.

Keywords: Catalan chub, Mediterranean barbel, endemic fish, biomarkers, perfluoroalkyl substances, baseline activities.

1. Introduction

Freshwater native fish in highly populated areas from the Mediterranean region are exposed to several anthropogenic threats due to: (1) close proximity to pollution sources that compromise water quality, (2) strong water flow fluctuations naturally inherent to the Mediterranean climate and (3) increasing pressure from competitive invasive species (Maceda-Veiga 2013). Despite recent efforts at reducing the amount of urban discharges into the aquatic ecosystems following the implementation of the Water Framework Directive (2013/39/EU), episodic events of fish mortality still occur. This is particularly relevant to small Mediterranean rivers where sustained episodic droughts may even worsen water quality parameters as an additional form of perturbation (Merciai et al. 2017; 2018).

Biochemical parameters with ecological implications in freshwater fish performance have been increasingly addressed (Colin et al. 2016a). Fish have developed biochemical mechanisms to confront threats and, modifications of some enzymatic activities have proved to be an adaptive response to stress. Muscular parameters, such as inhibition of acetylcholinesterase (AChE) activity, have largely been suggested as indicators of neurotoxicity while increases in pseudocholinesterases, such as butyrylcholinesterase (BuChE) are usually associated to enhanced protective defences (Sturm et al. 2000). Key enzymes involved in intermediary metabolism such as lactate dehydrogenase (LDH) and citrate synthase (CS) can also be altered under stress situations (Monteiro et al. 2007). LDH is an enzyme that catalyses the last step in anaerobic glycolysis and although energetically it is less efficient than the citric acid cycle, it plays a key role in conditions of urgent and high energetic demands. CS is the

first pace-maker enzyme of the citric acid cycle (Krebs cycle) and it is informative of the aerobic most efficient pathway in energy production. Liver is the main organ involved in biotransformation. Among the first steps of biotransformation, stand out the induction of 7-ethoxyresorufin-*O*-deethylase (EROD) activity related to CYP1A (Whyte et al. 2000) and benzyloxy-4[trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) activity associated to CYP3A (Celander 2011). The combination of both markers in fish has been applied in freshwater fish studies (Blanco et al. 2019; Quesada-Garcia et al. 2013; Soler et al. 2020). CEs are also phase I reactions involved in the hydrolysis of foreign chemicals as well as endogenous compounds (Wheelock et al. 2008) with a particular high stoichiometric affinity for organophosphorus pesticides for which a protective role, preventing AChE inhibition, has been awarded (Sanchez-Hernandez and Wheelock 2009). However, there are also *in vitro* evidences in fish that these enzymes could be affected by pharmaceuticals and personal care products (Solé and Sanchez-Hernandez 2015). Conjugation with GST is an important step of detoxification leading to more polar moieties that can be readily excreted (van der Oost et al. 2003). As a result of biotransformation reactions involving oxygen, reactive oxygen species (ROS) can be generated, which are counteracted by antioxidant enzymes (Livingstone 2001). The applicability of the former markers in cyprinid fish has been conducted in Mediterranean regions, such as Turkey using carp *Cyprinus carpio* (Gungordu et al. 2012), a French river using chub *Leuciscus cephalus*, barbel *Barbus barbus* but also rainbow trout *Oncorhynchus mykiss* (Aarab et al. 2004), in a Portuguese river using barbel *Luciobarbus bocagei*, chub *Squalius carolitertii* and nase *Pseudochondrostoma* sp (Pereira et al. 2013) and in large watercourses of the NW Mediterranean region (Fernandes et al. 2002; Lavado et al. 2006; Merciai et al. 2014; Olivares et al. 2010; Solé et al. 2000). In small watercourses from the same

geographical area, the cyprinid Catalan chub *Squalius laietanus* (hereafter, chub) and the Mediterranean barbel *Barbus meridionalis* (hereafter, barbel) are two native fish species that coexist in intermittent and permanent middle-stream rivers and may be the only resident fish in many of them (Maceda-Veiga et al. 2013; Mas-Martí et al. 2010). In the particular case of the small Ripoll river, the physical and biotic community characterisation (Colin et al. 2016b), the presence of bioaccumulated metals (Maceda-Veiga et al. 2012; 2013; Merciai et al. 2014) and that of organic contaminants (Blanco et al. 2019; Soler et al. 2020) in water and/or in fish has been previously documented. All these former river studies singled out an adequate reference site with respect to other downstream sites under the influence of industrial and domestic discharges. The two cyprinid species contrasted from the same reference site, show different habitat use and feeding behaviour. The chub is an omnivorous water-column dweller inhabiting both lentic and lotic waters (Casals 2005; Aparicio et al. 2016) and it is known to colonize preferentially deep pools and runs (Doadrio et al. 2011). The barbel is a clear benthic microhabitat dweller that feeds mainly on benthic invertebrates (Casals 2005; Mas-Martí et al. 2010). The two species cohabit in the Mediterranean basins of northeast Catalonia and southeast France, although the chub has a broader distribution in Catalonia (Aparicio et al. 2016). They may also express some individual natural evolutionary adaptive traits related to their habitat preference that make them particularly either more resilient or vulnerable to anthropogenic pressure. The analysis of bioaccumulated man-driven chemicals is a good indicator of exposure to lipophilic compounds ($\log K_{ow} > 3$) such as the persistent perfluoroalkyl substances (PFAS) of environmental concern (Scheringer et al. 2014). They were selected as supporters of the selection of the reference site as well as to reflect species differences, if any, between pelagic and benthic fish (Nania et al. 2012). Moreover, there are recent *in vitro*

suggestions that PFAs could interfere with hepatic detoxification by affecting CEs (Liu 2020).

The aim of the present study was to characterise the natural baseline enzymatic defences to environmental stressors of two closely related cyprinid species (chub and barbel). Additionally, the sensitivity to neurotoxic chemicals (e.g. dichlorvos) was contrasted *in vitro* to reveal particular species-dependant susceptibility. The results are discussed in terms of fish bioaccumulation and performance at lower water streams of the Mediterranean region habitats: water column (chub) and benthos (barbel) and resilience to face anthropogenic threats in their last natural habitats, key issues in their conservation.

2. Material and methods

2.1 Fish sampling

Fishing was performed in the mid-reaches of the Ripoll river (Besòs basin) in the NW of the Iberian Peninsula. Sampling coordinates for the reference site (Arenes) were: 2°3'24.07'' 41°38'45.05''. Eight individuals of each species, chub and barbel, were caught in spring (March) using a portable electrofishing unit which generated up to 200 V and 3 A pulsed DC. A portion of muscle was used for chemical analysis and another for biomarkers' determinations. Liver was frozen in liquid N₂ for biomarker analysis. Fish biometrics, such as size (as fork length, ± 0.1 mm) and mass (as total weight ± 0.01 g) and ratios: Fulton body condition calculated as $CF = \text{total body weight}/(\text{fork length})^3$ and the relative hepatic and gonad weight (HSI and GSI, respectively) were measured as liver or gonad weight/total weight $\times 100$. Fish collection was approved by

the Regional Government of Catalonia (Ref. AP/007). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures were conducted in accordance with the European Directive for animal experimentation (2010/63/EU).

2.2 Sample preparation for analysis of PFAS

Seventeen PFAS were analysed. Native compounds of perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluoronanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA), perfluorooctadecanoic acid (PFODA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS) and perfluorodecane sulfonate (PFDS) were supplied by Wellington Laboratories (Ontario, Canada). Stock standard solutions were prepared in acetonitrile at concentrations of 10 and 1 ng/ μ L and were stored at -18°C . Perfluoro-*n*-(1,2,3,4- $^{13}\text{C}_4$) octanoic acid (m-PFOA) and sodium perfluoro-1-(1,2,3,4- $^{13}\text{C}_4$) octane sulfonate (m-PFOS), also from Wellington Laboratories, were used as internal standards. HPLC grade water and acetonitrile were supplied by Merck (Darmstadt, Germany) and glacial acetic acid by Panreac (Barcelona, Spain). Active carbon 120/400 and ammonium acetate were provided by Supelco (Bellefonte, United States of America).

About 1 g of pooled muscle tissue was used for PFASs determination following Vicente et al. (2012) protocol using solid-liquid extraction with acetonitrile, clean-up

with activated carbon and analysis by an Ultra Performance Liquid Chromatography (UPLC) coupled to a Triple Quadrupole Mass Spectrometry Detector (Acquity TQD, Waters, USA) as described in detail in Carbajal et al. (2019). Concentrations are given in ng/g wet weight (ww). A supplementary Table (S1) is provided on the 17 compounds analysed by LC-MS/MS, indicating accuracy (% error) at a spiking level of 12 ng/g in fish, precision (as % coefficient of variation from a calibration standard at 100 ng/mL), and recoveries (% and standard deviation, SD) at a spiking level of 12 ng/g, in fish.

2.3 Sample preparation for biomarkers determination

A muscle portion, of about 0.3 g, from each fish was used for cholinesterases, LDH and CS determinations. The tissue was homogenized in a 50 mM buffer phosphate pH 7.4 in a 1:5 (w:v) ratio using a polytron® blender, centrifuged at 10,000 g x 30 min and the supernatant (S9) aimed to biochemical determinations. The liver from individual fish was homogenised with 100 mM buffer phosphate pH 7.4 in a 1:4 (w:v) ratio containing 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1mg/ml trypsin inhibitor and 1 mM ethylenediaminetetraacetic acid (EDTA), centrifuged at 10,000 g x 30 min and the supernatant (S9) was used for biochemical determinations.

2.4 Biochemical muscular parameters determination

Cholinesterases AChE, PrChE and BuChE were assayed in muscle at the substrate acetylthiocholine iodide, propionylthiocholine iodide and butyrylthiocholine iodide, as described in Solé et al. (2012). In each microplate well, 150 µl of 5,5'-dithio- bis-2-nitrobenzoate (DTNB; 270 µM) was mixed with 25 µl of adequately diluted sample and

after a 2 minute pre-incubation, the reaction was initiated by adding 50 μ l of each substrate at 1 mM final concentration. Activities were assayed according to the principle of Ellman et al. (1961) at 412 nm.

Lactate dehydrogenase (LDH) was measured using 150 μ l of NADH solution in phosphate buffer mixed with 25 μ l of diluted sample and 50 μ l of pyruvate solution in each microplate well. The final concentration in well was 200 μ M (NADH) and 1mM (pyruvate) and reading was done at 340 nm (Vassault 1983).

Citrate synthase (CS) was assayed using 25 μ l of diluted sample and final concentrations in well were 0.1 mM DTNB and 0.1 mM acethyl CoA. Reaction was started with the addition of oxalacetic acid (0.5 mM) in 100 mM Tris buffer pH 8.0. Reading was done at 412 nM (Childress and Somero 1990).

2.5 Biochemical liver parameters determination

7-ethoxyresorufin O-deethylase (EROD) activity was measured using 50 μ l of undiluted liver homogenate samples (S9) with a reaction mixture containing 0.2 mM NADPH, 3.3 μ M 7-ethoxyresorufin (ER) in 100 mM phosphate buffer pH 7.4 (Burke and Mayer, 1974). The reaction was followed over resorufin formation for 10 min with the fluorescence mode at 537 nm excitation and 583 nm emission. A six-point standard of resorufin (0-160 nM) was used to relate activity.

7-benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase (BFCOD) activity was measured using 50 μ l of undiluted liver homogenate samples (S9) with a reaction mixture containing 200 μ M BFC and 20 μ M NADPH, in 100 mM phosphate

buffer pH 7.4. A six-point standard of 7-hydroxi-4-[trifluoromethyl]-coumarin (0-160 nM) was used to relate activity.

Carboxylesterase (CE) activity was measured using 25 μ l of conveniently diluted sample and 200 μ l of α -naphthyl acetate (α NA) as substrate (250 μ M final concentration in well) or p-nitrophenyl acetate (pNPA) at 1 mM final concentration in well. Reading was measured during 5 min at 235 nm (Mastropaolo and Yourno 1981) and at 405 nm (Hosokawa and Sato 2002), respectively.

Glutathione S-transferases (GST) activity was measured using 25 μ l of diluted sample using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The final reaction mixture contained 1mM CDNB and 1 mM reduced glutathione (GSH). The activity was measured for 5 min at 340 nm (Habig et al. 1974).

Glutathione reductase (GR) activity was measured using 20 μ l of sample and 1 mM oxidized glutathione (GSSG) and 0.5 mM nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm for 3 min following Carlberg and Mannervik (1985) protocol.

Total glutathione-peroxidase (GPX) activity was determined using 20 μ l of sample and 2.5 mM GSH, 1 U of commercial GR, 0.625mM cumene hydroperoxide and 0.3 mM NADPH at 340 nm during 3 min according to the Gunzle and Flohe (1974) protocol.

Catalase (CAT) activity was measured using 10 μ l of sample and 50 mM H₂O₂ as substrate at 240 nm for 1 min (Aebi 1984).

Total protein content of the samples was determined by the Bradford method (1976) (Bradford 1976) adapted to microplate, using Bradford Bio-Rad Protein Assay

reagent and bovine serum albumin (BSA) as standard (0.1-1 mg/ml). Absorbance was read at 595 nm.

Assay conditions were kept similar and only the sample dilution was changed in order to maintain linearity of the enzymatic measurements. All assays were carried out in triplicate at 25°C, except EROD and BFCOD which were at 30°C, in 96-wellplates using a TECAN Infinite M200 microplate reader.

2.6 Esterases characterisation and *in vitro* exposure to the model esterase inhibitors

A range of twelve concentrations of acetylthiocholine iodide (ATC) from 0.01 to 20 mM was used to determine the kinetic parameters V_{max} and K_m for AChE. For CE characterisation of K_m and V_{max} values, a six-point range of the substrates α NA (0.03125-1 mM) and pNPA (0.3125-10 mM) were assayed. Sensitivity of AChE and CE activities to the model pesticide dichlorvos was evaluated in both species. Dichlorvos (2,2-dichlorovinyl dimethyl phosphate, CAS no. 62-73-7) was purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). *In vitro* exposures were carried out in diluted muscle and liver (S9) fraction. Stock solution was dissolved in water. The seven final incubation concentrations were in the range 0.0125 -12.8 μ M and a control. In each microplate well, 100 μ l of appropriately diluted sample were mixed with 5 μ l of the respective stock pesticide range to achieve the desired final concentration. Incubation was performed at room temperature for 30 min. Subsequently, AChE determinations in muscle and CE measurements in liver were performed as previously described. In addition, a selected 1mM concentration of other model esterase inhibitors, including dichlorvos, was chosen for its action on CE measures using the pNPA substrate. These chemicals were eserine hemisulfate (carbamate) and dichlorvos (OP pesticide) as

general esterase inhibitors whereas bis-(4-nitrophenyl)-phosphate (BNPP) is particular for CEs and tetraiso-propyl pyrophosphoramidate (iso-OMPA) as specific BuChE inhibitor to test their potential contribution in CE measures.

2.7 Statistical analysis

Prior to statistical analyses, data were checked to fulfil parametric requirements of normality (Shapiro-Wilks) and homogeneous variance (Levene's test). When this was not accomplished, they were log₁₀ transformed. Student's t-test was used to compare the enzymatic activities of both species. The V_{max} and K_m values were calculated from the 12 point substrate range for AChE and 6 points for CE determinations from Michaelis and Menten equation ($V = V_{max} [S]/K_m+[S]$), using the linearity transformation of Lineweaver-Burk plot. To calculate the 50% *in vitro* inhibition concentration values (IC₅₀) after pesticide exposure, the regression probability module of SPSS Systems at 95% of confidence with the log transformed data was used. All statistical analyses were carried out using SPSS System Software and the significance level for data analyses was $\alpha = 0.05$. Data are presented as mean $\pm$ SEM (standard error).

3. Results

3.1 Fish Biometrics and indices

Table 1 indicates the biometric and gross morphometric parameters: condition factor (CF), hepatosomatic index (HSI) and gonadosomatic index (GSI). These values lie within the range considered as normal for adult specimens of each species in the pre-spawning period.

3.2 PFAS in fish

Among the studied PFAS, compounds detected in muscle tissue of all fish samples were PFOS > PFUnA~ PFDoA > PFTriDA > PFDA > PFNA > PFDS, while other compounds were not detected. PFOS accounted for 42 and 50% of Σ PFAS, in chub and barbel, respectively, followed by PFUnA and PFDoA, being all of them long chain PFAS with high bioaccumulation potential which altogether made 82% in chub and 87% in barbel of Σ PFAS. Σ PFAS was 4.63 ng/g ww in chubs and 16.3 ng/g ww in barbels (Figure 1).

3.3 Esterases characterisation

In Table 2, kinetic parameters resulting from AChE characterisation using 12 concentrations of ATC, indicated similar V_{max} (in nmol/min/mg prot) and K_m (in μ M) values for both cyprinids, which resulted in a similar catalytic efficiency (V_{max}/K_m) of 55-58. CE's characterisation was undertaken in fish liver of both species using two recommended commercial substrates. In the chub, V_{max} was higher when using pNPA as substrate whereas in the barbel V_{max} was similar with any of the substrates. With both substrate measures, V_{max} was always higher in the chub whereas K_m was about one order of magnitude lower (higher substrate affinity) in the measures using the substrate α NA. Thus, the catalytic efficiency measured V_{max}/K_m was as follows: α NA-CE > pNPA-CE and for each substrate this ratio was higher in the chub by 4- (pNPA-CE) and 2-fold (α NA-CE).

An *in vitro* incubation (Table 2) of S9 fraction with a range of seven dichlorvos (selected model pesticide for *in vitro* studies as it does not require *in vitro* activation) concentrations was also attempted in order to calculate the IC₅₀ values (pesticide concentration that inhibits the control activity by 50%), for AChE in muscle and CE in liver. Muscular IC₅₀ for AChE was 6.5-times lower in the chub whereas hepatic CE was > 2-fold more sensitive in this cyprinid when using α NA and 1.2 higher in the case of measures with pNPA. As expected, IC₅₀ was lower (more sensitive) in both species when using α NA as substrate. The contrasted *in vitro* incubation with other model esterase inhibitors (at a single 1mM concentration) on pNPA-CE liver measures (Figure 2) indicated that incubations with eserine and dichlorvos, as broad esterase inhibitors, were more significant in chub (higher % of inhibition) in respect to the controls 100% activity. BNPP, as particular for CEs, clearly indicated that chub's CE contribution was much greater in this S9 fraction (>90% inhibition) while in barbel it was lower (68%) and BuChE contribution in the mixture revealed after iso-OMPA incubation was lower in chub (66%) and in barbel (41%).

3.4 Muscle and liver biomarkers in chub and barbel

Basal activities of muscular AChE, PrChE and BuChE, using the common 1 mM substrate concentration, only differed statistically between both species for BuChE measures (Table 3). Muscular LDH was significantly higher in the chub ($p < 0.001$) and, by contrast, CS was significantly elevated in the barbel ($p < 0.05$).

CYP-related activities in the liver S9 did not differ in terms of EROD activity but BFCOD activity was significantly higher in the barbel. GST activity was similar for

both species whereas the antioxidant defences GR and GPX were significantly higher in the barbel while CAT was so in the chub.

Some ratios were calculated between esterases (AChE/CE) and phase I and II reactions (EROD/GST and BFCOD/GST) and all these ratios were lower in the chub (Figure 3).

4. Discussion

The two small cyprinid fish selected for the study correspond to two native endemic species well adapted to the low stream conditions typical of Mediterranean rivers (Doadrio et al. 2011). Historically, their populations have been monitored in terms of species distribution and abundance in relation to other native and invasive fish populations in the NW Mediterranean region (Aparicio et al. 2000; Colin et al. 2018; Maceda-Veiga and Sostoa 2011; Maceda-Veiga et al. 2017a; 2017b; Merciai et al. 2017; 2018). Some of these former studies have identified and evaluated the most relevant threats for native fish species, including barbel and chub. Among them: poor water quality, water scarcity and competition triggered by the introduction of invasive species were identified as the most significant challenges these endemic species have to face (Maceda-Veiga 2013; Maceda-Veiga et al. 2017a; 2017b). Thus, the present study focuses on the biochemical *in vivo* and *in vitro* traits that may outline a better adaptability and plasticity of either cyprinid fish to face man-driven stressors.

Comparisons of baseline enzymatic activities were made in fish sampled in a confirmed reference area, all considering the undeniable anthropogenic fingerprint of NW Mediterranean watercourses (Blanco et al. 2019; Carbajal et al. 2019; Colin et al. 2016b; Maceda-Veiga et al. 2013; Marqueño et al. 2019; Soler et al. 2020). Markers of

neurotoxicity signs (AChE), protection (BuChE), energy metabolism (LDH and CS), xenobiotic metabolism and antioxidant defences (Colin et al. 2016a) were selected as suggestive of a particular microhabitat use and biochemical adaptation. Since fish were simultaneously sampled at the same site, physical and chemical water conditions (described in Soler et al. 2020) and the ecological characteristics of the habitat were common and only the influence of the particular diet, habitat use and physiological traits of each species will dictate the differences observed. In fact, the analysis of PFAS revealed higher bioaccumulation in barbel as formerly described for other organic contaminants and metals in the same fish species from the same geographical area (Maceda-Veiga et al. 2013; Blanco et al. 2019) and ascribed to the benthic habitat of barbel. The high correlation coefficient found between the seven individual PFAS levels in chub and barbel ($r=0.96$), supports the common source these contaminants and their association to the sediment, where they tend to store. The levels detected (Figure 1) are within the lower range of other studies in biota (0.63–274 ng/g) from the Jucar river (SE Spain) (Campo et al. 2016) or in various aquatic organisms from Catalonia with 0.23 to 144 ng/g ww of PFOS (Fernández-Sanjuan et al. 2010) and in the same cyprinids inhabiting rivers from nearby South-eastern France $\Sigma_{17}\text{PFAS} = 7\text{--}1811$ ng/g (Simmonet-Laprade et al., 2019) which support the reference identity of the selected site.

From a biochemical perspective, the relationship between baseline enzymatic activities and *in vitro* sensitivity to chemicals can provide an estimation of the fish susceptibility to xenobiotics (Sanchez-Nogue et al. 2013; Ribalta et al. 2015). Both fish species displayed similar baseline AChE and PrChE activities in muscle but the protective enzyme BuChE, although with a modest contribution, displayed higher activity in chub. A lower IC50 in chub muscular AChE after *in vitro* exposure to

dichlorvos identified this species as the most sensitive to neurotoxics. Nonetheless, the most meaningful protection in chub was most likely conferred by high hepatic CE activity and catalytic efficiency. Moreover, in chub, a demonstrated *in vitro* true and greater CE contribution in the S9 fraction was confirmed after BNPP incubations and by a lower IC₅₀ for dichlorvos (greater affinity) in liver when using the most suitable identified substrate (α NA) for pesticides in fish (Solé et al. 2012). Former *in vitro* evidences of liver CEs sensitivity to drugs in this two fish species were also seen as chemical dependent (Solé and Sanchez-Hernandez 2015). In several fish species, a negative relationship between baseline CE activities and IC₅₀ values has been described and regarded as compensatory mechanisms (Ribalta et al. 2015). Kinetic parameters such as K_m (the affinity for a given substrate), V_{max} (maximal hydrolysis rate) and catalytic efficiency (V_{max}/K_m) were similar for muscular AChE whereas they greatly differed for hepatic CE. In this case, V_{max} was 4.4-fold (pNPA) and 2.6-fold (α NA) higher in chubs, in line with the reported baseline activities. Substrate affinities (K_m) were similar for the two species and they fit within the range of those reported for marine fish using common substrates (Ribalta et al. 2015), and confirming the adequacy of the selected 1mM (pNPA) and 250 μ M (α NA) substrate concentrations. The corresponding catalytic efficiencies (V_{max}/K_m) were also higher in chub when using α NA for CE measures as revealed in other fish (Koenig et al. 2013; Nos et al. 2017; Ribalta et al. 2015; Sanchez-Nogue et al. 2013; Solé et al. 2012). On the other hand, the lower sensitivity of muscular AChE to inhibition by dichlorvos in barbel (expressed by a higher IC₅₀) points out this species as less vulnerable and likely seen a compensatory trait for the higher xenobiotics' bioaccumulation encountered in this species.

In terms of muscular activities related to energy production, LDH was significantly higher in chub and CS in barbel. This probably relates to their predator

avoidance strategy, consisting in swimming away in chub that would rely on the fast-responding anaerobic pathway, whereas barbel remains immobile, as strategy, and would use the more efficient aerobic CS pathway. In cyprinids, these enzyme activities are intimately related to swimming performance and to predation avoidance and therefore are of great ecological significance (He et al. 2013). Higher LDH coincides with a higher motility in pelagic fish with respect to those with more benthopelagic habits (Childress and Somero 1990; Koenig and Sole 2014).

Although both contrasted cyprinids are highly related from a phylogenetic perspective, a particular feeding behaviour and habitat preference can be at least partly responsible for differences in hepatic activities. In terms of enzymes involved in biotransformation, species differences were seen for BFCOD and CE activities although in an opposite way. EROD and BFCOD are CYP-related activities than can be influenced by chemical exposure but also by the physiological status of the fish. In this last case, individuals of chub (Soler et al. 2020) and barbel (personal observation) were all adults at the pre-maturity stage as confirmed by the histological analysis of gonads. In both species, maximal gonadal activity occurs from April onwards (Casals 2005). Higher catalytic activity in barbel with respect to chub from this same reference area was formerly described for EROD and BFCOD-activities in the liver microsomal fraction (Blanco et al. 2019). The antioxidant defences GR and GPX were also more elevated in barbel and in line with a higher ROS production mediated by CYP-dependent reactions. Coincident higher LDH and CAT activities in the chub are in line of a good response in this species under an acute stress situation. Bioaccumulation is the resulting balance between exposure and metabolism. In other cyprinids, including the chub *Squalius cephalus* and the barbel *Barbus plebejus*, higher presence of PCBs and DDTs was revealed in the barbel from the same Italian location (Vigano et al. 2000).

Higher presence of organic chemicals was in agreement with more antioxidant defences GR, GPX and GST (Vigano et al. 1998). In a French study, CAT activity was also higher for *S. cephalus* than for the barbel *B. barbus* collected at the same location (Aarab et al. 2004). Species differences in bioaccumulation and biochemical responses were also evidenced for another two cyprinids: carp (*C. carpio*) and barbel (*L. graellsii*) under the same chemical pressure (Lavado et al. 2006).

Some authors have proposed ratios among biomarkers to express susceptibility to chemicals. A ratio between AChE and CE was proposed by Kristoff et al. (2012) so that a lower ratio means more protection in front of neurotoxicity. Van der Oost (1998) suggested the biotransformation index (BTI) as a rate between phase I and phase II metabolism, being phase II GST a more strictly detoxification pathway. In the present species contrast approach, lower ratios were always attained in chub, which suggest a greater enzymatic protection in this fish.

From an overall biochemical perspective, it seems that chub is well equipped to face stressors and the higher metabolic demands of a water-column species. By contrast, barbel accumulates more lipophilic chemicals in line with its more benthic habitat but it also displays higher baseline EROD and BFCOD biotransformation activities in liver microsomes (Blanco et al. 2019), although in the present S9 fraction this was only significant for BFCOD, as well as elevated antioxidant defences to fight xenobiotic-mediated ROS production. Signs of reproductive disorders of high ecological relevance were revealed in barbel downstream a WWTP (Blanco et al. 2019) but also in chub sampled at the same polluted location (Soler et al. 2020). Thus, from a reproductive perspective both species seem equally susceptible to endocrine disrupting chemicals. However, the lipidomic composition was more seriously affected in barbel from the downstream sites, which lead the researchers to conclude that this species was more

sensitive to obesogens (Marqueño et al. 2019). However, fewer pathological responses in blood parameters were seen in chub downstream the same WWTP with respect to the barbel facing metal contamination of a comparable magnitude (Maceda-Veiga et al. 2013). The compensatory biochemical strategies of both cyprinids observed in our study could imply a similar environmental success. This is supported by a parallel decrease in contribution of the two native species to the fish community, mostly due to habitat loss and invasive species competition, described in more permanent water courses of the NW Mediterranean where they are still able to persist (Maceda-Veiga et al. 2010). However, due to differences in habitat preferences, the sensitivity of the chub to hydrological perturbations is greater than that reported for the barbel (Merciai et al. 2017). Chub is a species greatly affected by water abstraction, and this has caused its extinction from many intermittent courses where it was historically present (Aparicio et al. 2000; Benejam et al. 2010). Hydric stress makes the decline of vulnerable chub populations in smaller streams of Mediterranean areas even more pronounced (Murphy et al. 2013). In other European rivers with less hydric constrictions, species performance in relation to stressors has been addressed (Markovic et al., 2019).

In conclusion and from a biochemical perspective, chub and barbel express particular defences in front of anthropogenic pressure. A higher susceptibility to neurotoxics in chubs (lower IC₅₀ in muscle AChE to dichlorvos) is counteracted by higher protection by CEs, LDH and CAT. Barbel bioaccumulate more lipophilic compounds and displays higher CYP-related activities and antioxidant defences (GPX and GR) to face xenobiotic-associated ROS production. Overall, both species seem to handle stress by displaying particular compensatory mechanisms however, water constrains may particularly affect chub due to its higher hydric demands. Conservative measures related to water quality restoration, maintenance of a minimum ecological

hydric flow and the control of the invasive fish populations are needed to preserve the endemic fish populations of small Mediterranean rivers.

Acknowledgements

The authors acknowledge Victor Bonet for the English revision and José Domingo Rodríguez-Teijeiro for sampling help and the Erasmus student Davide Romeo under the E.U. Lifelong Learning Programme for his contribution.

References

- Aarab, N., Champeau, O., Mora, P., Daubeze, M., Garrigues, P., Narbonne, J.F., 2004. Scoring approach based on fish biomarkers applied to French river monitoring. *Biomarkers* 9, 258-270.
- Aebi, H., 1984. Catalase in vitro. *Methods in Enzymology* 105, 121-126.
- Aparicio, E., Vargas, M.J., Olmo, J.M., de Sostoa, A., 2000. Decline of native freshwater fishes in a Mediterranean watershed on the Iberian Peninsula: A quantitative assessment. *Environ. Biol. Fishes* 59, 11-19.
- Aparicio, E., Alcaraz, C., Carmona-Catot, G., García-Berthou, E., Pou-Rovira, Q., Rocaspana, R., Vargas, M.J., Vinyoles, D., 2016. *Peixos continentals de Catalunya. Ecologia, conservació i guia d'identificació*. Lynx Edicions, Barcelona.

- Benejam, L., Angermeier, P.L., Munne, A., Garcia-Berthou, E., 2010. Assessing effects of water abstraction on fish assemblages in Mediterranean streams. *Freshwater Biol.* 55, 628-642.
- Blanco, M., Rizzi, J., Fernandes, D., Colin, N., Maceda-Veiga, A., Porte, C., 2019. Assessing the impact of waste water effluents on native fish species from a semi-arid region, NE Spain. *Sci. Total Environ.* 654, 218-225.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin - direct fluorimetric assay of a microsomal o-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* 2, 583-588.
- Campo, J., Lorenzo, M., Perez, F., Pico, Y., Farre, M., Barcelo, D., 2016. Analysis of the presence of perfluoroalkyl substances in water, sediment and biota of the Jucar River (E Spain). Sources, partitioning and relationships with water physical characteristics. *Environ. Res.* 147, 503-512.
- Carbajal, A., Soler, P., Tallo-Parra, O., Isasa, M., Echevarria, C., Lopez-Bejar, M., Vinyoles, D., 2019. Towards Non-Invasive Methods in Measuring Fish Welfare: The Measurement of Cortisol Concentrations in Fish Skin Mucus as a Biomarker of Habitat Quality. *Animals* 9.
- Carlberg, I., Mannervik, B., 1985. Glutathione-reductase. *Methods in Enzymology* 113, 484-490.

- Casals, F., 2005. Les Comunitats íctiques dels rius mediterranis: relació amb les condicions ambientals. Dissertation, University of Barcelona.
<http://hdl.handle.net/2445/35897>
- Celander, M.C., 2011. Cocktail effects on biomarker responses in fish. *Aquat. Toxicol.* 105, 72-77.
- Colin, N., Porte, C., Fernandes, D., Barata, C., Padros, F., Carrasson, M., Monroy, M., Cano-Rocabayera, O., de Sostoa, A., Pina, B., Maceda-Veiga, A., 2016a. Ecological relevance of biomarkers in monitoring studies of macro-invertebrates and fish in Mediterranean rivers. *Sci. Total Environ.* 540, 307-323.
- Colin, N., Maceda-Veiga, A., Flor-Arnau, N., Mora, J., Fortuno, P., Vieira, C., Prat, N., Cambra, J., de Sostoa, A., 2016b. Ecological impact and recovery of a Mediterranean river after receiving the effluent from a textile dyeing industry. *Ecotox. Environ. Safe.* 132, 295-303.
- Colin, N., Villeger, S., Wilkes, M., de Sostoa, A., Maceda-Veiga, A., 2018. Functional diversity measures revealed impacts of non-native species and habitat degradation on species-poor freshwater fish assemblages. *Sci. Total Environ.* 625, 861-871.
- Childress, J.J., Somero, G.N., 1990. Metabolic scaling: a new perspective based on scaling of glycolytic enzyme activities. *Am. Zool.* 30, 161-173.
- Doadrio, I., Perea, S., Garzón-Heydt, P., González, J.L., 2011. Ictiofauna Continental Española, Ictiofauna Continental Española. Bases para su seguimiento. DG Medio Natural y Política Forestal. MARM. 616 pp. Madrid.

- Ellman, G.L., Courtney, K.D., Andres Jr, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7.
- Fernandes, D., Potrykus, J., Morsiani, C., Raldua, D., Lavado, R., Porte, C., 2002. The combined use of chemical and biochemical markers to assess water quality in two low-stream rivers (NE Spain). *Environ. Res.* 90, 169-178.
- Fernandez-Sanjuan, M., Meyer, J., Damasio, J., Faria, M., Barata, C., Lacorte, S., 2010. Screening of perfluorinated chemicals (PFCs) in various aquatic organisms. *Anal. Bioanal. Chem.* 398, 1447-1456.
- Gungordu, A., Erkmen, B., Kolankaya, D., 2012. Evaluation of spatial and temporal changes in biomarker responses in the common carp (*Cyprinus carpio* L.) for biomonitoring the Meric Delta, Turkey. *Environ. Toxicol. Phar.* 33, 431-439.
- Gunzler, W.A., Flohé, L., 1985. Glutathione peroxidase. In: Greenwald, R.A. (Ed.), *Handbook of Methods for Oxyradical Research*. CRC Press, pp. 285–290.
- Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-Transferases. *J. Biol. Chem.* 249, 7130-7139.
- He, W., Xia, W., Cao, Z.D., Fu, S.J., 2013. The effect of prolonged exercise training on swimming performance and the underlying biochemical mechanisms in juvenile common carp (*Cyprinus carpio*). *Comp. Biochem. Physiol. A* 166, 308-315.
- Hosokawa, M., Satoh, T., 2005. Measurement of carboxylesterase (CES) activities. In: Costa, L.G., Hodgson, E., Lawrence, D.A., Ozolins T.R., Reed D.J., Greenlee,

- W.F. (Eds.), Current Protocols in Toxicology, John Wiley & Sons, chapter 4, unit 4.7.
- Koenig, S., Guillen, K., Sole, M., 2013. Comparative xenobiotic metabolism capacities and pesticide sensitivity in adults of *Solea solea* and *Solea senegalensis*. *Comp. Biochem. Physiol. C* 157, 329-336.
- Koenig, S., Sole, M., 2014. Muscular cholinesterase and lactate dehydrogenase activities in deep-sea fish from the NW Mediterranean. *Mar. Environ. Res.* 94, 16-23.
- Kristoff, G., Barrionuevo, D.C., Cacciatore, L.C., Guerrero, N.R.V., Cochon, A.C., 2012. In vivo studies on inhibition and recovery of B-esterase activities in *Biomphalaria glabrata* exposed to "azinphos-methyl: Analysis of enzyme, substrate and tissue dependence. *Aquat. Toxicol.* 112, 19-26.
- Lavado, R., Urena, R., Martin-Skilton, R., Torreblanca, A., del Ramo, J., Raldua, D., Porte, C., 2006. The combined use of chemical and biochemical markers to assess water quality along the Ebro River. *Environ. Pollut.* 139, 330-339.
- Liu, Y.Z., Pan, L.H., Bai, Y., Yang, K., Dong, P.P., Liu, Y.Z., Fang, Z., 2020. Per- and polyfluoroalkyl substances exert strong inhibition towards human carboxylesterases. *Environ. Pollut.* 263 : 114463.
- Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar. Pollut. Bull.* 42, 656-666.
- Maceda-Veiga, A., 2013. Towards the conservation of freshwater fish: Iberian Rivers as an example of threats and management practices. *Rev. Fish Biol. Fisher.* 23, 1-22.

- Maceda-Veiga, A., Baselga, A., Sousa, R., Vila, M., Doadrio, I., de Sostoa, A., 2017a. Fine-scale determinants of conservation value of river reaches in a hotspot of native and non-native species diversity. *Sci. Total Environ.* 574, 455-466.
- Maceda-Veiga, A., de Sostoa, A., 2011. Observational evidence of the sensitivity of some fish species to environmental stressors in Mediterranean rivers. *Ecol. Indic.* 11, 311-317.
- Maceda-Veiga, A., Mac Nally, R., de Sostoa, A., 2017b. The presence of non-native species is not associated with native fish sensitivity to water pollution in greatly hydrologically altered rivers. *Sci. Total Environ.* 607, 549-557.
- Maceda-Veiga, A., Monleon-Getino, A., Caiola, N., Casals, F., de Sostoa, A., 2010. Changes in fish assemblages in catchments in north-eastern Spain: biodiversity, conservation status and introduced species. *Freshwater Biology* 55, 1734-1746.
- Maceda-Veiga, A., Monroy, M., de Sostoa, A., 2012. Metal bioaccumulation in the Mediterranean barbel (*Barbus meridionalis*) in a Mediterranean River receiving effluents from urban and industrial wastewater treatment plants. *Ecotox. Environ. Safe.* 76, 93-101.
- Maceda-Veiga, A., Monroy, M., Navarro, E., Viscor, G., de Sostoa, A., 2013. Metal concentrations and pathological responses of wild native fish exposed to sewage discharge in a Mediterranean river. *Sci. Total Environ.* 449, 9-19.
- Markovic, D., Walz, A., Kaercher, O., 2019. Scale effects on the performance of niche-based models of freshwater fish distributions: Local vs. upstream area influences. *Ecol. Model.* 411.

- Marqueño, A., Blanco, M., Maceda-Veiga, A., Porte, C., 2019. Skeletal Muscle Lipidomics as a New Tool to Determine Altered Lipid Homeostasis in Fish Exposed to Urban and Industrial Wastewaters. *Environ. Sci. Technol.* 53, 8416-8425.
- Mas-Marti, E., Garcia-Berthou, E., Sabater, S., Tomanova, S., Munoz, I., 2010. Comparing fish assemblages and trophic ecology of permanent and intermittent reaches in a Mediterranean stream. *Hydrobiologia* 657, 167-180.
- Mastropaolo, W., Yourno, J., 1981. An ultraviolet spectrophotometric assay for alpha-naphthyl acetate and alpha-naphthyl butyrate esterases. *Anal. Biochem.* 115, 188-193.
- Merciai, R., Bailey, L.L., Bestgen, K.R., Fausch, K.D., Zamora, L., Sabater, S., Garcia-Berthou, E., 2018. Water diversion reduces abundance and survival of two Mediterranean cyprinids. *Ecol. Freshw. Fish* 27, 481-491.
- Merciai, R., Guasch, H., Kumar, A., Sabater, S., Garcia-Berthou, E., 2014. Trace metal concentration and fish size: Variation among fish species in a Mediterranean river. *Ecotox. Environ. Safe.* 107, 154-161.
- Merciai, R., Molons-Sierra, C., Sabater, S., Garcia-Berthou, E., 2017. Water abstraction affects abundance, size-structure and growth of two threatened cyprinid fishes. *Plos One* 12.
- Monteiro, M., Quintaneiro, C., Nogueira, A.J.A., Morgado, F., Soares, A.M.V.M., Guilhermino, L., 2007. Impact of chemical exposure on the fish *Pomatoschistus microps* Kroyer (1838) in estuaries of the Portuguese Northwest coast. *Chemosphere* 66, 514-522.

- Murphy, C.A., Casals, F., Sola, C., Caiola, N., de Sostoa, A., Garcia-Berthou, E., 2013. Efficacy of population size structure as a bioassessment tool in freshwaters. *Ecol. Indic.* 34, 571-579.
- Nania, V., Pellegrini, G.E., Fabrizi, L., Sesta, G., De Sanctis, P., Lucchetti, D., Di Pasquale, M., Conia, E., 2009. Monitoring of perfluorinated compounds in edible fish from the Mediterranean Sea. *Food Chem* 115:951-7.
- Nos, D., Navarro, J., Barrie, C., Sole, M., 2017. Carboxylesterase activities in chondrichthyans of the western Mediterranean Sea. *Mar. Pollut. Bull.* 119, 332-335.
- Olivares, A., Quiros, L., Pelayo, S., Navarro, A., Bosch, C., Grimalt, J.O., Fabregat, M.D., Faria, M., Benejam, L., Benito, J., Sole, M., Barata, C., Pina, B., 2010. Integrated biological and chemical analysis of organochlorine compound pollution and of its biological effects in a riverine system downstream the discharge point. *Sci. Total Environ.* 408, 5592-5599.
- Pereira, S., Pinto, A.L., Cortes, R., Fontainhas-Fernandes, A., Coimbra, A.M., Monteiro, S.M., 2013. Gill histopathological and oxidative stress evaluation in native fish captured in Portuguese northwestern rivers. *Ecotox. Environ. Safe.* 90, 157-166.
- Quesada-Garcia, A., Valdehita, A., Torrent, F., Villarroel, M., Hernando, M.D., Navas, J.M., 2013. Use of fish farms to assess river contamination: Combining biomarker responses, active biomonitoring, and chemical analysis. *Aquat. Toxicol.* 140, 439-448.

- Ribalta, C., Sanchez-Hernandez, J.C., Solé, M., 2015. Hepatic biotransformation and antioxidant enzyme activities in Mediterranean fish from different habitat depths. *Sci. Total Environ.* 532, 176-183.
- Sanchez-Hernandez, J.C., Wheelock, C.E., 2009. Tissue distribution, isozyme abundance and sensitivity to chlorpyrifos-oxon of carboxylesterases in the earthworm *Lumbricus terrestris*. *Environ. Pollut.* 157, 264-272.
- Sanchez-Nogue, B., Varo, I., Solé, M., 2013. Comparative analysis of selected biomarkers and pesticide sensitivity in juveniles of *Solea solea* and *Solea senegalensis*. *Environ. Sci. Pollut. Res.* 20, 3480-3488.
- Scheringer, M., Trier, X., Cousins, I.T., de Voogt, P., Fletcher, T., Wang, Z., Webster, T.F., 2014. Helsingor Statement on poly- and perfluorinated alkyl substances (PFASs). *Chemosphere* 114, 337-339.
- Simmonet-Laprade, C., Budzinski, H., Babut, M., Le Menach, K., Munoz, G., Lauzent, M., Ferrari, B.J.D., Labadie, P., 2019. Investigation of the spatial variability of poly- and perfluoroalkyl substance trophic magnification in selected riverine ecosystems. *Sci. Total Environ.* 686, 393-401.
- Solé, M., Porte, C., Barcelo, D., 2000. Vitellogenin induction and other biochemical responses in carp, *Cyprinus carpio*, after experimental injection with 17 alpha-ethynylestradiol. *Arch. Environ. Contam. Toxicol.* 38, 494-500.
- Solé, M., Vega, S., Varo, I., 2012. Characterization of type "B" esterases and hepatic CYP450 isoenzymes in Senegalese sole for their further application in monitoring studies. *Ecotox. Environ. Safe.* 78, 72-79.

- Soler, P., Solé, M., Banon, R., Garcia-Galea, E., Durfort, M., Matamoros, V., Bayona, J.M., Vinyoles, D., 2020. Effects of industrial pollution on the reproductive biology of *Squalius laietanus* (Actinopterygii, Cyprinidae) in a Mediterranean stream (NE Iberian Peninsula). *Fish Physiol. Biochem.* 46, 247-264.
- Sturm, A., Wogram, J., Segner, H., Liess, M., 2000. Different sensitivity to organophosphates of acetylcholinesterase and butyrylcholinesterase from three-spined stickleback (*Gasterosteus aculeatus*): Application in biomonitoring. *Environ. Toxicol. Chem.* 19, 1607-1615.
- van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Phar.* 13, 57-149.
- van der Oost, R., Lopes, S.C.C., Komen, H., Satumalay, K., van den Bos, R., Heida, H., Vermeulen, N.P.E., 1998. Assessment of environmental quality and inland water pollution using biomarker responses in caged carp (*Cyprinus carpio*): Use of a bioactivation : detoxication ratio as a biotransformation index (BTI). *Mar. Environ. Res.* 46, 315-319.
- Vigano, L., Arillo, A., Aurigi, S., Corsi, I., Focardi, S., 2000. Concentrations of PCBs, DDTs, and TCDD equivalents in cyprinids of the middle Po River, Italy. *Arch. Environ. Contam. Toxicol.* 38, 209-216.
- Vigano, L., Arillo, A., Melodia, F., Arlati, P., Monti, C., 1998. Biomarker responses in cyprinids of the middle stretch of the River Po, Italy. *Environ. Toxicol. Chem.* 17, 404-411.

- Vassault, A., 1983. Lactate dehydrogenase. In: Bergmeyer, M.O. (Ed). Methods of Enzymatic Analysis, Enzymes: Oxidoreductases, Transferases. Academic Press, New York; p. 118-126.
- Wheelock, C.E., Phillips, B.M., Anderson, B.S., Miller, J.L., Miller, M.J., Hammock, B.D., 2008. Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). Rev. Environ. Contam. Toxicol. 195, 117-178.
- Whyte, J.J., Jung, R.E., Schmitt, C.J., Tillitt, D.E., 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit. Rev. Toxicol. 30, 347-570.

Table 1. Biometric parameters and gross morphometric indices: condition factor (CF), hepatosomatic index (HSI) and gonadosomatic index (GSI) of chub (*Squalius laietanus*) and barbel (*Barbus meridionalis*) collected from a relatively clean reference site in a NW Mediterranean river. Calculations of ratios described in M&M section. Results are given as the mean $\pm$ SD (n=8). Male: Female (M:F) ratio.

	Chub (5:3)	Barbel (3:5)
Total weight (g)	33.1 $\pm$ 4.3	16.1 $\pm$ 1.2
Total/fork length (cm)	14.7 $\pm$ 0.5	11.1 $\pm$ 0.3
CF (%)	1.0 $\pm$ 0.03	1.2 $\pm$ 0.02
HSI (%)	0.9 $\pm$ 0.10	1.8 $\pm$ 0.14
GSI (%)	4.7 $\pm$ 0.8	4.2 $\pm$ 0.6

Table 2. Kinetic parameters of maximal velocity (Vmax), substrate affinity (Km) and catalytic efficiency (Vmax/Km) for muscular acetylcholinesterase (AChE) and hepatic carboxylesterase (CbE) activities using the substrates α -naphthyl acetate (α NA) and p-nitrophenyl acetate (pNPA). Results correspond to mean $\pm$ SEM (n=4) for chub (*Squalius laietanus*) and barbel (*Barbus meridionalis*). The *in vitro* sensitivity to dichlorvos was calculated as the concentration causing 50% inhibition of the baseline activity (IC50) and probit results as the mean value recorded and the confidence interval (n=4).

		Vmax (nmol/min/mg prot)	Km (μ M)	Vmax/Km (ml/min/mg prot)	Dichlorvos IC50 (μ M)
AChE	Chub	18.12 $\pm$ 3.0	0.32 $\pm$ 0.03	55.44 $\pm$ 5.1	0.173 (0.101-0.332)
	Barbel	14.61 $\pm$ 1.1	0.26 $\pm$ 0.02	57.75 $\pm$ 4.3	1.118 (0.561-2.611)
CbE (α NA)	Chub	358.3 $\pm$ 11.7	0.15 $\pm$ 0.02	2458 $\pm$ 315	0.500 (0.373-0.688)
	Barbel	170.1 $\pm$ 15.2	0.14 $\pm$ 0.01	1181 $\pm$ 40.1	0.940 (0.658-1.413)
CbE (pNPA)	Chub	726.8 $\pm$ 99.4	1.17 $\pm$ 0.2	666.6 $\pm$ 111	3.514 (1.962-7.622)
	Barbel	164.9 $\pm$ 1.4	1.01 $\pm$ 0.1	174.0 $\pm$ 3.8	2.997 (1.528-7.687)

Table 3. Muscle and liver biomarkers in the two cyprinid species, chub (*Squalius laietanus*) and barbel (*Barbus meridionalis*) collected from a reference site in a small NW Mediterranean river. Acronym for the activities as in the M&M section. Results correspond to the mean $\pm$ SEM (n=8). All activities are in nmol/min/mg prot except for EROD and BFCOD (in pmol/min/mg prot) and CAT (in μ mol/min/mg prot).

Muscle	Chub	Barbel	t-test
AChE	52.5 $\pm$ 2.62	53.7 $\pm$ 5.07	n.s
PrChE	14.4 $\pm$ 0.63	13.2 $\pm$ 1.05	n.s
BChE	0.95 $\pm$ 0.06	0.58 $\pm$ 0.03	p<0.05
LDH	1141 $\pm$ 40.0	854 $\pm$ 40.9	p<0.001
CS	93.9 $\pm$ 17.2	157 $\pm$ 15.1	p<0.05
Liver			
EROD	1.91 $\pm$ 0.27	2.26 $\pm$ 0.44	n.s
BFCOD	1.66 $\pm$ 0.16	9.08 $\pm$ 2.05	p<0.05
CE (pNPA)	477 $\pm$ 43.5	270 $\pm$ 21.3	p<0.001
CE (α NA)	295 $\pm$ 14.2	177 $\pm$ 10.6	p<0.001
GST	660 $\pm$ 71.8	608 $\pm$ 20.9	n.s
GR	25.16 $\pm$ 1.09	36.8 $\pm$ 1.34	p<0.001
GPX	191 $\pm$ 4.6	360 $\pm$ 20.2	p<0.001
CAT	1106 $\pm$ 27.4	787 $\pm$ 64.5	p<0.001

Figure 1. Levels of perfluorinated compounds (PFCs) corresponding to pooled muscle of *Squalius laietanus* (chub) and *Barbus meridionalis* (barbel) collected from a reference small stream in a NW Mediterranean river. In order of abundance: perfluorooctane sulfonate (PFOS), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorodecanoic acid (PFDA), perfluorotridecanoic acid (PFTriDA), perfluoronanoic acid (PFNA) and perfluorodecane sulfonate (PFDS).

Figure 2. Percentage (%) of residual CE activity in respect to controls (100% activity) after 1mM *in vitro* incubations of several model esterase inhibitors in hepatic S9 CE (using pNPA as substrate) of chub (*Squalius laietanus*) and barbel (*Barbus meridionalis*) collected from a reference site in a small NW Mediterranean river. Acronym for the full name of chemicals in the M&M section. Results correspond to the mean $\pm$ SEM (n=4).

Figure 3. Ratios between esterases: AChE/CE using two substrates α NA and pNPA. Ratios between phase I (BFCOD and EROD) and phase II (GST) activities were calculated from the mean activity values from Table 3. Acronyms as in the M&M section.

Declaration of interests

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.

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

Click here to access/download
Supplementary Material
Table S1.docx

Cyprinid fish

- Biaccumulation PFAS: Barbel>Chub
- Phase I oxidation : Barbel>Chub
- Phase I hydrolysis: Chub> Barbel
- Protection defenses: Chub>Barbel