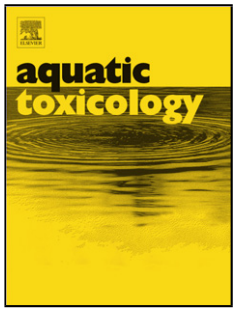

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Authors: A. Barreto, L.G. Luis, P. Paíga, L.H.M.L.M. Santos, C. Delerue-Matos, A.M.V.M. Soares, K. Hylland, S. Loureiro, M. Oliveira

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A multibiomarker approach highlights effects induced by the human pharmaceutical gemfibrozil to gilthead seabream *Sparus aurata*

A. Barreto[1*], L.G. Luis[1], P. Paíga[2], L.H.M.L.M. Santos[2,3], C. Delerue-Matos[2], A.M.V.M. Soares[1], K. Hylland[4], S. Loureiro[1], M. Oliveira[1]

[1] Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

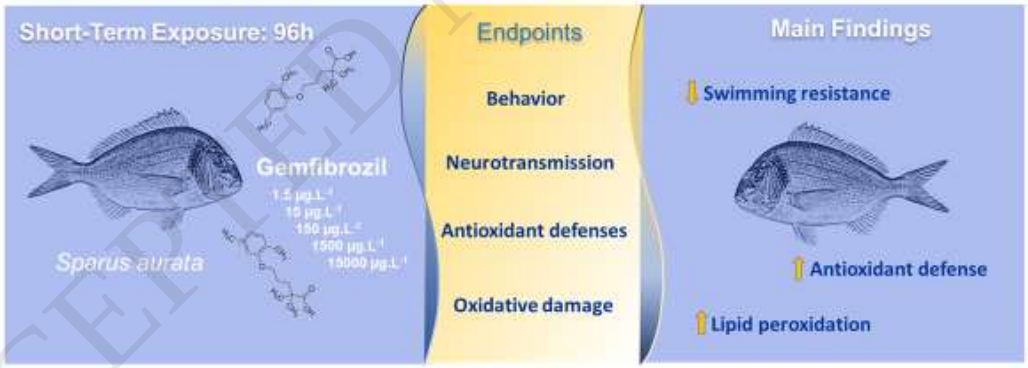
[2] REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal

[3] Present affiliation: Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain

[4] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Graphical abstract



Highlights:

- Gemfibrozil affected *Sparus aurata* even at an environmentally relevant concentration;

- Gemfibrozil decreased the capability of *Sparus aurata* to swim against a water flow;
- Gemfibrozil induced hepatic oxidative damage in gilthead seabream.

Abstract

Lipid regulators are among the most prescribed human pharmaceuticals worldwide. Gemfibrozil, which belongs to this class of pharmaceuticals, is one of the most frequently encountered in the aquatic environment. However, there is limited information concerning the mechanisms involved in gemfibrozil effects to aquatic organisms, particularly to marine organisms. Based on this knowledge gap, the current study aimed to assess biochemical and behavioral effects following a sublethal exposure to gemfibrozil (1.5, 15, 150, 1500 and 15000 $\mu g.L^{-1}$) in the estuarine/marine fish *Sparus aurata*. After the exposure to 1.5 $\mu g.L^{-1}$ of gemfibrozil, fish had reduced ability to swim against a water flow and increased lipid peroxidation in the liver. At concentrations between 15 to 15000 $\mu g.L^{-1}$, the activities of some enzymes involved in antioxidant defense were induced, appearing to be sufficient to prevent oxidative damage. Depending on the organ, different responses to gemfibrozil were displayed, with enzymes like catalase being more stimulated in gills, whereas glutathione peroxidase was more activated in liver. Although there were no obvious concentration-response relationships, the integrated biomarker response version 2 (IBRv2) analysis revealed that the highest concentrations of gemfibrozil (between 150 to 15000 $\mu g.L^{-1}$) caused more alterations. All the tested concentrations of gemfibrozil induced effects in *S. aurata*, in terms of behavior and/or oxidative stress responses. Oxidative damage was found at a concentration that is considered environmentally relevant, suggesting a potential of this pharmaceutical to impact fish populations.

Keywords: fibrates; seabream; behavior; biomarkers; oxidative damage

1. Introduction

Pharmaceuticals are considered emerging environmental contaminants of concern due to their high consumption and continuous environmental release (as parental compound and/or metabolites). This is both due to inefficient wastewater treatment processes and, for some substances, high environmental persistence and low degradation rates (Andreozzi et al. 2003; Fent et al. 2006, Schmidt et al. 2011). The prescription rates of lipid regulators are continually increasing and gemfibrozil (GEM) is among the most widely used (Al-Habsi et al. 2016; Prindiville et al., 2011). GEM was approved by the Food and Drug Administration (FDA) in 1976 for use by humans to reduce serum lipids. It reduces the levels of triglycerides, very low-density lipoprotein (VLDL, “bad cholesterol”) and low-density lipoprotein (LDL, “bad cholesterol”) and increases high-density lipoprotein (HDL, “good cholesterol”) (Kim et al. 2017). In North America and Europe these drugs are widely used to control hyperlipidaemia resulting from the western diet (Ido et al. 2017). In the United States, in 2009, GEM was prescribed over 500 000 times (Bulloch et al. 2012; Jackevicius et al. 2011). Being among the most prescribed human pharmaceuticals, lipid regulators are frequently reported in wastewater and surface waters (Andreozzi et al. 2003; Gros et al. 2006; Lin and Reinhard 2005; Sanderson et al. 2003; Schmidt et al. 2011; Togola and Budzinski 2007). In Europe, GEM has been found at concentrations up to 4.76 $\mu g.L^{-1}$ in wastewater treatment plant effluents (Andreozzi et al. 2003) and up to 1.5 $\mu g.L^{-1}$ in surface waters (Fang et al. 2012). In marine ecosystems, GEM is also among the most frequently detected compounds, with concentrations between 1 and 758 $ng.L^{-1}$ in seawater (Gaw et al. 2014; Vidal-Dorsch et al. 2012). Despite its presence in aquatic ecosystems, there is still limited information concerning mechanisms of toxicity for GEM to aquatic organisms, particularly for marine fish.

Earlier studies on GEM exposure to aquatic organisms have revealed a potential of this pharmaceutical to induce alterations of biochemical and behavioral endpoints (Al-Habsi et al. 2016; Fraz et al. 2018; Henriques et al. 2016; Mimeault et al. 2006; Prindiville et al. 2011; Quinn et al. 2011, 2008; Schmidt et al. 2011; Skolness et al. 2012; Zurita et al. 2007). In zebrafish (*Danio rerio*), GEM was found to impair hatching success and embryonal development, change locomotor activity and reduce survival, with a reported 96-h LC_{50} (50% lethal effect concentration) of 11.01 $mg.L^{-1}$ (Henriques et al. 2016). GEM

activated cholinesterase in the PLHC-1 cell lines of the fish clearfin livebearer (*Poeciliopsis lucida*) (Zurita et al. 2007) and enzymes involved in oxidative stress of goldfish (*Carassius auratus*), as well as increased lipid peroxidation (Mimeault et al. 2006). GEM has furthermore been reported to decrease plasma testosterone levels in freshwater goldfish (*Carassius auratus*) (Mimeault et al. 2005) and to activate antioxidant enzymes and modulate metallothionein expression in blue mussel (*Mytilus* spp.) (Schmidt et al. 2011). The ability of GEM to induce behavioral alterations has also been reported for the freshwater cnidarian *Hydra attenuata* (Quinn et al. 2008). GEM exposure increased growth and reproduction of *Daphnia magna* (Salesa et al. 2017, Steinkey et al. 2018). The effect of GEM on lipid metabolism was previously reported for the freshwater fish fathead minnow (*Pimephales promelas*) (Skolness et al. 2012) and rainbow trout (*Oncorhynchus mykiss*) (Prindiville et al. 2011). In marine fish, GEM has been reported to affect antioxidant defenses in sole (*Solea senegalensis*) (Solé et al. 2014) and to inhibit the activity of P450-catalysed pathways of yellow European eel (*Anguilla anguilla*) (Lyssimachou et al. 2014). In these studies fish were exposed through intraperitoneal injection. In the gilthead seabream (*Sparus aurata*), GEM has been reported to induce genotoxic effects at a concentration frequently detected in the environment (1.5 $\mu g.L^{-1}$) (Barreto et al. 2017). It affected transcription of key genes involved in lipid homeostasis and was characterized as a stress-inducing agent (Teles et al. 2016). It is, however, not known if GEM alters enzymatic activities associated with oxidative stress and biotransformation and whether exposure to GEM also affects behavior.

Considering the existing knowledge gaps concerning the mechanistic effects of GEM exposure to marine fish, the gilthead seabream (*Sparus aurata*) was selected as a model species and several biomarkers were included in an integrated assessment of possible effects. This top predator is widespread in Atlantic and Mediterranean coastal waters, with a high economic importance for both fishery and aquaculture, being one of the most consumed fish in the Mediterranean area (Teles et al. 2016). Furthermore, *S. aurata* has previously been shown to be sensitive to short-term exposure to GEM as demonstrated by increased DNA damage and increased cortisol levels (Barreto et al. 2017; Teles et al. 2016). Effects of GEM were determined following 96-h waterborne

exposure by assessing swimming ability, which may provide information on the ability of fish to escape predators, to chase prey and escape pernicious conditions, and biomarkers involved in neurotransmission (cholinesterase - ChE), biotransformation and antioxidant defenses (catalase (CAT), glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and glutathione reductase (GR)) as well as oxidative damage, i.e. lipid peroxidation (LPO). This set of biomarkers was chosen to assess the general health status of fish focusing on the ability to respond to oxidative challenge, maintain biotransformation and prevent damage in order to maintain fitness. This approach has been previously adopted by other authors using a battery of behavioral and biochemical biomarkers, such as ChE, GST, CAT activities and LPO levels to assess the effects of toxic metals and bisphenol A to *S. aurata* (Souid et al. 2013, 2015). The purpose of the present study was thus to understand the potential effects of GEM to the marine fish *S. aurata* and the mechanisms of toxicity involved.

2. Material and Methods

2.1. Chemicals

All reagents used were of analytical grade obtained from Sigma-Aldrich (Germany), Bio-Rad (Germany) and Merck (Germany). GEM was acquired from Tokyo Chemical Industry Co., Ltd. (TCI) and the isotopically labelled standard gemfibozil-d6 was purchased from Santa Cruz Biotechnology (USA).

2.2. Test organisms and acclimation

Juvenile gilthead seabream (*Sparus aurata*), with a length of 9±0.5 cm and a weight of 8.1±0.6 g, from an aquaculture facility (Santander, Spain), were acclimated for 4 weeks in aquaria with aerated and filtered (Eheim filters) artificial seawater (ASW, Ocean Fish, Prodac). This water was prepared by dissolving the salt in reverse osmosis purified water to obtain a salinity of 35, in a controlled room temperature (20ºC) and natural photoperiod. During the acclimation period, animals were fed daily with commercial fish food (Sorgal, Portugal) at a ratio of 1 g per 100 g of fish. The ASW used to maintain fish during the acclimation period was also used during the toxicity test.

2.3. Experimental design

All experimental procedures were carried out following the Portuguese and European legislation (authorization N421/2013 of the Portuguese legal authorities). Animal handling was performed by an accredited researcher. The bioassay followed, in general, the OECD guidelines for fish acute bioassays (OECD 1992). A stock solution of GEM (50 $g.L^{-1}$) was prepared, daily, in dimethyl sulfoxide (DMSO) due to its limited water solubility. DMSO was selected as a solvent due to its widespread use in several toxicological studies (Mimeault et al. 2006; Zurita et al. 2007; Quinn et al. 2008; Schmidt et al. 2011). Test solutions of GEM were prepared by dilution of the stock solution in ASW.

After the acclimation period, 70 fish were randomly distributed in the experimental aquaria, with ten fish per condition (n=10) in the ratio 1 g of fish per 1 L of ASW. The experimental design included a negative control (seawater only), a solvent control (0.03% DMSO, the maximal concentration of DMSO used in the GEM treatments) and five GEM concentrations: 1.5, 15, 150, 1500 and 15000 $\mu g.L^{-1}$. Fish were exposed for 96 h as recommended by the OECD guideline for fish acute toxicity testing (203), without feeding. The lowest tested concentration of GEM was chosen because it is considered an environmentally relevant concentration, based on levels detected in surface waters (Fang et al. 2012). The concentration range used was based on 10-fold increases.

Daily, after checking fish mortality, behavior alterations and assessing the water parameters (temperature, salinity, conductivity, pH and dissolved oxygen), approximately 80% of the experimental media was renewed to circumvent GEM degradation and to reduce the build-up of excretion products. During the exposure time, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period.

2.4. Quantification of GEM in the experimental media

Water samples were collected daily (at 0 and 24 h) from each aquarium. GEM was extracted using solid phase extraction (SPE). Briefly, Strata X cartridges (200 mg, 3 mL) (Phenomenex, USA) were conditioned with 5 mL methanol and 5 mL ultra-pure water. Then, 10 mL of water sample was percolated through the cartridge (3-5 $mL.min^{-1}$), rinsed with 5 mL ultra-pure water and dried under vacuum (20 min). Finally, GEM was eluted from the cartridges with methanol (10 mL). Extracts were evaporated until dryness under

a gentle stream of nitrogen and reconstituted with 1 mL acetonitrile/ultra-pure water (30:70, v/v). Gemfibrozil-d6 (10 µL of 5 $mg.L^{-1}$ in methanol) was added to the extract as internal standard. GEM analysis was performed on a Nexera UHPLC system with a triple-quadrupole mass spectrometer detector LCMS-8030 (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved using a Kinetex C18 column (2.1 x 150 mm i.d., 1.7 µm particle size) from Phenomenex (USA) using 5 mM ammonium acetate/ammonia buffer (pH 8) as solvent A and acetonitrile as solvent B at a flow rate of 0.22 $mL.min^{-1}$. The gradient elution was performed as follows: initial conditions: 30% B; 0-2.0 min, 30%-100% B; 2.0-4.5 min maintained at 100% B, 4.5-5.5 min return to initial conditions; and from 5.5-9.5 min, re-equilibration of the column. Column oven was set at 30ºC and the autosampler was operated at 4ºC. The injection volume was 5 µL.

GEM was analysed in the negative ionization mode and quantification was performed in multiple reaction monitoring mode (MRM) using two transitions between the precursor ion and the most abundant fragment ions (MRM1: 249.00>121.15 and MRM2: 249.00>127.05). Quantification was performed by the internal standard calibration method. The method detection limit (MDL) for GEM in water was 4.0 $ng.L^{-1}$.

2.5. Assessment of swimming performance

After 96 h exposure, each fish was gently transferred to a 1.5 m long track race flume with 7 cm diameter with a running water flow of 20 $L.min^{-1}$ and induced to swim, generally following the procedure described by Oliveira et al. (2012). The time that animals were able to swim against the water flow was recorded. After this test, fish were put back into their original test aquaria where they stayed for 2 h before being used to determine biochemical endpoints.

2.6. Preparation of biological material for biomarker determination

After the recovery period, animals were anesthetized with tricaine methanesulfonate (MS-222), their length measured, weighed and euthanized by spinal section. Liver, gills, brain and muscle were taken from each animal, snap frozen in liquid nitrogen to prevent enzyme degradation and stored at -80ºC until further processing.

2.6.1. Liver and gills

Liver and gills were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4), using an ultrasonic homogenizer (Sonifier 250, Branson sonicator). One aliquot of homogenate, for LPO determination, was transferred to a microtube with 4% BHT (2,6-dieter-butyl-4-metylphenol) in methanol, to prevent oxidation and stored at -80 ºC until analysis. The remaining homogenate was used for post-mitochondrial supernatant (PMS) isolation. PMS was accomplished by centrifugation at 12 000 x g for 20 min at 4ºC. PMS aliquots were stored at -80ºC until the determination of CAT, GST, GPx and GR activities.

2.6.2. Muscle and brain

Muscle and brain were used for ChE activity determination. Tissues were homogenized in potassium phosphate buffer (0.1 mM, pH 7.2), centrifuged at 3 300 x g for 3 min at 4ºC, supernatant was collected and stored at -80ºC.

2.7. Biochemical biomarker analysis

Protein concentration was determined according to Bradford (1976), adapted to microplate, using bovine γ - globulin as a standard.

2.7.1. ChE activity

ChE activity was determined according to the Ellman's method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996). The rate of thiocholine production was assessed at 412 nm as nmol of thiocholine formed per min per mg of protein using acetylthiocholine as substrate.

2.7.2. CAT activity

CAT activity was assayed as described by Claiborne (1985) and the variations in absorbance at 240 nm, caused by the dismutation of hydrogen peroxide (H_2O_2), were recorded. CAT activity was calculated as µmol H_2O_2 consumed per min per mg of protein.

2.7.3. GST activity

GST activity was determined spectrophotometrically by the method of Habig et al. (1974) adapted to microplate (Frasco and Guilhermino 2002), following the conjugation of the substrate, 1-chloro-2, 4-dinitrobenzene (CDNB), with reduced glutathione. Absorbance was recorded at 340 nm (25 ºC) and activity expressed as nmol CDNB conjugate formed per min per mg of protein.

2.7.4. GPx activity

GPx activity was measured according to the method described by Mohandas et al. (1984) as modified by Athar and Iqbal (1998). Oxidation of NADPH was recorded spectrophotometrically at 340 nm and the enzyme activity results expressed as nmol NADPH oxidized per min per mg of protein.

2.7.5. GR activity

GR activity was estimated according the method of Carlberg and Mannervik (1975) adapted to microplate (Lima et al. 2007), measuring NADPH disappearance at 340 nm. GR activity was expressed as nmol of $NADP^+$ formed per min per mg of protein.

2.7.6. LPO levels

LPO levels were estimated by the formation of thiobarbituric acid reactive substances (TBARS) based on Ohkawa et al. (1979), adapted by Wilhelm Filho et al. (2001). Absorbance was measured at 535 nm and LPO levels were expressed as nmol of TBARS formed per mg of protein.

2.8. Integrated biomarker response (IBR)

To integrate all the results from the different tested biomarkers and to understand global responses, the IBR index was calculated according to Sanchez et al. (2013), using IBR version 2 (IBRv2). IBRv2 was designed to modify the IBR previously developed by Beliaeff and Burgeot (2002). The IBR was chosen to integrate the different biomarker responses into a numeric value (Devin et al. 2014). The assessed endpoints were combined into one general ‘‘stress index’’ to integrate biomarker data into a value representing the stress level at each tested concentration, based on the principle of reference deviation.

Overall, data were log-transformed (Yi) and the overall mean (μ) and standard deviation (s) calculated. Data was further standardized by subtracting the overall mean and dividing by the standard deviation as presented in the following equation:

$$Zi = \left(\frac{yi - \mu}{s}\right)$$

The difference between Zi and Z0 (control) was calculated in order to determine A values. Representative results are shown as star plot charts indicating the deviation of all biomarkers in relation to the control (0) (Sanchez et al. 2013). In addition, data were analyzed using a weighing procedure for endpoints as previously described (Liu el al. 2013, 2015), assuming that a biochemical alteration has lower impact on the organism health than changes at an individual level. Behavior is considered as the outcome of many biological processes resultant from interactions between the organisms and the surrounding environment (Oliveira et al. 2015). Thus, biochemical biomarkers were weighted with a factor of one and behavior with a factor of three. More information about IBRv2 and the difference between this version and version 1 can be found in the supplementary information.

2.9. Data analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test), using Sigma Plot 12.0 software package. Differences between controls (negative and solvent) were carried out using a Student t-test ($p<0.05$). Differences between treatments and controls were compared using one-way analysis of variance (ANOVA), followed by Dunnett's comparison test whenever applicable ($p<0.05$).

3. Results and Discussion

3.1. Quantification of GEM in the experimental media

The analysis of GEM concentrations revealed that nominal concentrations of GEM differed 6 to 37% from the results obtained by chemical analyses (Table S1). After 24 h, GEM degradation was higher in the aquaria with the lowest concentrations, as previously presented (Barreto et al. 2017). Other authors

also reported a decrease of GEM concentrations more evident in the lowest concentrations after 14-d exposure of goldfish (Mimeault et al. 2005). In that study, the initial concentrations 1.5 and 1500 $\mu g.L^{-1}$ gave measured concentrations of 0.34 and 851.9 $\mu g.L^{-1}$ after 14 d, respectively. The observed decrease of GEM concentrations in the water can also be explained by the incorporation of GEM in the fish. In the study of Mimeault et al. (2005), the quantification of GEM in the plasma of goldfish exposed to 1500 and 10000 $\mu g.L^{-1}$ revealed that, after 96 h, GEM was present in concentrations higher than 75000 $\mu g.L^{-1}$ for both treatments. After 14-d exposure, plasma concentrations of animals exposed to nominal concentrations of 1.5 and 1500 $\mu g.L^{-1}$ were 170 and 78000 $\mu g.L^{-1}$, respectively (Mimeault et al. 2005).

3.2. Biological responses

The solvent DMSO did not induce significant effects when compared to the negative control (t-test, $p>0.05$) for any reported endpoint. Therefore, all GEM exposure data were compared to the negative control.

The ability of *S. aurata* to swim against a water flow (in terms of time of swimming) was significantly decreased (between 50 and 65%; $p<0.05$) in individuals exposed to GEM, relative to the control group (Figure 1), highlighting behavior as a sensitive endpoint. The detected swimming performance impairment may have serious environmental consequences (Wolter and Arlinghaus 2003). The basic activities of fish, such as predator–prey interactions, reproduction and migration, are completely dependent on the individuals' capacity for locomotion (Svendsen et al. 2015; Vieira et al. 2009). A decrease in locomotion was also reported for zebrafish larvae exposed to GEM concentrations equal to or higher than 1500 $\mu g.L^{-1}$ (Henriques et al. 2016). However, unlike in the present study, the locomotor activity decreased when GEM concentration increased (Henriques et al. 2016).

Altered swimming behavior may be associated with effects on neurotransmission. ChE activity is essential for the degradation of the neurotransmitter acetylcholine in cholinergic synapses and thus involved in a correct transmission of nerve impulses both in vertebrates and invertebrates (Pan et al. 2012; Sureda et al. 2018). Thus, it could be hypothesized that a decrease in ChE might be a possible explanation for the observed decrease in

the swimming performance (Hernández-Moreno et al. 2011). However, in the present study, ChE activity was not significantly altered at the tested concentrations (Fig. 2A and B), suggesting that other factors (e.g., decreased available energy associated with the need to metabolize GEM and to activate enzymatic processes or inadequate capacity to supply oxygen to tissues (Kennedy and Farrell 2006)) may be involved in the detected behavioral alteration.

Oxidative stress is a mechanism of toxicity described for several environmental contaminants such as metals and pesticides (Lushchak 2016). In the present study, CAT activity significantly increased (between 50 and 93%) in the gills of fish exposed to concentrations higher than 1.5 $\mu g.L^{-1}$ ($p<0.05$) (Fig. 3A). GR activity also significantly increased between 46 and 72% in gills ($p<0.05$) in individuals exposed to concentrations of 15, 150 and 1500 $\mu g.L^{-1}$ (Fig. 3D), but not under the highest exposure concentration. However, no significant alterations were found in terms GST and GPx activities of gills (Fig. 3B and 3C, respectively).

In the liver, CAT activity was significantly increased (150%) ($p<0.05$) in animals exposed to 15000 $\mu g.L^{-1}$ (Fig. 4A) whereas no significant alterations were found in GST activity (Fig. 4B). GPx and GR activities significantly increased ($p<0.05$), between 156 and 243% (Fig. 4C) and 42-75% (Fig. 4D), respectively, in concentrations higher than 1.5 $\mu g.L^{-1}$ of GEM. The activity of GST, involved in the detoxification of many xenobiotics and playing an important role in protecting tissues from oxidative stress, was not affected by exposure to GEM. However, the assessed enzymes involved in antioxidant defense (CAT, GPx and GR) were activated both in gills and liver in a tissue- and concentration-dependent manner. At concentrations of GEM higher than 15 $\mu g.L^{-1}$, some enzymatic activities were maintained (gill CAT and liver GR) or decreased (gill GR). These observed responses may be due to the negative feedback from excess of substrate or direct damage by oxidative modifications (Ceyhun et al. 2010; Rodrigues et al. 2016). On other hand, at concentrations between 150 and 15000 $\mu g.L^{-1}$, fish may have also cope with this xenobiotic compound, resulting in similar responses at these three concentrations.

As the present study, previous studies with aquatic organisms have demonstrated the induction of oxidative stress by GEM (Mimeault et al. 2006;

Schmidt et al. 2014, 2011), but a direct comparison between results is not straightforward due to differences in exposure duration, test organisms (species and their natural environment, including freshwater versus seawater), and *in vivo* versus *in vitro* studies. Teles et al. (2016) reported that the hepatic transcription of CAT, GPx and GST in *S. aurata* was not altered following 96 h exposure to GEM. However, the present study demonstrated that antioxidant enzymes (CAT, GPx and GR) were responsive to GEM exposure showing that evaluation of enzyme activity is key considering the complex regulatory mechanisms for gene expression that occurs at both post-transcriptional and post-translational levels.

As shown in Figure 5B, peroxidative damage (assessed as TBARS levels) was only found in liver at 1.5 $\mu g.L^{-1}$. This concentration led to a 57% increase in TBARS levels compared to liver from the control group. In gills from fish exposed to 15 and 150 $\mu g.L^{-1}$ GEM there was a significant ($p<0.05$) decrease in LPO levels, corresponding to 24 and 30% when compared to the control, respectively (Fig. 5A). The decreased LPO levels observed after exposure to GEM have earlier been reported for the digestive gland of marine mussels (*Mytilus* spp.) and shown to depend on exposure time (Schmidt et al. 2014). Increased (Mimeault et al. 2006) or unaltered (Quinn et al. 2011) LPO levels after the exposure to GEM were also previously reported for freshwater organisms, suggesting that the mechanisms of GEM toxicity are to a large extent species-specific.

In the present study, 1.5 $\mu g.L^{-1}$ of GEM was able to induce oxidative damage in *S. aurata* without leading to significant alteration of antioxidant enzyme activity. At GEM concentrations higher than 1.5 $\mu g.L^{-1}$, activation of antioxidant defences appeared to be sufficient to prevent oxidative damage. Previous data involving GEM and other lipid regulators showed a high prevalence of peroxisome proliferation (even as an acute effect), indicating the possibility of occurrence of oxidative stress, which may lead to irreversible damage by lipid peroxidation (Nunes et al. 2004; Qu et al. 2001; Quinn et al. 2011). On other hand, the reported ability of GEM to reduce lipids may have also contributed to the observed LPO decrease (Ozansoy et al. 2001; Roy and Pahan 2009; Sutken et al. 2006).

The analysis of antioxidant status and other stress responses in different tissues of organisms exposed to pollutants helps to understand the associated mechanisms of toxicity and predict the degree of effects at different levels of biological organization (Franco et al. 2006; Oliveira et al. 2008). In the present study, responses in gills and liver were very different following exposure to GEM. CAT appeared as more responsive in gills than in the liver whereas GST and GR displayed overall similar profiles of response in both tissues. GPx, however, was more responsive in liver. These detected differences may be explained by the enzymatic basal activities. CAT basal activity was lower in gills than in the liver and GPx basal activity was lower in liver than in gills. Oxidative damage was only detected in liver at a concentration unable to activate enzymatic defences.

Although the mechanisms responsible for the effects of GEM is not known in detail, it is considered that many of the above-mentioned effects are mediated by GEM interaction with peroxisome proliferator-activated receptor α (PPARα) (Al-Habsi et al. 2016; Marija et al. 2011; Staels et al. 1992), which is involved in the regulation of lipid metabolism in liver, heart, kidney and muscle (Marija et al. 2011; Pyper et al. 2010; Schoonjans et al. 1996). PPARα may be activated by natural ligands and synthetic agents, including fibrates (such as GEM) (Marija et al. 2011; Touyz and Schiffrin 2006). Fibrates are known to induce proliferation of peroxisomes in liver cells with associated coordinated transcriptional activation of peroxisomal fatty acid β-oxidation system and production of reactive oxygen species (ROS) (Lores Arnaiz et al. 1997, 1995; Marija et al. 2011; Moody et al. 1991; Palma et al. 1991; Pyper et al. 2010; Schoonjans et al. 1996). Elevated concentrations of H_2O_2 stimulate lipid peroxidation, which this may explain the increase of LPO levels in the liver and the absence in the gills. On the other hand, gills are key organs for the direct action of waterborne pollutants since they are involved in a range of processes critical to survival (e.g. respiration, osmoregulation, excretion of nitrogenous residual products and regulation of the acid-base balance) (Evans 1987; Oliveira et al. 2008, 2012), and also in immune functions involving oxidative processes (Rodrigues et al. 2016; Tkachenko et al. 2014). Gills are highly vulnerable to toxic chemicals, because their large surface area facilitates toxicant interaction and absorption (Evans 1987; Oliveira 2008), so it is

expected that some enzymatic responses are activated more and primarily in gills than in the liver.

The integration of the data using IBR allows to visualize more clearly the specific responses of biomarkers for each tested condition (Beliaeff and Burgeot 2002). The IBR provides a combination of a graphical synthesis of the different biomarker responses and a numeric value which integrates all these responses at once (Devin et al. 2014). Based on the IBRv2 values, the effects of the different concentrations of GEM would be ordered as follows: 150 $\mu g.L^{-1}$≈15000 $\mu g.L^{-1}$≈1500 $\mu g.L^{-1}$>15 $\mu g.L^{-1}$>1.5 $\mu g.L^{-1}$ (Fig. 6A). The similar IBRv2 values observed for 150, 1500 and 15000 $\mu g.L^{-1}$ may be explained by the similarity of the fish responses independent of the GEM concentration due to reasons described above. Although there was no concentration-response relationship for the tested biomarkers (Fig. 6A), the results show that exposure to GEM at concentrations between 150 and 15000 $\mu g.L^{-1}$ caused more effects than exposure to 1.5 and 15 $\mu g.L^{-1}$. In general, analyzing the assessed endpoints star plots obtained with IBRv2, for each experimental condition (Fig. 6B1-B5), it seems clear that GEM had more effects in terms of capability to swim against a flow of *S. aurata*, CAT and GR activities in gills and GPx and GR activities in liver. If the data analyses takes into account a weighing factor attributed to different biological levels of organization as suggested by Liu et al. (2013, 2015), the effects of the different concentrations of GEM would be ordered as follows: 150 $\mu g.L^{-1}$≈15000 $\mu g.L^{-1}$≈1500 $\mu g.L^{-1}$>1.5 $\mu g.L^{-1}$≈15 $\mu g.L^{-1}$ (Table S2). This data analysis, attributing a higher weighing factor to behavior, did, however, not alter the ranking of GEM impact. Considering the integration of the data from biochemical endpoints (CAT, GST, GPx and GR activities and LPO levels) per tissue (gills *versus* liver) – Table S3 – the IBRv2 values were higher in gills than in liver for 1.5, 15 and 150 $\mu g.L^{-1}$ of GEM. However, the IBRv2 values were similar between the two tissues for 1500 $\mu g.L^{-1}$ of GEM and for 15000 $\mu g.L^{-1}$ the IBRv2 value was higher in liver than in gills.

The detection of GEM toxicity at an environmentally relevant concentration may be of concern, taking into account that fish are exposed to a variety of contaminants in their natural habitat, including pharmaceuticals sharing the toxicological properties of GEM. Further studies assessing effects of low GEM concentrations and longer exposure periods are encouraged to improve the

knowledge about the mechanisms involved on the toxicity of fibrates to non-target organisms like marine fish and its ability to adapt to these compounds.

4. Conclusions

A multibiomarker approach showed that short-term exposure to an environmentally relevant concentration of gemfibrozil (1.5 $\mu g.L^{-1}$) induced behavioral alterations and oxidative damage in the liver of the marine fish *Sparus aurata*. At higher concentrations the activities of some enzymes involved in antioxidant defense (catalase, glutathione peroxidase and glutathione reductase) were induced. Although there was no concentration-response relationship for responses, it was clear that higher concentrations (150, 1500, 15 000 $\mu g.L^{-1}$) had more effects on fish than lower concentrations (1.5 and 15 $\mu g.L^{-1}$). The integrated biomarker response version 2 (IBRv2) was found to be a useful tool to combine the results from many biomarkers.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Fig. 1 – Gemfibrozil affects the capability to swim against a water flow (expressed as time in seconds that the fish are able to swim=swimming resistance (s)) of *Sparus aurata* (n=10 per condition) against a water flow after 96 h exposure. Results are expressed as mean ± standard error.

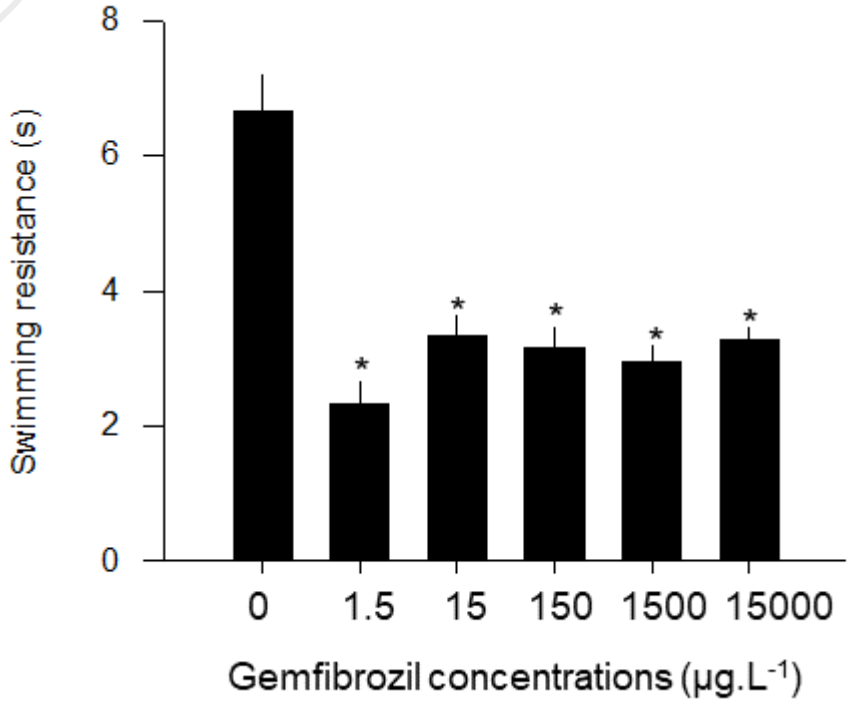

Fig. 2 – Gemfibrozil effects on the brain **(A)** and muscle **(B)** cholinesterase (ChE) activity of *Sparus aurata* (n=10 per condition) after 96 h exposure. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p < 0.05$). (**1.5 -column)**

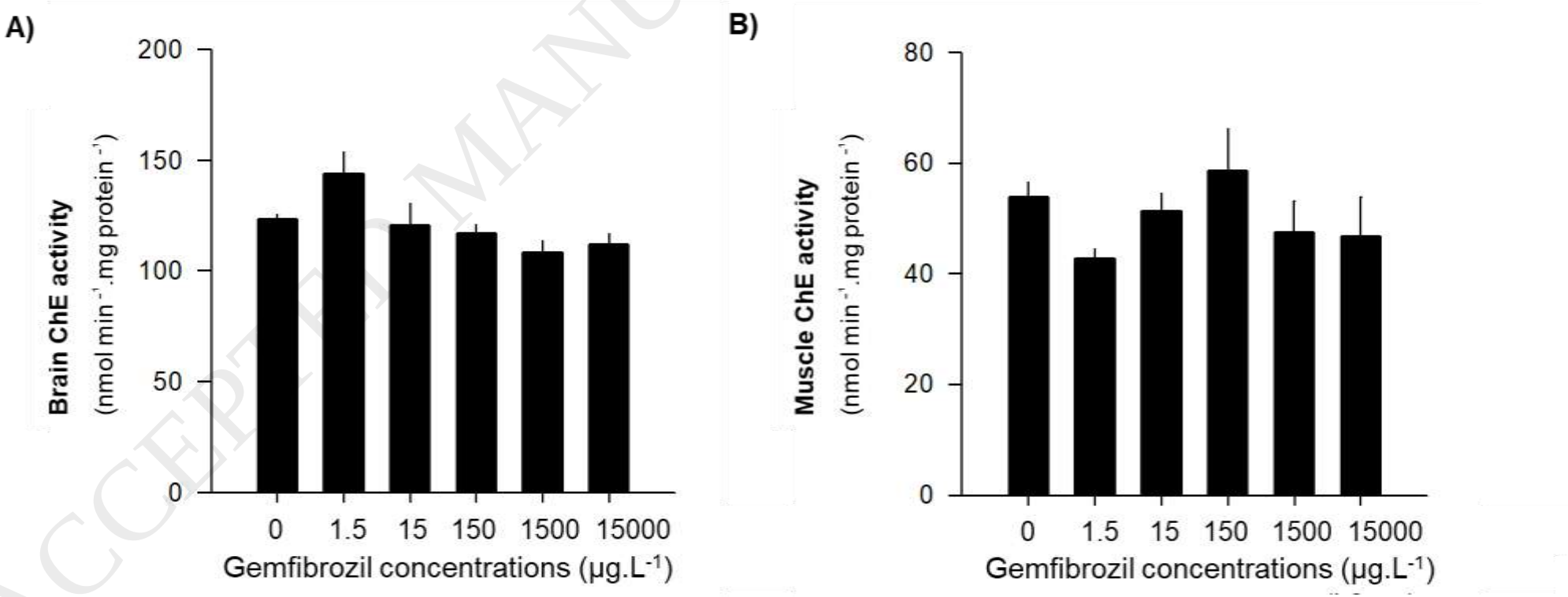

Fig. 3 – Gemfibrozil effects on the gills of *Sparus aurata* (n=10 per condition) after 96 h exposure: **A)** Catalase (CAT) activity; **B)** Glutathione *S*-transferases (GST) activity; **C)** Glutathione peroxidase (GPx) activity; **D)** Glutathione reductase (GR) activity. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p < 0.05$). (**2 -column)**

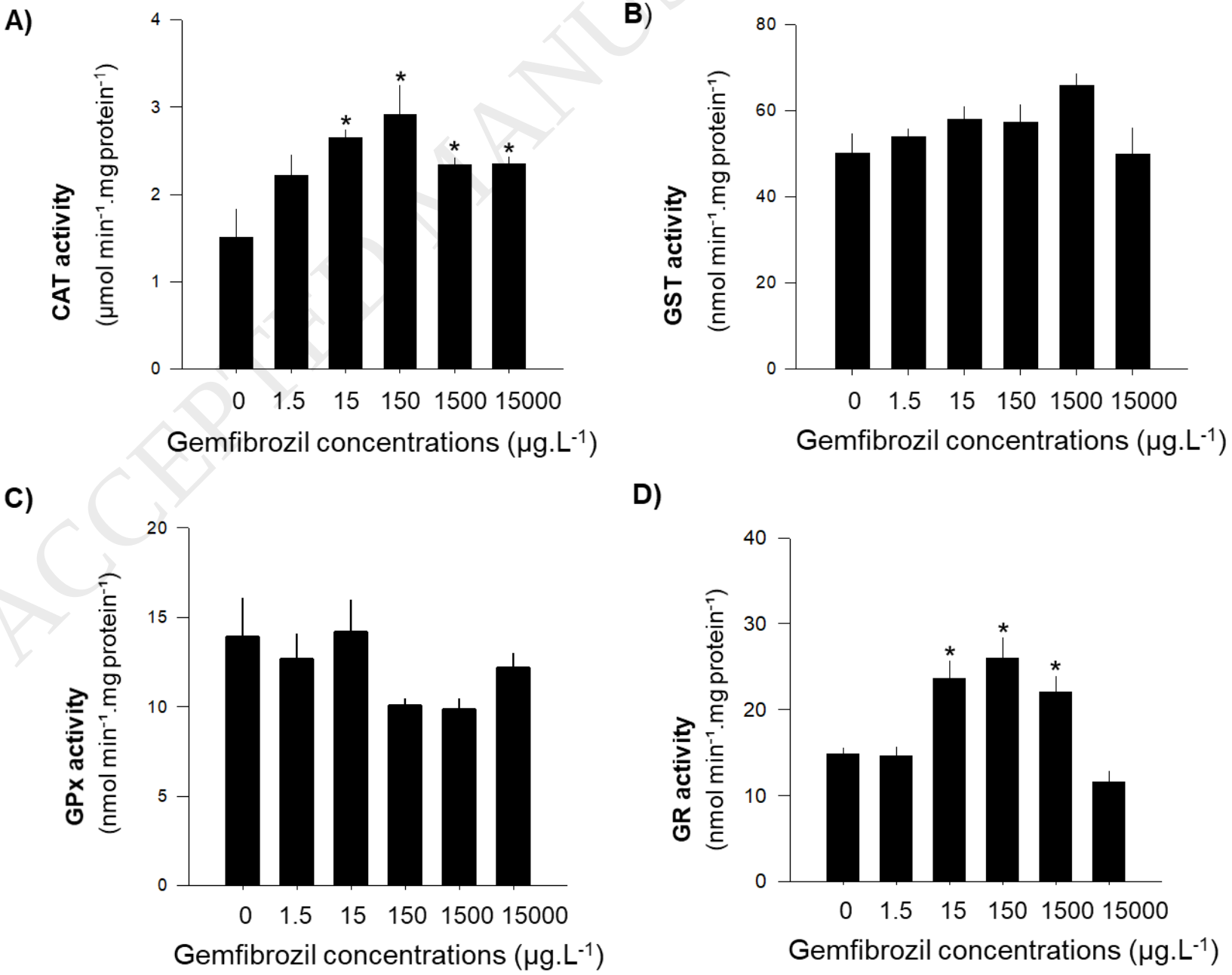

Fig. 4 – Gemfibrozil effects on the liver of *Sparus aurata* (n=10 per condition) after 96 h exposure**: A)** Catalase (CAT) activity; **B)** Glutathione *S*-transferases (GST) activity; **C)** Glutathione peroxidase (GPx) activity; **D)** Glutathione reductase (GR) activity. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p < 0.05$). (**2 -column)**

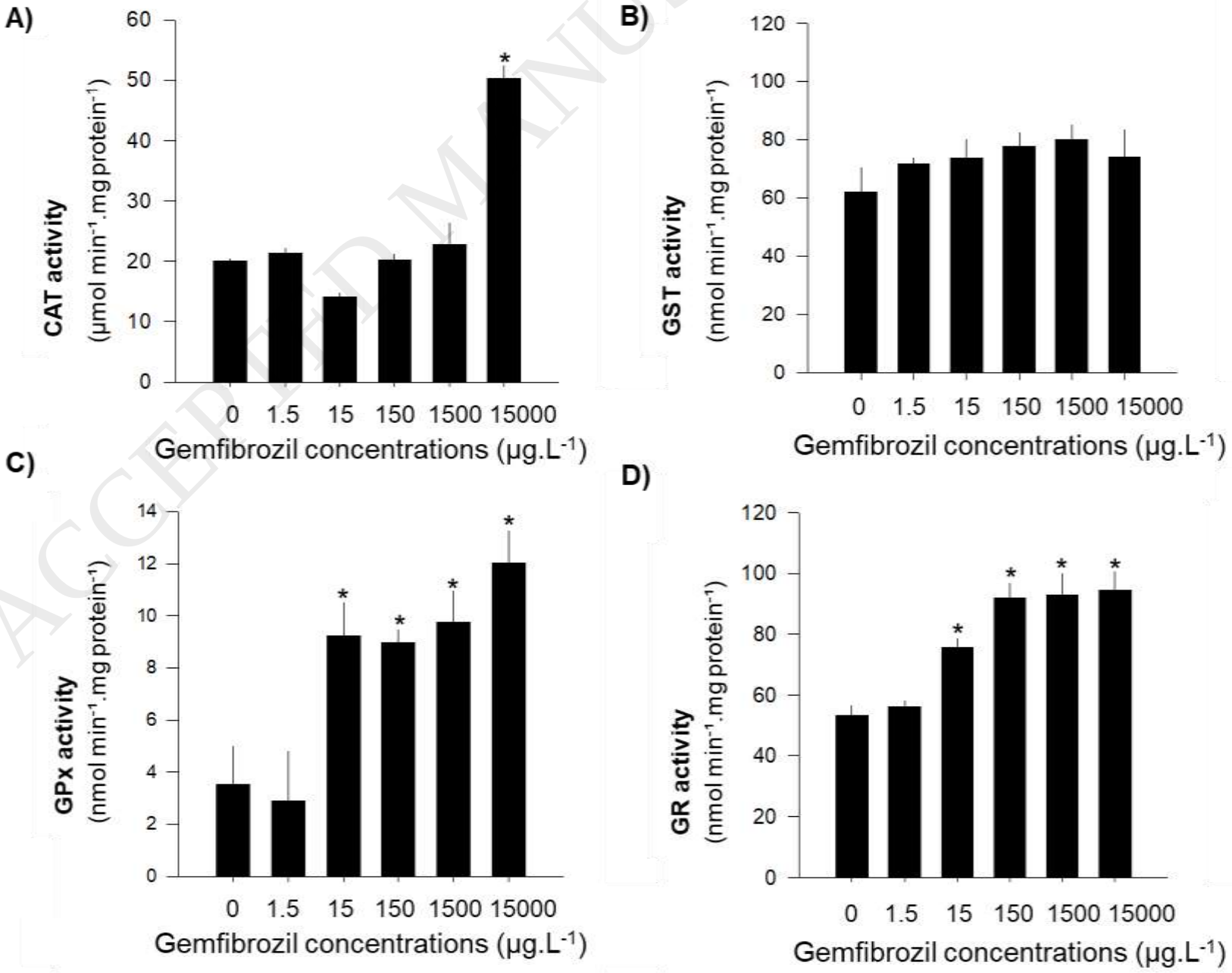

Fig. 5 – Gemfibrozil effects on the gills **(A)** and liver **(B)** lipid peroxidation (LPO) of *Sparus aurata* (n=10 per condition) after 96 h exposure. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p < 0.05$). (**1.5 -column)**

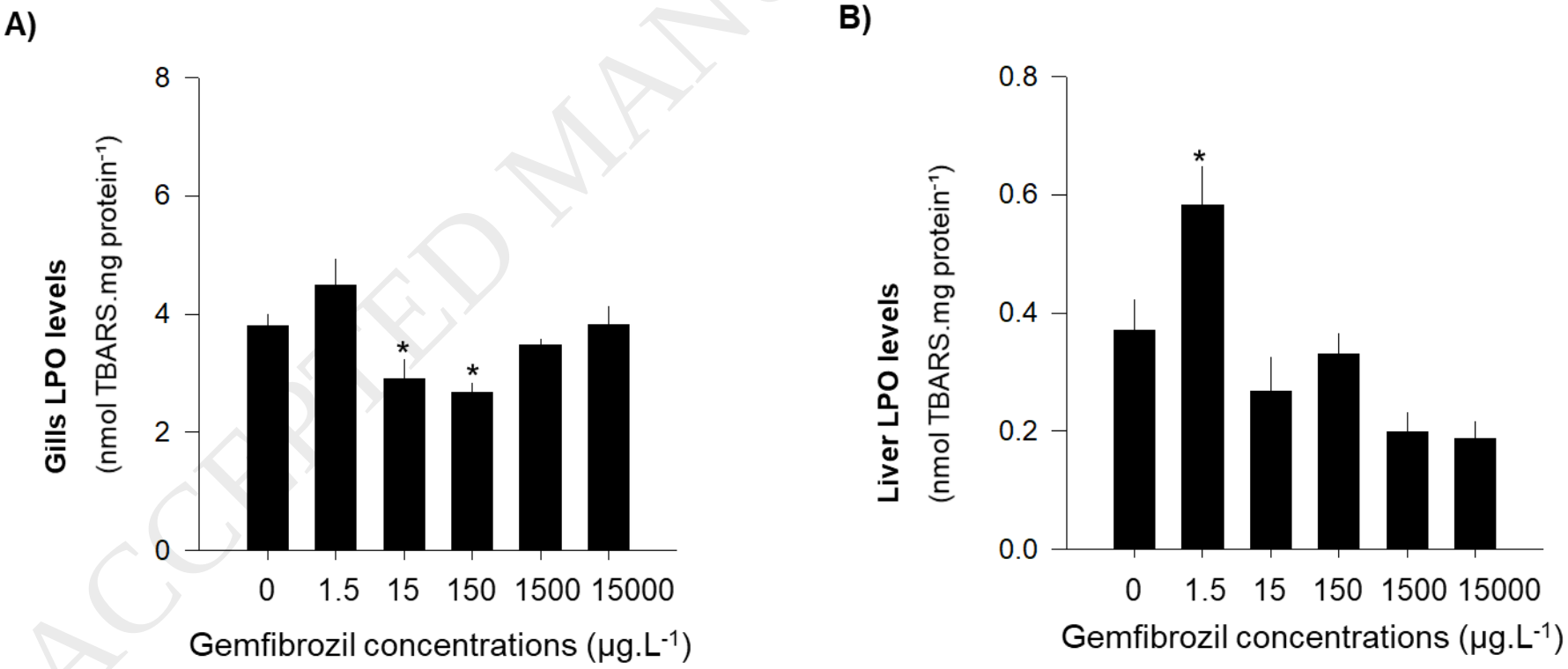

Fig. 6 – Integrated biomarker response version 2 (IBRv2) (**A**) and assessed endpoints star plots for each experimental condition (**B1-B5**). Gemfibrozil (GEM); Cholinesterase (ChE); Catalase (CAT); Glutathione *S*-transferases (GST); Glutathione peroxidase (GPx); Glutathione reductase (GR); Lipid peroxidation (LPO) (**2 -column**)

Fig. 6 – Integrated biomarker response version 2 (IBRv2) (**A**) and assessed endpoints star plots for each experimental condition (**B1-B5**). Gemfibrozil (GEM); Cholinesterase (ChE); Catalase (CAT); Glutathione *S*-transferases (GST); Glutathione peroxidase (GPx); Glutathione reductase (GR); Lipid peroxidation (LPO) (**2 -column**)