Accepted manuscript with doi: https://doi.org/10.1016/j.scitotenv.2021.144976

Long-term exposure to virgin and seawater exposed microplastic enriched-diet causes liver oxidative stress and inflammation in gilthead seabream *Sparus aurata,* Linnaeus 1758.

Capo, X[1]; Company, JJ[1]; Alomar, C[1]; Compa, M[1]; Sureda, A[2,3]; Grau, A[4]; Hansjosten, B[4]; López-Vázquez, J[5]; Quintana, JB[5]; Rodil, R[5]; Deudero, S[1].

1- Instituto Español de Oceanografía, Centro Oceanografico de Baleares, Muelle de Poniente s/n, 07015, Palma de Mallorca, Balearic Islands.
2- Research Group in Community Nutrition and Oxidative Stress, and Health Research Institute of Balearic Islands (IdISBa), University of Balearic Islands, E-07122, Palma de Mallorca, Balearic Islands, Spain.
3- CIBEROBN (Physiopathology of Obesity and Nutrition), University of the Balearic Islands, E-07122, Palma de Mallorca, Balearic Islands, Spain.
4- Laboratorio de Investigaciones Marinas y Acuicultura, LIMIA-Govern de les Illes Balears, Port d'Andratx, Balearic Islands, Spain.
5- Department of Analytical Chemistry, Nutrition and Food Sciences, Institute of Research on Chemical and Biological Analysis (IAQBUS), Universidad de Santiago de Compostela, Constantino Candeira S/N, 15782 Santiago de Compostela, Spain

Correspondence

Xavier Capó Fiol
Instituto Español de Oceanografía, Centro Oceanografico de Baleares, Muelle de Poniente s/n, 07015, Palma de Mallorca, Balearic Islands.
Email: xavier.capo@ieo.es

Abstract

Plastics accumulation in marine ecosystems has notable ecological implications due to their long persistence, potential ecotoxicity, and ability to adsorb other pollutants or act as vectors of pathogens. The present work aimed to evaluate the physiological response of the gilthead seabream (*Sparus aurata*) fed for 90 days with a diet enriched with virgin and seawater exposed low-density polyethylene microplastics (LDPE-MPs) (size between 100-500 μM), followed by 30 days of depuration, applying oxidative stress and inflammatory markers in liver homogenates. No effects of LDPE-MPs treatments on fish growth were observed throughout this study. A progressive increase in antioxidant enzyme activities was observed throughout the study in both treatments, although this increase was higher in the group treated with seawater exposed MPs. This increase was significantly higher in catalase (CAT), glutathione reductase (GRd), and glutathione-s-transferase (GST) in the seawater exposed MPs group, with respect to the virgin group. In contrast, no significant differences were recorded in superoxide dismutase (SOD) and glutathione peroxidase (GPx) between both groups. Exposure to MPs also caused an increase in the oxidative damage markers (malondialdehyde and carbonyls groups). Myeloperoxidase activity significantly increased because of MPs treatments. After 30 days of depuration, antioxidant, inflammatory enzyme activities and oxidative damage markers returned to values similar to those observed in the control group. In conclusion, MPs exposure induced an increase of antioxidant defences in the liver of *S. aurata.* However, these elevated antioxidant capabilities were not enough to prevent oxidative damage in the liver since, an increased oxidative damage marker was associated with MPs ingestion. The treatment with seawater exposed MPs caused a more significant antioxidant response (CAT, GRs, and GST). Although after a depuration period of 30 days a tendency to recover the initial values of the biomarkers was observed this does not seem to be time enough for a complete normalization.

Keywords

Microplastics, Pollution, Oxidative stress, Inflammation, *Sparus aurata*

1. Introduction

Nowadays, marine litter contamination is one of the most relevant pressing issues affecting numerous aquatic and marine ecosystems worldwide(UNEP., 2009). Marine litter encompasses elements of different compositions: metals, glass, paper, cloth or plastic, but among these, plastic is considered the most persistent and problematic, representing up to 80-85% of the marine litter present in the oceans (Andrady, 2011). In this sense, the amount of plastic released into marine environments is increasing considerably each year (Andrady, 2011; Boteler, 2017; UNEP., 2009). Microplastics (MPs) are plastic particles with a diameter smaller than 5 mm (Andrady, 2011; Cózar et al., 2014). MPs can be classified as primary MPs (that are expressly manufactured with a size less than 5 mm in diameter, commonly used to produce pellets, cosmetics personal care and household products, synthetic fibres from washing clothes, etc.), and secondary MPs (plastics derived from the fragmentation of larger plastics into smaller particles in the marine environment due to the action of erosive agents, including the combination of ultraviolet radiation, the abrasive force of waves and temperature, etc.)(Andrady, 2011; Cózar et al., 2014).

MP can be made up of a variety of compounds such as nylon, polypropylene (PP), polyethylene terephthalate (PET), polyethylene (PE), bisphenols, or polyvinyl chloride (PVC) and can be toxic for marine organisms due to contaminants like phthalates and bisphenols added during their manufacturing processes (Hidalgo-Ruz et al., 2012; Rochman et al., 2013; Vert et al., 2012). Also, MPs consist of long hydrophobic chains, which in aquatic and marine environments, can accumulate several pollutants present, at water in higher concentrations than in the sediments (Andrady, 2011). Furthermore, due to its physicochemical properties, MPs can accumulate several environmental pollutants in their surface, which can potentially increase their toxicity across the food web (Rochman et al., 2013; Yuan et al., 2020). Consequently, recent studies have identified that MPs can accumulate on their surface several substances such as heavy metals, polycyclic aromatic hydrocarbons, antibiotics, aromatic and aliphatic compounds, etc (Zhang et al., 2020). Due to their small size, MPs particles can be ingested or filtrated by a wide range of marine organisms, and for this reason, MPs can enter the food web of marine ecosystems (Au et al., 2017; Gallo et al., 2018; Li et al., 2019). Moreover, considering their size, colour and buoyancy, MPs can be ingested by many fish species due to their similarity to preys (Güven et al., 2017; Jovanović, 2017). In addition, MPs can be partially digested and their degradation products can be absorbed and distributed throughout the organism and incorporated into different tissues or cells (Barboza et al., 2018). MPs or their derivates can cause adverse health effects in marine organisms, including metabolic disorders,

neurotoxicity, hepatic stress, oxidative stress, inflammation, decreased growth, blockage of feeding appendages, behaviour alterations, obstruction of the gastrointestinal tract or cause pseudo-satiation resulting in reduced food intake (Barboza et al., 2018).

Most pollutants, including MPs, together with additives or other adsorbed elements, accumulated in marine organisms can induce the overproduction of reactive oxygen species (ROS) (Alomar et al., 2017; Barboza et al., 2018; Sillero-Rios et al., 2018; Sureda et al., 2018). If the excess of ROS is maintained along the time, an oxidative stress situation is established that can induce oxidative damage to cellular components, as DNA, proteins and lipids (Livingstone, 2001). Oxidation of biomolecules generates several end products, as malondialdehyde (MDA), carbonyl derivates, and 8-hydroxy-2-deoxyguanosine, that can be used as biomarkers of oxidative stress (Bartoskova et al., 2013; Box et al., 2007). ROS overproduction can disrupt vital functions, altering mitochondrial function and inducing apoptosis (Halliwell, 2007; Regoli et al., 2004). However, all organisms, including fishes, have developed a complex network of antioxidant and detoxifying mechanisms to scavenge or avoid ROS generation, detoxify pollutants and repair or remove damaged molecules (Barboza et al., 2018; Livingstone, 2001). This antioxidant system includes enzymatic and non-enzymatic antioxidants. Enzymatic antioxidant mechanisms include enzymes such as superoxide dismutase (SOD), which eliminates superoxide anion; catalase (CAT) and glutathione peroxidases (GPx) that remove hydrogen peroxide; and glutathione reductase (GRd) that regenerates glutathione (GSH), an essential non-enzymatic antioxidant. Glutathione-*s*-transferase (GST) is a phase II detoxification enzyme that contributes to eliminate xenobiotic compounds and oxidation products conjugating toxic compounds with glutathione, and producing more hydrophilic and less toxic compounds (Cunha et al., 2005; Sureda et al., 2006).

The gilthead seabream (*Sparus aurata* Linnaeus, 1758) is a common species in the Mediterranean Sea, although it is also present along the eastern Atlantic coasts from Great Britain to Senegal. It is a highly appreciated and consumed fish in Southern Europe, and, in this sense, gilthead seabream has experienced an increased growth in the aquaculture industry (FAO, 2001-2020). In addition, this species is one of the most used model fish species in aquaculture studies and in the natural environment. *S. aurata* is also often used as a model in bioaccumulation studies (Grigorakis, 2007),as a bioindicator for toxicity(DelValls et al., 1998; Espinosa et al., 2017),and endocrine studies (Forner-Piquer et al., 2018). Moreover, *S. aurata* is a

very voracious predator in the wildlife, which suggests that it could bioaccumulate MPs or other pollutants (Balart et al., 2009).

Nowadays, fishing resources are limited and are being overexploited. For this reason, aquaculture is a growing industry supplying the world's increasing demand for seafood (FAO, 2020). According to data from the Food and Agriculture Organization (FAO) of The United Nations (UN), about 82.1 million tons of aquatic animals were produced by aquaculture (FAO, 2020). This data gives an idea of the significant impact aquaculture has in the world. Considering this, fish growing in aquaculture facilities are exposed to the pollutants present in the sea but also from the infrastructure itself. Plastic materials have surrounded these fishes throughout their life cycle as most of the materials used in aquaculture facilities are plastic derived: from the fattening farms, juvenile tanks, nets, ropes, pipes, buoys and the structures that form the cages (Wu et al., 2020; Zhu et al., 2019). Moreover, the continuous maintenance and cleaning to avoid fouling organisms on aquaculture gear (nets, ropes, cages, etc..) also contributes to the release of fibres and particles in the form of microplastics into the environment (FAO, 2020).

Consequently, the first objective of this study was to determine long term physiological effects in reared *Sparus aurata* caused by the ingestion of MPs and assessed throughout oxidative stress and inflammatory markers determined in fish liver. The secondary objective was to assess differential biochemical response to pure polyethylene, compared to seawater exposed polyethylene enriched diets. Additionally, the study also aimed to evaluate the effects of 30 days depuration period on the biochemical response of fish liver.

2. Material and Methods

2.1 Diet preparation

For the experiment, three different types of diets were formulated: 1) a control diet (90% food and 10% filler), 2) a diet with virgin MPs (90% food and 10% LDPE-MP) and 3) a diet with seawater exposed MPs (90% food and 10% LDPE-MP previously exposed to seawater for 2 months in an area of high anthropic impact (39º32'38.04" N 2º22'50,97"E). LDPE-MPs 022 pellets f were purchased from a Spanish distributor, Repsol. This plastic was additive free.

Seawater exposed MPs were stored in nylon nets, hung from the aquaculture cages located in the Port of Andratx and submerged at a depth of 0.5-1m from the sea surface. Weekly, the nets were manually shaken to remove any large biofouled organisms. After two months, the pellets were removed from the nets, cleaned with distilled water and oven-dried at 60ºC for 48 h. The ingredients for the control treatment consisted of 37 g of fishmeal LT94, 9.2 g wheat gluten, 21.5 g soybean meal, 8.3 g fish oil, 0.9 g soy lecithin, 9.1 g wheat flour, 0.5 g choline chloride, 0.1 g vitamin C, 0.9 g vitamin and mineral mixes, 1.9 g binders (½ guar gum + ½ carboxymethyl), 0.5 g amino acid mixture, mixture (methionine-lysine, 1: 1) and 10 g filler (sepiolite-cellulose-guar gum, 1:1:1) in 100 g dw. LDPE pellets for the virgin and seawater exposed MPs enriched diet treatments were dipped in liquid nitrogen and ground. After grinding, plastics were sieved in pre-cleaned stainless-steel metal sieves to collect fragments smaller than 0.5 mm. Additionally, LDPE pellets (thereafter MPs) were ground into 500 - 100 µm fragments, using conical separation grinders for conventional use. MPs obtained were mixed with the same ingredients as the control diet, but for 100g of food, 10 g of it were substituted with 10 g of virgin and seawater exposed MPs.

2.2 Pollutants detection

A subsample of seawater exposed MPs was collected from the harbour after two months submerged in the water. Pollutants present in MPs (both virgin and seawater exposed) were determined by gas chromatography coupled to quadrupole-time-of-flight mass spectrometry (GC-QTOF) following standardized procedures. In order to decrease background contamination of phthalates, all glass material were baked at 300ºC for 12 hours, 3% (w/w) alumina was added to ethyl acetate (Worsfold, 2019). Next, 0.2 g of MPs seawater exposed pellets were sonicated with 2 mL of ethyl acetate for 30 min at room temperature in vials covered with aluminium foil. The ethyl acetate was transferred to a new vial and the

extraction was repeated with a fresh 2 mL of ethyl acetate aliquot. The 4 mL of solvent was mixed and evaporated under a nitrogen stream and the dry residue was redissolved in 200 µL of ethyl acetate. An aliquot of ethyl acetate extracts was derivatized with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, 60:40) at 60ºC for 1 hour. One microliter of each extract was injected in split less mode using an Agilent 7693B series (Agilent Technologies, Santa Clara, CA, USA) into the gas chromatograph 7890A interfaced with a 7200 QTOF mass spectrometer (Agilent Technologies). The separation was performed in aHP-5MS column (Agilent Technologies J&W, 30 m x 0.25 mm x 0.25 µm) with helium (99.9999%, Nippon gases, Madrid, Spain) as carrier gas at a flow rate1 mL/min. The temperature programme startedat 50ºC for 1 minute, then ramped until 290ºC at 10ºC/min and held for 15 minutes. The acquisition was performed in electron impact ionization (EI) mode at 70 eV with an emission current filament set at 5 µA. QTOF MS was operated at 5 spectra/s in the mass range 40 to 1000 Da and the resolution was about 3714 FWHM (full width half maximum) at 68.9947 m/z and 7875 FWHM at 501.9708 m/z. Mass calibration was performed each three samples, in accordance with the recommendation of the manufacturer, using perfluoro tributyl amino (PF-43).

A suspect screening was performed by comparison of MS spectra for each feature with those of spectral libraries. The libraries used for data processing were (1) an in-house empirical high-resolution pollutants library (356compounds in total, 236 underivatized compounds and 120 derivatized compounds),(2) a commercial high-resolution library of pesticides containing 844 compounds supplied by Agilent, (3) a low-resolution plastic additives composed of 626 compounds created as of the information obtained from the European Chemicals Agency (ECHA, 2020)and AccuStandard pages (Bolgar, 2015) and (4) library NIST.17(306,622 compounds).

2.3 Experimental procedure

The experimental procedure was carried out at the LIMIA (Laboratorio de Investigaciones Marinas y Acuicultura) in Andratx (Mallorca, Balearic Islands) laboratory facilities between June 2018 and February 2019. A total of 2000 specimens of cultured alevine of*S. aurata* of about 3 g were allocated into a large 2000 L seawater tank and left to acclimate for 4 months prior to commencing the experimental procedure. After the acclimation period, 600 alevins of *S. aurata* (7 months old, total length 11.8 ± 0.3 cm and weight 44.9 ± 3.1 g) were randomly assigned to 12 tanks of 1000 L with an equal stocking rate of 100 fish per tank. Tanks were located inside the laboratory and were protected from climate and weather, ensuring the

same physical and chemical conditions in each tank. The photoperiod was of 16 h light / 8 h dark cycle. There was no difference in the average daily temperature between the tanks, and it was maintained at 19 ± 2 °C, with a water flow rate of ~1L/minute to ensure 1.5 water-cycles per day, and the oxygen concentration was of 5.9-6.1 ppm. Three different diet treatments were applied: control diet (Control) without MPs, virgin MPs diet enriched with 10% MPs, and seawater exposed MPs diet (enriched with 10% of seawater exposed MPs). Three replicate tanks were randomly assigned to each treatment. The experimental procedure lasted 120 days, including a 90 days exposure period in which the MPs treatment group was exposed to a diet enriched in MPs and followed a 30 days depuration period. During the depuration period, all tanks were fed with the same original control diet. During the exposure, feeding of fish was done twice daily at a 2% body weight rate $\cdot day^{-1}$ using an automatic feeder (FIAP Belt Feeder with clock (12 hours).

2.4 Fish sampling

For each sampling period (T_0, T_{30}, T_{60}, T_{90} and T_{120}) and treatment, 9 randomly selected fish (3 from each tank) were sampled after a period of 24 h of feed deprivation from each of three treatments. Individuals from each treatment were randomly caught using a hand net, placed into a smaller tank and anesthetized with tricaine methanesulfonate/MS-222 (1g in 10 L of water) to minimize stress. Individuals were sacrificed by decapitation and immediately dissected. From each fish, the liver was placed into a microtube and stored individually at −80 °C until further processing. One liver slice of each fish was stored at room temperature in 10% phosphate-buffered formalin until histological analysis. The total weight (g) and the total length (cm) of each individual were recorded at 0, 30, 60 90 and 120 days of the beginning of the experiment. To investigate the possible effect of MPs ingestion on fish's fitness or physical condition, Fulton's condition factor (CF) was calculated (Nash et al., 2006). CF was subsequently calculated individually (total weight in g / (total length in cm)^3) x 100.

2.5 Histological analysis

Portions of liver which had been fixed in 10%(w/v) phosphate-buffered formalin, were dehydrated using a graded series of ethanol solutions and cleared with X-Free® (Bio-Optica), embedded in paraffin wax at 60ºC and sectioned at 3-4 μm thickness, using a rotation microtome (MICROM HM330). One section of each sample was taken and stained with Mayer's Hematoxylin and Eosin (H&E). All sections were then

mounted using Eukitt ® (Sigma-Aldrich), and observed under a light microscope (Olympus BX51) with an attached photographic camera (Olympus DP20).

2.6 Gastrointestinal tract analysis for microplastic ingestion

Gastrointestinal tract samples were used to determine MPs ingestion. All instruments were cleaned with acetone previously before the start of each analysis. To isolated MPs in fish's gastrointestinal tract, tissues were dissolved in 10% of potassium hydroxide(KOH) (20 mL of KOH / g tissue) as described by Dehaut and collaborators (Dehaut et al., 2016). The chemical digestion took place during 48-96 h at room temperature inside a fume hood. The digested solution was filtered through polycarbonate membrane filters (20.0 µm, diameter 47 mm) and left to dry at room temperature for 24 h. Filters were observed under the stereomicroscope for microplastic identification. Given the high number of plastic pellets observed in the filters, the ingestion of MPs by *S. aurata* was assessed by applying a Covering Index following the Braun-Blanquet system (Boudouresque, 1971). For this, the filter was divided into quadrants and the percentage coverage of the quadrants by MPs was estimated (microplastic ingestion index).

2.7 *Sample preparation for biochemical determinations*

Around 0.15 g of liver sample was 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). Each homogenate was briefly sonicated (2–3 s) using an ultrasonic processor. Homogenates were centrifuged at 9000 ×g, 10 min, 4 °C and supernatants were recovered and used for biochemical assays. All biochemical analyses were normalised per mg of total protein, measured with a commercial kit (Biorad®), using bovine serum albumin as a standard.

2.8 Enzymatic activities

Antioxidant (CAT, SOD, GRd and GPx), inflammatory (Myeloperoxidase (MPO)) and detoxification (GST) enzymatic activities were measured with a Shimadzu UV-2401 PC spectrophotometer at 25°C. CAT activity was measured following the method described by Aebi (Aebi, 1984). GRd activity was measured by a modification of the Goldberg and Spooner's spectrophotometric method (Goldberg DM Spooner R. , 1984). GPx activity was determined using the method described by Flohe (Flohe and Gunzler, 1984).

Measurements were recorded at a wavelength of 550 nm. SOD activity was determined by the degree of inhibition of the reduction of cytochrome C through the superoxide anion generated by the xanthine oxidase/hypoxanthine system (Flohe and Otting, 1984). MPO activity was measured by guaiacol oxidation, following the method described by Capeillere (Capeillère-Blandin, 1998). GST activity was determined at 314 nm, using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Habig et al., 1974).

2.9 Malondialdehyde determination

MDA levels, a marker of lipid peroxidation, were analysed using a colorimetric assay based on the reaction of MDA with a chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm. Briefly, samples or standards were placed in polypropylene tubes containing n-methyl-2-phenylindole (10.3 mM) in acetonitrile:methanol (3:1). Then, HCl (12 N) was added, and the samples were incubated for 1 h at 45°C. The absorbance was measured at 586 nm. MDA concentration was calculated using a standard curve of known concentration.

2.10 Protein carbonyls derivates

Protein carbonyl derivatives were measured in homogenate supernatants by an adaptation of the method Levine et al. (1994) (Levine et al., 1994). Samples were deproteinised with metaphosphoric acid. Precipitates were resuspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37°C. Then, samples were precipitated with 20% trichloroacetic acid, and centrifuged for 10 min at 1000 g and 4°C. The precipitate was washed twice with ethanol:ethyl acetate (1:1). Precipitated were resuspended with Guanidine 6 M in phosphate buffer 2 mM, pH 2.3. The concentration of carbonyl groups was calculated from the absorbance at 340 nm using the value of 22000 M^{-1} cm^{-1} for the molar absorption of aliphatic DNPH derivatives. Samples were analysed against a blank of guanidine solution.

2.11 Statistical analysis

In order to study differences in the physiological response of *S. aurata* to a MPs enriched diet, a two-way analysis of variance (ANOVA) was performed for each biomarker. The two analysed factors were the diet treatment (control diet, virgin MPs diet and seawater exposed MPs diet) and the time of MPs exposure (T_0,

T_{30}, T_{60}, T_{90} and T_{120}). Normality of distribution and equality of variance were evaluated by Shapiro-Wilk test and Levene's test, respectively. Least significant difference t-test (LSD) post hoc tests were conducted to determine the statistical differences of biological parameters between control and treatment (Solomando et al., 2020). Differences between treatments and times were considered significant at P value < 0.05. The results were presented as mean ± standard error of the mean (S.E.M.). Statistical analyses were carried out using the SPSS statistical analysis package (version 25.0).

2.12 Ethics statement

Care, maintenance, handling and sampling of fish was carried out following the protocols previously established by LIMIA, according to legal qualifications, in strict accordance with the recommendations from the Directive 2010/63/UE, and in compliance with the Spanish law (RD53/2013, BOE n. 34 February 8th, 2013). All experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of the Balearic Islands (Reference CEEA 96/05/18).

3. Results

3.1 Biometric parameters

All fish maintained an adequate state of health with no signs of any specific disease, and no mortality was observed at any time during the experimental period. No significant differences ($p \geq 0.05$) were observed between total fish length (11.9±0.23 cm at T_0, 13.2±0.25 cm at T_{30}, 14.1±0.18 cm at T_{60}, 15.3±0.15 cm at T_{90} and 16.9±0.23 cm at T_{120}) and total weight (44.9±2.6 g at T_0, 64.8±3.11 g at T_{30}, 81.9±3.5 g at T_{60}, 102 ±18.1g at T_{90} and 128±4.9 g at T_{120}) among the three treatment diets and throughout the experiment. No differences (Freedom degrees (3, 46) F=1.21 p=0.315) in Fulton's Condition factors (CFs) were observed between T_0 and T_{120} (2.59±0.03 at T_0, 2.68±0.05 in the control group at T_{120}, 2.63±0.05 g at T_{120} in virgin MPs group and 2.58±0.05 at T_{120} in MPs seawater exposed group.

3.2 Pollutants analysis

The presence of compounds adhered in seawater exposed MPs is presented in table 1. A total of 31 compounds were detected in MPs after 2 months of seawater exposure in an area characterized by high anthropic impact. Of these compounds, only 13 of these compounds were identified in the original virgin MPs. There was a wide variety of compounds, including phthalates, chemical antioxidants, fatty acids, personal care products, benzenes, surfactants, and phenols.

3.3 Microplastic ingestion

Table 2 shows the gastrointestinal MPs contents. No plastic ingestion was observed in the control group throughout the study. However, a progressive and significant increase (Freedom degrees (12, 224) F=19.454 p=0.00) was observed in MPs treatments, virgin and seawater exposed MPs, at times T_{30}, T_{60} and T_{90}. Both MPs treatments showed a higher plastic ingestion after 90 days of MPs exposure (42.6±6.8% in the virgin MPs group and 44.9±6.8% in the seawater exposed MPs group). After the 30-day depuration period, at which MPs were eliminated from the diet of both MPs treatments, plastic ingestion was not observed in the gastrointestinal tract content analysis.

3.4 Histological analysis

No liver lesions were observed in any of the fish exposed to MPs enriched diets, nor in the control fish (Fig. 1 A, B, C) nor in those subjected to treatments (Fig. 1C, D, F, G). Figure 1 shows liver histological samples at T_0, T_{90} and T_{120} in the three different treatments. At T_{90}, control samples (Fig. 1B) did not show significant damage compared to T_0 samples (Fig. 1A), but both MPs treatment caused an increase in the number of lymphocytes and eosinophils. These were located around pancreatic islets (Fig.1H), although lymphocytes were mainly observed around bile ducts in the epithelium of which rodlet cells were also detected (Fig. 1I). Likewise, no structural damage was seen in any of the treatments at T_{120} (Fig. 1 E, F, G), observing the same histological findings as in the T_{90} exposure.

3.5 Biochemical determinations

Biomarkers of oxidative stress and detoxification mechanism after MPs exposure measured in the liver of *S. aurata* are presented in Figure 2. In general, a progressive increase was observed in antioxidant enzyme activities due to MPs treatment (both virgin and seawater exposed MPs) throughout the study, reaching a maximum value at T_{90}. However, this increase was slightly higher in seawater exposed MPs. CAT activity remained stable throughout the study in the control group. However, a progressive increase in CAT activity was observed in the MPs-treated groups. In $T_{30,}$ both virgin and seawater exposed MPs significantly increase CAT activity in fish liver compared to T_0 and control T_{30}. However, this increase was significantly higher in seawater exposed MPs both in T_{60} and T_{90}. It is interesting to highlight a decrease in CAT activity in the seawater exposed MPs group in $T_{120}$with respect to T_{60} and T_{90} (Fig. 2A). SOD activity (represented in Figure 2B) followed a similar pattern as the response of CAT activity throughout the study. In this case, no differences in SOD activity throughout the study were observed within the control group. MPs treatment, both virgin and seawater exposed, caused an increase in SOD activity throughout the study after T_{60}. GRd enzyme activity was modulated by MPs, and GRd activity in the control group remained stable throughout the study. Seawater exposed MPs caused a significant increase in GRd activity at T_{30}, T_{60}, T_{90} and T_{120}, in comparison to the control group. Fish from the virgin MPs treatment only showed a significant increase of GRd activity in T_{90} (Fig. 2C). MPs treatment causes an increase in GPx activity both in virgin and seawater exposed groups. This increase was significant from T_{60} to T_{120} in both treatments (Fig.2D).

GST activity as a marker of detoxification is presented in Figure 3. No variations in GST activity in the control group were detected throughout the study. However, MPs supplementation significantly increased GST activity in fish liver. In this sense, both virgin and seawater exposed MPs caused an increase in GST

activity with respect to the control in T_{30}, T_{60}, T_{90} and T_{120}. In addition, GST activity in T_{60} and T_{90} was significantly higher than GST activity in T_0, in both treatments. GST activity in virgin MPs at T_{120} decrease in comparison to T_{60} and T_{90}. However, this decrease was not observed in the seawater exposed MPs group.

MPO activity in liver homogenate supernatants, a marker of inflammation, is represented in Figure 3. MPO activity did not change throughout the study in the control group. However, exposure of fish to virgin and seawater exposed MPs caused an increase in MPO activity. Different responses between the two treatments were observed. While, virgin MPs treatment causes a significant increase in MPO activity at T_{30}; MPO activity decreased at T_{60}, T_{90} and T_{120} with respect to T_{30}. On the other hand, MPO in seawater exposed MPs reached its highest value at T_{60}, remained stable at T_{90} and decreased at T_{120}.

The effects of MPs in liver oxidative damage markers (MDA and Carbonyl protein derivates) are indicated in Figure 4. MDA levels remained stable throughout the study in the control group. MPs ingestion increased MDA levels in both virgin and seawater exposed treatments. Specifically, MP intake induced an increase in MDA at T_{30} with respect to T_0; this increase was also significant in T_{60} with respect to T_0 and the control group at T_{60}. However, no differences between virgin MPs and the control group were detected between T_{90} and T_{120}. Both MPs treatment causes an increase in carbonyls protein derivates. An increase in carbonyl protein derivates was also observed in the control group at T_{90}, although its values remained consistently lower than for both MPs treatments. Virgin and seawater exposed MPs caused an increase in carbonyl protein derivates at T_{60} and T_{90}, and these values significantly decreased at T_{120}.

4. Discussion

The amount of plastics released to the seas and oceans is a growing problem and with a complex solution in the short term due to society's significant dependence on these materials. It was estimated that in 2017 near 8 million tons of plastics were discarded into the sea, and it is believed that this amount could increase year by year (UNEP. 2009; Boteler 2017). Risks derived from MPs come from the material itself and their high persistence in the environment, as well as from the chemicals and pollutants adsorbed to the surface (Rochman et al., 2013).

The effects of ingestion of MP in marine organisms have been widely studied; however, there is a lack of knowledge about the effects at the molecular level of long-term MPs intake as well as the effects derived from a depuration period. A growing number of studies mostly report a food-borne exposure to MPs within a great diversity of marine organisms from plankton (Cole et al., 2013), to bivalves (Berglund et al., 2019), crustaceans (Hara et al., 2020), fish (Alomar et al., 2017; Mancia et al., 2020), marine turtles (Duncan et al., 2019) and cetaceans (Lusher et al., 2015). The present results showed that a 90-day exposure to a diet enriched with MPs significantly increases the plastic content in the gastrointestinal tract of *S. aurata*. In addition, the 30 day of depuration period reduces MPs content in the gastrointestinal tract.

There is growing scientific evidence supporting the presence of pollutants, including MPs, which can cause ROS overproduction and induce an oxidative stress situation in the exposed organisms (Alomar et al., 2017; Capo et al., 2015b; Sureda et al., 2018). The present study evaluated the possible toxic effects in the liver of *S. aurata* exposed for 90 days to a diet enriched with virgin MPs and MPs which had been exposed for two months to seawater in a busy and impacted harbour (seawater exposed MPs). Additionally, the effects of a 30-day depuration period were also evaluated to determine whether the adverse effects of MPs intake could be reversible. All organisms are provided with antioxidant and detoxifying mechanisms to prevent deleterious effects associated with pollutants and excessive ROS production (Barboza et al., 2018; Livingstone, 2001). In this sense, it has been hypothesised that variations in this antioxidant, and detoxifying mechanism can be used as biomarkers of aquatic and marine pollution (Capo et al., 2015b; Sureda et al., 2011). The current results evidenced a progressive increase in the activities of CAT, SOD, GPx and GRd in fish liver as a consequence of the exposure to a diet enriched with MP, both virgin and seawater exposed. The results are in agreement with biochemical determinations quantified in the liver of other species as it is the case of red tilapia which was fed with a diet enriched with 1,8 x 10^8MPs particles

per mL for 14 days (Ding et al., 2018), zebrafish (*Danio renio*, Hamilton, 1822) (Lu et al., 2016) and the sea bass (*Dicentrarchus labrax*) fed with a diet enriched with between 0,25-0,69 mg of MPs per L for 96 hours (Barboza et al., 2018). An increase in SOD activity was also found in *O. niloticus* liver samples after an exposure to 0.1 µm polystyrene MPs for 14 days (Ding et al., 2018). Similarly, an increase in liver CAT and SOD activities has been reported in *D. renio* after 7-day exposure to5 µm polystyrene-MPs (Lu et al., 2016). In the same line, a study performed with sea bass observed an increase in muscle and brain oxidative damage after ingestion of MPs at 0.25-0.69 mg/L, during 96 hours (Barboza et al., 2018). On the other hand, studies performed with *D. labrax* after 3 weeks of PVC-MPs supplementation did not show an increase in antioxidant enzymes (Espinosa et al., 2019). The absence of differences in antioxidant enzymes was also observed in striped red mullet (*Mullus surmuletus;* Linnaeus, 1758) when comparing fish with MPs present in their digestive tract to fish without MPs (Alomar et al., 2017). However, all these studies were carried out within short periods of exposure to MPs, with a wide range of MPs concentrations, or with samples collected from the wild, while our study consisted of 90-day MPs exposure. Our results show that antioxidant (CAT, GRd and GPx) and detoxification (GST) activities are slightly higher in seawater exposed MPs, treatment in comparison to virgin MPs. In this sense, a previous study has reported an increase in the toxicity response of fish species as a consequence of chemicals adsorbed on the surface of plastics (Rochman et al., 2013). In this work, several compounds were found adhered to the MPs after two months exposed to seawater in an area of high anthropogenic impact, and between these compounds a wide variety of compounds as plastic, food and paint additives, aromatic hydrocarbons and industrial antioxidants were detected. The presence of these compounds in seawater exposed MPs is a consequence and indication of anthropic pressure in marine environments, which is particularly high in port areas (Buruaem et al., 2012; Solis-Weiss et al., 2004). These results are in accordance with several studies that have detected several compounds as metals, organic pollutants, or polycyclic aromatic hydrocarbons sorbing and accumulating on the surface of marine MPs, typically in concentrations higher than those present in the marine environment (Rios et al., 2007; Rochman et al., 2013; Rochman et al., 2014).

MDA is a biomarker widely used to determine lipid oxidative damage (Capo et al., 2015b; Sureda et al., 2011). A significant increase in liver MDA levels was found after the MPs exposure, and it is interesting to highlight that this increase was more evident in the seawater exposed MPs group. Similarly, carbonyl protein derivates are used to indicate oxidative protein damage (Capó et al., 2020). We have evidenced a considerable increase in liver protein damage as a consequence of MPs exposure; however, this increase

438 was only significant from T_{60}, both in virgin and seawater exposed treatments. These results are in
439 accordance with previous studies (Alomar et al., 2017; Barboza et al., 2018; Ding et al., 2018) suggesting
440 that the increase in antioxidant defences observed after MPs ingestion was not enough to prevent oxidative
441 damage in *S. aurata* liver.

442 Several studies have demonstrated a direct relationship between pollutants and inflammation (Auffret,
443 1988; Bayne and Lowe, 1985), and it has also been observed that MPs can induce inflammation and immune
444 system alterations (Brown et al., 2001). MPO is a pro-inflammatory enzyme located in neutrophils, and its
445 primary function is to produce hypochlorous acid, with antimicrobial activity, which is released into
446 extracellular space during degranulation (van der Veen et al., 2009). It is evidenced that an excessive MPO
447 activity is related to an increase in oxidative damage and inflammation (Capo et al., 2015a; van der Veen
448 et al., 2009). An increase in MPO activity in tissues is directly related to immune cell infiltration and
449 inflammation (Solomando et al., 2020). Our results showed an increase in MPO activity as a consequence
450 of MPs exposure, suggesting that MPs induces an inflammatory response in *S. aurata* liver. Our results are
451 in accordance with previous studies evidencing that microplastics exposure induces an inflammatory
452 response in *S. aurata* and *D. renio* gut (Qiao et al., 2019; Solomando et al., 2020), in copepods (Jeong et
453 al., 2017) and bivalves gills (von Moos et al., 2012).

454 MPs ingestion not only causes oxidative stress but also can causes toxicity (Barboza et al., 2018; Rochman
455 et al., 2013; Rochman et al., 2014). Toxic molecules should be rapidly metabolized and/or excreted by
456 organisms in order to avoid its accumulation in their body (Sureda et al., 2006). The metabolization of toxic
457 compounds is carried out in two phases. In the first phase, compounds are metabolized by cytochrome P450
458 system, which catalyses the addition of an oxygen atom to the molecule; in the second phase, the compound
459 generated by the cytochrome P450 system is conjugated with endogenous substances as glutathione,
460 glucuronic acid or sulphate, to convert them in more hydrophilic compounds (Falfushynska et al., 2019;
461 Uno et al., 2012). GST is an enzyme involved in phase II detoxification process that produces a more
462 hydrophilic glutathione conjugate of the toxic compound, therefore favouring their elimination (Sureda et
463 al., 2006). An increase in GST activity has been reported as a consequence of MPs ingestion in wild *M.*
464 *surmuletus* showing MPs in their gastrointestinal tract (Alomar et al., 2017) and in *D. labrax* juveniles,
465 exposed during 96 h to MPs (Barboza et al., 2018). Our results showed an increase in GST activity as a
466 consequence of MPs diet supplementation. In this sense, the higher GST activity was observed after 90

467 days of MPs exposure. However, no significant differences were found between virgin or seawater exposed
468 MPs, even though GST activity was slightly higher in the seawater exposed treatment in all sampling times.

469 In addition, the effects of 30-day depuration period on oxidative stress and detoxification markers were also
470 investigated. A decrease in oxidative stress and detoxification markers was identified in the liver of *S.*
471 *aurata* after the depuration period. CAT activity also decreased after the depuration period, but this decrease
472 was only significant in the seawater exposed MPs treatment. In the same way, GRd activity also decreased
473 after the 30-day depuration period. However, this decrease was only significant in virgin MPs treatment,
474 whereas SOD, GPx and MPO activities did not decrease as a consequence of the depuration period.
475 Carbonyl protein derivates also decreased after a 30 days depuration period, reaching levels like those
476 detected on the 30^{th} day of the study. On the other hand, no effects of the depuration period were found in
477 MDA levels.

478 Our results did not show evident histological damage due to the MPs exposure and intake at T_{90}. However,
479 the increase in the infiltration of immune cells observed in T_{90} could explain the increase in MPO activity
480 observed in T_{90}. In this sense, histological analysis in samples from T_{120} shows a reduction in the infiltration
481 of immune cells in the liver that would be related to the decrease in enzymatic activities observed at the
482 same time.

483 Moreover, a significant decrease in GST activity was also found after the depuration period, although this
484 decrease was only significant in the virgin MPs treatment. The GST activity in the virgin MPs treatment
485 after 30 days of depuration period is similar to the GST activity measured at day 30 of the study. The results
486 suggest that 30-days of depuration can slightly reduce the oxidative stress and hepatotoxicity status in *S.*
487 *aurata*, but it is not enough to observe a complete recovery from MPs exposure in the liver. In this sense,
488 there is a lack of literature considering the effects of depuration periods in the antioxidant and detoxification
489 mechanism in fish. A study performed in liver from juveniles of catfish (*Clarias gariepinus,*Burchell, 1822)
490 did not find any differences in the antioxidant defences after a depuration period (Iheanacho and Odo,
491 2020). Other studies performed on bivalves exposed to MPs for 14 days, followed by 7 days period
492 depuration, did not find effects in SOD and CAT activities, while a decrease in GPx and GST activities
493 after the depuration period was reported (Ribeiro et al., 2017).

In conclusion, long-term MPs exposure induced an increase in the antioxidant defences in the liver of *S. aurata*. However, this increase in antioxidant capabilities was not enough to prevent oxidative damage since there was an increase in MDA levels due to MPs ingestion. MPs exposure also caused an increase in GST activity; these results suggest an activation of detoxification systems as a consequence of MPs ingestion. Seawater exposed MPs cause a higher antioxidant response (CAT, GRd and GST). A depuration period of 30 days induces a tendency to recover initial values of biomarkers determinations in fish liver, although it does not seem to be enough for its complete normalization.

5. Acknowledgements

José Maria Valencia, Elvira Álvarez, Beatriz Ríos, Alberto Aparicio, Karlos Garcia for all of the laboratory support. Aurelio Ortega from the Centro Oceanográfico de Murcia for supplying fish. Acknowledgments to the research project Plásticos derivados de la acuicultura: impactos y efectos en las redes tróficas marinas" (ACUIPLASTIC), with reference number CTM2017-88332-R and funded by the Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad, en el marco del Plan Estatal de Investigación Científica y Técnica y de Innovación 2013-2016, from the Spanish Government. X Capó was supported by a postdoctoral contract with Juan de la Cierva-Formación (2018-037395-I). A. Sureda was supported by the Instituto de Salud Carlos III (CIBEROBN - CB12/03/30038). MC is the recipient of a pre-doctoral FPI Fellowship from the Conselleria d'Innovació, Recerca i Turisme, of the regional government of the Balearic Islands co-financed by the European Social Fund as part of the FSE 2014-2020 operational programme. We extend the acknowledgements to Xunta de Galicia (ED431C2017/36), the Spanish Agencia Estatal de Investigación (ref. CTM2017-84763-C3-R-2 and CTM2017-90980-REDT) and FEDER/ERDF funds. Acknowledgements to Net4sea: Red científica de análisis de riesgo y exposición a contaminantes emergentes en el medio marino de la Agencia Nacional de Investigación, Ministerio de Ciencia, Innovación y Universidades (CTM2017-90890-REDT).

6. References

Aebi H. Catalase in vitro. Methods Enzymol 1984; 105: 121-6.

Alomar C, Sureda A, Capó X, Guijarro B, Tejada S, Deudero S. Microplastic ingestion by *Mullus surmuletus* Linnaeus, 1758 fish and its potential for causing oxidative stress. Environ Res 2017; 159: 135-142.

Andrady AL. Microplastics in the marine environment. Mar Pollut Bull 2011; 62: 1596-605.

Au SY, Lee CM, Weinstein JE, van den Hurk P, Klaine SJ. Trophic transfer of microplastics in aquatic ecosystems: Identifying critical research needs. Integr Environ Assess Manag 2017; 13: 505-509.

Auffret MM. Histopathological changes related to chemical contamination in *Mytilus edulis* from field and experimental conditions MARINE ECOLOGYPROGRESS SERIES. Mar. Ecol. Prog. Ser 1988; 46: 101-107.

Balart E, Pérez-Urbiola J, Campos-Dávila L, Monteforte-Sanchez M, Ortega-Rubio A. On the first record of a potentially harmful fish, *Sparus aurata* in the Gulf of California. Biological Invasions 2009; 11: 547-550.

Barboza LGA, Vieira LR, Branco V, Figueiredo N, Carvalho F, Carvalho C, et al. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquat Toxicol 2018; 195: 49-57.

Bartoskova M, Dobsikova R, Stancova V, Zivna D, Blahova J, Marsalek P, et al. Evaluation of ibuprofen toxicity for zebrafish (*Danio rerio*) targeting on selected biomarkers of oxidative stress. Neuro Endocrinol Lett 2013; 2: 102-8.

Bayne BL, Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A., Livingstone, D.A.,, Lowe DM, Moore, M.N., Atebbing, A.R.D., Widdings, J. The Effects of Stress and Pollution on Marine Animals. Praeger, New York. 1985.

Berglund E, Fogelberg V, Nilsson PA, Hollander J. Microplastics in a freshwater mussel (*Anodonta anatina*) in Northern Europe. Sci Total Environ 2019; 697: 30.

Bolgar MH, J; Groeger J; Meronek, S. Handbook for the chemical analysis of plastic and polymer additives: CRC Press, 2015.

Boteler BS, LO; Bodle, R; Donat, L. The UN Ocean Conference - June 2017, Guidance to the ENVI Committee of the European Parliament. European Parliament: Brussels 2017.

577 Boudouresque C. Méthodes d'étude qualitative et quantitative du benthos (en particulier du
578 phytobenthos). Tethys 1971; 3: 79-104.

579 Box A, Sureda A, Galgani F, Pons A, Deudero S. Assessment of environmental pollution at Balearic
580 Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis*. Comp Biochem
581 Physiol C Toxicol Pharmacol 2007; 146: 531-9.

582 Brown DM, Wilson MR, MacNee W, Stone V, Donaldson K. Size-dependent proinflammatory effects of
583 ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of
584 ultrafines. Toxicol Appl Pharmacol 2001; 175: 191-9.

585 Buruaem LM, Hortellani MA, Sarkis JE, Costa-Lotufo LV, Abessa DM. Contamination of port zone
586 sediments by metals from Large Marine Ecosystems of Brazil. Mar Pollut Bull 2012; 64: 479-88.

587 Capeillère-Blandin C. Oxidation of guaiacol by myeloperoxidase: a two-electron-oxidized guaiacol
588 transient species as a mediator of NADPH oxidation. Biochem J 1998; 336: 395-404.

589 Capo X, Martorell M, Sureda A, Tur JA, Pons A. Effects of docosahexaenoic supplementation and in
590 vitro vitamin C on the oxidative and inflammatory neutrophil response to activation. Oxid Med Cell
591 Longev 2015a; 187849: 19.

592 Capo X, Tejada S, Box A, Deudero S, Sureda A. Oxidative status assessment of the endemic bivalve
593 *Pinna nobilis* affected by the oil spill from the sinking of the Don Pedro. Mar Environ Res 2015b; 110:
594 19-24.

595 Capó X, Tejada S, Ferriol P, Pinya S, Mateu-Vicens G, Montero-González I, et al. Hypersaline water
596 from desalinization plants causes oxidative damage in *Posidonia oceanica* meadows. Sci Total Environ
597 2020; 736: 22.

598 Cole M, Lindeque P, Fileman E, Halsband C, Goodhead R, Moger J, et al. Microplastic ingestion by
599 zooplankton. Environ Sci Technol 2013; 47: 6646-55.

600 Cózar A, Echevarría F, González-Gordillo JI, Irigoien X, Ubeda B, Hernández-León S, et al. Plastic
601 debris in the open ocean. Proc Natl Acad Sci U S A 2014; 111: 10239-44.

602 Cunha I, Garcia LM, Guilhermino L. Sea-urchin (*Paracentrotus lividus*) glutathione S-transferases and
603 cholinesterase activities as biomarkers of environmental contamination. J Environ Monit 2005; 7: 288-94.

604 Dehaut A, Cassone AL, Frère L, Hermabessiere L, Himber C, Rinnert E, et al. Microplastics in seafood:
605 Benchmark protocol for their extraction and characterization. Environ Pollut 2016; 215: 223-233.

606 DelValls TA, Blasco J, Sarasquete MC, Forja JM, Gomez-Parra A. Evaluation of heavy metal sediment
607 toxicity in littoral ecosystems using juveniles of the fish *Sparus aurata*. Ecotoxicol Environ Saf 1998; 41:
608 157-67.

609 Ding J, Zhang S, Razanajatovo RM, Zou H, Zhu W. Accumulation, tissue distribution, and biochemical
610 effects of polystyrene microplastics in the freshwater fish red tilapia (*Oreochromis niloticus*). Environ
611 Pollut 2018; 238: 1-9.

612 Duncan EM, Broderick AC, Fuller WJ, Galloway TS, Godfrey MH, Hamann M, et al. Microplastic
613 ingestion ubiquitous in marine turtles. Glob Chang Biol 2019; 25: 744-752.

614 ECHA. Describing uses of additives in plastic material for articles and estimating related exposure -
615 Practical Guide for Industry 2020: 1-19.

616 Espinosa C, Cuesta A, Esteban M. Effects of dietary polyvinylchloride microparticles on general health,
617 immune status and expression of several genes related to stress in gilthead seabream (*Sparus aurata* L.).
618 Fish Shellfish Immunol 2017; 68: 251-259.

619 Espinosa C, Esteban M, Cuesta A. Dietary administration of PVC and PE microplastics produces
620 histological damage, oxidative stress and immunoregulation in European sea bass (*Dicentrarchus labrax*
621 L.). Fish Shellfish Immunol 2019; 95: 574-583.

622 Falfushynska H, Sokolov EP, Haider F, Oppermann C, Kragl U, Ruth W, et al. Effects of a common
623 pharmaceutical, atorvastatin, on energy metabolism and detoxification mechanisms of a marine bivalve
624 *Mytilus edulis*. Aquat Toxicol 2019; 208: 47-61.

625 FAO. Aquaculture topics and activities. Acuicultura. In: Departamento de Pesca y Acuicultura de la FAO
626 2001-2020.

627 FAO. The State of World Fisheries and Aquaculture 2020. . 2020.

628 Flohe L, Gunzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984; 105: 114-21.

629 Flohe L, Otting F. Superoxide dismutase assays. Methods Enzymol 1984; 105: 93-104.

630 Forner-Piquer I, Mylonas CC, Calduch-Giner J, Maradonna F, Gioacchini G, Allarà M, et al. Endocrine
631 disruptors in the diet of male *Sparus aurata*: Modulation of the endocannabinoid system at the hepatic
632 and central level by Di-isononyl phthalate and Bisphenol A. Environ Int 2018; 119: 54-65.

633 Gallo F, Fossi C, Weber R, Santillo D, Sousa J, Ingram I, et al. Marine litter plastics and microplastics
634 and their toxic chemicals components: the need for urgent preventive measures. Environ Sci Eur 2018;
635 30: 018-0139.

636 Goldberg DM Spooner R. p. Glutathione reductase. Methods of enzymatic analysis. Enzymes 1:
637 oxidoreductases, transferases, 1984; 3: 258-265.

638 Grigorakis K. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus*
639 *aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. Aquaculture 2007; 272:
640 55-75.

641 Güven O, Gökdağ K, Jovanović B, Kıdeyş AE. Microplastic litter composition of the Turkish territorial
642 waters of the Mediterranean Sea, and its occurrence in the gastrointestinal tract of fish. Environ Pollut
643 2017; 223: 286-294.

644 Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic
645 acid formation. J Biol Chem 1974; 249: 7130-9.

646 Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans 2007; 35: 1147-50.

647 Hara J, Frias J, Nash R. Quantification of microplastic ingestion by the decapod crustacean *Nephrops*
648 *norvegicus* from Irish waters. Mar Pollut Bull 2020; 152: 15.

649 Hidalgo-Ruz V, Gutow L, Thompson RC, Thiel M. Microplastics in the Marine Environment: A Review
650 of the Methods Used for Identification and Quantification. Environmental Science & Technology 2012;
651 46: 3060-3075.

652 Iheanacho SC, Odo GE. Dietary exposure to polyvinyl chloride microparticles induced oxidative stress
653 and hepatic damage in *Clarias gariepinus* (Burchell, 1822). Environ Sci Pollut Res Int 2020; 27: 21159-
654 21173.

655 Jeong CB, Kang HM, Lee MC, Kim DH, Han J, Hwang DS, et al. Adverse effects of microplastics and
656 oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod
657 *Paracyclopina nana*. Sci Rep 2017; 7.

658 Jovanović B. Ingestion of microplastics by fish and its potential consequences from a physical
659 perspective. Integr Environ Assess Manag 2017; 13: 510-515.

660 Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively
661 modified proteins. Methods Enzymol 1994; 233: 346-57.

662 Li J, Lusher AL, Rotchell JM, Deudero S, Turra A, Bråte ILN, et al. Using mussel as a global
663 bioindicator of coastal microplastic pollution. Environ Pollut 2019; 244: 522-533.

664 Livingstone DR. Contaminant-stimulated reactive oxygen species production and oxidative damage in
665 aquatic organisms. Mar Pollut Bull 2001; 42: 656-66.

Lu Y, Zhang Y, Deng Y, Jiang W, Zhao Y, Geng J, et al. Response to Comment on "Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (*Danio rerio*) and Toxic Effects in Liver". Environ Sci Technol 2016; 50: 12523-12524.

Lusher AL, Hernandez-Milian G, O'Brien J, Berrow S, O'Connor I, Officer R. Microplastic and macroplastic ingestion by a deep diving, oceanic cetacean: the True's beaked whale *Mesoplodon mirus*. Environ Pollut 2015; 199: 185-91.

Mancia A, Chenet T, Bono G, Geraci ML, Vaccaro C, Munari C, et al. Adverse effects of plastic ingestion on the Mediterranean small-spotted catshark (*Scyliorhinus canicula*). Mar Environ Res 2020; 155: 14.

Nash R, Valencia AH, Geffen A. The origin of Fulton's condition factor - Setting the record straight. Fisheries 2006; 31: 236-238.

Qiao R, Sheng C, Lu Y, Zhang Y, Ren H, Lemos B. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. Sci Total Environ 2019; 662: 246-253.

Regoli F, Frenzilli G, Bocchetti R, Annarumma F, Scarcelli V, Fattorini D, et al. Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. Aquat Toxicol 2004; 68: 167-78.

Ribeiro F, Garcia AR, Pereira BP, Fonseca M, Mestre NC, Fonseca TG, et al. Microplastics effects in *Scrobicularia plana*. Mar Pollut Bull 2017; 122: 379-391.

Rios LM, Moore C, Jones PR. Persistent organic pollutants carried by synthetic polymers in the ocean environment. Mar Pollut Bull 2007; 54: 1230-7.

Rochman CM, Hoh E, Kurobe T, Teh SJ. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Sci Rep 2013; 3.

Rochman CM, Kurobe T, Flores I, Teh SJ. Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. Sci Total Environ 2014; 493: 656-61.

Sillero-Rios J, Sureda A, Capo X, Oliver-Codorniu M, Arechavala-Lopez P. Biomarkers of physiological responses of *Octopus vulgaris* to different coastal environments in the western Mediterranean Sea. Mar Pollut Bull 2018; 128: 240-247.

695 Solis-Weiss V, Aleffi F, Bettoso N, Rossin P, Orel G, Fonda-Umani S. Effects of industrial and urban
696 pollution on the benthic macrofauna in the Bay of Muggia (industrial port of Trieste, Italy). Sci Total
697 Environ 2004; 328: 247-63.

698 Solomando A, Capó X, Alomar C, Álvarez E, Compa M, Valencia JM, et al. Long-term exposure to
699 microplastics induces oxidative stress and a pro-inflammatory response in the gut of *Sparus aurata*
700 Linnaeus, 1758. Environ Pollut 2020; 266: 115295.

701 Sureda A, Box A, Enseñat M, Alou E, Tauler P, Deudero S, et al. Enzymatic antioxidant response of a
702 labrid fish (*Coris julis*) liver to environmental caulerpenyne. Comp Biochem Physiol C Toxicol
703 Pharmacol 2006; 144: 191-6.

704 Sureda A, Box A, Tejada S, Blanco A, Caixach J, Deudero S. Biochemical responses of *Mytilus*
705 *galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill
706 (Eivissa Island, Spain). Aquat Toxicol 2011; 101: 540-9.

707 Sureda A, Capo X, Busquets-Cortes C, Tejada S. Acute exposure to sunscreen containing titanium
708 induces an adaptive response and oxidative stress in *Mytillus galloprovincialis*. Ecotoxicol Environ Saf
709 2018; 149: 58-63.

710 UNEP. Marine Litter: A Global Challenge. Nairobi: UNEP 2009.

711 Uno T, Ishizuka M, Itakura T. Cytochrome P450 (CYP) in fish. Environ Toxicol Pharmacol 2012; 34: 1-
712 13.

713 van der Veen BS, de Winther MP, Heeringa P. Myeloperoxidase: molecular mechanisms of action and
714 their relevance to human health and disease. Antioxid Redox Signal 2009; 11: 2899-937.

715 Vert M, Doi Y, Hellwich K-H, Hess M, Hodge P, Kubisa P, et al. Terminology for biorelated polymers
716 and applications (IUPAC Recommendations 2012). Pure and Applied Chemistry 2012; 84: 377.

717 von Moos N, Burkhardt-Holm P, Köhler A. Uptake and effects of microplastics on cells and tissue of the
718 blue mussel *Mytilus edulis* L. after an experimental exposure. Environ Sci Technol 2012; 46: 11327-35.

719 Worsfold PT, A; Poole, C; Miró, M. Encyclopedia of Analytical Science. Elsevier, Plymouth, England,
720 2019, pp. 5109.

721 Wu F, Wang Y, Leung JYS, Huang W, Zeng J, Tang Y, et al. Accumulation of microplastics in typical
722 commercial aquatic species: A case study at a productive aquaculture site in China. Sci Total Environ
723 2020; 708: 22.

724 Yuan W, Zhou Y, Chen Y, Liu X, Wang J. Toxicological effects of microplastics and heavy metals on the
725 *Daphnia magna*. Sci Total Environ 2020; 746: 141254.

726 Zhang H, Pap S, Taggart MA, Boyd KG, James NA, Gibb SW. A review of the potential utilisation of
727 plastic waste as adsorbent for removal of hazardous priority contaminants from aqueous environments.
728 Environ Pollut 2020; 258: 30.

729 Zhu J, Zhang Q, Li Y, Tan S, Kang Z, Yu X, et al. Microplastic pollution in the Maowei Sea, a typical
730 mariculture bay of China. Sci Total Environ 2019; 658: 62-68.

731

732

733

734

735

736

737

738

739

740

741

742

743

744

745

746

747

748

Figure 1

Histopathology analysis of the effects of MPs ingestion in the liver at different sampling times.H&E staining. A) Histological section of the liver at T_0. Bar 500µm. B-D) Micrographs of the liver at T_{90}, corresponding to B) Control, C) Virgin and D) Sea water exposed MPs treatments. Bar 500µm. E-G) Micrographs of the liver at T_{120}, corresponding toE) Control, F) Virgin and G) sea water exposed MPs. Bar 500µm. H) Presence of eosinophils (white star) surrounding a pancreatic islet. Bar 50µm. I) Detail of a rodlet cell (arrowhead) in the epithelium of a bile duct. Bar 20µm.Pancreatic islet (black arrow), blood vessel (white arrow), bile ducts (black star) are indicated.

Figure 2

Effects of MPs treatment on Antioxidant enzymes activities

Statistical analysis: two-way ANOVA. MPs mean significant effect of treatment (# means differences respect control group; % means differences respect Sea water exposed treatment); T, significant effect of time (* means differences respect T0, $ reveals differences respect T30, @ reveals differences respect T60, º means differences respect T90) MPsxT significant interaction between both factors. When interaction exists, different letters reveals significant differences.

Figure 3

Effects of MPs treatment on GST and MPOactivities

Statistical analysis: two-way ANOVA. MPs mean significant effect of treatment (# means differences respect control group; % means differences respect Sea water exposed treatment); T, significant effect of time (* means differences respect T0, $ reveals differences respect T30, @ reveals differences respect T60, º means differences respect T90) MPsxT significant interaction between both factors. When interaction exists, different letters reveals significant differences.

Figure 4

Effects of MPs treatment on oxidative damage markers

Statistical analysis: two-way ANOVA. MPs mean significant effect of treatment (# means differences respect control group; % means differences respect Sea water exposed treatment); T, significant effect of time (* means differences respect T0, $ reveals differences respect T30, @ reveals differences respect T60, º means differences respect T90) MPsxT significant interaction between both factors. When interaction exists, different letters reveals significant differences.

Table 1. Compounds isolated from MPs after 2 months exposed to sea water

Compound	Virgin MPs	Seawater exposed MPS	Use
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (DIBP)	X	X	Plasticizer
Butylated Hydroxytoluene (BHT)		X	Antioxidant additive
1-Hexadecanol	X	X	Emulsifier
Isopropyl palmitate		X	Personal care product
Triacetin	X	X	Plasticizer
Di-n-butyl phthalate (DnBP)	X	X	Plasticizer
Bis(2-ethylhexyl) phthalate (DEHP)	X	X	Plasticizer
Diethyl phthalate (DEP)	X	X	Plasticizer
Dimethyl phthalate (DMP)	X	X	Plasticizer
Galaxolide		X	Personal care product
Benzophenone	X	X	Photostabilizer (UV filter)
Triisobutyl phosphate (TiBP)	X	X	Flame retardant
Diphenyl Ether		X	Personal care products additive and precursor plastic synthesis
Phenanthrene		X	Polycyclic aromatic hydrocarbon (PAH)
Benzothiazole	X	X	Multiples uses (dye, pesticide and drug)
Diethyl succinate		X	Personal care product
1,2,4-Trichlorobenzene		X	Multiples uses (dye, pesticide and lubricant)
9,12 (Z,Z)-Octadecadienoic acid		X	Lubricant
2-Ethylhexyl salicylate	X	X	Photostabilizer (UV filter)
Bisphenol A		X	Plasticizer
Lactic acid		X	Multiples uses (personal care products and textile)
(Z)-Oleic acid	X	X	Surfactant
Palmitic acid		X	Surfactant
Hexanoic acid		X	Lubricant
Myristic acid	X	X	Surfactant
Stearic acid		X	Lubricant
Phenol		X	Precursor plastic synthesis
2-Dodecanol		X	Lubricant
2-Decanol		X	Lubricant
1-Monopalmitin		X	Emulsifier
1-Octadecanol	X	X	Emulsifier

Table 2. MPs index ingestion during the experimental procedure

		T0	T30	T60	T90	T120	ANOVA		
							MPs	Time	MPs X T
MPs Index	Control	0.05±0.002a	0.034±0.003a	0.05±0.002a	0.02±0.0002a	0.05±0.02a	X	X	X
	Virgin		9.94±3.54b	18.2±7.6b	42.6±6.8c	0.05±0.02a			
	Marinated		11.1±3.80b	12.5±5.7b	44.9±6.8c	0.16±0.02a			

Statistical analysis: two-way ANOVA. MPs mean significant effect of treatment (# means differences respect control group; % means differences respect Marinated treatment); T, significant effect of time (* means difernces respect T0, $ reveals differences respect T30, @ reveals differences respect T60, º means differences respect T90) MPsxT significant interaction between both factors. When interaction exists, diferent letters reveals significant differences.

Figure 1

Click here to access/download;Figure;Figure 1 .jpg

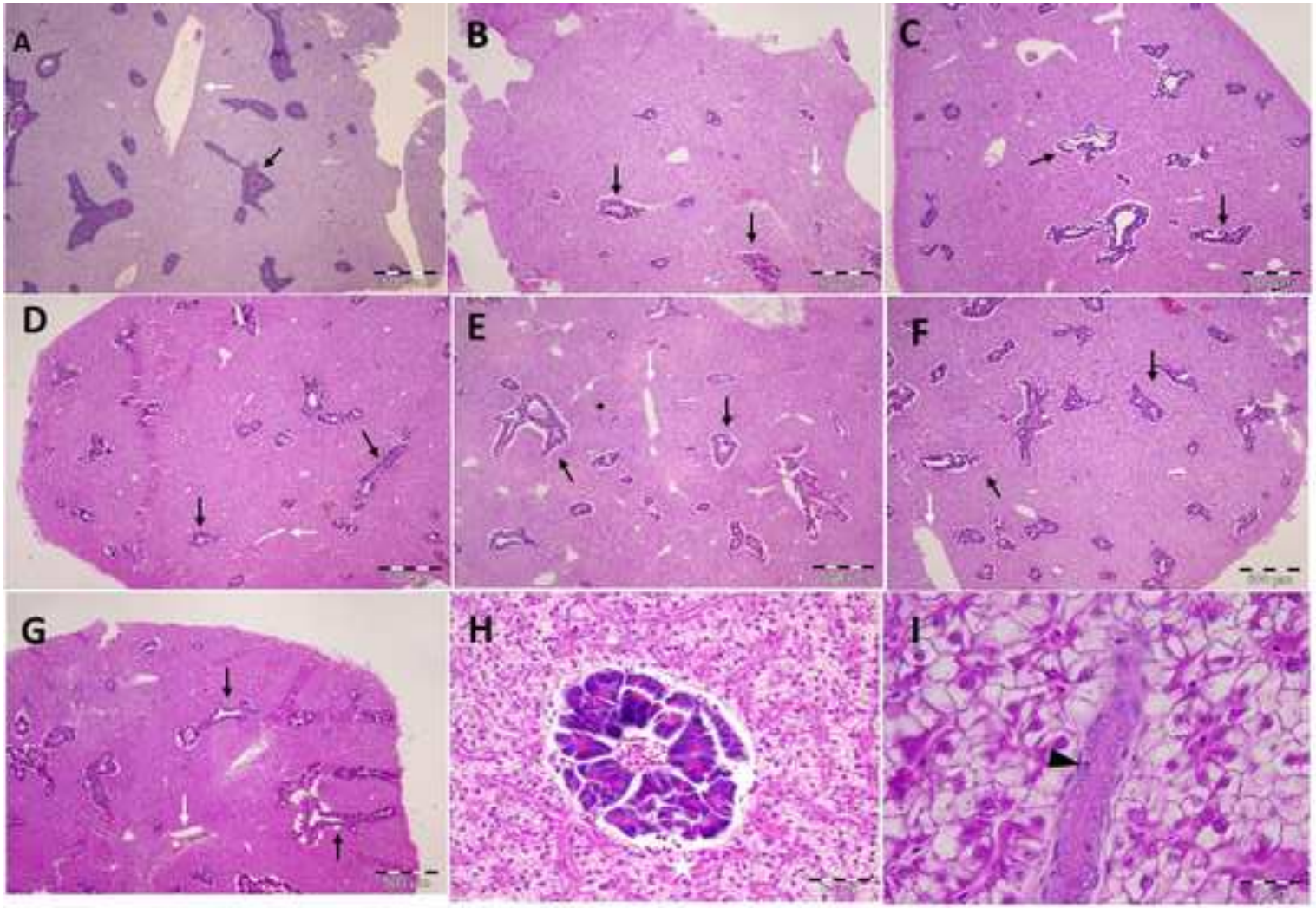


Figure 2

Click here to access/download;Figure;Figure 2.jpg

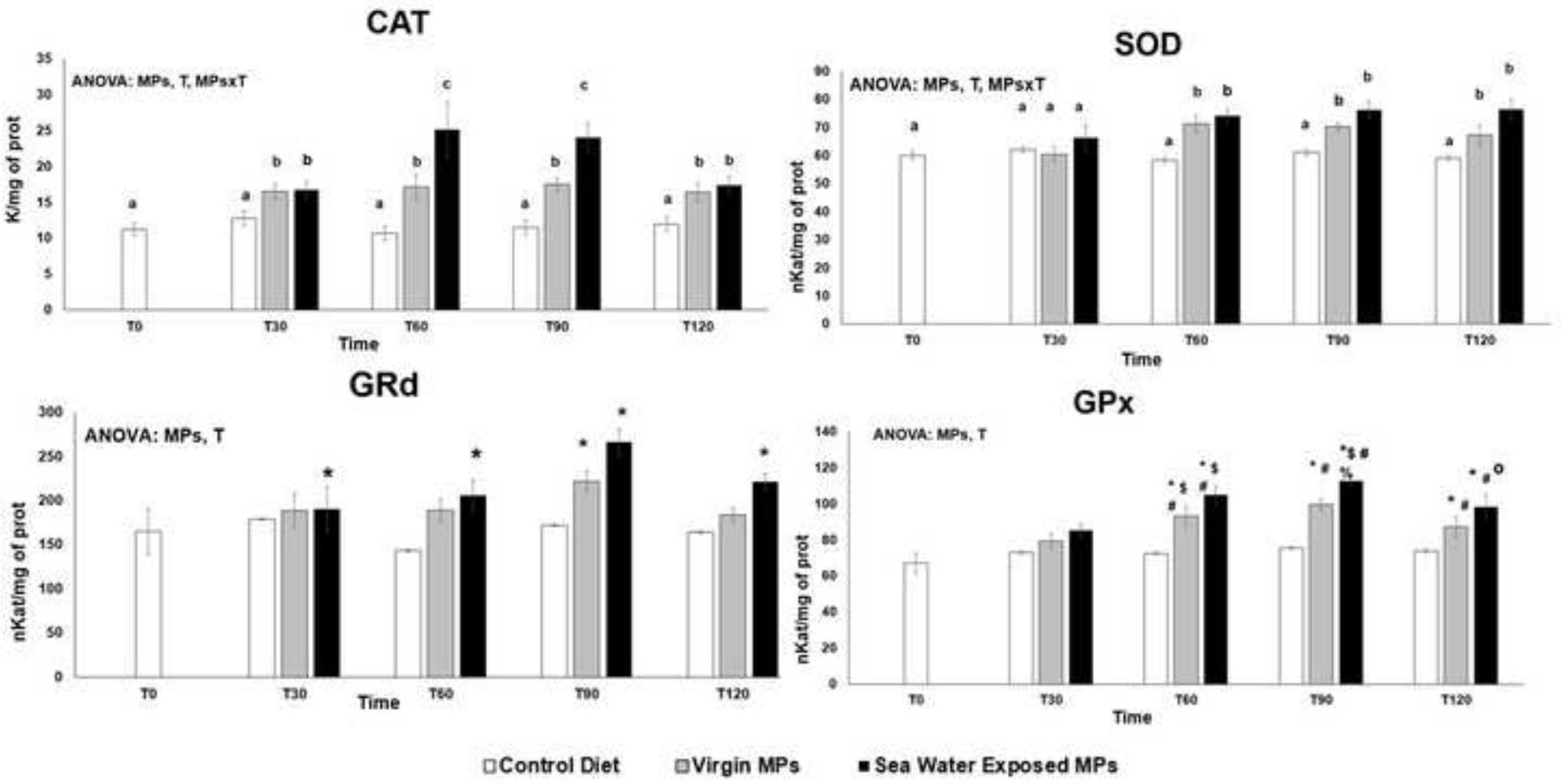


Figure 3

Click here to access/download;Figure;Figure 3.jpg

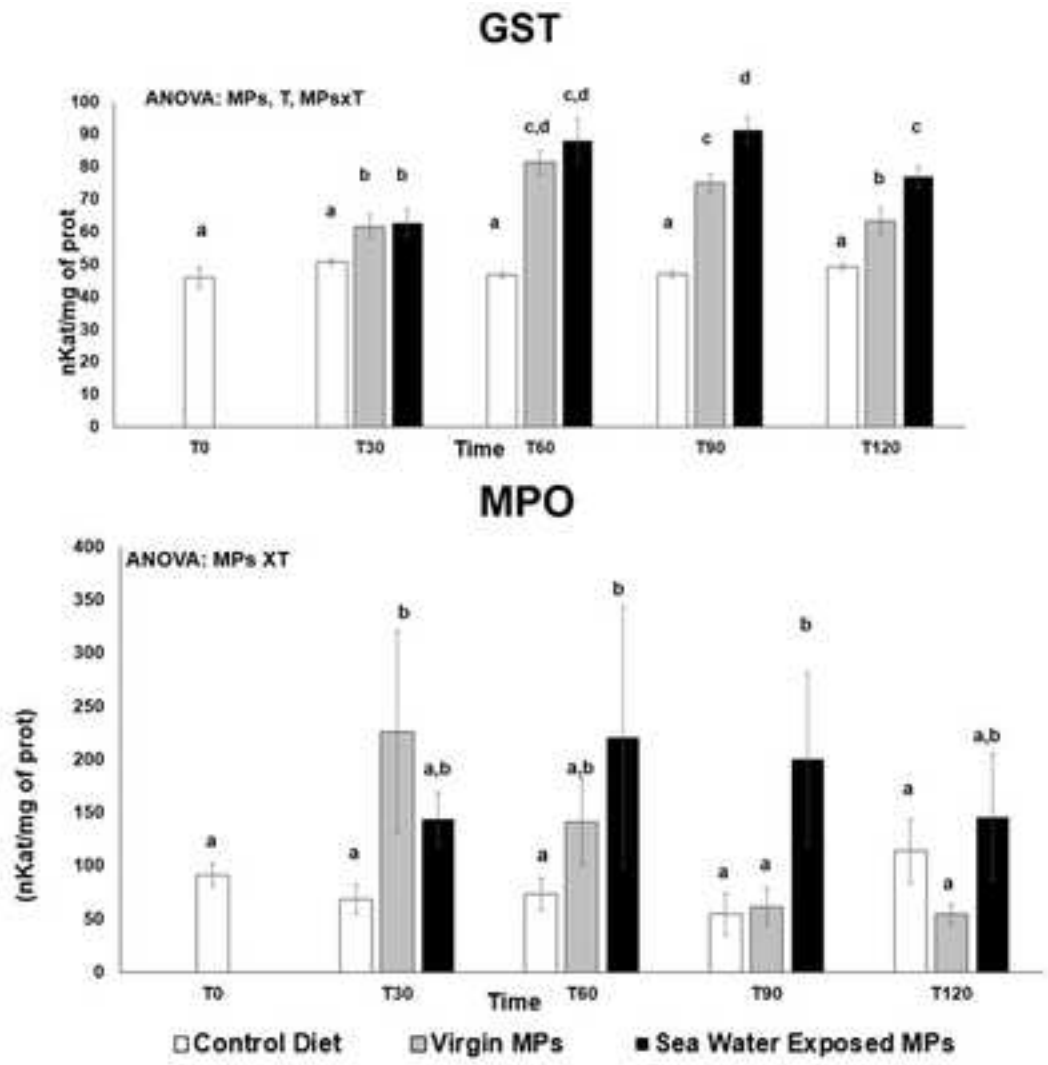
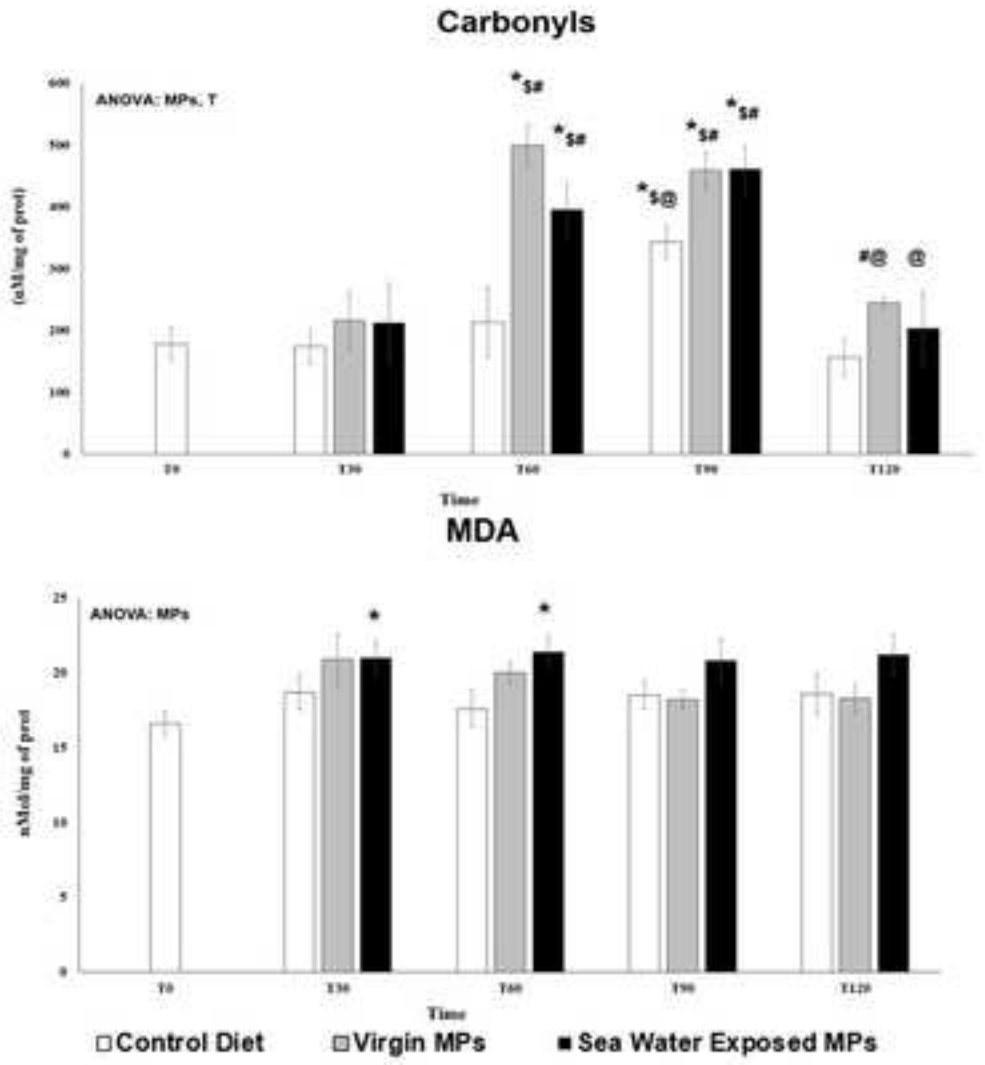


Figure 4

Click here to access/download;Figure;Figure 4.jpg



Authors contribution

XC, MC, CA AS, and SD conception and design of research; XC, JJC, MC, AG, BH, JLV, JBQ, RR and SD, performed experiments; XC, JJC, MC, CA, AS, AG, BH, JLV, JBQ, RR and SD analysed data; XC, JJC, MC, CA, AG, BH and SD interpreted results of experiments; XC, MC, CA, AS and SD drafted manuscript; XC, CA, MC, AS and SD edited and revised manuscript; XC, JJC, CA, MC, AS, AG, BH, JLV, JBQ, RR and SD approved final version of manuscript. All authors read and approved the final manuscript

Declaration of interests

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