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Assessment of the effect of long-term exposure to microplastics and depuration period in *Sparus aurata* Linnaeus, 1758: Liver and blood biomarkers

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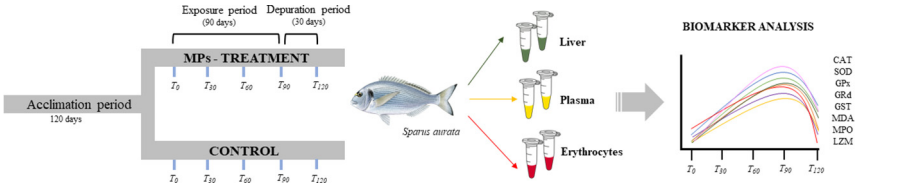
HIGHLIGHTS

- Microplastic exposure induced antioxidant enzymes and oxidative damage in liver and plasma of *S. aurata*.
- Microplastic intake increased plasma levels of lysozyme and myeloperoxidase activity in plasma.
- None of the parameters determined in erythrocytes-antioxidants and MDA– were altered.
- Stress and inflammatory biomarkers in plasma and liver tended to recover at the depuration period.

GRAPHICAL ABSTRACT



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ABSTRACT

The constant increase in plastic pollution has attracted great attention in recent years due to its potential detrimental effects on organisms and ecosystems. While the consequences of ingestion of large plastic litter are mostly understood, the impacts resulting from a long-term exposure and a recovery period of microplastics (MPs) are still limited. The aims were to monitor oxidative stress, detoxification and inflammatory biomarkers in liver, plasma and erythrocytes of *Sparus aurata* exposed during 90 days to low-density polyethylene (LDPE)-MPs enriched diet (10% by weight) followed by 30 days of depuration. Exposure to LDPE-MPs progressively activates the antioxidant and detoxification system and induces an inflammatory response in liver and plasma, whereas no significant changes were observed in erythrocytes. The plasma activities of catalase, myeloperoxidase (MPO), lysozyme and the levels of malondialdehyde (MDA) as maker of lipid peroxidation significantly increased after exposure to LDPE-MPs for 90 days compared to the control group. The activities of all antioxidant enzymes – catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase-, the detoxification enzyme glutathione *s*-transferase, MPO, the production of reactive oxygen species and the levels of MDA were also significantly increased in liver after MPs exposure. Additionally, all these biomarkers tended to recover during the depuration period, most of them reaching similar levels to those of the control group. In conclusion, the ingestion of a diet containing LDPE-MPs for 90 days induced a progressive increase in oxidative stress and inflammation biomarkers in liver and plasma of *S. aurata* but not in erythrocytes, which tended to regain control values

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when not exposed to MPs for 30 days. The present study contributes to a better understanding of the toxic effects of MPs in *S. aurata* and highlights the usefulness of plasma that can be obtained in a minimally invasive way to monitor these effects.

1. Introduction

Microplastics (MPs) are usually defined as plastic fragments smaller than 5 mm in diameter (Arthur et al., 2009). These MPs can have two origins: primary MPs, those plastic particles manufactured directly with a microscopic size (Browne, 2015; Cole et al., 2011), and secondary MPs, formed by the fragmentation of larger plastics into smaller pieces as a result of mechanical action, photo-oxidation and biodegradation (Alimba and Faggio, 2019; Auta et al., 2017; Gewert et al., 2015; Prokić et al., 2021, 2019; Strungaru et al., 2019). MPs are emerging pollutants in marine habitats and have been considered a serious threat to marine species (Collard et al., 2017; Dehaut et al., 2016; Galloway et al., 2017; Ogonowski et al., 2018). Although MPs are ubiquitous, they accumulate predominantly in semi-enclosed basins, such as the Mediterranean Sea, to a greater degree than in the open oceans (Cózar et al., 2015; Eriksen et al., 2014). It has been estimated that the average densities of floating MPs in the Mediterranean Basin exceed 100,000 items/km^2, which shows the importance of the risk they can pose for the marine environment (Collignon et al., 2012). Alomar et al. (2016) also reported that coastal shallow sediments from Marine Protected Areas (MPAs) in the Balearic Islands (western Mediterranean), concentrations of MPs of up to 0.90 ± 0.10 MPs/g, highlighting the availability of MPs for benthic organisms. Linked to their small size, MPs can be ingested by an extensive range of marine organisms ranging from pelagic and benthic fishes to marine mammals, and thus entering into marine food-webs (Courtene-Jones et al., 2017; Dehaut et al., 2016; Fossi et al., 2016; Karlsson et al., 2017; Neves et al., 2015; Pellini et al., 2018; Renzi et al., 2018; Tanaka and Takada, 2016; Van Cauwenberghe et al., 2015; Zhu et al., 2019).

Available data suggest that MPs chemical polymers, such as polyvinyl chloride (PVC), polyethylene (PE), polystyrene (PS) and polypropylene (PP) are considered hazardous for marine organisms (Horton et al., 2017; Lithner et al., 2011; Obbard et al., 2014; PlasticsEurope, 2018). Once ingested, these polymers can be distributed through the circulatory system and be incorporated into the cells and tissues causing severe toxic effects on the consumer organisms (Mattsson et al., 2015; Teuten et al., 2009). Negative effects of MPs ingestion include decreased feeding capacity (Cole et al., 2015; Schirinzi et al., 2020), fecundity suppression (Sussarellu et al., 2016), inflammatory responses (Burgos-Aceves et al., 2021; Jin et al., 2018), endocrine system disruptions (Pagano et al., 2019; Rochman et al., 2014; Teuten et al., 2009) and oxidative stress (Choi et al., 2018; Solomando et al., 2020). Specifically, exposure of marine organisms to MPs, can induce the overproduction of reactive oxygen species (ROS) leading to oxidative damage to macromolecules of tissues (Capo et al., 2021; Sureda et al., 2006). In order to avoid ROS damage, fish present a complex system of antioxidants and detoxifying mechanisms which involves enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRd), that could prevent ROS production (Espinosa-Diez et al., 2015). Some investigations have evidenced significant increases in antioxidant enzymes after exposure to MPs in different fish species such as *Symphysodon aequifasciatus* (Pellegrin, 1904), exposed for 15 days, and *Oreochromis niloticus* (Linnaeus, 1758), exposed for 30 days (Hamed et al., 2019; Wen et al., 2018).

Immune system of fish is the first defense system against foreign substances comprising cellular and humoral components (Burns-Naas et al., 2001; Galloway and Handy, 2003). The immune system is very sensitive to toxic compounds and alterations in innate immunity can be used as a bioindicator of environmental toxic stressors (Aliko et al., 2018; Burgos-Aceves et al., 2018, 2016; Galloway and Handy, 2003; Jiang et al., 2016; Li et al., 2013). Lysozyme (LZM) is an immune humoral enzyme present in fish serum (Lee and Yang, 2002; Smith et al., 2000) that cleaves the peptidoglycan component of bacterial cell walls (Luo et al., 2018). Some studies reported an increase in LZM activity in different species of *Mytilus* spp. after exposure to MPs (Canesi et al., 2015; Wang et al., 2020). However, there has been scarce literature available with regard to the effects of MPs exposure on LZM activity in fish species. In *Oncorhynchus mykiss* (Walbaum, 1792) fish exposed to PS-MPs (100–400 μm) for 4 weeks, no clear effects were observed in plasma LZM (Ašmonaite et al., 2018). Myeloperoxidase (MPO), an enzyme present in cells of the innate immune system, is secreted to plasma after infection or in presence of an inflammatory response during the acute phase immune response. MPO uses H_2O_2 as substrate to generate the bactericidal hypochlorous acid (Sureda et al., 2005). Also, this enzyme could be used as bioindicator of immune system activation and inflammation in front of plastic pollution (Chen et al., 2019).

Most studies focus on evaluating the effects of MPs on liver and gut, while there is little information on plasma and erythrocytes under a long-term exposure in laboratory conditions (Ding et al., 2018; Lu et al., 2016; Qiao et al., 2019). The assessment of the balance between overall oxidant and antioxidant defenses in liver, plasma and erythrocytes can be a valuable tool for evaluating the potential damaging effects of MPs on fish (Collard et al., 2017; Mattsson et al., 2017). In addition, blood samples allow to monitor studies with minimally invasive sampling and without the need to sacrifice animals thus, it is of interest to determine their potential use as biomarkers.

The concern about the strong impact that MPs pollution can have on aquatic biota has led to the development of various studies. However, there are important gaps in the knowledge about the toxicological potential of long-term exposure to these pollutants and whether these effects are reversed after a recovery time, without exposure to MPs. In fact, to our knowledge, the longest exposure time to MPs was 45 days followed by 30 days of depuration using juvenile *Clarias gariepinus* (Burchell, 1822) as a model (Iheanacho and Odo, 2020). In the present study, gilthead seabream (*Sparus aurata* Linnaeus, 1758) was used as a model species to fill the gap in understanding the mechanisms of MPs effects on liver, plasma and erythrocytes. The gilthead seabream is widespread in Atlantic and Mediterranean coastal waters and it is one of the most commercially important fishery and aquaculture species. Furthermore, *S. aurata* has already proved its suitability as a bioindicator in toxicity testing (DelValls et al., 1998; Faggio et al., 2014b; Fazio et al., 2013a; Teles et al., 2005; Zena et al., 2015). Moreover, in a previous study it was evidenced that antioxidant and detoxification enzymes increased in gut of *S. aurata* after 90 days exposed to MPs (Solomando et al., 2020). Hence, to better understand the potential effects of long-term exposure to MPs, the aim of the current study was to analyze the physiological response through the evaluation of antioxidant enzymes and immune parameters in plasma, erythrocyte and liver of *S. aurata* after 90 days of exposure to an enriched diet with 10% LDPE-MPs, followed by a 30-day depuration period. Since PE and PP are the two most widespread forms of plastic in the marine environment (Cole et al., 2011; Law, 2017), LDPE-MPs were selected as a compound model for the present study.

2. Materials and methods

2.1. Reagents

All reagents except those where specified were purchased from Merck Life Science S.L.U., Madrid, Spain.

2.2. Experimental design, diet and sampling

The experimental procedure and dietary preparation was previously described in Solomando et al. (2020). Briefly, 2000 juvenile gilthead seabream of 3 g were transported from the *Instituto Español de Oceanografía, Centro Oceanográfico de Múrcia* (Spain) and were acclimatized for 120 days at LIMIA (Laboratorio de Investigaciones Marinas y Acuicultura) laboratory in Andratx (Mallorca, Balearic Islands) in a 2000 L tank to recover from handling and transportation stress effects (Petochi et al., 2011) and to adapt to the new environment (Kayali et al., 2011). Then, a subsample of 90 fish (weight 44.9 ± 3.1 g and body length 11.8 ± 0.3 cm) were assigned to six (2 Treatments (Control and MPs-treatment) × 3 Replications) 1000 L tanks (n = 100 each tank). Each tank was located inside the laboratory with a 16:8 h light-dark regime system, a water flow rate of ~1 L/min, an oxygen concentration of 5.9–6.1 ppm and the temperature was adjusted at 19 ± 2 °C. The experimental tanks were part of a semi-open flow seawater system integrating mechanical filters which prevent input of MPs from the exterior, UV sterilization and compressed air supply. After this, the experiment lasted 120 days, including a 90 days exposure period in which the MPs-treatment group was exposed to a diet enriched in LDPE-MPs (size 200–500 μm, range comprising the usual size of primary MPs used in industrial and personal care processes) purchased from Quimidroga, S.A. (Barcelona, Spain) and free of additives, followed by a 30 days depuration period. During the depuration period fish in both treatments were fed with the control diet. Two diets were prepared for the study, the control diet (90% food and 10% filler) and the MPs-treatment diet (90% food and 10% LDPE-MPs, 10 g MPs in 100 g food), which were similar to the concentration ranges (0.5–10% of diet) reported in previous experiments (Jovanović et al., 2018; Rochman et al., 2013; Santana et al., 2016). During the acclimatization, fish were fed twice a day at a rate of 2% body weight·day^{-1} with a combination of Gemma and Mar Vitalis CV2 pellets (Trouw Nutrition España S.A). The ingredients of the control and MPs-treatment diet are previously described in Solomando et al. (2020) and presented in the Supplementary material. The specimens used in the study were juveniles during the entire experimental procedure and the maximum weight and size reached by the fish was 117.1 ± 6.4 g and 16.3 ± 0.2 cm, respectively.

Blood and tissue sampling were carried out at time 0 and every 30 days for four months (Solomando et al., 2020). On each sampling day, blood was withdrawn for oxidative stress analysis with 1 mL sterile syringe through the caudal sinus. Blood was transferred to an Eppendorf tube coated with lithium heparin (0.5 mg/mL) as anticoagulant for plasma and erythrocyte isolation. After blood extraction, individuals were sacrificed and liver was immediately dissected from all fishes sampled by opening the abdominal cavity. Then, 0.1 g of liver was placed into an Eppendorf tube, stored in liquid nitrogen and afterwards at −80 °C until biochemical analysis. Furthermore, to check if MPs ingestion occurred during the exposure period, gastrointestinal tracts of fish were analyzed: MPs isolation and extraction was carried out by applying a potassium hydroxide (KOH) 10% chemical digestion of samples as described by Dehaut et al. (2016). Visual identification of digested organic matter was conducted under the stereomicroscope (Euromex NZ 1903-S) to identify ingested MPs in the gastrointestinal tracts of *S. aurata*. To avoid external contamination by MPs during the procedure, laboratory coats (100% cotton) and gloves were used and all the material used and the work surfaces were sealed twice before using it with distilled water and alcohol. In addition, the absence of contamination during the analysis of the contents of the gastrointestinal tract, two glass Petri dishes were placed on each side of the stereomicroscope and the absence of MPs was verified before and after each sample.

2.3. Preparation of samples for biochemical analysis

To analyze the effects of the treatment diet, with and without MPs, on the oxidative stress, inflammatory response and antioxidant and detoxification system, liver and blood samples of 90 specimens of *S. aurata* (n = 10 of each treatment for each time period) were collected. Blood samples were immediately centrifugated (1700*g*, for 15 min, 18 °C; Orto Alresa Biocen 22R) to obtain plasma and erythrocytes and were kept at −80 °C until biochemical analyses at the laboratory. Liver samples were homogenized in ten volumes (w/v) of 100 mM Tris–HCl buffer (pH 7.5) using a small sample dispersing system (Ultra-Turrax® Disperser, IKA) and centrifuged (9000*g*, for 10 min, 4 °C; Sigma 3K30) (Sureda et al., 2006). After centrifugation, supernatants were collected and immediately used for the biochemical analyses.

For all samples, total protein content was determined by a colorimetric method (Biorad Protein Assay), using bovine serum albumin (BSA) as a standard to normalize all biochemical results.

2.4. Biochemical analysis

Antioxidant enzymes activities of catalase (CAT) (Aebi, 1984), superoxide dismutase (SOD) (Flohé and ötting, 1984), glutathione peroxidase (GPx) (Flohé and Günzler, 1984) and glutathione reductase (GRd) (Goldberg and Spooner, 1984), the glutathione S-transferase (GST) (Habig et al., 1974) and the inflammatory biomarker myeloperoxidase (MPO) (Capeillère-Blandin, 1998) were determined with a Shimadzu UV-2401 PC spectrophotometer at 25 °C (Supplementary material).

ROS generation was measured in liver using 2,7-dichlorofluorescin diacetate (DCFH-DA) as indicator. DCFH-DA (30 μg/mL) in PBS was added to a 96-well microplate containing liver homogenates and the fluorescence (Ex, 480 nm; Em, 530 nm) was recorded at 25 °C for 1 h in FL 9800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.).

Malondialdehyde (MDA) levels in liver, plasma and erythrocytes, as a lipid peroxidation marker (Pinya et al., 2016; Sureda et al., 2018), were assayed by using commercial colorimetric kit for MDA determination (Sigma-Aldrich, Spain).

Lysozyme (LZM) activity in fish plasma was assayed by the method of Lee and Yang (2002) using a bacterial suspension of *Micrococcus lysodeikticus* cells. The activity was monitored at 450 in a microplate reader (BioTek®, PowerWaveXS) (Supplementary material).

2.5. Data analysis

Normality and homogeneity of variance were evaluated using Shapiro-Wilks and Levene's tests, respectively. The statistical significance of the biomarkers activities was assessed by a two-way ANOVA. The two analyzed factors were: diet treatment (control diet and MPs-treatment diet) and MPs exposure time 0, 30, 60, 90 and 120 days (T_0, T_{30}, T_{60}, T_{90} and T_{120}). Least significant difference t-test (LSD) post hoc paired comparison was further made to recognize differences between groups. Results were expressed as mean ± standard error of the mean (S.E.M.) and all differences were considered significant at $p < 0.05$. All statistical analysis was carried out using a statistical package (SPSS version 25.0 for Windows®).

2.6. Ethics approval

This study was approved by the Animal Experimentation Ethics Committee of the University of the Balearic Island (Reference CEEA 96/05/18). The methods of all experiments were carried out following the protocols previously established by LIMIA according to legal qualifications, in strict accordance with the Directive 2010/63/UE,

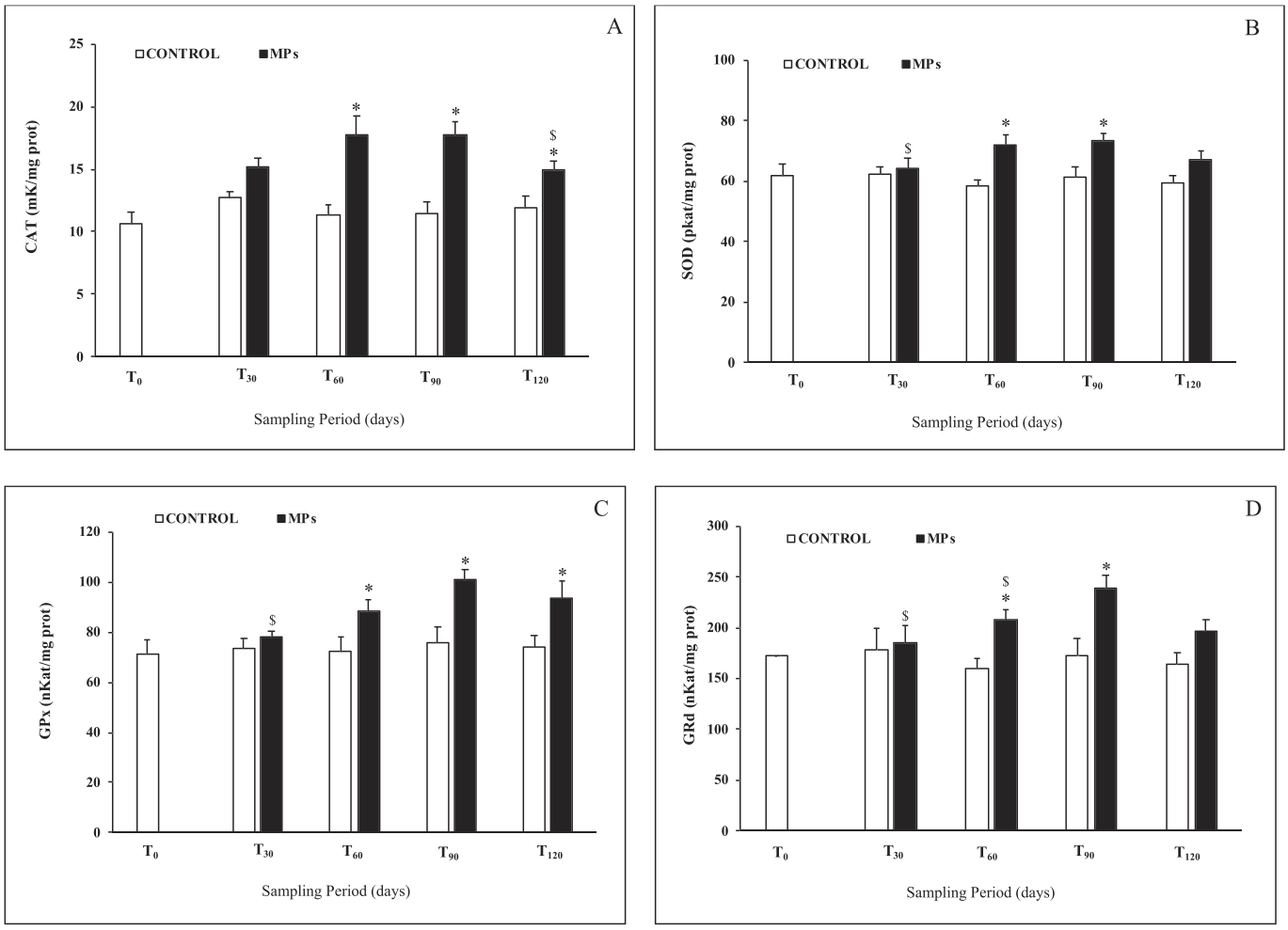

Fig. 1. Catalase (CAT) (A); Superoxide dismutase (SOD) (B); Glutathione peroxidase (GPx) (C); Glutathione reductase (GRd) (D) activities determined in *S. aurata* liver exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data are presented as mean ± s.e.m. * indicates significant differences ($p < 0.05$) between values of control group and MPs group and $ indicates significant differences respect to T_{90} MPs (two-way ANOVA analysis).

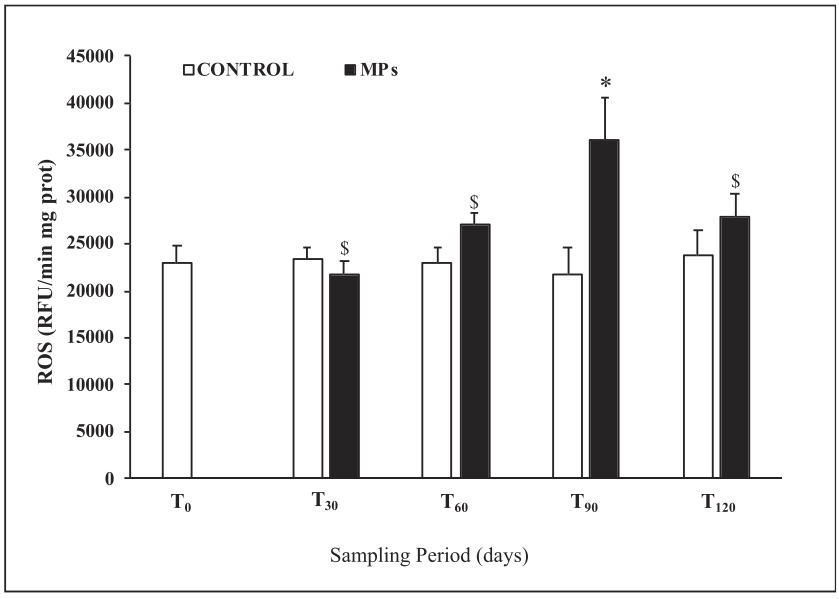

Fig. 2. Reactive oxygen species (ROS) production determined in *S. aurata* liver exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data are presented as mean ± s.e.m. * indicates significant differences ($p < 0.05$) between values of control group and MPs group and $ indicates significant differences respect to T_{90} MPs (two-way ANOVA analysis).

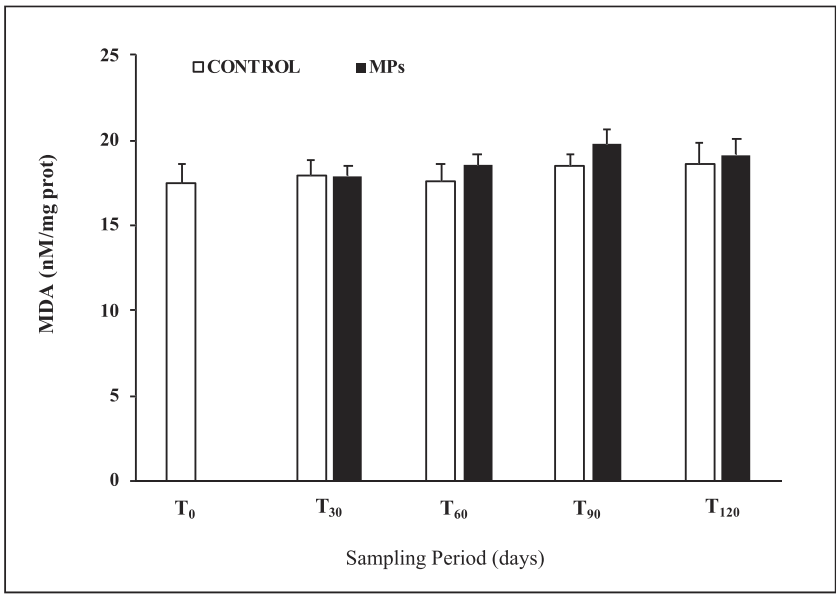

Fig. 3. Malondialdehyde (MDA) biomarker activity determined in *S. aurata* liver exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}) (two-way ANOVA analysis). Data are presented as mean ± s.e.m. (two-way ANOVA analysis). No significant differences were evidenced.

and in compliance with the Spanish law (RD53/2013, BOE n. 34 February 8th 2013).

3. Results

Mortality and the possible existence of alterations in the behavior of the fish were verified daily throughout the study. No mortality was observed from the MPs exposure treatment or evidence of altered behavior, indicating that LDPE-MPs do not cause evident acute toxicity to *S. aurata*.

3.1. Biomarkers in liver

The activities of the antioxidant enzymes, CAT, SOD, GPx and GRd, in liver of *S. aurata* exposed to LDPE-MPs are shown in Fig. 1. All enzymatic activities progressively increased in the MPs-treatment throughout the study, reaching a maximum value after 90 days of exposure (T_{90}), while the values remained similar in the control group. Significant differences in the activity of CAT were found at T_{30}, T_{60}, T_{90} and T_{120} in the MPs-treatment respect to T_0 (LSD, $p < 0.05$). Moreover, the CAT values of the MPs-treatment at T_{60}, T_{90} and T_{120} were significantly increased than those of the control group, by 56.4%, 54.4% and 25.3%, respectively (LSD, $p < 0.05$). At T_{120} CAT activity showed a significant decrease respect to T_{90} in the MPs-treatment (LSD, $p < 0.05$). A similar trend was observed in the SOD activity, evidencing higher values in the MPs-treatment at T_{60} and T_{90} than T_0 (LSD, $p < 0.05$). Furthermore, the values of the MPs-treatment at T_{60} and T_{90} were significantly increased by 23.7% and 19.9%, respectively compared with the control group (LSD, $p < 0.05$), while at T_{120} both treatments had similar activities (LSD, $p > 0.05$). In addition, GPx activity at T_{60}, T_{90} and T_{120} was significantly higher than at T_0 (LSD, $p < 0.05$), whereas in the case of GRd significant differences were

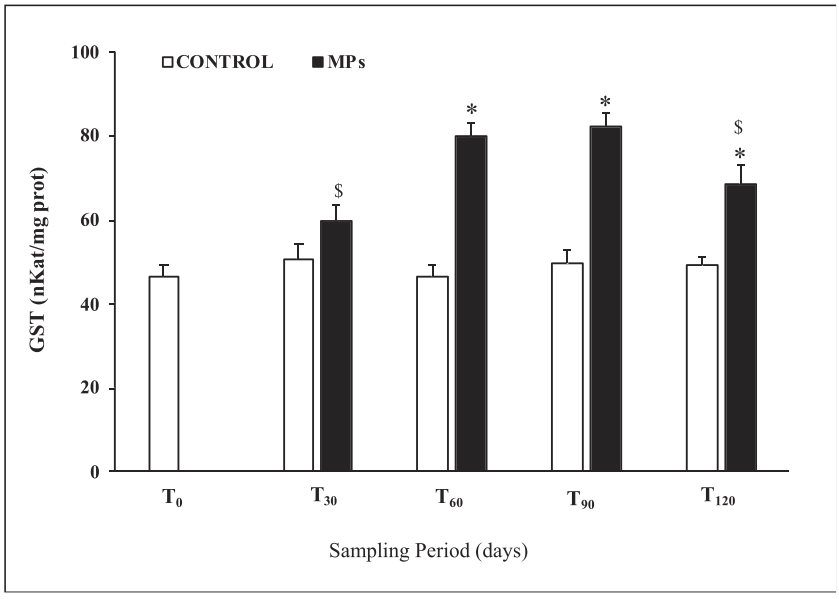

Fig. 4. Glutathione S-transferase (GST) activity determined in *S. aurata* liver exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data are presented as mean ± s.e.m. * indicates significant differences ($p < 0.05$) between values of control group and MPs group and $ indicates significant differences respect to T_{90} MPs (two-way ANOVA analysis).

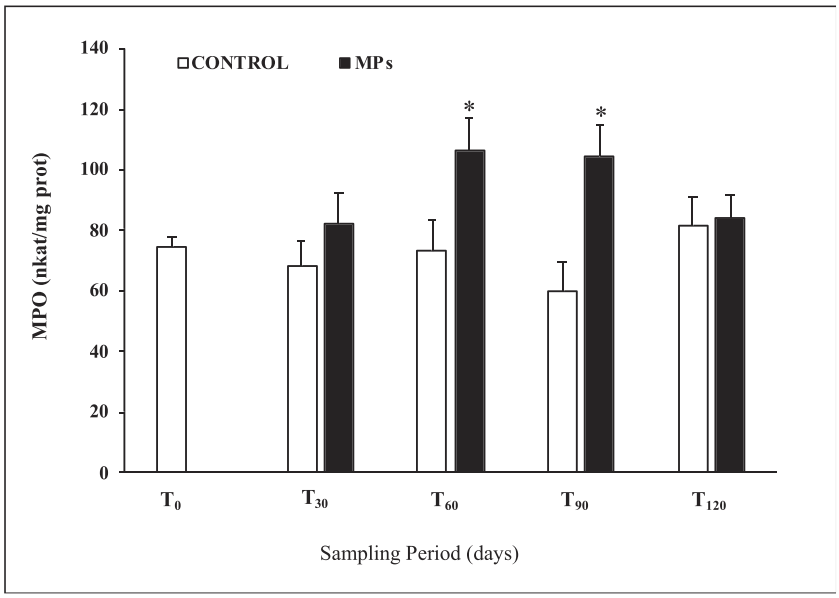

Fig. 5. Myeloperoxidase (MPO) activity determined in *S. aurata* liver exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data are presented as mean ± s.e.m. * indicates significant differences ($p < 0.05$) between values of control group and MPs group and $ indicates significant differences respect to T_{90} MPs (two-way ANOVA analysis).

only found at T_{60} and T_{90} with respect to T_0 (LSD, $p < 0.05$). For both enzymes, the values observed after 60 and 90 days were significantly higher in the MPs-treatment compared to the control group, 22% and 34% at T_{60} and T_{90} in GPx, and 29.8% and 38.6% at T_{60} and T_{90} in GRd (LSD, $p < 0.05$). Additionally, for GPx and GRd the activity at T_{90} was significantly higher respect to T_{30}, and also respect to T_{60} in the case of GRd (LSD, $p < 0.05$).

The production of ROS in the liver of *S. aurata* exposed or not exposed to enriched MPs diet is presented in Fig. 2. The production of ROS progressively increased in the MPs-treatment as the exposure time progresses reporting a maximum value after 90 days of exposure (T_{90}). The values of the MPs-treatment at T_{90} were significantly increased by 65.6% compared with the control group. At T_{120} ROS production reported a significant decrease respect to T_{90} in the MPs-treatment recovering values similar to the control group.

MDA values (Fig. 3) remained similar in both treatments during the study without significant differences between *S. aurata* exposed or not exposed to enriched MPs diet (LSD, $p > 0.05$).

Significant differences in GST activity (Fig. 4) were evidenced at T_{30}, T_{60} and T_{90} with respect to T_0 in the MPs-treatment (LSD, $p < 0.05$), with the highest values at T_{90}. In addition, the activity in the MPs-treatment group at T_{60}, T_{90} and T_{120} was significantly increased by 71.1%, 65.9% and 39.2%, respectively, compared with the controls (LSD, $p < 0.05$).

In addition, MPO activity (Fig. 5) at T_{60} and T_{90} in the MPs-treatment was significantly higher compared to T_0 and were significantly higher than those of the control group by 45% and 73.2%, respectively, but without completely recovering the values of the control group at T_{120} (LSD, $p > 0.05$).

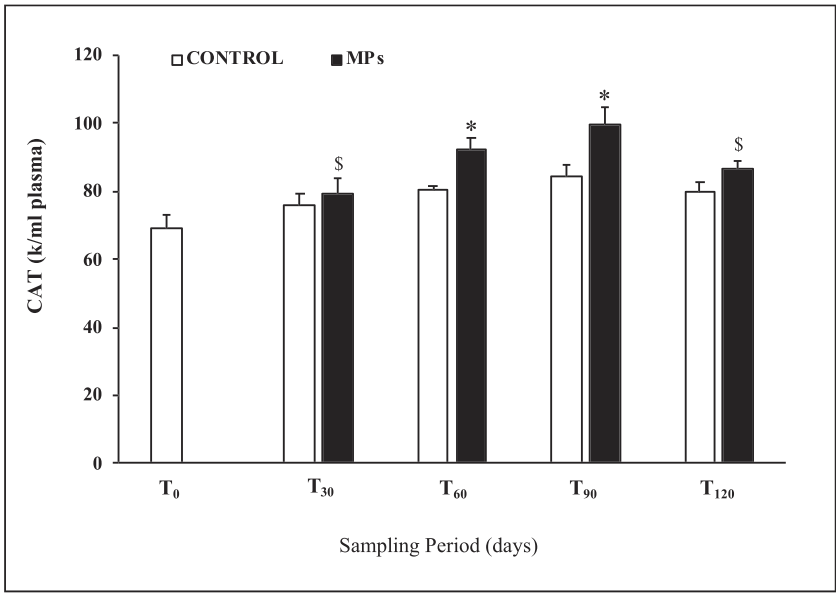

Fig. 6. Catalase (CAT) activity determined in *S. aurata* plasma exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data are presented as mean ± s.e.m. * indicates significant differences ($p < 0.05$) between values of control group and MPs group and $ indicates significant differences respect to T_{90} MPs (two-way ANOVA analysis).

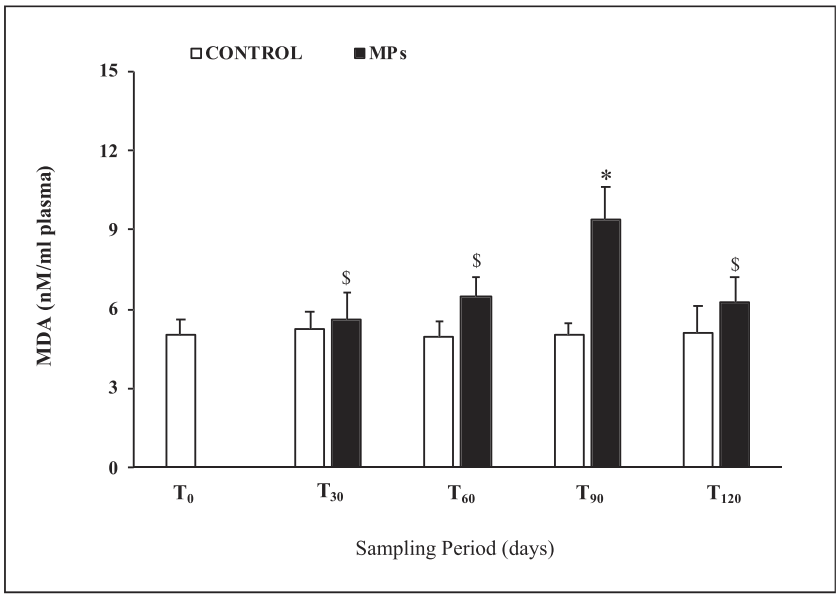

Fig. 7. Malondialdehyde (MDA) activity determined in *S. aurata* plasma exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data are presented as mean ± s.e.m. * indicates significant differences ($p < 0.05$) between values of control group and MPs group and $ indicates significant differences respect to T_{90} MPs (two-way ANOVA analysis).

3.2. Biomarkers in plasma

The activity of CAT in plasma progressively increased in the MPs-treatment throughout the exposure period, reaching a maximum value at T_{90}, while the values remained similar in the control group (Fig. 6). CAT activity at T_{30}, T_{60}, T_{90} and T_{120} was significantly higher than at T_0 (LSD, $p < 0.05$) and significant increases were found at T_{60} and T_{90} in the MPs-treatment by 14.7% and 18.3%, respectively, compared to the control group (LSD, $p < 0.05$).

Plasma MDA levels increased over time, evidencing significant differences at T_{90} with respect to T_0, T_{30}, T_{60} and T_{120} in the MPs-treatment (LSD, $p < 0.05$) (Fig. 7). Moreover, the level of MDA at T_{90} was significantly increased by 87.1% in the MPs-treatment compared to the control group (LSD, $p < 0.05$). The 30 days depuration period (T_{120}) evidenced a significant decrease in MDA activity in the MPs-treatment, reaching similar values to those in the control group.

MPO activity significantly increased only in the MPs-treatment at T_{90} compared to T_0 and T_{30} (LSD, $p < 0.05$) (Fig. 8). Furthermore, the MPO activity showed significant differences between the MPs and the control group at T_{90}, with an increase of 58.6% (LSD, $p < 0.05$). At T_{120} MPO activity was recovered in the MPs group with values similar to those from the control group (LSD, $p > 0.05$). Similarly, plasma LZM activity (Fig. 8) increased during the MPs exposure period compared to the control diet, reaching significant maximum values at T_{90} respect T_0 (LSD, $p < 0.05$). In addition, the values of the MPs-treatment at T_{90} were significantly increased by 79.4%, than the observed in the control group (LSD, $p < 0.05$). At T_{120}, after the depuration period, LZM values decreased significantly respect to T_{90} (LSD, $p < 0.05$), recovering values similar to the control group and no differences, but no differences were found between treatments at T_{120} (LSD, $p > 0.05$).

3.3. Biomarkers on erythrocytes

None of the parameters determined in erythrocytes of *S. aurata* - antioxidant enzymes and MDA - have reported significant differences associated with the intake of MPs (LSD, $p > 0.05$) (Table 1).

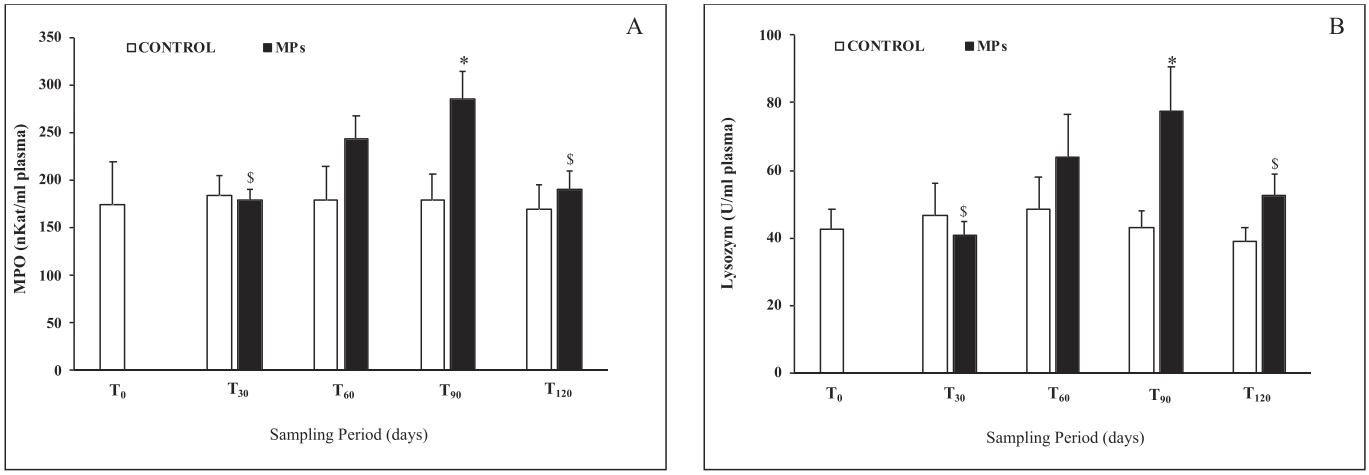

Fig. 8. Myeloperoxidase (MPO) (A) and Lysozyme (LZM) (B) activities determined in *S. aurata* plasma exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data are presented as mean ± s.e.m. * indicates significant differences ($p < 0.05$) between values of control group and MPs group and $ indicates significant differences respect to T_{90} MPs (two-way ANOVA analysis).

Table 1
Enzymatic activities (CAT, SOD, GPx and GRd) and MDA levels in *S. aurata* erythrocytes exposed to LDPE-MPs for 90 days (0, 30, 60 and 90 days) and followed by a no-exposure period to LDPE-MPs of 30 days (T_{120}). Data represent mean ± s.e.m. (two-way ANOVA analysis). No significant differences were reported in any parameter.

		Sampling period (days)				
		T_0	T_{30}	T_{60}	T_{90}	T_{120}
CAT	Control	126.6 ± 25.9	157.6 ± 16.7	162.9 ± 36.9	174.5 ± 31.3	169.3 ± 32.4
(mK/mg prot)	MPs		177.8 ± 38.7	200.8 ± 43	220.1 ± 48.9	187.8 ± 43.6
SOD	Control	0.9 ± 0.02	0.9 ± 0.02	0.9 ± 0.02	0.9 ± 0.1	0.9 ± 0.02
(pKat/mg prot)	MPs		0.9 ± 0.1	0.9 ± 0.02	0.9 ± 0.01	0.9 ± 0.02
GPx	Control	12.9 ± 0.5	14.1 ± 0.6	13.3 ± 0.6	13.8 ± 0.6	14.5 ± 0.5
(nKat/mg prot)	MPs		13.2 ± 0.5	13.1 ± 0.7	14.1 ± 0.3	14 ± 0.5
GRd	Control	2.9 ± 0.3	3.1 ± 0.6	3.3 ± 0.4	3.8 ± 0.6	2.9 ± 0.5
(nKat/mg prot)	MPs		2.6 ± 0.3	3.4 ± 1	3 ± 0.5	3 ± 0.4
MDA	Control	3.7 ± 0.4	4.1 ± 0.3	3.2 ± 0.3	3 ± 0.2	3.4 ± 0.4
(nM/mg prot)	MPs		3.6 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	3.2 ± 0.2

4. Discussion

In the current work, the potential negative effects of MPs in liver, plasma and erythrocytes of *S. aurata* fed for 90 days with a diet enriched with 10% LDPE-MPs under controlled laboratory conditions were investigated through biochemical analysis. Furthermore, the effects of a 30 days depuration period were inspected to determine whether the toxic effects detected by the ingestion of LDPE-MPs are irreversible or on the other hand, fish are able to bounce back once the toxins are removed from their diet.

The results of the present research evidenced that long-term exposure to LDPE-MPs activated the antioxidant and detoxification system, increased the production of ROS and induced an inflammatory response in plasma and liver, whereas no significant changes were observed in erythrocytes. The liver has been widely used to assess the metabolic disturbances induced by MPs in fish because this organ is the main responsible for the detoxification and inactivation of exogenous molecules (Alomar et al., 2017; Ding et al., 2018). In addition, exposure to xenobiotics also induces changes in the blood parameters of fish, making them suitable biomarkers of exposure to different toxins (Burgos-Aceves et al., 2019; Faggio et al., 2014b; Fazio et al., 2013b; Forouhar Vajargah et al., 2019; Gomes et al., 2015; Ibrahim et al., 2021; Ribeiro et al., 2011). The analyzed biochemical parameters in plasma and liver showed a significant increase after exposure to LDPE-MPs for 90 days compared to the control group. Additionally, all biomarkers from liver and plasma tended to recover during the depuration period, most of them reaching similar levels to those of the control group, suggesting that the *S. aurata* are able to recover their basal physiological status after of one month of detoxification. Furthermore, no significant differences were reported in Fulton's condition factor (Faggio et al., 2014a) when comparing MPs-treatment and control group (results published in Solomando et al. (2020)), suggesting that MPs enriched diet did not affect survival, development and growth of *S. aurata* and, both groups can be compared with respect to the biomarkers determined.

According to (EFSA, 2016), MPs with a size greater than 150 μm will probably not be absorbed and pass from the intestinal cavity to the lymphatic and circulatory system. However, diverse studies have shown that MPs can be transformed into nanoplastics through digestive fragmentation and have even observed few particles of more than 250 μm in the liver of various species of fish, such as *Mugil cephalus*, *Saurida tumbil* and *Danio rerio* suggesting a paracellular route of entry (Abbasi et al., 2018; Avio et al., 2015; De Sales-Ribeiro et al., 2020; Lu et al., 2016). MPs intake and translocation have been reported to induce negative effects in the liver of *D. rerio*, including oxidative stress, inflammation and lipid accumulation (Lu et al., 2016). Also, Ding et al. (2018) reported that the antioxidant enzyme SOD was progressively increasing in the liver of *Oreochromis niloticus* (Linnaeus, 1758) fish throughout a exposure period of 14 days to 0.1 μm PS-MPs. Moreover, Wen et al. (2018) recently found that CAT, SOD and GPx activities increased in liver after 30 days of 500 mg/L PS-MPs exposure in another fish species, *Symphysodon aequifasciatus* (Pellegrin, 1904). Our results are in agreement with these previous observations, since the activity of antioxidant enzymes CAT and SOD but also the GPx and GRd in the liver of *S. aurata* significantly increased throughout the LDPE-MPs exposure period. The increase in the liver activities of antioxidant enzymes occurs in parallel with a progressive increase in the production of ROS in the group exposed to MPs. Similarly, increases in ROS generation in the liver of *D. rerio* exposed to PS-MPs (10 and 100 μg L^{-1}) for 35 days and in *Carassius auratus* exposed to PVC-MPs (0.1 or 0.5 mg/L) for four days were also reported (Romano et al., 2020; Umamaheswari et al., 2020). As in many studies published, besides antioxidant enzymes (CAT, SOD, GPx and GRd), GST activity was analyzed because they usually act in a coordinated manner to antioxidant enzymes in order to ensure the optimal protection against oxidative stress. GST is involved in phase II biotransformation process of xenobiotics, producing a more hydrophilic glutathione conjugate of the exogenous substance helping their elimination (Sureda et al., 2006). In field conditions, Alomar et al. (2017) found a slight increase in GST activity in liver of *Mullus surmuletus* (Linnaeus, 1758) which had ingested MPs, suggesting an induction of the detoxification systems. Similarly, Barboza et al. (2018) observed an increase in liver GST activity of *Dicentrarchus labrax* (Linnaeus, 1758) but in laboratory conditions, fed with a controlled diet containing MPs. In the present study, an increased GST activity was observed in the liver of *S. aurata* during the 90 days of MPs exposure. Our findings, in concordance to the previous studies, suggest that MPs intake induces an important detoxification activity in fish liver in order to avoid their accumulation in the organism (Sureda et al., 2006).

In addition, MDA, a by-product of lipid peroxidation, was analyzed to evaluate the capacity of the antioxidant system to avoid oxidative damage associated with the intake of MPs (Ferreira et al., 2016; Karami et al., 2016). Most of studies evaluating the levels of MDA did not observe significant increase in fish liver after MPs exposure (Barboza et al., 2018; Ding et al., 2018; Fonte et al., 2016; Luís et al., 2015; Oliveira et al., 2013). However, the works by Ferreira et al. (2016) and Solomando et al. (2020) revealed statistically significant higher MDA levels in fish exposed to diets containing MPs than in fish exposed to diets without MPs. In our study MDA content progressively increased in liver until the end of the MPs exposure period. These results suggest that the induction of the antioxidant enzymatic system in *S. aurata* was probably not enough to prevent oxidative damage to liver lipids after 90 days of LDPE-MPs exposure.

Another potential consequence of ingesting MPs is the alteration of the immune system with an impact on fish health and welfare (Espinosa et al., 2018). MPO is a ferrous lysosomal enzyme commonly used as indicator for evaluating the immunity status, since it is involved in the removal of extracellular foreign matters (Chen et al., 2019). Therefore, increases in MPO levels are correlated, in studies of the effects of MPs, with an infiltration of immune cells in the tissues and an activation of the inflammatory response (Jeong et al., 2017; Qiao et al., 2019; Van Der Veen et al., 2009; Von Moos et al., 2012). An in vitro

assay with continuous exposure of head-kidney leukocytes from *S. aurata* and *D. labrax* to polyvinylchloride (PVC) and polyethylene (PE) MPs (1, 10 and 100 mg/mL for 1 and 24 h) resulted in no significant alterations of their innate immune responses (phagocytic and peroxidase activities and respiratory burst) (Espinosa et al., 2018). However, another study from the same research group reported that the feeding of *D. labrax* with PVC and PE MPs (0, 100 and 500 mg kg^{-1}) for three weeks increased the oxidative burst of head kidney leukocytes (Espinosa et al., 2019). Conversely, inflammatory and histological changes in the liver were reported in the freshwater fish *C. auratus* (Linnaeus, 1758) after six weeks of MPs exposure (Jabeen et al., 2018). Likewise, histological analysis of *S. aurata* exposed to LDPE-MPs for 90 days demonstrated an increase in the infiltration of immune cells in liver (Capo et al., 2021). Also, Espinosa et al. (2019) reported that the intake of PE and PVC-MPs produced histopathological lesions in liver, after 21 days of exposure in *D. labrax*. In the present research, we could detect a significant increase in MPO activity in the liver induced by LDPE-MPs exposure. Results from this study seem to suggest that long-term exposure of *S. aurata* to LDPE-MPs could induce oxidative stress and tissue disruption causing an inflammatory response and immune cells recruitment and infiltration, confirming that MPs act as inflammatory agents and stressors in fish (Barboza et al., 2018; Ferreira et al., 2016; Greven et al., 2016).

There is a scarcity of knowledge about the impacts of MPs in blood of fish fed under controlled diets enriched with MPs (Hamed et al., 2019; Iheanacho and Odo, 2020). The use of hematological parameters is a useful tool for monitoring the impact of different environmental pollutants on fish since blood samples can be obtained without sacrificing specimens (Fazio, 2019; Sayed and Authman, 2018). MPs by transcellular route or minor fragments derived from digestion could be absorbed and pass through the intestine and can be distributed to tissues and organs through the circulatory system causing disease, tissue damage and inflammatory responses (Browne et al., 2008; Dawson et al., 2018; Niklasson et al., 2011; Sundh et al., 2010; Sundh and Sundell, 2015). In the present study, no significant effects of the ingestion of MPs were observed in the activities of antioxidant enzymes (CAT, SOD, GPx and GRd) and in the levels of MDA in erythrocytes at none of the analyzed times, indicating the absence of oxidative stress and oxidative damage in these cells. In this sense, erythrocytes are not sensitive enough bioindicators to show the possible effects of the long-term consumption of LDPE-MPs in *S. aurata*.

Plasma has been suggested to reflect the global oxidative stress state of an individual because it receives ROS produced by damaged tissues and activated inflammatory cells (Iheanacho and Odo, 2020; Marrocco et al., 2017).Banaee et al. (2019) investigated the effects of cadmium chloride (Cd) alone and in combination with MPs on *Cyprinus carpio* (Linnaeus, 1758) through plasma biochemical parameters after 30 days of exposure. Results showed significant changes in plasma enzymes activities, acetylcholinesterase, gamma-glutamyl-transferase and alkaline phosphatase, especially when Cd and MPs were combined, indicating a possible disruption in the cell signaling pathways, oxidative stress and tissue damage. Also, in *O. niloticus* exposed to 1, 10, and 100 mg/L of MPs for 15 days, increased activities of SOD, CAT, MDA, total peroxides and oxidative stress indices in blood were reported (Hamed et al., 2020). The present study revealed that CAT and MDA activities in plasma showed a significant and progressive increase in the MPs-treatment throughout the exposure period, reaching a maximum value after 90 days, while the values remained similar in the control group. The findings of the present study suggest that the significant rise of ROS after 90 days of LDPE-MPs exposure induced an antioxidant response which was not enough to avoid the rise in the lipid peroxidation.

Immune status and inflammatory response after LDPE-MPs exposure were measured in plasma of *S. aurata* through LZM and MPO activities respectively. Similarly to oxidative stress biomarkers, the results revealed that MPs exposure for 90 days induced an increase in both biomarkers, while remained unchanged in the control group. Increased LZM activity was noticed in the bivalve *Mytilus galloprovincialis* (Lamarck, 1819) exposed to PS-nanoparticles (50 nm) at 0–50 μg/mL (Canesi et al., 2015). Recently, an assay showed significantly higher LZM levels in *Mytilus coruscus* (Gould, 1861) after 14-day exposure to PS-MPs (Wang et al., 2021). However, there has been little literature available with regard to the effects of MPs exposure on the LZM activity in fish. In *Oncorhynchus mykiss* (Walbaum, 1792) exposed to PS-MPs (100–400 μm) for a period of 4 weeks, no variations of immune system parameters (serum LZM activity and blood immune cells counts) were observed in the systemic circulation (Ašmonaite et al., 2018). Hence, it could not be demonstrated that PS-MPs induced inflammatory responses upon ingestion in this fish species. The observed increase in the activity of LZM in *S. aurata* suggests that this enzyme participates in the immune response to face the presence of LDPE-MP, which gives evidence of the activation of LZM in front of exogenous molecules in the circulatory system (Galloway and Handy, 2003; Jiang et al., 2016; Li et al., 2013). In addition, the activity of MPO as an indicator of immune system activation was also elevated in the MPs-treatment group. MPO produces hypochlorous acid, a strong oxidant, whose primary role is to eliminate the bacteria but also is capable of oxidizing host component contributing to oxidative stress and tissue damage (AshaRani et al., 2009; Mahmoudi et al., 2011). Greven et al. (2016) stated that PS (41.0 nm) and polycarbonate (158.7 nm) nanoplastic particles exposure induced damage in their innate immune system by altering MPO levels and increase in oxidative burst in plasma of *Pimephales promelas* (Rafinesque, 1820). These findings demonstrate that MPs exposure could potentially induce a stress response interfering with innate immune responses in fish.

The depuration period was designed to assess the capacity of fish to recover their basal physiological status when MPs particles were removed from their diet. Interestingly, after a depuration period for 30 days all biomarkers determined in liver and plasma showed a significant decline in the LDPE-MPs exposed group recovering values similar to those of the control group. Results from the recent study published by Solomando et al. (2020) evidenced that after the recovery period of 30 days no MPs were observed in the gut content of *S. aurata*, indicating the ability of fish to eliminate MPs. Moreover, all the biomarkers analyzed (CAT, SOD, GP_x, GSH, MDA, Protein carbonyl contents, GST and MPO) in gut also tended to normalize their values after a one-month depuration period. The exposure of *O. niloticus* early juveniles to 1, 10 or 100 mg/L MPs during 15 followed by 15 of recovery has been found to induce anemia, alterations in blood haemato-biochemical parameters such as increases in serum levels of aminotransferases and creatinine and increased oxidative stress (Hamed et al., 2020). Although the recovery period significantly reduced the levels of MPs, the altered parameters were not fully recovered and only oxidative stress was normalized in the group exposed to 1 mg/L MPs. In the study of Iheanacho and Odo (2020), *Clarias gariepinus* fed a diet containing to PVC particles (0, 0.5, 1.5 and 3.0%) for 30 days, presented a reduction in erythrocyte mean cell volume, cell hemoglobin and neutrophil counts and induced oxidative stress in gills and brain. A depuration period for 45 days allowed the recovery of most of the parameters analyzed. The results from the present study evidenced the capability of *S. aurata* to recover from the situation of oxidative stress and pro-inflammatory state induced by LPD-MPs, when these are eliminated from the diet. While this study shows that *S. aurata* was able to recover normal values after a depuration period of 30 days, future studies are necessary to determine the accumulation and depuration kinetics of MPs in fish tissues, in order to increase the knowledge on the physiological impacts of MPs after a long-term exposure (Paul-Pont et al., 2016; Ribeiro et al., 2017).

5. Conclusion

Our research provides new insights in assessing the impact of LDPE-MPs on *S. aurata* physiological response in liver, erythrocytes and

plasma after a long-term exposure followed by a depuration period. The MPs-enriched diet induced oxidative stress and a pro-inflammatory status in liver and in plasma after 90 days of intervention, whereas no evidence of effects was observed in erythrocytes. These results suggest that some of the MP particles or fragments resulting from digestion may pass through the enterocyte cells, enter the circulatory system and reach the liver, inducing a stressful situation. However, additional studies are necessary to confirm the route of entry and subsequent distribution. In addition, the changes evidenced in plasma may reflect the global effects of MPs on the organism and could be a suitable bioindicator of chronic MPs exposure. Furthermore, after a 30-day purification period, the different biomarkers analyzed tend to normalize, suggesting the ability of *S. aurata* to regain their normal physiological situation. Nonetheless, further research is needed to provide information on the intake and translocation of MPs and its potential long-term effects.

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CRediT authorship contribution statement

Antònia Solomando: Writing - original draft, Methodology, Investigation, Formal analysis. Xavier Capó: Methodology, Investigation. Carme Alomar: Conceptualization, Methodology, Investigation. Montserrat Compa: Methodology, Investigation. José María Valencia: Conceptualization, Methodology, Investigation Antoni Sureda: Conceptualization, Methodology, Investigation, Project administration. Salud Deudero: Conceptualization, Methodology, Project administration, Funding acquisition. All authors participated in the final writing - review & editing of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147479.

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