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Immunotoxicological effects of perfluorooctanesulfonic acid on European seabass are reduced by polyethylene microplastics

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ABSTRACT

Marine environments receive plastic waste, where it suffers a transformation process into smaller particles. Among them, microplastics (MPs; <5 mm) are ingested by aquatic organisms leading to negative effects on animal welfare. The interactions between MPs, contaminants and organisms are poorly understood. To clarify this issue, European seabass (*Dicentrarchus labrax* L.) were fed with diets supplemented with 0 (control), polyethylene (PE) MPs (100 mg/kg diet), perfluorooctanesulfonic acid (PFOS, 4.83 μg/kg diet) or PFOS adsorbed to MPs (MPs-PFOS; final concentrations of 4.83 μg and 100 mg of PFOS and MP per kg of feed, respectively). Samples of skin mucus, serum, head-kidney (HK), liver, muscle, brain and intestine were obtained. PFOS levels were high in the liver of fish fed with the PFOS-diet, and markedly reduced when adsorbed to MPs. Compared to the control groups, liver EROD activity did not show any significant changes, whereas brain and muscle cholinesterase activities were decreased in all the groups. The histological and morphometrical study on liver and intestine showed significant alterations in fish fed with the experimental diets. At functional level, all the experimental diets affected the humoral (peroxidase, IgM, protease and bactericidal activities) as well as cellular (phagocytosis, respiratory burst and peroxidase) activities of HK leukocytes, being more marked those effects caused by the PFOS diet. Besides, treatments produced inflammation and oxidative stress as evidenced at gene level. Principal component analysis demonstrated that seabass fed with MPs-PFOS showed more similar effects to MPs alone than to PFOS. Overall, seabass fed with MPs-PFOS diet showed similar or lower toxicological alterations than those fed with MPs or PFOS alone demonstrating the lack of additive effects or even protection against PFOS toxicity.

1. Introduction

Great amounts of plastic debris end in the marine environment, where they undergo a process of weathering and fragmentation into smaller fragments, called microplastics (MPs) and nanoplastics (NPs). MPs (5 mm–100 nm) comprise a very heterogeneous assemblage of particles in composition, size, and shape. Among all polymers, polyethylene (PE) represents the polymer with the greatest global production for plastic manufacturing [1] being frequently found in the open sea [2]. The worldwide presence and persistence of MPs in aquatic environments is of particular concern since these pollutants represent an increasing threat to marine organisms and ecosystems. Due to their size, MPs can be ingested by marine organisms. The MPs absorption pathway on fish can occur through oral, gill and skin interaction, being rapidly

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accumulated in the gills and intestines [3]. Several reports on field and experimental studies have described the disrupting effects that ingested MPs have on development, growth, reproduction, immunity and behaviour of different marine species by impacting energy distribution, producing oxidative stress, or neurotoxicity [4–6]. In addition to the potential negative effects of the MPs *per se*, plastic particles can act as vectors for other pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, pharmaceuticals and heavy metals [7–9], which may influence their toxicity and bioaccumulation [10,11].

Recently, perfluoroalkyl compounds (PFAS) have called the attention due to their global occurrence in the environment and their toxicity to aquatic organisms [12]. PFAS have been widely used in many industrial activities as surfactants, surface protectors, and performance chemicals in firefighting foams, leather, or food packing among other uses. They are highly resistant to biological degradation, persistent, and bioaccumulate in the environment [13]. Among PFAS, perfluorooctanesulfonic (PFOS) and perfluorooctanoic acid (PFOA) are the most representative compounds [13]. As consequence, these compounds are gaining increased regulatory and public attention and have been listed as persistent organic pollutants (POPs) in Annex B of the Stockholm Convention since 2009. For PFOS bioaccumulation, it has been registered concentrations ranging from 0.16 to 9.2 μg/kg in marine fish and concentrations up to 14.1 μg/kg in freshwater fish's species [14]. The direct toxicity of PFOS and PFOA has been relatively well studied in laboratory toxicity experiments with freshwater aquatic animals [reviewed by Ref. [15]. However, the effect of these compounds in marine organisms is less studied. Some studies in fish have evaluated the exposure to PFOS in fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes)*, sheepshead minnow (*Cyprinodon variegatus*), bluegill sunfish (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*) or zebrafish (*Danio rerio*) evidencing an impact on fish immunity, fertility and normal liver and gonadal functions [16–19]. Since both MPs and PFASs migrate from different environmental compartments (e.g., soil) to aquatic environments [20], their interaction deserves systematic investigation. In addition, the adsorption of PFASs to MPs could change the transport of PFASs in aquatic environments due to the ingestion of MPs by marine organisms. A recent study reported that PFOS adsorbed to PE MPs enhanced the bioaccumulation of this pollutant in clams [10]. It is necessary to understand the behave of multiple pollutants in natural environments to assess its real risks to marine organisms.

Polyethylene was chosen since it has been commonly considered as one of the reference polymers to investigate chemical sorption to MPs [21]. Our collaborators recently investigated the potential sorption of PFOS onto MPs and its potential desorption process using an artificial gut fluid (AGF) [22]. Ingestion of plastics has been identified as one of the main source of MPs entry in fish [23]. In order to identify these processes in the ocean, this experiment was replicated in the laboratory simulating natural conditions. The effects caused by the dietary administration of PE-MPs, PFOS alone and PFOS associated to PE-MPs was investigated on European seabass (*Dicentrarchus labrax*), selected as a model of marine fish, and the bioaccumulation, biomarkers, immunity and histopathology were studied.

2. Material and methods

2.1. Animals

Forty-eight (10 ± 4 g) juveniles of European seabass, obtained from Culmarex (Murcia, Spain), were randomly distributed in eight running seawater aquaria (250 L, flow rate 900 L/h) in the Marine Fish Facilities at the University of Murcia at 28‰ salinity, 20 ± 2 °C and photoperiod (12:12 L/D). Fish were fed at a rate of 1.5% body weight per day with a commercial pellet diet (Skretting). Animal housing, management and procedures were approved by the Ethical Committee on Animal Experimentation of the University of Murcia.

2.2. Experimental design and sampling

Particle size of PE MPs was from 150 to 500 μm (mean 267.4 ± 89.6 μm) and provided by the Man-Technology-Environment Research Centre of Örebro University (Örebro, Sweden). Perfluorooctane sulfonate (PFOS, solid powder; IUPAC: 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluorooctane-1-sulfonic acid, CAS 2785-37-3; purity ≥98%, dissolved in water) was purchased from Sigma-Aldrich. PFOS was adsorbed to PE MPs (MPs-PFOS) at the Örebro University by using 50 g/L of plastic that was weighed into 1 L polypropylene bottle filled with 500 mL of double-deionized water and gently shacked on a rotary shaker for 7 days. The samples were filtrated using a funnel and glass microfiber filter (1.0 μm, Whatman® glass microfiber filters, GE Healthcare Life Sciences). Subsequently, the samples were rinsed with double-deionized water and dried by vacuum evaporation. MPs were extracted in methanol (>99.9% purity, Fisher Scientific) by ultra-sonication followed by centrifugation (7000 g). Detailed description of the PFOS quantification sorbed on the plastic can be found in previous publications [22,24].

In each aquarium, fish received one of the following experimental diets as elsewhere [25] using two replicates per treatment: I. Control (C): commercial diet alone II. MPs: diet containing, per kg of feed, 100 mg of virgin PE MPs, III. PFOS: diet containing 4.83 μg of PFOS/kg feed and IV. MPs-PFOS: diet with PFOS adsorbed to PE MPs (final concentrations of 4.83 μg and 100 mg of PFOS and MPs, respectively). Feeds were daily prepared. For this, the appropriate quantity of MPs, PFOS or MPs-PFOS were added to cod oil, vigorously shaken, and then sprayed onto the commercial pellets, which were shaken again to allow uniform distribution in the final diet. The control diet was sprayed with the same quantity of cod oil alone. Cod oil never exceeded 1% of the diet. Fish were closely observed during feeding to ensure they adequately ate all the diet. Fish were fed the diets for twenty-one days and then sampled after sacrifice by an overdose of MS222 (100 mg/L; Sandoz).

Blood samples and mucus were collected following established protocols [26]. Serum and mucus were stored at −20 °C until use. Head-kidney (HK) leucocytes were isolated, suspended in sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride, 2% foetal calf serum (FCS, Gibco), 100 i.u/mL penicillin (Flow) and 100 mg/mL streptomycin (Flow)] and adjusted to 10^7 cells/mL [27]. Fragments of gut, HK and liver were stored in TRIzol Reagent (Invitrogen) at −80 °C for gene expression analysis. Liver samples were processed for ethoxyresorufin-O-deethylase (EROD) activity and PFOS levels, while brain and muscle samples were used for acetylcholinesterase (AChE) and cholinesterase (ChE) determinations, respectively. Gut and liver samples were processed for histopathological analysis.

2.3. Analysis of PFOS levels

Levels of PFOS in the liver were determined by the ion-pair extraction technique. Pools of 2–3 livers per condition were homogenized by crushing samples in methanol (MeOH, LC/MS/MS grade, Sigma-Aldrich) with the addition of tetrabutylammonium hydrogen sulphate (TBAHS, 0.5 M, pH = 10, Sigma-Aldrich), used as ion pairing agent, and vortexed. Then, crushed samples were transferred into 15 mL polypropylene tubes with glass pipette and extracted following the next steps. To extract PFOS, 1 mL of TBAHS was added followed by 3 mL of methyl *tert*-butyl ether (Sigma-Aldrich), then extracts were vortexed, let on the orbital shaker for 10 min and centrifugated (6 min at 4000 g). Supernatants were transferred (approx. 3 mL) to a new polypropylene tube and the extraction was repeated twice. Collected supernatants were evaporated to dryness and reconcentrated in 50 μL of MeOH. Finally, extracts were vortexed and centrifuged (2 min at 60 g) to avoid liver particles and 35 μL of extracts were transferred to LC vial with the addition of 1 ng of mass-labelled $^{13}C_8$ PFOS (Wellington Laboratories Inc.) and 60 μL of mobile phase (2 mM ammonium acetate in methanol).

The LC vial was vortexed for 30 s and extracts analysed using LC method as previously described [24].

2.4. Determination of biomarkers

EROD activity was measured in S9 fraction of fish liver. Liver samples were individually homogenized in 100 mM phosphate buffer and centrifuged (4 °C, 10,000 g, 20 min). Total protein content of this S9 fraction obtained was measured using the Lowry protein assay. Each well of a 96-well microplate received 10 μL of S9 fraction diluted to about 5 mg/mL of protein, 7- Ethoxyresorufin (final well concentration of 0.2 μg/mL) and NADPH (final well concentration of 0.17 mg/mL). Fluorescence was measured during 10 min at 38 s intervals ($\lambda = 530$ nm for excitation, 590 nm for detection) using a Synergy HT microplate reader (BIOTEK). Readings were compared to a resorufin standard curve including blanks. EROD activity was expressed as pmol resorufin per minute per mg protein (pmol/min/mg protein).

In the experimental conditions used, the main cholinesterase activity in fish brain samples is from the AChE enzymes, whereas in the muscle both AChE and pseudocholinesterase contribute considerably. Therefore, AChE will be used to indicate the activity in the brain and ChE activities in the muscle, as in other studies with the same species [28]. To do this, brain or muscle samples from each individual fish were homogenized in cold phosphate-buffer (0.1 M, pH = 7.2) in a ratio 1 g wt tissue/10 mL buffer and centrifuged at 4 °C (3300 g for 3 min). The supernatant was collected and the protein concentration determined and standardized [28]. AChE and ChE activities were determined by the Elman's technique adapted to microplates [29], using acetylcholine as substrate and readings at 412 nm. The enzymatic activity was expressed in nanomoles of substrate hydrolysed per minute per mg of protein (nmol/min/mg protein).

2.5. Immunological analysis

The protein concentration in skin mucus and serum samples was determined by Bradford's dye binding method. Protease activity in the skin mucus was determined using 2% azocasein (Sigma-Aldrich) as substrate and data presented as % of activity compared to trypsin (5 mg/mL; Sigma-Aldrich). Peroxidase activity in the skin mucus was determined using 20 mM 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich) and 5 mM H_2O_2 as substrates, where a change of 1 OD at 450 nm is related to one unit of peroxidase activity [26]. Total immunoglobulin M (IgM) levels were determined in the skin mucus by an enzyme-linked immunosorbent assay (ELISA) using commercial anti-IgM antibodies (Aquatic Diagnostics) and expressed as optical density at 450 nm [30]. Bactericidal activity against the pathogenic *Vibrio anguillarum* was analysed in skin mucus and serum and bacterial viability determined by the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 1 mg/mL; Sigma-Aldrich) test [26]. Bactericidal activity was expressed as percentage of non-viable bacteria. All the assays were performed in triplicates and with respective blanks without sample.

Regarding the cellular innate immune parameters of European seabass HK leucocytes, phagocytosis, respiratory burst, and peroxidase activities were determined as detailed elsewhere [31,32]. Phagocytosis against *Saccharomyces cerevisiae* yeast cells was evaluated by flow cytometry whereby the phagocytic ability is represented by the percentage of HKLs with ingested yeast cells and the phagocytic capacity by the relative amount of ingested yeast cells per leucocyte. Respiratory burst was determined by a chemiluminescent method upon stimulation with phorbol myristate acetate (PMA). Peroxidase activity in HKLs was determined after cell lysis as for the skin mucus. All analyses were made in triplicate.

2.6. Gene expression study

Total RNA was extracted from the HK, liver, and gut using TRIzol Reagent (Invitrogen). The RNA was then quantified, and the purity was assessed by spectrophotometry using a nanodrop before treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesised from 1 μg of total RNA using the SuperScript IV reverse transcriptase (Invitrogen) with an oligo-dT18 primer. The expression of eight selected immune- and stress-relevant genes [interleukin 1b (*il1b*), interleukin 6 (*il6*), interleukin 10 (*il10*), transforming growth factor beta (*tgfb*), heat shock protein 70 (*hsp70*), nuclear factor (erythroid-derived 2)-like 2 (*nrf2*), superoxide dismutase (*sod*), and catalase (*cat*)] was analysed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μL of SYBR Green supermix, 5 μL of primers [0.4 μM each] and 5 μL of cDNA template) were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C and, finally, 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. The primers used are shown in Supplementary Table 1. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method [33], using *D. labrax* ribosomal *18s* (*rps18*) and elongation factor 1 alpha (*ef1a*) as the endogenous references.

2.7. Histopathological and morphometric study of gut and liver

Gut and liver samples were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 h. The samples were then dehydrated using increasing concentrations of ethanol and embedded in paraplast (Thermo Scientific). Sections were cut at 3 μm, dewaxed, rehydrated, and stained with haematoxylin-eosin (HE). Slides were studied under a light microscope (Leica 6000B). Images were obtained with a Leica DFC280 digital camera and Leica Application Suite v.2.5.0 R1 acquisition software, which were then used for the morphometric analysis by ImageJ 1.46r software (National Institutes of Health, USA). In the gut, the loss of epithelial integrity, the presence of cell debris in the lumen, villus height, the presence of goblet cells, mucus secretion, intraepithelial and lamina propia leucocytes, micro-villi disorganization/disruption and oedema were monitored. Measurements included villus height (μm, measured from the tip to the base of villus), intestinal diameter (μm) and number of goblet cells per area of epithelium layer. The villus height was corrected to intestinal diameter to consider the variations that may occur because of increased intestinal diameter. In the case of liver, the presence of vacuoles and congestion were monitored. Results from 5 independent fish specimens were obtained and analysed (three slides from each specimen and six images from each slide).

2.8. Statistical analyses

The normality of the variables was evaluated by the Shapiro–Wilk test and homogeneity of the variance by the Levene test (Supplementary Table 2). Statistical differences among the groups were assessed by the Kruskal-Wallis nonparametric analysis or by the one-way ANOVA, depending on the normality of the variables. The ANOVA was followed by the Tukey or Games-Howell test, depending on the homogeneity of the variables. Principal component analysis (PCA) was applied to test the relations among the evaluated parameters. The significance level was 95% in all cases ($p < 0.05$). Statistical analysis and graphs were carried out using either SPSS or GraphPad software.

3. Results

3.1. MPs reduce the PFOS levels in the liver

The levels of PFOS were determined in the liver of European seabass

fed MPs and/or PFOS. Although detected, liver levels of PFOS were low and roughly similar in control fish and in those receiving virgin MPs for 21 days (Fig. 1). As expected, the level of PFOS in those fish fed for 21 days with a diet containing PFOS was significantly much higher than in control fish (Fig. 1). Interestingly, fish fed the same amount of PFOS adsorbed to PE MPs resulted in low PFOS levels, and similar to those found in fish fed MPs alone, indicating no accumulation.

3.2. Both MPs and PFOS show similar biomarker alterations

EROD activity, measured in the S9 fraction from seabass liver, was increased by all the tested diets though it did not reach significance when compared to fish fed the control diet (Fig. 2A). By sharp contrast, AChE and ChE activities were decreased by MPs and/or PFOS in the brain and the muscle (Fig. 2B). Brain AChE activity was significantly reduced by all the treatments showing a reduction of 33%, 20.6% and 21.1% compared to controls in treatments with MPs, PFOS and MPs-PFOS, respectively. Muscle ChE activity was only significantly reduced by the MPs (25.44 ± 0.98 nmol/min/mg protein) or PFOS alone (27.51 ± 1.43 nmol/min/mg protein) respect to controls (34.95 ± 1.51 nmol/min/mg protein; Fig. 2B).

3.3. Immunotoxicological effects of PFOS are not impaired by MPs

Regarding the humoral immune parameters, seabass skin mucus protease activity (Fig. 3A) and IgM levels (Fig. 3B) were not altered by MPs and/or PFOS. However, peroxidase activity analysed in mucus (Fig. 3C) was significantly increased in fish fed the PFOS diet, doubling the value obtained in control fish (64.29 ± 8.32 Units/mg protein). By contrast, skin mucus bactericidal activity was significantly decreased in a 51% in fish fed the diet containing MPs compared to controls (Fig. 3D) but increased in a 93% in the serum of fish fed MPs with adsorbed PFOS (Fig. 3D).

Regarding the leucocyte immune parameters, phagocytic ability was significantly increased in seabass fed PFOS and MPs-PFOS compared to control fish (Fig. 3E) with increases of 41% and 26%, respectively, though the phagocytic capacity did in fish fed MPs or PFOS alone with increases of 12% and 16%, respectively (Fig. 3F). Leucocyte respiratory burst was significantly increased in seabass fed PFOS alone compared to all the treatments (Fig. 3G) though the leucocyte' peroxidase activity was significantly increased in all the treated seabass specimens when compared to the controls (Fig. 3H).

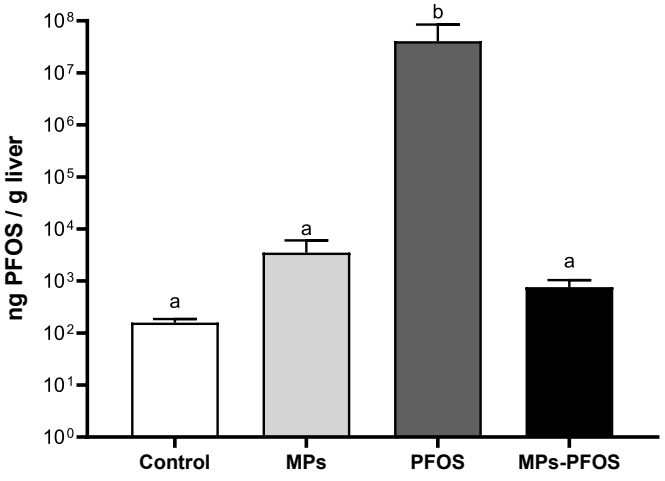

Fig. 1. Perfluorooctane sulfonate (PFOS) levels determined in the liver of European seabass fed with commercial diet alone (control) or containing, per kg of fed, 100 mg of virgin polyethylene MPs (MPs), 4.83 μg of PFOS or PFOS adsorbed to MPs (MPs-PFOS; final concentrations of 4.83 μg and 100 mg of PFOS and MPs, respectively) for 21 days. Data represent the mean ± SEM (n = 5). Letters indicate statistical differences between treatments ($p < 0.05$).

3.4. MPs reduce the transcriptional alterations provoked by PFOS

The expression of some immune and stress-related genes studied in the HK, liver and gut of seabass specimens was affected by the dietary intake of MPs, PFOS and MPs-PFOS (Fig. 4). MPs alone significantly up-regulated the transcription of *il6*, *il10* and *hsp70* in the gut, and of *nrf2* in the HK, whilst down-regulated that of *il1b* in the HK and of *cat* in the liver when compared to the control group. PFOS diet significantly up-regulated the transcription of *il6* in all the tissues, *il1b* in the gut and *nrf2* in the HK when compared to the controls. In addition, PFOS provoked a down-regulation of *sod* and *cat* in the liver and of *il1b* and *hsp70* in the HK. Interestingly, European seabass specimens fed the MPs-PFOS diet showed lower regulation in the transcription of tested genes than the pollutants alone (Fig. 4).

3.5. Histological study

Potential histopathological alterations produced in the gut and liver from European seabass caused by the dietary exposure to MPs, PFOS or MP-PFOS were studied by light microscopy and morphometrical analysis. In a general view of the gut, all the treatments provoked a more irregular and disorganized intestine wall (Fig. 5B and C) compared to controls. In fish fed the MP diet, gut images revealed the presence of areas with a high vacuolization of enterocytes, which were more detectable in the apical parts of the villus (Fig. 5F). Concomitantly, MPs provoked a significant reduction in the number of goblet cells and villus height whilst the villus thickness and intestinal diameter: villus height ratio were increased when compared to controls (Table 1). Surprisingly, PFOS-fed gut showed similar morphological status of control fish, although mucosa displayed incipient signs of infiltration (Fig. 5C,G) and much increased density of goblet cells (Table 1). Fish fed the MPs-PFOS diet showed areas with high levels of enterocyte vacuolization (Fig. 5D, H). In fact, gut morphometric analyses denoted a significant increase in the number of goblet cells, villus thickness and intestinal diameter: villus height ratio when compared to the control group (Table 1) while the villus height was reduced.

Regarding the histopathological analysis in the liver, the general view identifies an increased vacuolation (Fig. 5J-L). The main alterations observed were loss of the parenchyma organization, increased hepatocyte vacuolation, displacement of the nuclei of the hepatocytes to the cell periphery, and congestion of blood sinusoids by either MPs, PFOS and MPs-PFOS diets (Fig. 5N-P), which was confirmed by the morphometric analysis (Table 1). No significant differences between MPs and/or PFOS were observed.

3.6. PCA demonstrates that MPs-PFOS show similar effects to MPs alone

The principal component analysis with all the parameters evaluated identified two components explaining 80.3% of the variance (Fig. 6). Very interestingly, when data were clustered by the treatment group seabass fed with MPs-PFOS clustered closer to those fed with MPs alone than to those exposed to PFOS alone, reinforcing that MPs decrease the impact of PFOS.

4. Discussion

In our previous works, we described slight effects of dietary MPs in the immune system of gilthead seabream (*Sparus aurata*) [31], and European seabass [25], suggesting the oxidative stress as the main mechanism involved in the MPs toxicity, as has been supported by other authors [34]. At the same time, although several toxicological studies have been published regarding the toxicity of PFOS [10,19], the impact of this compound in the immune system of fish has been scarcely reported. It has been reported that PFAS exposure produced

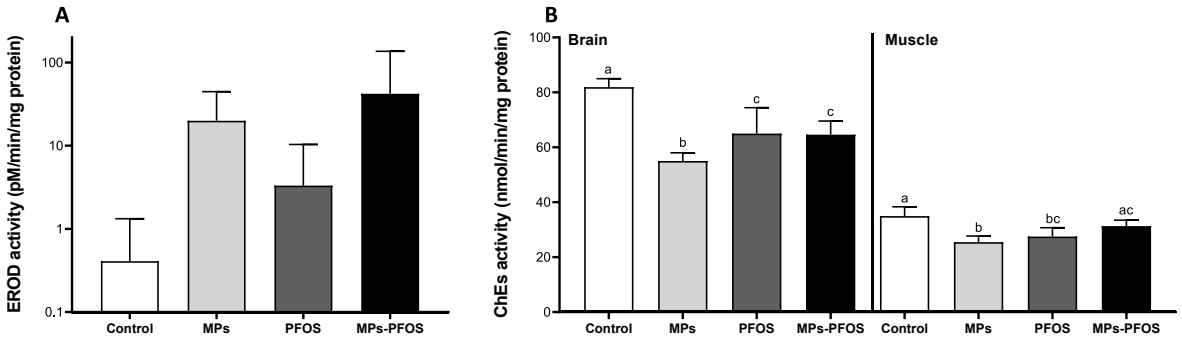

Fig. 2. Liver EROD activity (A) and brain and muscle ChE activities (B) from European seabass fed with commercial diet alone (control) or containing, per kg of fed, 100 mg of virgin polyethylene MPs (MPs), 4.83 μg of PFOS or PFOS adsorbed to MPs (MPs-PFOS; final concentrations of 4.83 μg and 100 mg of PFOS and MPs, respectively) for 21 days. Data represent the mean ± SEM (n = 5). Letters indicate statistical differences between treatments ($p < 0.05$).

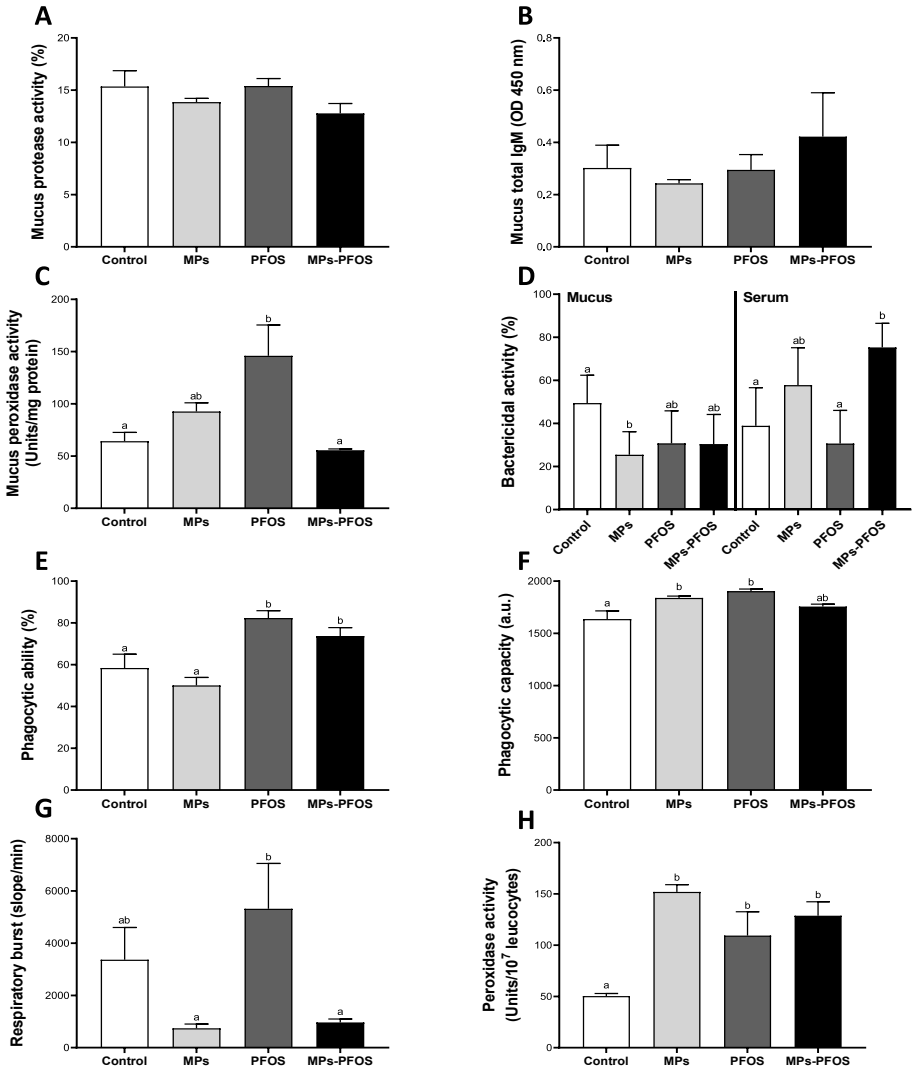

Fig. 3. Immunological parameters of European seabass fed with commercial diet alone (control) or containing, per kg of fed, 100 mg of virgin polyethylene MPs (MPs), 4.83 μg of PFOS or PFOS adsorbed to MPs (MPs-PFOS; final concentrations of 4.83 μg and 100 mg of PFOS and MPs, respectively) for 21 days. **A**. Skin mucus protease activity. **B**. Skin mucus immunoglobulin M (IgM) levels. **C**. Skin mucus peroxidase activity. **D**. Bactericidal activity against *Vibrio anguillarum* in skin mucus and serum. **E**. Phagocytic ability of head-kidney leucocytes (HKLs). **F**. Phagocytic capacity of HKLs. **G**. Respiratory burst activity of HKLs. **H**. Peroxidase activity of HKLs. Data represent the mean ± SEM (n = 5). Letters indicate statistical differences between treatments ($p < 0.05$).

immunotoxicity on green mussels (*Perna viridis*) [35], bottlenose dolphins (*Tursiops truncatus*) [36], and immunosuppression in marine medaka (*Oryzias melastigma*) larvae [37]. To the best of our knowledge, this is the first work that reported the effects of PFOS on European seabass and, in addition, their combined effects with the MPs. The results of the present study clearly showed that dietary MPs and PFOS had a significant influence on the immune parameters and biomarker levels in European seabass. Contrary to expected results, the supply of MP-PFOS did not increase the toxic effects of this compound, evidencing the lack of additive effects or even protection against PFOS toxicity of some biological responses addressed by different biomarkers as was illustrated by the PCA analysis.

4.1. Bioaccumulation and histopathology

Previous studies reported that MPs spiked with PFOS induced higher

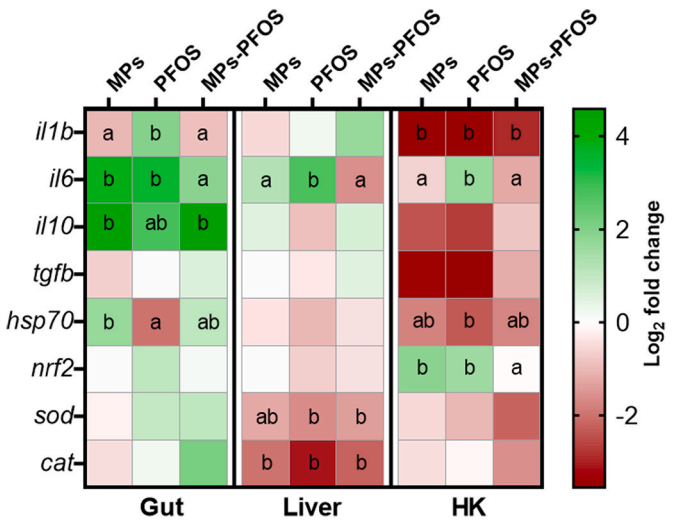

Fig. 4. Transcription of genes related to inflammation and oxidative stress in the gut, liver and head-kidney (HK) of European seabass fed with commercial diet alone (control) or containing, per kg of fed, 100 mg of virgin polyethylene MPs (MPs), 4.83 μg of PFOS or PFOS adsorbed to MPs (MPs-PFOS; final concentrations of 4.83 μg and 100 mg of PFOS and MPs, respectively) for 21 days. Data represent the mean ± SEM (n = 5) fold change respect to the control group. Letters indicate statistical differences between treatments ($p < 0.05$).

Table 1
Histopathological determinations in the gut and liver of European seabass fed with commercial diet alone (0, control) or containing 100 mg polyethylene microplastics (MPs)/kg diet, 4.83 μg perfluorooctane sulfonic acid (PFOS)/kg diet or MPs-PFOS (100 mg MP and 4.83 μg PFOS/kg diet) for 21 days. Values are the mean ± SEM (n = 5). Statistical differences ($p < 0.05$) between groups are indicated by different letters.

Tissue	Histological character	Control	MPs	PFOS	MPs-PFOS	*p* value
Gut	Goblet cells per 100,000 μm^2	62.3 ± 6.2^a	46.0 ± 8.5^b	167.1 ± 5.7^c	170.2 ± 4.2^c	***0.01***
	Villus thickness (μm)	48.2 ± 1.40^a	84.2 ± 3.1^b	59.8 ± 2.5^{ab}	88.7 ± 3.3^b	***0.02***
	Villus height (μm)	300.7 ± 23.8^a	265.0 ± 11.6^b	307.7 ± 8.1^a	170.5 ± 5.5^c	***0.01***
	Intestinal diameter (μm)	1009.5 ± 21.7^a	1154.2 ± 2.4^a	1224.4 ± 22.4^a	1050.4 ± 54.5^a	*0.124*
	Intestinal diameter: Villus height ratio	3.8 ± 0.3^a	4.53 ± 0.2^b	4.0 ± 0.1^a	6.8 ± 0.5^b	***0.01***
Liver	Vacuole presence (%)	10.2 ± 1.3^a	45.8 ± 7.4^b	32.6 ± 0.3^b	44.7 ± 5.1^b	***0.001***
	Congestion (%)	0.02 ± 0.007^a	2.6 ± 0.6^b	3.1 ± 0.5^b	4.0 ± 0.7^b	***0.001***

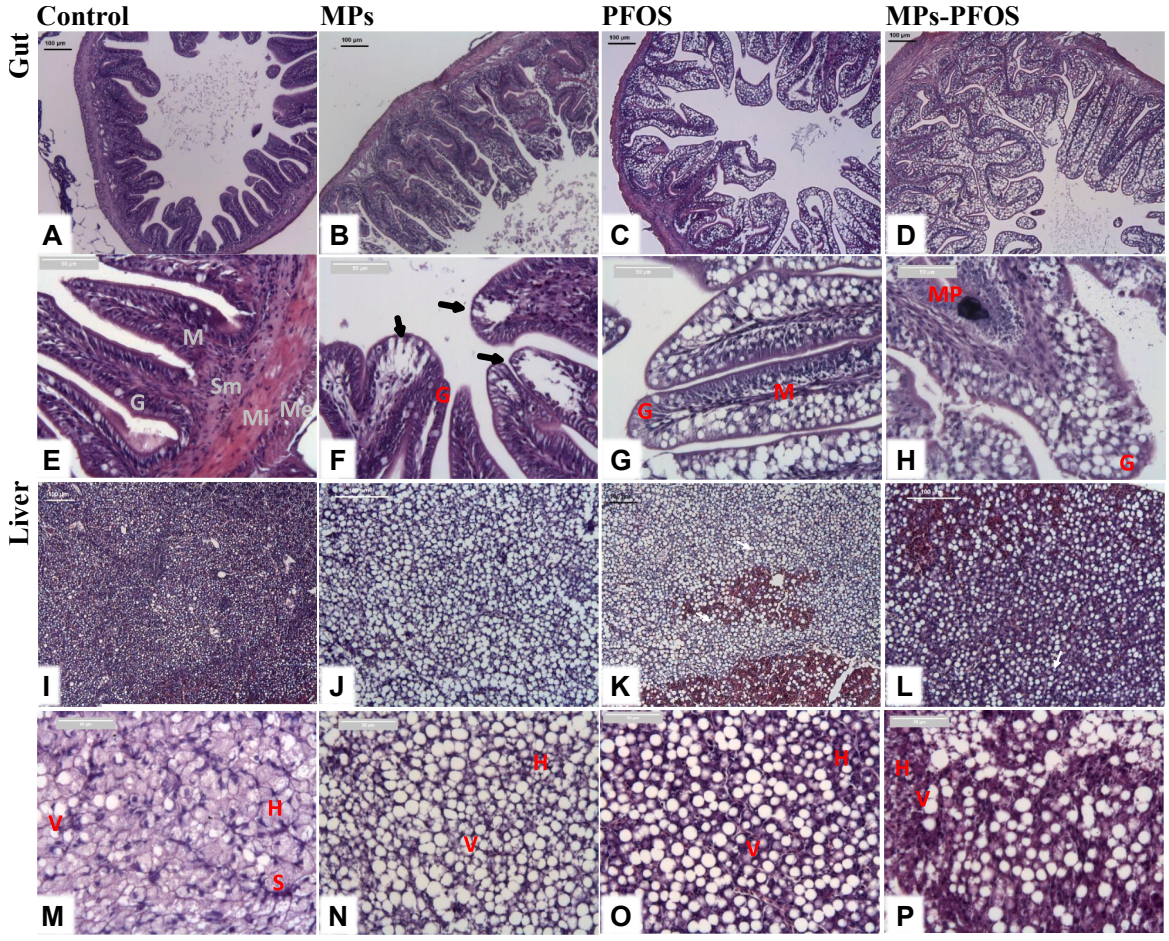

Fig. 5. Histopathological analysis of gut (**A-H**) and liver (**I-P**) of European seabass fed with commercial diet alone (control; **A,E,I,M**) or containing, per kg of fed, 100 mg of virgin polyethylene MPs (MPs; **B,F,J,N**), 4.83 μg of PFOS (**C,G,K,O**) or PFOS adsorbed to MPs (MPs-PFOS; final concentrations of 4.83 μg and 100 mg of PFOS and MPs, respectively; **D,H,L,P**) for 21 days. Sections were stained with haematoxylin-eosin. M, mucosa; Sm, submucosa; Mi, muscularis interna; Me, muscularis externa; G, globet cells; H, hepatocytes; S, sinusoid; V, vacuolization; * Focal necrosis; MP, microplastic. Black arrows indicate mucosa disorganization.

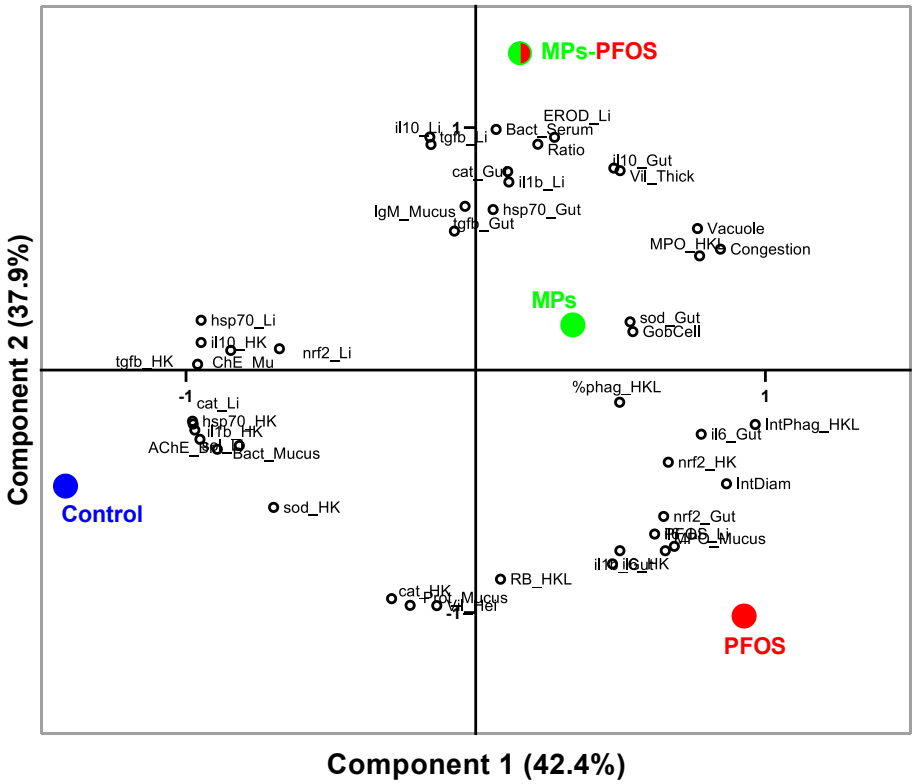

Fig. 6. Principal components analysis (PCA) of the main parameters (PFOS level, biomarkers, immunity, gene expression and histopathology) of European seabass fed with commercial diet alone (control) or containing, per kg of fed, 100 mg of virgin polyethylene MPs (MPs), 4.83 μg of PFOS or PFOS adsorbed to MPs (MPs-PFOS; final concentrations of 4.83 μg and 100 mg of PFOS and MPs, respectively) for 21 days.

bioaccumulation of the compound in clams [10] and fish whole body after longer exposition [38], which contrasts with our results. Similar results regarding a reduced bioaccumulation of other pollutants in presence of MPs has been evidenced also for arsenic, mercury, benzo[a] pyrene and pyrene (reviewed by Ref. [39]). The diversity of results obtained highlights the difficulty of standardising a pattern of plastic toxicity, which must be studied in each specific case. Bioaccumulation of PFAS may display different patterns depending on the ingestion capacity of MPs by aquatic organisms [7]. Recently, a review [20] suggested that, once ingested, PFAS sorbed onto MPs might be released from MPs-PFAS due to the low pH in the fish stomach leading to enhanced bioavailability of cationic PFASs and reduced bioavailability of anionic PFASs, increasing its toxicity [20]. Interestingly, the presence of bile salts or surfactants in the stomach might have also a very relevant implication in the desorption process of PFOS-spiked particles. Thus, desorption of PFOS from MPs, regardless the quantity of exposed PFOS-spiked particles, in Atlantic Cod (*Gadus morhua*) artificial gut fluid was more than 75% after 4 days of exposure [22]. Our results showed histopathological alterations in the intestine and liver from European seabass exposed to MPs, PFOS and MPs-PFOS, with respect to the structure observed in the control animals. The animals fed MPs diet showed different intestinal alterations, generally in the mucosa cells, enterocytes and goblet cells, suggesting disorganization of the intestinal barrier or mechanical abrasion, which agrees with previous observations in seabass fed PE-MPs [25]. This disorganization would favor the entry of MPs into the body and tissue distribution since MPs could be isolated from liver or muscle, among others, though the entry and translocation mechanisms are unclear. Findings relating the size of MPs with the negative impacts might be controversial. While it is documented that small MPs produce greater impact on fish, larger MPs increase the concentration and retention in the intestines provoking more chances to impact the fish. On the other hand, the animals fed the PFOS diet showed very few alterations in the intestine morphology, showing different incipient signs of infiltration, disorganization in the lamina propria and a slightly increase in the mucus layer. These alterations, although incipient, are in accordance to those reported in zebrafish chronically exposed to PFOS [40], which failed to affect the intestine morphology from females, but included an increase of goblet cells and a decrease of columnar cells in males. Interestingly, MPs-PFOS diet showed various signs of intestinal damage in a higher degree, being all the epithelium with a large enterocyte vacuolization. These observations suggested that PFOS attached to the MPs might produce its toxic effects in a higher degree, exacerbating the toxic effects of the PFOS. In addition, PFOS could be transferred from the MPs to the organism, as has been reported with other contaminants in fish [41].

In the liver, fish fed MPs diet showed loss of parenchyma organization and increased hepatocyte vacuolation. Previous studies reported necrosis, infiltration, and lipid accumulation of droplets in zebrafish liver following exposure to MPs and nanoplastics. These facts indicate inflammation and lipid accumulation as well as increased levels of fatty acids, and decreased levels of amino acids [42]. Recently, research carried out with goldfish (*Carassius auratus*) evidenced some necrosis and hemorrhages in liver after a diet supply with 0.5 mg/L Polyvinyl chloride (PVC) MPs [34]. These results suggested that the ingestion of MPs could affect the liver at both morphological and functional levels, which agree with our previous observations in seabass [25]. On the other hand, fish fed the PFOS diet showed liver alterations including hypertrophied and vacuolated hepatocytes, a greater number of vacuolated hepatocytes or congestion of blood sinusoids, which agree with other works [40]. Interestingly, although the bioaccumulation of PFOS was not significantly different from controls, fish fed the MPs-PFOS diet showed similar liver alterations, which suggest an interaction between PFOS attached to MPs and liver tissue. As commented above, total or partial liberation of PFOS sorbed to MPs may occur during the digestion process due to the weakly lipophilic behaviour of PFOS, pH or even bile salts. As consequence, it is reasonable to find in the liver these kind and

degree of damage produced by PFOS in our experiment.

4.2. Biomarkers

The hepatic EROD activity was measured in the liver from seabass fed the different diets. We did not observe a significant effect in the liver EROD activity of fish exposed to MPs, PFOS or their combination. Similar results were observed in medaka and zebrafish exposed for 4 months to MPs [38]. Nevertheless, other studies have evidenced an increase of EROD activity in red tilapia (*Oreochromis niloticus*) upon MPs exposure [43]. The comparison between studies with MPs is difficult due to the dissimilarities on MPs composition, pollutant sorption, the size of the particles (0.1 μm), the exposure route (dissolved in the water) or the concentration (100 μg/L). The duration of the exposure could impact the evaluation of this biomarker. Further research is needed to evaluate this biomarker under these exposure conditions.

Brain AChE and muscle ChE activities were reduced in fish exposed to MPs, PFOS or both contaminants indicating neurotoxicity. Previous studies with the seabass and other species documented AChE and/or ChE activity inhibition under exposure to different types of MPs alone [28] or in combination with other contaminants [44]. Therefore, the findings of the present study showed the ability of MPs present in the food to induce neurotoxicity and locomotor alterations. These findings reinforce the use of these enzymes as environmental biomarkers for neuronal and locomotor toxicity in relation to MPs and other pollutants, and increase the concerns regarding the potential effects of MPs on human health and wellbeing.

4.3. Immunity

Regarding the immune responses of seabass, the results indicate that MPs slightly affected the immune parameters by increasing serum bactericidal and leucocyte phagocytic and peroxidase activities, which is consistent with our previous works both in the seabream [31] and seabass [25]. Phagocytosis and bactericidal activity are key functions of the innate immune system. Their increase in seabass by MPs treatments may suggest that inflammatory reactions are taking place, due to the ingestion of MPs, activating a battery of immune responses including bactericidal and phagocytosis activities. On the other hand, PFOS diet was able to increase the mucus peroxidase activity and HKL phagocytic and peroxidase activities, which could contrast with other works that mostly described immunosuppressive properties of PFOA both *in vitro* and *ex vivo* [45]. However, MPs-PFOS diet provoked effects more similar to MPs than to PFOS alone, suggesting some kind of interaction by MPs in the response produced by the fish against PFOS. Interestingly, MPs-PFOS diet increased the serum bactericidal activity respect to the controls, but not to the MPs alone. These might be because liver, as the main producer of complement and antimicrobial peptides that are secreted to the bloodstream, is severely damaged and suffered inflammation, what is suggested by the histopathology study and expression of the *il1b* gene. Further studies are needed to understand the MP effects on fish immunity and how they could reduce the negative toxic effects of other contaminants.

In addition, we also investigated the expression of different genes related to inflammation and oxidative stress on head-kidney, liver, and gut. The expression of pro-inflammatory factors showed a significant decrease of expression of *il1b* in head-kidney of fish fed the MPs, PFOS and MPs-PFOS diets at 21 days. Previous studies carried out with NPs in seabass also pointed out the reduction of this genetic marker suggesting the mobilization of immune cells to other target organs where higher inflammation was registered [46]. Similarly, *il6* showed to be affected in the three analysed tissues for 21 days on fish fed the MPs and PFOS diet. Our results denoted that MPs and PFOS alone promoted inflammatory processes after long exposure, but this response did not happen in the fish fed the MPs-PFOS diet. Regarding oxidative stress, the expression of *sod* and *cat* showed to be down-regulated in liver from fish fed the MPs, PFOS and MPs-PFOS diets, suggesting that the antioxidant defense was severally compromised. In addition, the expression of *nrf2* was up-regulated while *hsp70* was down-regulated in head-kidney and gut from fish fed the MPs and PFOS diets. Our results are consistent with previous reports where PFOS exposure increased the expression of *nrf2* [47] while MPs exposure decreased the expression of *hsp70* in head-kidney [32]. Interestingly, it has been found in common cormorants (*Phalacrocorax carbo*) a negative correlation between the expression of heat shock proteins and PFOS hepatic levels [48]. Considering the role of these protein stabilizers, the down regulation produced by PFOS could entail a decrease in the protein stability and, eventually, increase abnormal protein accumulation. In this sense, the significant increase of *hsp70* expression in gut after 21 days denoted the susceptibility to the MPs exposure, as was supported by the histological and morphometric assay. Thus, we suggest that stress may be caused by mechanical damage produced by MPs [6]. These results are in the line with previous studies showing the combined effect of PFOS and MPs evidencing the ability of MPs to sorb pollutants altering its toxicology and impact on marine organisms.

5. Conclusion

Overall, our results indicate that both MPs and PFOS diets were able to affect some biomarkers and immune parameters and severally affected liver and gut tissues from European seabass. However, the intake of diet containing PFOS attached to MPs produced similar effects than MPs alone, or at least, lower than PFOS alone. This study is in line with previous data demonstrating that MPs are able to adsorb and reduce the harmful and toxicological effects produced by certain pollutants.

Credit author statement

CE: Methodology, Investigation, Formal analysis, Writing – original draft; **CGF**: Investigation, Formal analysis, Writing – original draft, Writing – review & editing; **BC**: Methodology, Formal analysis; **SHK**: Methodology, Writing – review & editing, Funding acquisition; **LRV**: Methodology; **LG**: Methodology, Writing – review & editing, Funding acquisition; **CC**: Methodology, Formal analysis; **JC**: Methodology, Writing – review & editing, Funding acquisition; **MAE**: Conceptualization, Writing – review & editing, Funding acquisition; **AC**: Conceptualization, Investigation, Formal analysis; Writing – original draft; Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.108793.

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