UAB
Universitat Autònoma de Barcelona

Dipòsit digital de documents de la UAB

This is the **accepted version** of the journal article:

Teles, Mariana [et al.]. «Evaluation of gemfibrozil effects on a marine fish (Sparus aurata) combining gene expression with conventional endocrine and biochemical endpoints». *Journal of hazardous materials*, Vol. 318 (November 2016), p. 600-607 DOI 10.1016/j.jhazmat.2016.07.044

This version is available at https://ddd.uab.cat/record/323790

under the terms of the BY-NC-ND license.

Evaluation of gemfibrozil effects on a marine fish (Sparus aurata) combining gene expression with conventional endocrine and biochemical endpoints

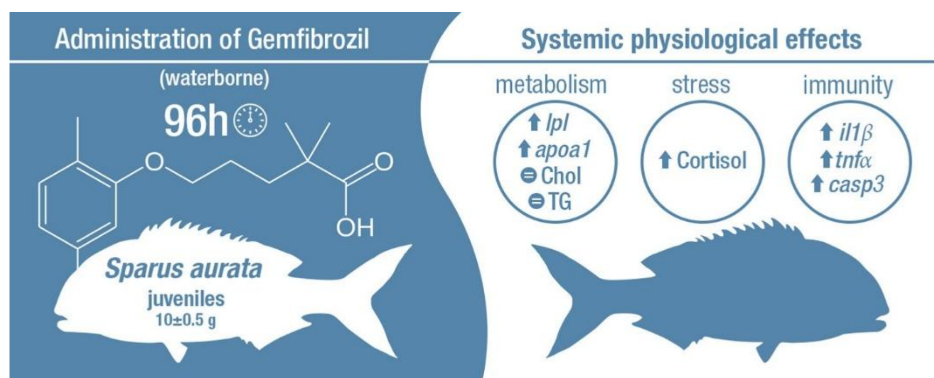
M. Teles [a,*], C. Fierro-Castro [a], P. Na-Phatthalung [b], A. Tvarijonaviciute [c], A.M.V.M. Soares [d], L. Tort [a], M. Oliveira [d]

[a] Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193, Barcelona, Spain
[b] Department of Microbiology and Excellent Research Laboratory on Natural Products, Faculty of Science and Natural Product Research Center of Excellence, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand
[c] Department of Medicine and Animal Surgery, Universitat Autònoma de Barcelona, 08193, Barcelona, Spain
[d] Department of Biology & CESAM, University of Aveiro, 3810-193, Aveiro, Portugal

highlights

- Gemfibrozil (GEM) effects were assessed at molecular, endocrine and physiological levels on Sparus aurata.
- Hepatic mRNA levels of apolipoprotein AI and lipoprotein mRNA levels were increased.
- Proinflammatory cytokines mRNA levels increased after GEM exposure.
- High cortisol levels indicate that GEM is recognized as stressor by the fish.

graphical abstract



article info

Keywords:
Human pharmaceuticals
Gemfibrozil
Marine fish
Biomarkers

abstract

The information on the potential hazardous effects of gemfibrozil (GEM) on marine fish is extremely scarce. In the current study, molecular, endocrine and biochemical parameters were assessed in Sparus aurata after 96 h waterborne exposure to a GEM concentration range. Hepatic mRNA levels of target genes known to be regulated via peroxisome proliferator-activated receptor a (ppara) in mammals, such as apolipoprotein AI (apoa1) and lipoprotein (lpl) were significantly increased, without a concomitant activation of the ppar pathways. GEM (15 ¡.cg L^{-1}) induced an upregulation in mRNA levels of interleukin 1i3 (il1ˇ), tumour necrosis factor-a (tnf) and caspase 3 (casp3), suggesting an activation of proinflammatory processes in S. aurata liver. However, mRNA levels of genes related with the antioxidant defence system and cell-tissue repair were unaltered under the tested experimental conditions. Higher levels of GEM induced a cortisol rise, an indication that it is recognized as a stressor by S. aurata. Cortisol levels and the mRNA levels of il1ˇ, tnf and casp3 may be suggested as potential biomarkers of GEM effects in marine fish.

* Corresponding author at: Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193, Barcelona, Spain.
E-mail address: mteles0@gmail.com (M. Teles).

1. Introduction

Substantial amounts of pharmaceuticals are permanently entering water bodies mainly due to inefficient removal during wastewater and slurries treatments [1]. Thus, these compounds represent a potential environmental threat to non-target organisms and ultimately, to human health. Predators, such as some fish species, may be particularly vulnerable to the presence of pharmaceuticals in the environment due to their capacity to bioaccumulate and bioconcentrate xenobiotics [2,3] and to their position at the top of the aquatic food chain that make them potential targets of biomagnified compounds.

Fibrate drugs, such as gemfibrozil (GEM), are among the most prescribed pharmaceuticals in human medicine [4]. GEM helps to reduce cholesterol and triglycerides in the blood and, in this way, reduce the risk of atherosclerosis [5]. Due to its widespread use, continuous release and persistence in the environment, GEM is consistently detected in the aquatic environment at concentrations ranging from ng L^{-1} in surface waters up to 5 μg L^{-1} in the effluents of sewage treatment plants from different European countries [6,7]. Previous studies in freshwater species showed that GEM is taken up and bioconcentrate in Carassius auratus, causing an increase in antioxidant defence enzymes [3]. GEM has also been reported to induce genotoxicity in Danio rerio [8], reduced fecundity in Pimephales promelas [9], and cause behavioural changes in D. rerio [10]. In estuaries and sea, the concentrations of pharmaceuticals have been poorly characterized. In the case of GEM, according to Gaw et al. (2014), detected levels varied from 1 up to 758 ng L^{-1} [11]. There is also a need of information concerning GEM effects on marine fish, since, to the author's knowledge, there are only two available studies with marine fish (Anguilla anguilla and Solea senegalensis) [12,13]. Furthermore, in these studies fish were exposed through intraperitoneal injection, a procedure that does not represent an environmentally occurring exposure.

Regarding molecular effects, it has been shown in mammals that fibrates mimic fatty acids and bind to the peroxisome proliferator-activated receptors (ppar), ligand-inducible transcription factors belonging to the nuclear hormone receptor superfamily. There are three types of ppar (α, β, γ), though fibrates bind preferentially to pparα [14]. Pparα regulates gene expression by binding, as a heterodimer with the retinoid X receptor (rxr), to specific DNA sequences, called peroxisome proliferator response elements (ppre). This results in the transcriptional activation of target genes, such as apolipoproteins (apo), lipoprotein lipase (lpl) and acyl-CoA oxidase [15]. In addition, pparα has been shown to modulate glucose metabolism, liver inflammation and hepatocyte proliferation in mammals [16].

Similar to mammals, three isoforms of ppar have been identified in different fish species, including Sparus aurata [17]. However, divergent findings have been reported in fish regarding ppar expression after exposure to fibrates. It was shown that GEM induced ppar$_{<}$ expression in D. rerio [18], whereas in C. auratus ppar$_{<}$ pathways were not activated [19]. Thus, further studies are needed to clarify this topic in fish.

Changes in the hypothalamo–pituitary–interrenal (HPI) axis function, measured as cortisol levels or selected stress-related genes, are important indicators of fish condition [20]. Generally, fish respond to stress with increases in plasma cortisol, the main corticosteroid in fish [21]. However, this adaptive cortisol response in fish may be impacted by environmental chemicals [22,23]. A large amount of the available studies concerning HPI alterations in fish have focused on metals or environmental contaminated waters but little is known about the effects of pharmaceuticals at the endocrine level on marine fish.

The aim of the present study was to investigate GEM effects at different levels of biological organization on a marine fish species, S. aurata (gilthead sea bream), in order to produce knowledge on possible toxicity pathways, and evaluate potential biomarkers of GEM exposure and effects on this species. On this regard, molecular, endocrine and biochemical parameters were selected as endpoints representing a component of individual fitness, useful to address the effects of this emergent contaminant of concern on S. aurata. This teleost fish is widespread in Atlantic and Mediterranean coastal waters and has a high commercial importance for fishery and aquaculture. As one of the most consumed fish in the Mediterranean area, it may represent an important route of possible entry of contaminants into humans. Thus, the study of effects on this species becomes high relevant. Therefore, fish were waterborne exposed, during 96 h to different concentrations of GEM, and a set of biomarkers from different levels of biological organization were measured. In order to get insights into GEM mechanisms of action, mRNA levels of key genes were assessed in the liver, a target organ of the majority of xenobiotics, including GEM [13]. Liver also has an important role in the redox metabolism of xenobiotics, as well as in the lipid and carbohydrate metabolism. Selected genes belong to the following categories: peroxisome receptors, lipid regulators, antioxidant enzymes, cell-tissue repair, immune system and apoptosis regulation. To evaluate the potential endocrine-disruptive effects of GEM, cortisol was measured in plasma. Biochemical indicators of lipid metabolism (cholesterol, triglycerides), carbohydrate metabolism (glucose) and hepatic health indicators (aspartate aminotransferase – AST, alanine transaminase – ALT, and alkaline phosphatase – ALP) were also assessed in plasma.

2. Materials and methods

2.1. Test organisms

S. aurata specimens with 9 ± 0.5 g (mean ± standard deviation) were commercially acquired from an aquaculture in the North of Spain. Once in the laboratory, fish were acclimated to laboratorial conditions for three weeks in 250 L aquaria containing aerated and filtered (Eheim filters) artificial saltwater (35 g L^{-1}) in a 16:8 photoperiod and temperature controlled (20 ± 1 °C) room. Fish were fed daily, with commercial fish food, up to 48 h before the bioassay. All experimental procedures involving fish were carried out according to the 3 R's principles of Animal Experimentation following the actual Portuguese legislation that agrees with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63). Animal handling was performed with accredited researchers.

2.2. Fish bioassay

A stock solution of GEM was prepared by dissolving the pharmaceutical in dimethyl sulfoxide. Test solutions were prepared by dilution of the stock solution in artificial saltwater (salinity 35) to test a concentration range from 1.5 to 15000 μg L^{-1}. Fish were randomly distributed in 80 L tanks containing the test solutions and exposed for 96 h. No food was provided to fish during the bioassay. During the experiment, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period. Every 24 h, after checking fish mortality, water temperature, salinity, conductivity, pH and dissolved oxygen in the tanks, 80% of the test media was carefully changed, to reduce the build-up of metabolic residues. At the end of the exposure period blood was collected from the posterior cardinal. After blood sampling, fish were sacrificed by spinal section and the livers were excised, flash frozen in liquid nitrogen and stored at −80 °C until analysis. Plasma was

isolated by centrifugation (5 min at 13,000g) and stored at −80 °C until analysis.

2.3. Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from each liver (n = 7) using TRI Reagent (Sigma). All procedures were performed following the manufacturer's protocols. The concentration and purity of RNA were assessed with a NanoDropND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). An aliquot of RNA was run on a 1% agarose gel and post-stained with ethidium bromide to verify the RNA integrity. Reverse transcription (RT), to generate cDNA, was performed using 1 μg of total RNA, denatured 70 °C, 10 min, Oligo dT_{15} primer (Promega) and SuperScript™ III Reverse Transcriptase enzyme (Invitrogen), in presence of the recombinant ribonuclease inhibitor RNaseOUT™ (Invitrogen) in a final volume of 20 μL. The reaction was performed at 37 °C for 1 h, heat inactivated at 70 °C for 15 min.

2.4. Transcriptional analysis

The GenBank identification, primer sequences and efficiencies are shown in Table 1. The set of genes included nuclear receptors of fibrates (pparα, pparβ, pparγ), rxr, markers of lipid and lipoprotein metabolism (lpl, apoa1), antioxidant status, specifically glutathione peroxidase 1 and 4 (gpx1, gx4), superoxide dismutase [Zn] (sod2), glutathione reductase (gr), catalase (cat), glutathione S-transferase 3 (gst3) and peroxiredoxin 6 (prdx6); cell-tissue repair, including heat-shock protein 70 (hsp70) and glucose regulated protein-75 (grp75); innate immune function, such as interleukin 1β (il1β), tumour necrosis factor-α (tnfα) and transferrin (tf); and apoptosis, namely Bcl-2 associated X protein (bax) and caspase 3 (casp3). Efficiency of the amplification was determined for each primer pair using serial 5-fold dilutions of pooled cDNA. The efficiency was calculated as $E = 10^{(-1/s)}$ where s is the slope generated from the serial dilutions, when Log dilution is plotted against ΔCt (threshold cycle number) [24]. RT-qPCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). Reactions were assembled according to manufacturer's instructions with individual 20 μL reactions consisting of 10 μL SYBR® Green PCR master mix (2x), 200 nM primers and 2 μL of cDNA template. All reactions were run in the Bio-RadCFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, USA), following the protocol: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s, followed by melting curve to verify the amplification of a single product. All samples were run in triplicate. The expression data obtained from three independent technical replicates were used to calculate the Ct value. After checking for primers efficiency, RT-qPCR analysis of all the individual samples was determined following the same protocol described above.

2.5. Normalization strategy

The most commonly used normalization strategy involves standardization to a single constitutively expressed control gene. In recent years, it has become clear that the expression of the housekeeping genes can change, for example, between tissues and experimental conditions. Accordingly, the expression stability of the potential housekeeping gene should be evaluated in each experimental assay (Andersen et al., 2004). In the present study, we used the NormFinder application to evaluate the most appropriate housekeeping gene among four (β-actin, elongation factor-1α – ef1α; 18 s ribosomal RNA gene – 18s, and glyceraldehyde 3-phosphate dehydrogenase – gapdh). This application is based on an algorithm for identifying the most appropriate normalization gene among a set of

Table 1
Sequences and efficiencies of primers used for quantitative real-time PCR analysis in *Sparus aurata* liver.

Gene name	Acronym	GenBank accession No.	Forward	Reverse	Efficiency (%)
Elongation factor-1α	ef1α	AF184170	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	99.5
18 S ribosomal RNA gene	18s	AY993930	GCATTTATCAGACCCAAAACC	AGTTGATAGGGCAGACATTCG	96.7
β-Actin	β-actin	X89920	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATGCT	98.9
Glyceraldehyde 3-phosphate dehydrogenase	gapdh	DQ641630	TGCCCAGTACGTTGTTGAGTCCAC	CAGACCCTCAATGATGCCGAAGTT	98
Peroxisome proliferator-activated receptor alpha	pparα	AY590299	GCAGCCTGTGAGTCTTGTGAGTGA	CTCCATCAGGTCTCCACACAGC	99.7
Peroxisome proliferator-activated receptor beta	pparβ	AY590301	CGTGTTCGGGATTCGGGACT	CACCCTGTCGTGCTGCTCTGTA	99.0
Peroxisome proliferator-activated receptor gama	pparγ	AY590304	CGGAGAGAGAAGCAAGAACAAGAA	GAGGAGGAGGAGATGGAGGTGTA	96.5
Retinoid X receptor	rxr	HS092100	GGGCTTCTTCAAGAGGACAGT	TGCACCGCTTCTCTCTTCAT	102.1
Apolipoprotein AI	apoa1	AF013120	GAATACAAGGAGCAGATGAAGCAGATG	TGGTGACGGAGGCAGCGATG	100.0
Lipoprotein lipase	lpl	AY495672	CGTTGCCAAGTTTGTGACCTG	AGGGTGTTCTGGTTGTCTGC	94.5
Glutathione peroxidase 1	gpx1	DQ524992	GAAGGTGGATGTGAATGGAAAAGATG	CTGACGGGACTCCAAATGATGG	99.2
Glutathione peroxidase 4	gpx4	AM977818	TGCGTCTGATAGGGTCCACTGTC	GTCTGCCAGTCCTCTGTCGG	98.3
Glutathione reductase	gr	AJ937873	TGTTCAGCCACCCACCCATCGG	GCGTGATACATCGGAGTGAATGAAGTCTTG	91.5
Catalase	cat	JQ308823	TGGTCGAGAACTTGAAGGCTGTC	AGGACGCAGAAATGGCAGAGG	97.8
Superoxide dismutase [Mn]	sod2	JQ308833	CCTGACCTGACCTACGACTATGG	AGTGCCTCCTGATAT TTCTCCTCTG	99.0
Glutathione-S-transferase3	gst3	JQ308828	CCAGATGATCAGTACGTGAAGACCGTC	CTGCTGATGTGAGGAATGTACCGTAAC	100.3
Peroxiredoxin 6	prdx6	GQ252684	AGAGACAAGGACGGAATGC	TGTGGCGACCTTCTTCTG	99.2
Heat-shock protein 70	hsp70	EU805481	AATGTTCTGCGCATCATCAA	GCCTCCACCAAGATCAAAGA	100.1
Glucose-regulated protein, 75 kDa	grp75	DQ524993	TCCGGTGTGGATCTGACCAAAGAC	TGTTTAGGCCCAGAAGCATCCATG	100.2
Interleukin 1β	il1β	AJ277166	GGGCTGAACAACAGCACTCTC	TTAACACTCTCCACCCTCCA	102.4
Tumour necrosis factor-α	tnfα	AJ413189	CAGGCGTCGTTCAGAGTCTC	CTGTGGCTGAGAGGTGTGTG	99.3
Transferrin	tf	JF309047	CAGGACCAGCAGACCAAGTT	TGGTGGAGTCCTTGAAGAGG	100.2
Bcl-2 associated X protein	bax	AM963390	CAACAAGATGGCATCACACC	TGAACCCGCTCGTATATGAAA	100.3
[illegible]	casp3	EU722334	[illegible]	[illegible]	99.8

1. Introc

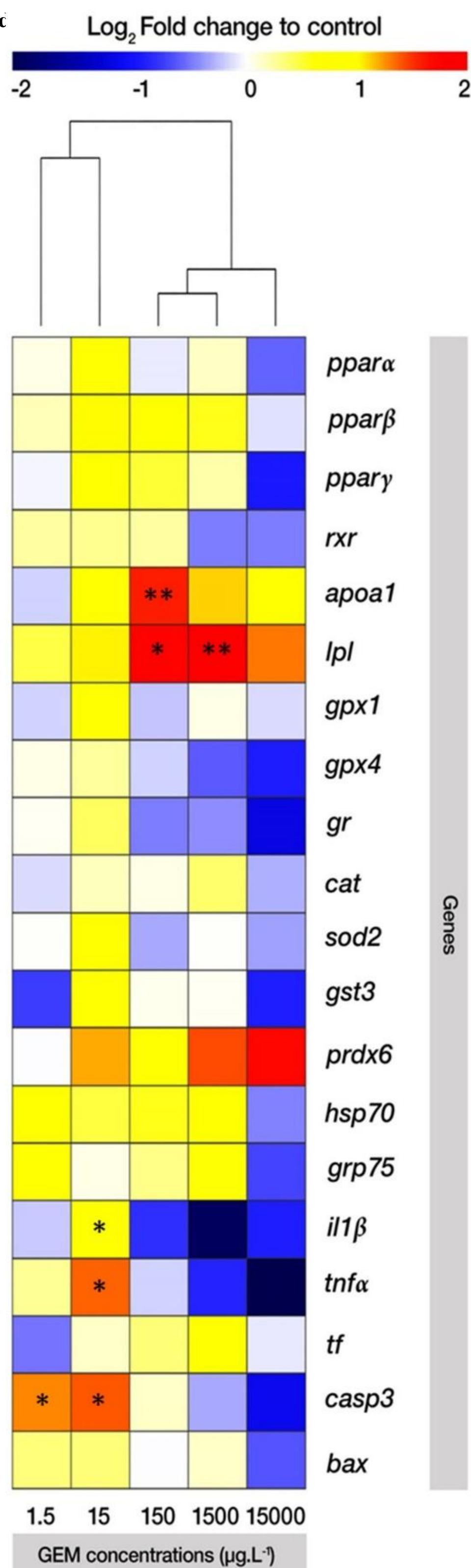


Fig. 1. Overall S. aurata mRNA response profile to gemfibrozil represented as a heatmap. Target genes were: ppar˛, ppar˘, ppary (peroxisome proliferator-activated receptors), apoa1 (apolipoprotein A-I), lpl (lipoprotein lipase), gpx1, gpx4 (glutathione peroxidase 1 and 4), gr (glutathione reductase), cat (catalase), sod2 (superoxide dismutase [Mn]), gst3 (glutathione-S-transferase 3), prdx6 (peroxiredoxin 6), hsp70 (heat-shock protein 70), grp75 (glucose-regulated protein, 75 kDa), il1˘ (interleukin 1i3), tnf˛ (tumor necrosis factor-a), tf (transferrin), bax (Bcl-2 associated X protein), casp3 (caspase 3). Differences were determined by one-way ANOVA followed by Tukey's test. An asterisk indicates statistically significant differences from the control group (*p < 0.05; **p < 0.01).

S. aurata (gilthead sea bream), in order to produce knowledge gene in a given sample set and experimental design is performed. Intra- and inter-group variation calculations are another feature of this program [25]. According to NormFinder results, the expression of the target genes was normalized using the best combination of two housekeeping genes. Relative gene expression was calculated with the ��Ct method including the PCR efficiencies of the target and housekeeping gene according to Pfaffl (2001) [24].

2.6. Biochemical analysis in plasma

Cholesterol, triglycerides, AST, ALT and ALP were determined in the plasma of seven fish per group using commercially available kits (Olympus Systems Reagents; Olympus life and Material Science Europe GmbH, Hamburg, Germany) following manufacturers indications. Intra- and inter-CV were below 10% in all cases. All parameters were performed with an automatic analyser (Olympus Diagnostica, GmbH, Freiburg, Germany). Cortisol was measured in the plasma by radioimmunoassay. The antibody for the assay was purchased from M.P. Biomedicals LLC (OH, USA) and used in a final dilution of 1:4500. Antibody cross-reactivity with cortisol is 100%. The radioactivity was quantified using a liquid scintillation counter. The lower detection limit of the cortisol assay is 0.16 ng mL^{-1}. Glucose was determined by enzymatic colorimetric analysis in ELISA plates using commercial kits (Biomérieux, France).

2.7. Integrated biomarker response (IBR)

The tested endpoints were combined into one general index termed IBR [26] and results presented divided by n, as suggested by Broeg and Lehtonen (2006) [27]. Results of data standardization procedure needed for IBR calculation were presented in experimental conditions star plot.

2.8. Statistical analysis

Results are expressed as mean ± SD (standard deviation). Statistical analysis was done using SPSS software 22 (SPSS Inc, IBM, IL, USA). The assumptions of normality and homogeneity of data were verified. One-way analysis of variance (one-way ANOVA) was performed in order to assess significant effects of the different concentrations of GEM. This analysis was followed by the post-hoc Tukey test to signal significant differences between groups [28]. Significance of results was ascertained at a = 0.05.

3. Results

In the present study, the variation of each housekeeping gene was determined in the liver of S. aurata, prior to the analysis of the target genes. According to NormFinder calculations the stability values of the candidate housekeeping genes were: 0.016 for ef1˛, 0.022 for i3-actin, 0.025 for gapdh and 0.035 for 18s. The most stable gene was ef1˛, and thus this gene was considered the best gene to be used in RT-qPCR data analysis. Considering that multiple housekeeping gene approaches are recommended as normalization strategy [29], the best combination of two-genes, ef1˛ and 18s (stability value 0.017), was used for the calculations in the current study.

The overall S. aurata mRNA response profile to GEM was represented as a heatmap (Fig. 1). The hepatic mRNA abundance of the nuclear receptors (ppar and rxr) was at control level in fish exposed to GEM, regardless of the tested concentration (Fig. 1 and Supplementary material). Transcripts encoding for proteins involved in lipid metabolism, such as lpl were upregulated after 96 h exposure to 150 and 1500 j.cg L^{-1} GEM. Similarly, apoa1 presented an increased mRNA abundance after 96 h exposure to 150 j.cg L^{-1} GEM. With respect to the genes belonging to the antioxidant defence

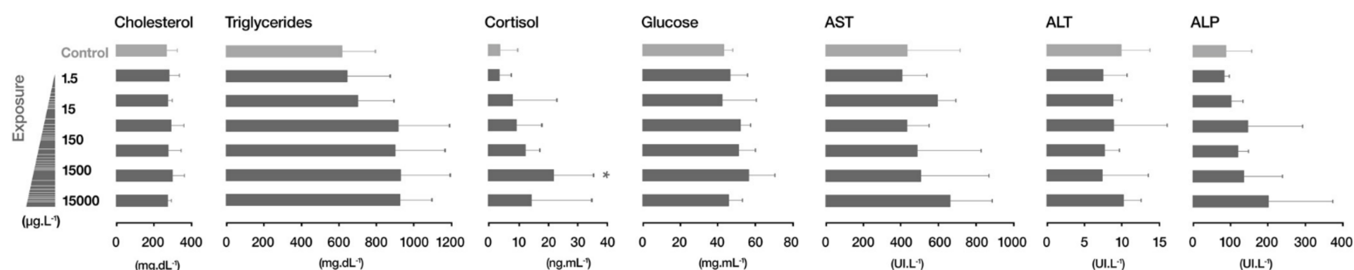


Fig. 2. Levels of cholesterol, triglycerides, cortisol, glucose, AST (aspartate aminotrasferase), ALT (alanine transaminase) and ALP (alkaline phosphatase) in the plasma of Sparus aurata after 96 h waterborne exposure to gemfibrozil. Values represent the means ± SD (n = 7 per group). Differences were determined by one-way ANOVA followed by Tukey's test. An asterisk indicates statistically significant differences from the control group (*p < 0.05; **p < 0.01).

(gpx1, gpx4, gr, cat, sod2, gst3, prdx6) and tissue-repair (hsp70, grp75) categories, mRNA levels were unchanged for all GEM tested concentrations. Immune-related genes, namely il1β and tnfα presented an increased mRNA abundance after 96 h exposure to 15 μg L^{-1}. On the other hand, tf mRNA transcriptional levels were unaltered in the liver of S. aurata exposed to GEM. The apoptosis-related gene casp3, also presented an increased mRNA abundance in the liver of S. aurata after 96 h exposure to the two lowest tested concentrations (1.5 and 15 μg L^{-1}). On the other hand, bax mRNA abundance was similar to that of the control group. In summary, from the six categories of genes evaluated, GEM waterborne exposure induced changes in mRNA transcripts mainly related with lipid metabolism, inflammation and apoptotic response.

After exposure to 1500 μg L^{-1} GEM, significantly higher plasma cortisol levels were observed, when compared to the control group. However, the levels of total cholesterol and triglycerides, as well as glucose levels in plasma showed no significant differences among study groups. Similarly, the hepatic health indicators, ALP, ALT and AST, were unaltered in plasma (Fig. 2).

The IBR index depicted 15 μg L^{-1} as the GEM concentration inducing more alterations in S. aurata followed by 150 and 1500, 15,000 and then 1.5. The overall integrated response pattern is displayed in Fig. 3.

4. Discussion

In mammals, GEM mechanism of action is mediated, in part, through activation of the pparα, which regulates the transcriptional levels of genes involved for example in lipid metabolism [30]. In the present study, mRNA abundance of ppar and rxr was unaltered in animals exposed for 96 h to GEM, which is in line with other studies with freshwater fish showing no hepatic activation of ppar pathways. This was observed in Oncorhynchus mykiss after 15 days injection with 100 mg kg^{-1} GEM [4]. Similarly, pparα and pparγ mRNA hepatic levels were unaffected in male C. auratus waterborne exposed to 1500 μg L^{-1} GEM for 14 and 28 days, whereas pparβ mRNA abundance was significantly reduced [19]. Nonetheless, increased whole-body pparα mRNA transcript levels were observed in female D. rerio exposed through diet during 30 d, although not in males, with decreased pparγ mRNA transcript abundance in both males and females [18]. Despite the unaffected levels of ppar mRNA abundance, the transcriptional levels of genes known to be regulated via pparα in mammals, such as apoa1 (that encodes for a protein involved in cholesterol homeostasis) and lpl (that encodes for lipoprotein lipase, which is involved in the hydrolysis of triglyceride molecules present in circulating lipoproteins) [16], were significantly increased in the present study. The obtained results also agree with previous findings showing unaltered ppar gene expression in O. mykiss after 15 days injection with GEM, despite the increased lpl mRNA levels [4]. Similarly, Cyprinus carpio exposed to the fibrate clorofibric acid displayed increased mRNA levels of both apoa1 and lpl in liver, without activation of PPARα gene expression [30]. Thus, if the lack of change in pparα mRNA abundance observed in the present study could suggest that GEM is not regulating pparα expression in S. aurata, the increased transcriptional levels of apoa1 and lpl may suggest a previous pparα transient activation, with a peak of expression occurring before the sampling moment. Results appear to reflect a time gap between receptor activation and the activation of downstream pathways. To clarify GEM effects in ppar pathways, further studies are needed at shorter, as well as longer exposure times.

The S. aurata 96 h exposure to GEM did not induce changes in the hepatic expression of antioxidant related-genes, unlike in other studies with freshwater fish [19] and rodents [31]. This result suggests that under the tested conditions, the pool of available basal non enzymatic and enzymatic defences were enough to cope with GEM potential pro-oxidant challenge. In other studies, induction of enzymatic activities of hepatic cat and gpx has been reported in male C. auratus after 14 and 28 days exposure to 1.5 or 1500 μg L^{-1} GEM, without oxidative damage [19]. Under the present experimental conditions GEM did not induce changes in hsp70 and grp75 transcriptional levels, indicating an absence of cellular stress strong enough to induce de novo synthesis of these cell chaperones. Previous studies showed increases in both hepatic transcripts and protein levels of hsp70 and grp75 in different fish species, including S. aurata, after exposure to pesticides or confinement stress [32,33].

The expression of genes related to the innate immune function was studied in order to clarify the potential immunotoxicity of GEM. Present results showed an upregulation of mRNA abundance of transcripts encoding proinflammatory mediators, such as il1β and tnfα, in the liver of S. aurata exposed to 15 μg L^{-1} GEM. This finding is particularly important since it happens when fish were exposed to low concentrations, but not at high concentrations. The effects on the transcriptional levels of il1β and tnfα suggest a link between GEM exposure and altered immune response, probably through ppar independent mechanisms. As suggested in this study for marine fish, in marine bivalves, the immune system can be a target for GEM action. GEM injection induced lysosomal destabilisation and lysosomal enzyme release, as well as an increased phagocytic activity in marine mussels [34]. The present result also shows that the immune response of fish to GEM is highly dependent on the concentration. The expression of apoptosis related genes, examined in the present study, revealed no alterations in expression of bax but increased casp3 mRNA levels in the groups exposed to 1.5 and 15 μg L^{-1} GEM, suggesting an activation of part of the cell-death cascade. Similar results were found in human erythrocytes, where GEM induced casp3 increase [35]. The activation of proinflammatory cytokines and apoptosis-related genes at GEM concentrations, in the same order of magnitude of levels previously reported in effluents of sewage treatment plants [36], suggest that this pharmaceutical may represent a risk to fish, rendering them more susceptible to other internal or external insults.

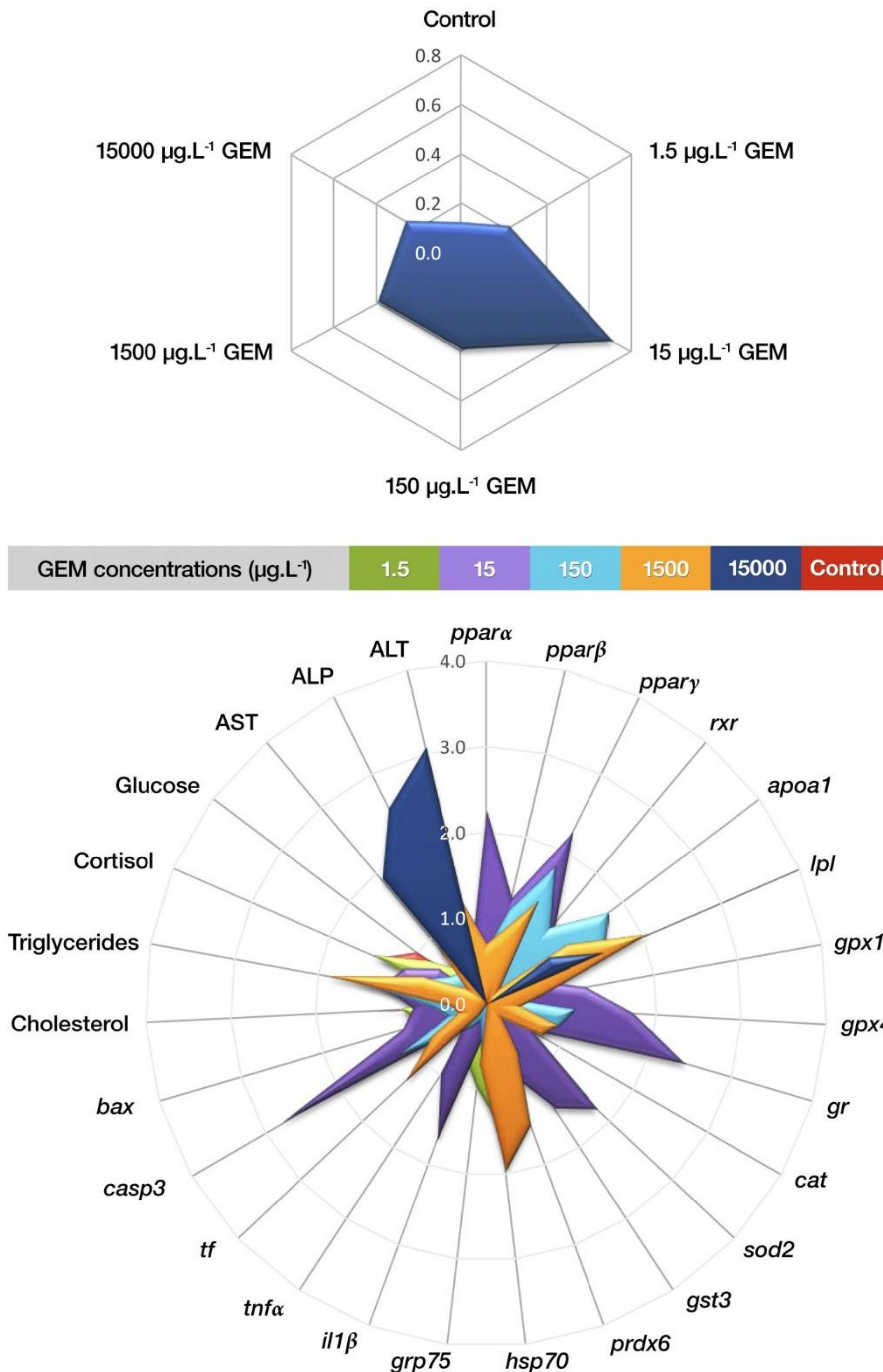


Fig. 3. Integrated biomarker response (IBR) and assessed endpoints star plots for each experimental condition. Cholesterol, triglycerides, cortisol, glucose, AST (aspartate aminotransferase), ALP (alkaline phosphatase, ALT (alanine transaminase), pparα, pparβ, pparγ (peroxisome proliferator-activated receptors), apoa1 (apolipoprotein A-I), lpl (lipoprotein lipase), gpx1, gpx4 (glutathione peroxidase 1 and 4), gr (glutathione reductase), cat (catalase), sod2 (superoxide dismutase [Mn]), gst3 (glutathione-S-transferase 3), prdx6 (peroxiredoxin 6), hsp70 (heat-shock protein 70), grp75 (glucose-regulated protein, 75 kDa), il1β (interleukin 1β), tnfα (tumor necrosis factor-a), tf (transferrin), bax (Bcl-2 associated X protein) and casp3 (caspase 3).

In humans, GEM chronic administration leads to decreased plasma triglycerides and cholesterol levels. Under the present conditions, GEM did not induce changes in plasma concentrations of triglycerides and cholesterol in S. aurata. Similarly, previous studies showed no changes (for 1.5 $\mu g\,L^{-1}$) or inconsistent results (for 600 $\mu g\,L^{-1}$) in these parameters in the plasma of P. promelas waterborne exposed to GEM for 2, 8 and 21 days [9]. However, O. mykiss sampled 15 days after GEM injection presented decreased triglycerides and cholesterol concentrations in plasma [4]. Al-Habsi et al. (2015) [18] reported decreased plasma levels of triglycerides and cholesterol in the whole-body of D. rerio exposed to GEM through diet during 30 d. These inconsistent results may be attributed to differences in experimental methodologies like exposure duration, administration method, species used, organ studied, among other factors. Furthermore, in the present study it is possible that the mRNA increase in lpl was not high enough to induce changes reflected at the systemic level.

Acute stressors, including handling, transport, confinement as well as xenobiotics, induce an elevation of plasma cortisol in most fish species [20,37,38]. The results of the present study support

this premise, since GEM (1500 $\mu g\,L^{-1}$) induced an increase in S. aurata plasma cortisol. To the author's knowledge, the only available study concerning GEM effects in fish cortisol response [18] reported unaltered cortisol in the whole-body of zebrafish fed with GEM. Studies in humans showed that GEM treatment caused an elevation in serum levels of cortisol in men with dyslipidemia [39]. The present results, demonstrated that S. aurata was able to mount a normal physiological response to the chemical stressor by increasing plasma cortisol levels. Nevertheless, the magnitude of the response may be considered moderate taking into account that acute handling stressors may induce 20 fold increases, and in the present study, although significantly, a limited 4–5 fold increase was observed after 1500 $\mu g\,L^{-1}$ GEM exposure. Generally, a fish stress response includes plasma cortisol and glucose increase. However, different patterns of response have been found [38], indicating that the relation between both parameters is not always straightforward. In the present study, the cortisol increase was not accompanied by glucose variation which seems to support the idea that cortisol action on fish carbohydrate metabolism may be influenced by a number of other factors, since glucose is not the only fuel for fish energy metabolism [40]. Alternatively, it may also be considered that the cortisol increase was not high enough to induce a detectable increase in plasma glucose levels. The present results do not point out to liver injury induced by GEM exposure, since AST, ALP and ALT activities were unaltered in the plasma.

The star plots diversity shapes obtained for each GEM concentration, reflected a dissimilar pattern of response, without a clear relation with concentration. Further studies should focus on other responses (e.g. linking gene expression with enzymatic activities). Overall, despite the clear differences between the cited studies and the present study (e.g. species, concentrations, exposure methodology), it appears that GEM may be recognized as a stressor to fish and able to induce molecular alterations. Nonetheless, the consequences of these alternations should be better explored by assessing effects at longer exposures. According to the present data, GEM appears to induce in S. aurata a non-monotonic response pattern, that may involve different molecular mechanisms (e.g., stress response, general toxicity, negative feed-back). It should be taken into consideration that S. aurata is an estuarine/marine fish, that will unlikely be exposed to the maximum reported levels in the environment (effluents of sewage treatment plants) tested in this study. Nonetheless, the maximum reported level of GEM in seawater was, according to Gaw et al. (2014) [11], 758 ng L^{-1}, approximately half of the lowest concentration tested in the present study. Thus, more studies should be performed on the effects of this pharmaceutical to fish, testing lower concentrations and also using endpoints with more obvious reflexes at the population level such as behaviour alterations (e.g. swimming performance).

5. Conclusions

Present study demonstrated that 96 h exposure to GEM, a human pharmaceutical, caused effects on a non-target species. It induced an increase in the transcriptional levels of key genes involved in lipid homeostasis, even without a concomitant activation of ppar pathways. However, GEM does not appear to induce an endocrine disruptive effect, since cortisol increased in plasma which suggests that GEM may be recognized as a stressing agent by fish although without affecting the intermediary metabolism. Altogether present results suggest that S. aurata is sensitive to GEM exposure thus producing a response that involves lipid homeostasis, endocrine response and immune activation. According to the obtained data, $il1\beta$, $tnf\alpha$ and casp3 genes and cortisol plasma levels appear as potential early warning indicators of GEM exposure, but further studies should be performed in order to clarify effects both at short- and long-term exposures.

Acknowledgements

This research was supported through the COMPETE – Operational Competitiveness Program and national funds through FCT – Foundation for Science and Technology, under the project "NANOAu – Effects of Gold Nanoparticles to Aquatic Organisms" (FCTPTDC/MAR-EST/3399/2012) (FCOMP-01-0124-FEDER-029435); CESAM: UID/AMB/50017/2013 and by the "Plan Nacional de Investigación", Government of Spain (AGL2013-48835-C2-2-R).; M. Teles and M. Oliveira have a post-doctoral fellowships from FCT (SFRH/BPD/85107/2012 and SFRH/BPD/109219/2015, respectively) supported by the European Social Fund and national funds from the "Ministério da Educação e Ciência (POPH – QREN – Tipologia 4.1)" of Portugal. J.C. Balasch and A. Barreto are thankfully acknowledged for the graphic design of the figures and technical support, respectively. A.T was granted by the Program "Juan de la Cierva" of Ministerio de Economia y Competitividad, Spain.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.07.044.

References

[1] L.H.M.L.M. Santos, A.N. Araújo, A. Fachini, A. Pena, C. Delerue-Matos, M.C.B.S.M. Montenegro, Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment, J. Hazard. Mater. 175 (2010) 45–95.

[2] R. van der Oost, J. Beyer, N.P.E. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ. Toxicol. Pharmacol. 13 (2003) 57–149.

[3] C. Mimeault, A.J. Woodhouse, X.S. Miao, C.D. Metcalfe, T.W. Moon, V.L. Trudeau, The human lipid regulator gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus, Aquat. Toxicol. 73 (2005) 44–54.

[4] J.S. Prindiville, J.A. Mennigen, J.M. Zamora, T.W. Moon, J.-M. Weber, The fibrate drug gemfibrozil disrupts lipoprotein metabolism in rainbow trout, Toxicol. Appl. Pharmacol. 251 (2011) 201–208.

[5] M.J. Chapman, Fibrates in 2003: therapeutic action in atherogenic dyslipidaemia and future perspectives, Atherosclerosis 171 (2003) 1–13.

[6] A.M.P.T. Pereira, L.J.G. Silva, L.M. Meisel, C.M. Lino, A. Pena, Environmental impact of pharmaceuticals from Portuguese wastewaters: geographical and seasonal occurrence, removal and risk assessment, Environ. Res. 136 (2015) 108–119.

[7] V. Osorio, A. Larrañaga, J. Aceña, S. Pérez, D. Barceló, Concentration and risk of pharmaceuticals in freshwater systems are related to the population density and the livestock units in Iberian Rivers, Sci. Total Environ. 540 (2016) 267–277.

[8] L. Rocco, G. Frenzilli, G. Zito, A. Archimandritis, C. Peluso, V. Stingo, Genotoxic effects in fish induced by pharmacological agents present in the sewage of some Italian water-treatment plants, Environ. Toxicol. 27 (2012) 18–25.

[9] S.Y. Skolness, E.J. Durhan, K.M. Jensen, M.D. Kahl, E.A. Makynen, D.L. Villeneuve, G.T. Ankley, Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (Pimephales promelas), Environ. Toxicol. Chem. 31 (2012) 2615–2624.

[10] J.F. Henriques, A.R. Almeida, T. Andrade, O. Koba, O. Golovko, A.M.V.M. Soares, M. Oliveira, I. Domingues, Effects of the lipid regulator drug gemfibrozil: a toxicological and behavioral perspective, Aquat. Toxicol. 170 (2016) 355–364.

[11] S. Gaw, K.V. Thomas, T.H. Hutchinson, Sources, impacts and trends of pharmaceuticals in the marine and coastal environment, Philos. Trans. R. S. London B: Biol. Sci. 369 (2014).

[12] A. Lyssimachou, R. Thibaut, E. Gisbert, C. Porte, Gemfibrozil modulates cytochrome P450 and peroxisome proliferation-inducible enzymes in the liver of the yellow European eel (Anguilla anguilla), Environ. Sci. Pollut. Res. Int. 21 (2014) 862–871.

[13] M. Solé, A. Fortuny, E. Mañanós, Effects of selected xenobiotics on hepatic and plasmatic biomarkers in juveniles of Solea senegalensis, Environ. Res. 135 (2014) 227–235.

[14] C. Blanquart, O. Barbier, J.C. Fruchart, B. Staels, C. Glineur, Peroxisome proliferator-activated receptor alpha (PPARalpha) turnover by the ubiquitin-proteasome system controls the ligand-induced expression level of its target genes, J. Biol. Chem. 277 (2002) 37254–37259.

[15] M.C. Sugden, E.A. Sugden, M.J. Holness, Peroxisome proliferator-activated receptors A2 – Lennarz, in: J. William, M.D. Lane (Eds.), Encyclopedia of Biological Chemistry, Elsevier, New York, 2004, pp. 239–245.
[16] M. Rakhshandehroo, B. Knoch, M. Müller, S. Kersten, Peroxisome proliferator-activated receptor alpha target genes, PPAR Res. (2010) 20.
[17] M.J. Leaver, E. Boukouvala, E. Antonopoulou, A. Diez, L. Favre-Krey, M.T. Ezaz, J.M. Bautista, D.R. Tocher, G. Krey, Three peroxisome proliferator-activated receptor isotypes from each of two species of marine fish, Endocrinology 146 (2005) 3150–3162.
[18] A.A. Al-Habsi, A. Massarsky, T.W. Moon, Exposure to gemfibrozil and atorvastatin affects cholesterol metabolism and steroid production in zebrafish (Danio rerio), Comp. Biochem. Physiol. (2015) (in press).
[19] C. Mimeault, V.L. Trudeau, T.W. Moon, Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARß) mRNA levels in male goldfish (Carassius auratus), Toxicology 228 (2006) 140–150.
[20] M. Teles, S. Boltaña, F. Reyes-Lopez, M.A. Santos, S. Mackenzie, L. Tort, Effects of chronic cortisol administration on global expression of GR and the liver transcriptome in Sparus aurata, Mar. Biotechnol. 15 (2013) 104–114.
[21] C. Fierro-Castro, M.C. Santa-Cruz, M. Hernandez-Sanchez, M. Teles, L. Tort, Analysis of steroidogenic pathway key transcripts in interrenal cells isolated by laser microdissection (LMD) in stressed rainbow trout, Comparative biochemistry and physiology. Part A, Mol. Integr. Physiol. 190 (2015) 39–46.
[22] H.M. Levesque, J. Dorval, A. Hontela, G.J. Van Der Kraak, P.G. Campbell, Hormonal, morphological, and physiological responses of yellow perch (Perca flavescens) to chronic environmental metal exposures, J. Toxicol. Environ. Health A 66 (2003) 657–676.
[23] M. Teles, M. Pacheco, M.A. Santos, Biotransformation, stress and genotoxic effects of 17 beta-estradiol in juvenile sea bass (Dicentrarchus labrax L.), Environ. Int. 32 (2006) 470–477.
[24] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.
[25] C.L. Andersen, J.L. Jensen, T.F. Orntoft, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Res. 64 (2004) 5245–5250.
[26] B. Beliaeff, T. Burgeot, Integrated biomarker response: a useful tool for ecological risk assessment, Environ. Toxicol. Chem./SETAC 21 (2002) 1316–1322.
[27] K. Broeg, K.K. Lehtonen, Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach, Mar. Pollut. Bull. 53 (2006) 508–522.
[28] J.H. Zar, Biostatistical Analysis, Prentice Hall, 1999.
[29] R. Urbatzka, S. Galante-Oliveira, E. Rocha, L.F. Castro, I. Cunha, Normalization strategies for gene expression studies by real-time PCR in a marine fish species, Scophthalmus maximus, Mar. Geonomics 10 (2013) 17–25.
[30] J. Corcoran, M.J. Winter, A. Lange, R. Cumming, S.F. Owen, C.R. Tyler, Effects of the lipid regulating drug clofibric acid on PPARa-regulated gene transcript levels in common carp (Cyprinus carpio) at pharmacological and environmental exposure levels, Aquat. Toxicol. 161 (2015) 127–137.
[31] M.L. O'Brien, T.P. Twaroski, M.L. Cunningham, H.P. Glauert, B.T. Spear, Effects of peroxisome proliferators on antioxidant enzymes and antioxidant vitamins in rats and hamsters, Toxicol. Sci. 60 (2001) 271–278.
[32] A. Bermejo-Nogales, L. Benedito-Palos, A. Saera-Vila, J.A. Calduch-Giner, A. Sitja-Bobadilla, J. Perez-Sanchez, Confinement exposure induces glucose regulated protein 75 (GRP75/mortalin/mtHsp70/PBP74/HSPA9B) in the hepatic tissue of gilthead sea bream (Sparus aurata L.), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 149 (2008) 428–438.
[33] I. Varó, J.C. Navarro, B. Nunes, L. Guilhermino, Effects of dichlorvos aquaculture treatments on selected biomarkers of gilthead sea bream (Sparus aurata L.) fingerlings, Aquaculture 266 (2007) 87–96.
[34] L. Canesi, L.C. Lorusso, C. Ciacci, M. Betti, F. Regoli, G. Poiana, G. Gallo, A. Marcomini, Effects of blood lipid lowering pharmaceuticals (bezafibrate and gemfibrozil) on immune and digestive gland functions of the bivalve mollusc, Mytilus galloprovincialis, Chemosphere 69 (2007) 994–1002.
[35] E. Tellone, S. Ficarra, B. Giardina, R. Scatena, A. Russo, M.E. Clementi, F. Misiti, E. Bellocco, A. Galtieri, Oxidative effects of gemfibrozil on anion influx and metabolism in normal and beta-thalassemic erythrocytes: physiological implications, J. Membr. Biol. 224 (2008) 1–8.
[36] R. Andreozzi, M. Raffaele, P. Nicklas, Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment, Chemosphere 50 (2003) 1319–1330.
[37] A. Hontela, C. Daniel, A.C. Ricard, Effects of acute and subacute exposures to cadmium on the interrenal and thyroid function in rainbow trout, Oncorhynchus mykiss, Aquat. Toxicol. 35 (1996) 171–182.
[38] M. Teles, V.L. Maria, M. Pacheco, M.A. Santos, Anguilla anguilla L. plasma cortisol lactate and glucose responses to abietic acid, dehydroabietic acid and retene, Environ. Int. 29 (2004) 995–1000.
[39] A. Hautanen, M. Manttari, V. Manninen, H. Adlercreutz, Gemfibrozil treatment is associated with elevated adrenal androgen, androstanediol glucuronide and cortisol levels in dyslipidemic men, J. Steroid Biochem. Mol. Biol. 51 (1994) 307–313.
[40] T.P. Mommsen, M.M. Vijayan, T.W. Moon, Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation, Rev. Fish Biol. Fish. 9 (1999) 211–268.