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Integrated biomarker responses in European seabass Dicentrarchus labrax (Linnaeus, 1758) chronically exposed to PVC microplastics

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Abstract:	Few studies evaluated long-term effects of polyvinyl chloride (PVC) microplastics (MPs) ingestion in fish. The present study aimed to investigate the integrated biomarker responses in the liver and blood of 162 European seabass, Dicentrarchus labrax , exposed for 90 days to control, virgin and marine incubated PVC enriched diets (0.1% w/w) under controlled laboratory condition. Enzymatic (EROD) and tissue alterations (Histopathology), oxidative stress (CAT and LPO), gene expression alterations (TRAF3, PPAR-α, PPAR-γ and ER-α) and genotoxicity (ENA assay) were examined. Additives and environmental contaminants levels in PVC-MPs, control feed matrices and in seabass muscles were also detected. The results showed that the chronic exposure at environmentally realistic PVC-MPs concentrations in seabass, cause early warning responses of toxicological harm in liver by induction of oxidative stress, the histopathological alterations and also by the modulation of the PPARs and Er-α genes expression. A trend of increase of DNA alterations and the observation of some neoformations attributable to lipomas suggest also genotoxic and cancerogenic effects of PVC. This investigation provides important data to understand the regulatory biological processes affected by PVC-MPs ingestion in marine organisms and may also support the interpretation of results provided by studies on wild species.
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Journal of Hazardous Materials Editorial Office

To whom it may concern,

Please find enclosed the manuscript "Integrated biomarker responses in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) chronically exposed to PVC microplastics" for its consideration for publication in the Journal of Hazardous Materials.

To date, the microplastics (MPs) ingestion by marine organisms is one of the main global concerns. Exposure to MPs may induce complex responses and their potential environmental and ecological consequences are a challenging task. Although several laboratory studies investigated the toxicological effects of different polymer-type MPs on marine biota, more experiments are needed to assess long-term effects of MPs effects at environmentally relevant concentrations and the role of MPs contaminants.
In this respect, this research represents one of the few studies, that investigates the toxicity induced over different time endpoints in the liver and blood of European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758), by chronic exposure (up to 90 days) to and ingestion of virgin and marine incubated polyvinyl chloride (PVC) MPs. The present paper explores under controlled laboratory condition the impact of environmentally realistic PVC-MPs concentration (0.1% w/w) on species of ecological and commercial importance and sensitive to the exposure of several pollutants analyzing integrated biomarker responses (at molecular, subcellular and tissue levels) and chemical contaminants of MPs (additives and POPs level).
These results provide an important contribution to better understand the regulatory biological processes affected by MPs ingestion in marine organisms; they may support the interpretation of results provided by studies on wild species; they underline the importance of long-term studies also considering the inter-relationship of plastic polymers, additives, and other contaminants as inescapable parameters for reliable MPs toxicity assessment in fish.

Overall, the authors believe that this research fits well with the aims and the scope of the journal *Journal of Hazardous Materials*. Indeed, our study give new inputs to improve understanding of synergistic effects of MPs and their vehicled pollutants on marine biota health status.

The authors provide the following as possible reviewers of this manuscript:

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The publication of this research article is approved by all co-authors.
The content of this manuscript has not been previously submitted or published.

Thank you for opportunity to submit this research to the *Journal of Hazardous Materials*.

Best regards,

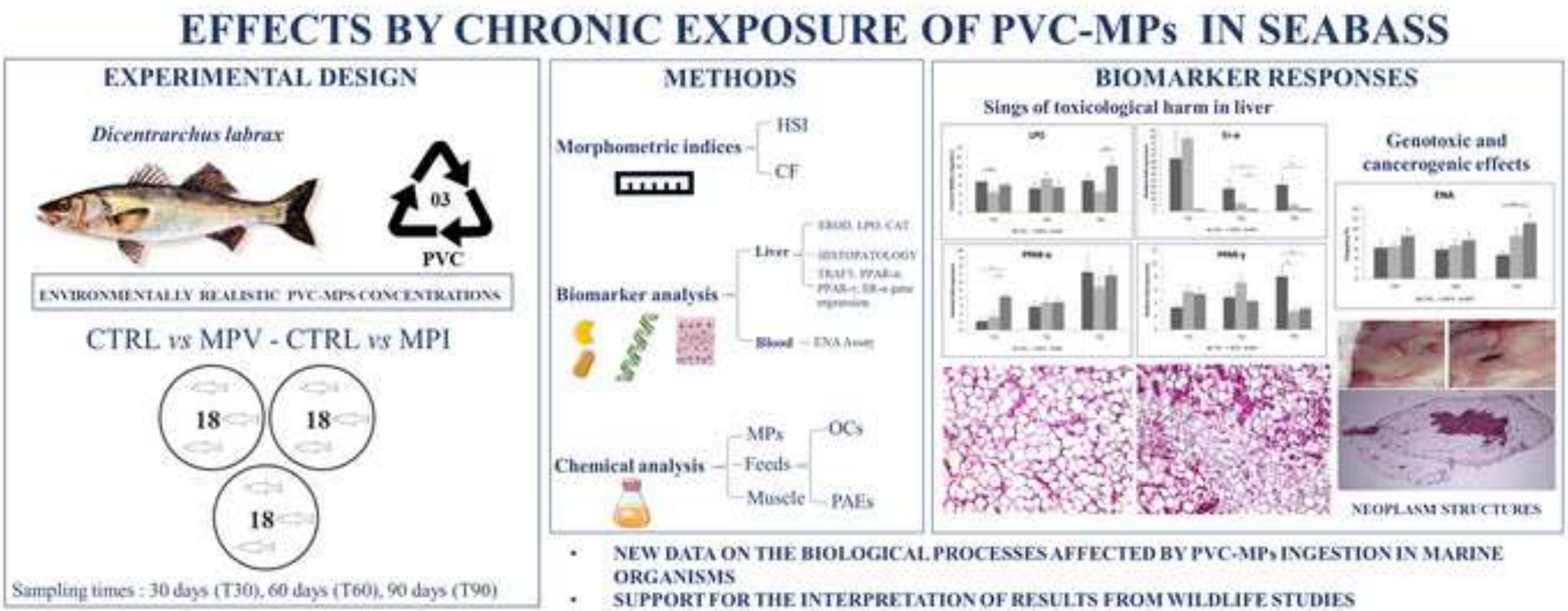
Teresa Romeo
(on behalf of all Authors)

STATEMENT OF "ENVIRONMENTAL IMPLICATION"

Microplastics (MPs) ingestion may cause physical/mechanic and chemical harms to marine organisms. For this, studies under controlled laboratory condition are needed to investigated MPs effects at environmentally relevant concentrations and the role of MPs contaminants.

In light of this, present paper investigates the integrated biomarker responses, induced over different time endpoints in *Dicentrarchus labrax*, by chronic exposure to ingestion of virgin and marine incubated polyvinyl chloride MPs. This investigation provides important data to understand the regulatory biological processes affected by MPs ingestion in marine organisms and may also support the interpretation of results provided by studies on wild species.

Graphical Abstract



Highlights

Exposure to PVC-MPs cause early warning responses of toxicological harm in liver.

Exposure to PVC-MPs cause genotoxic and cancerogenic effects.

Development of neoplasm tissues could be related to the PVC chronic exposure.

Additives and POPs levels in PVC-MPs, control feed and muscles were detected.

Chronic exposure studies are an important tool to clarify the MPs impact in fish.

Integrated biomarker responses in European seabass *Dicentrarchus labrax* (Linnaeus, 1758) chronically exposed to PVC microplastics

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Abstract

Few studies evaluated long-term effects of polyvinyl chloride (PVC) microplastics (MPs) ingestion in fish. The present study aimed to investigate the integrated biomarker responses in the liver and blood of 162 European seabass, *Dicentrarchus labrax*, exposed for 90 days to control, virgin and marine incubated PVC enriched diets (0.1% w/w) under controlled laboratory condition.

Enzymatic (EROD) and tissue alterations (Histopathology), oxidative stress (CAT and LPO), gene expression alterations (TRAF3, PPAR-α, PPAR-γ and ER-α) and genotoxicity (ENA assay) were examined. Additives and environmental contaminants levels in PVC-MPs, control feed matrices and in seabass muscles were also detected.

The results showed that the chronic exposure at environmentally realistic PVC-MPs concentrations in seabass, cause early warning responses of toxicological harm in liver by induction of oxidative stress, the histopathological alterations and also by the modulation of the PPARs and Er-α genes expression. A trend of increase of DNA alterations and the observation of some neoformations attributable to lipomas suggest also genotoxic and cancerogenic effects of PVC.

This investigation provides important data to understand the regulatory biological processes affected by PVC-MPs ingestion in marine organisms and may also support the interpretation of results provided by studies on wild species.

Keywords: Marine litter, polyvinyl chloride, ingestion, biomarker, ecotoxicology

Introduction

The occurrence of plastic litter in marine ecosystems and their potential hazard to cause harm to biota represent a great problem for marine biodiversity conservation (Bucci et al., 2020; Kühn and Van Franeker, 2020; Savoca et al., 2021).

Particular consideration is paid by the scientific community to the environmental threat of microplastics (MPs), small plastic fragments lower than 5 mm in size (NOAA, 2014).

Due to their features (composition, persistence, small size and chemical/physical property), these "emerging" contaminants are ubiquitous in the marine habitat worldwide (Cózar et al., 2014; Lusher et al., 2015; Waller et al., 2017; Xu et al., 2020) and they can be ingested by marine fauna at different trophic levels and also transferred along the food-web (Fossi et al., 2018).

MPs ingestion and their consequent occurrence in tissues may cause physical/mechanic and chemical harms to marine organisms (Foley et al., 2018; Gola et al., 2021; Palmer and Herat, 2021; Pedà et al., 2016; Prata et al., 2020; Wright et al., 2013).

Particularly, chemical harm is due to the potential transfer of toxic substances from plastic to biota. As a matter of fact, in the marine environment persistent organic pollutants (POPs) and metals may adhere on MPs surface, and under favourable physical/chemical condition MPs are able to leach plastic additives such as phthalates and bisphenol A (Amelia et al., 2021; Kannan and Vimalkumar, 2021; Lee et al., 2014; Liu et al., 2019; Mohamed Nor and Koelmans, 2019; Rochman et al., 2014a, 2013a; Wang et al., 2021).

Marine fauna can, thus, be exposed to contaminants through leaching of plastic additives or other contaminants sorbed on MPs surface (e.g. POPs) and its health status may be affected (Rochman et al., 2014b; Tanaka et al., 2015).

Although the additives and POPs are toxic and can also bio-accumulate (Koelmans et al., 2016; Lithner et al., 2011), the importance of MPs's role in the transfer of these compounds in marine organisms is still debatable (Syberg et al., 2015) and the synergistic effects of MPs and other environmental contaminants on biota health need to be clarified.

Most of toxicity studies investigated the effects of exposure to polyolefins (polyethylene and polypropylene) and polystyrene MPs (De Sá et al., 2018; Vijayaraghavan et al., 2022). The polyvinyl chloride (PVC) represents one the most common polymers in the marine environment (Andrady, 2011) and also the second type of plastic most produced in the world after the polyolefins (Plastics Europe. Plastics - The fact 2020, 2020). Furthermore, PVC is classified as a hazardous polymer due to the carcinogenic properties of its monomer and the high amount of additives integrated during their production processes (Lithner et al., 2011).

At the same time, a large number of studies, focus mainly on short-term and/or acute exposure, underestimating the MPs toxicity at environmentally realistic concentrations and the deriving endpoints (Cormier et al., 2021; De Sá et al., 2018; Vijayaraghavan et al., 2022; Wang et al., 2020). To the best of our knowledge there is limited information on the long-term effects of PVC MPs in teleost (Boyle et al., 2020; Cormier et al., 2021; Ebrahimpour et al., 2021; Espinosa et al., 2019, 2017; Iheanacho et al., 2020; Iheanacho and Odo, 2020; Jovanović et al., 2018; Lei et al., 2018; Pedà et al., 2016; Rochman et al., 2017; Romano et al., 2018; Vijayaraghavan et al., 2022; Xia et al., 2022, 2020) and only Cormier et al. (2021) assessed the physiological effects of PVC MPs during 4 months' exposure in a fish species.

PVC particles ingestion by fish was found to cause inhibition of growth (Cormier et al., 2021; Vijayaraghavan et al., 2022; Xia et al., 2020), decrease in the reproductive output (Cormier et al., 2021), behaviour (Cormier et al., 2021; Vijayaraghavan et al., 2022), enzymatic and tissue alterations (Cormier et al., 2021; Ebrahimpour et al., 2021; Espinosa et al., 2019; Iheanacho and Odo, 2020; Lei et al., 2018; Pedà et al., 2016; Xia et al., 2020), oxidative stress (Cormier et al., 2021; Espinosa et al., 2019; Iheanacho et al., 2020; Iheanacho and Odo, 2020; Vijayaraghavan et al., 2022; Xia et al., 2020), physical toxicity (Xia et al., 2022), immunoregulation (Espinosa et al., 2019, 2017), neurotoxicity (Iheanacho et al., 2020), and gene expression alterations (Boyle et al., 2020; Espinosa et al., 2017; Xia et al., 2022, 2020). However, more experiments are needed to evaluate long-term effects of PVC MPs ingestion and its potential consequences on fish health status.

In this respect, the present research aims to investigate the integrated biomarker responses (at molecular, subcellular and tissue levels) induced over different time endpoints in the liver and blood of European seabass, *Dicentrarchus labrax* (Linnaeus, 1758), by chronic exposure (up to 90 days) to and ingestion of virgin and marine incubated PVC microplastics.

Material and methods

PVC-MPs and treatment diet preparation

Virgin PVC was purchased from a local company and its polymeric nature has been confirmed by Fourier transform infrared (FTIR) spectroscopy technique using an Agilent Cary 630 spectrophotometer (Figure S1). Samples of virgin PVC pellets have been deployed for three months in a Contaminated Site of National Interest (SIN; Italian Directive 23 December 2005 n. 266, art. 1 com. 561; Milazzo harbour, Sicily (IT)) to simulate the natural contamination processes of plastics in the marine environment (incubated PVC pellets). To ensure a uniform distribution of plastic into the feeds, both types of PVC (virgin and incubated) were treated and grinded according to the procedure reported by Pedà et al. (2016). The diets were formulated and prepared in the laboratory at the Institute of Science of Food Production of the CNR as described in previous study (Pedà et al., 2016). The control treatment diet contained 0% plastic while the virgin and incubated PVC treatment diets contained 0.1% (w/w) irregularly shaped plastic fragments lower than 0.3 mm in size. Plastic and feed samples were stored at -20 °C for chemical analysis. Representative images of both types of PVC-MPs surfaces after grinding, obtained by scanning electron microscopy are shown in the supplementary material (Figures S2a and S2b).

Ethical statement

Experiments were authorized by the Italian Ministry of Health and conducted according to the ethical principles indicated by the European Union Directive (2010/63/UE) and Legislative Decree No 26/2014 on the use of animals for scientific purposes. Experiments were carried out in the authorized Aquaculture Experimental Facility of IAMC (now IRBIM) of Messina (IT).

Test organisms

The marine teleost *D. labrax* was selected as test organism for this study because it is very sensitive to the exposure of several pollutants and it is also easy to maintain in laboratory conditions (Ferreira et al., 2010). Moreover, European seabass is a species of ecological and commercial importance, which may be subject to MPs ingestion both in natural environment and in the aquaculture facilities. A total of 162 European seabass specimens (140 ± 8.42 g mean ± SD body weight) were obtained from a commercial fish farm. The fish were randomly placed into 9 indoor tanks (1350 L) and after one month of acclimation, three replicate tanks (18 fish per group) were randomly assigned to each treatment (54 fish per group). Seabass were kept under a natural photo and thermo period and the physico-chemical parameters were daily monitored.

Experimental design and sampling

The fish were exposed for 90 days to three different treatment diets: control (CTRL), virgin microplastics (MPV), incubated microplastics (MPI) and were daily fed by hand at 1.4% of body weight supplied in 2 meals. During all the experimental time, fish were monitored for any possible signs of impaired health status (i.e., feeding behaviour, swimming activity, condition of skin and fins, external lesions). Sampling was carried out after 30 days (T30), 60 days (T60) and at the end of the experiment, 90 days (T90). A total of 18 animals per treatment (6 fish per replica) were sacrificed at random by percussive stunning (followed by rapid destruction of brain) at each sampling. Immediately, blood samples were collected from the caudal vein and few blood drops were used for genotoxicity biomarkers. After, fish were weighed (total weight, TW), measured (total length, TL) and dissected. Livers were removed immediately, weighed and divided in two aliquots. A part of the liver was stored at -80 °C for enzymatic, oxidative stress and gene expression analysis and the other aliquot was fixed in Bouin solution for histological analysis. Muscle samples were collected and then stored at -20 °C for analysis of organochlorine compounds (OCs), and phthalate esters (PAEs).

Morphometric indices

To assess the potential liver stress and the general health status of all seabass exposed to PVC-MPs, Hepatosomatic index (HSI) and Fulton's Condition factor (CF) were calculated as follows:

HSI = liver weight (g) / total weight (g) ×100 (Slooff et al., 1983)

CF = total weight (g) / total length (cm^3) × 100 (Lloret et al., 2013)

Biomarker analysis

Liver ethoxyresorufin-*o*-deethylase (EROD) activity was measured in the S9 fraction according to the methodology developed by Lubet et al. (1985) The EROD activity was expressed as pmol min^{-1} mg $prot^{-1}$.

Lipid peroxidation (LPO) was estimated in liver through the quantification of the malondialdehyde (MDA), a secondary product of the peroxidation process, according to the procedure of Ohkawa et al. (1979) and Bird and Draper (1984) with modifications. The absorbance was measured at 535 nm and the LPO levels were expressed as nmol TBARS mg $prot^{-1}$ using a molar extinction coefficient of a 1.56×10^5 M^{-1} cm^{-1}.

Catalase activity (CAT) in liver samples was determined in cytosol as described by Aebi (1984). The activity was expressed in nmol min^{-1}.

The erythrocytic nuclear abnormalities (ENA) assay was performed in mature erythrocytes as reported by Pacheco and Santos (2002). A total of 1000 mature erythrocytes were counted per individual. The total frequency of anomalies was expressed as the mean value (‰) of the sum for the 4 types of anomalies observed (lobed, kidney, segmented and micronuclei).

These analyses were carried out on tissue samples collected from 81 fish, 3 fish per each replicated tank, at 30, 60 and 90 days of exposure.

TNF receptor-associated factor 3 (TRAF3); Peroxisome proliferator-activated receptor alpha and gamma (PPAR-α, PPAR-γ) and the Estrogen receptor alpha (ER-α) were selected as genes of interest (GOIs) to be analysed in the liver. Gene expression analysis was performed as previously described by Limonta et al. (2019) (see SM). Total RNA was extracted from liver aliquots of 20-70 mg (w. w.) of 54 seabass, 6 fish per each treatment group at each exposure time (2 fish per each replica), using Aurum Total RNA kit (Bio-Rad), in accordance with the manufacturer's protocol. Specific primers were designed using Beacon Designer (Premier Biosoft International), and the amplification efficiency for each primer pair was assessed through a 5-points calibration curve (Table S1). The gene expression was quantified using the software iQ5 optical System Software v. 2.0 (Bio-Rad) according to the ΔΔCt method (Livak and Schmittgen, 2001). The values were expressed as Relative fold expression.

Histopathology investigations were carried out on liver samples collected from 54 fish, 2 fish per replica replicated tank, at 30, 60 and 90 days of exposure. Samples were processed according to Pedà et al. (2016). A semi-quantitative analysis was performed on *at random* section of the liver slide by assigning a score (from 0 to 5) depending on the severity of the tissue alterations (Table S2). Details are reported in supplementary material.

In addition, a macroscopic external and internal (coelomic cavity) examination was performed on each sampled fish in order to detect morphological and tissues abnormalities. The observed abnormal structures were recorded, sampled and then processed for histopathological analysis (See supplementary material).

Chemical analysis

Detection of organochlorine compounds (OCs) and phthalate ester (PAEs) concentrations in PVC-MPs and CTRL feed matrices

OCs contaminants were analysed according to the U.S. Environmental Protection Agency (EPA) 8081/8082 method with laboratory modifications (Marsili and Focardi, 1997) using 1 g of PVC-MPs and CTRL feed matrices. The detection of OCs was performed by gas-chromatography (GC) using a high-resolution capillary gas chromatograph equipped with an electron capture detector (63Ni ECD) (AGILENT 6890/N) and has found HCB, total DDTs (op' and pp'DDT, op and pp' DDE, op' and pp'DDD) and PCBs (30 congeners). The results were expressed as ng/g dry weight (d.w.). Details are reported in supplementary material.

The bis (2-ethylhexyl) phthalate (DEPH) extraction from 1 g of PVC-MPs and CTRL feed matrices was carried out according to the method of Di Bella et al. (2004). Each sample was diluted with n-hexane before GC-MS analysis and each analysis was conducted in triplicate. A HRGC-MS Shimadzu QP2010 System equipped with Supelco SPB-5MS (30 m x 0.25 mm, 0.25 mm film thickness) capillary column was used to detect the DEHP concentrations. The results were expressed as μg/g dry weight (d.w.).

Detection of organochlorine compounds (OCs) and phthalate ester (PAEs) concentrations in seabass muscle tissues

OCs and PAEs levels were investigated in muscle samples (10 g) pooled from 18 fish (6 per replicate) per each treatment and at each sampling time (9 muscle pool) and extracted according to the methods of Marsili and Focardi (1997) and Baini et al. (2017), respectively. Details are reported in supplementary material.

In accordance with Baini et al. (2017) method (see SM), PAEs (mono-benzyl phthalate (MBZP), mono-butyl phthalate (MBP), mono (2-ethylhexyl) phthalate (MEPH); di-n-hexylphthalate (DNHP), benzyl butyl phthalate (BBzP), bis(2-ethylhexyl) phthalate (DEPH) diisooctylisophthalate (DIOIP) and di-n-decyl phthalate (DNDP)), concentrations were measured by Agilent gas chromatograph equipped with a mass spectrometer (GC-MS). The OCs and PAEs results were expressed as μg/g dry weight (d.w.).

Statistical analyses

Before analyses, box plot method has been used to identify outliers' values for all biomarkers data. In the box plots, any data that lies outside the upper or lower fence lines were considered outliers. According to the winsorization method, the outlier's values have been replaced with the largest or lowest value in the data excluding outliers, respectively (Caliani et al., 2019). This analysis was carried out using StatSoft. Statistica.v.10.0. software.

Statistical differences among treatments (CTRL, MPV, MPI) at different exposure times (T30, T60, T90) were assessed by a non-parametric multivariate analysis (one-way PERMANOVA) for the morphometric indices and for each biomarker. The data matrices were square root transformed and analysed on the basis of Euclidean distance, using 4999 permutations. Pair-wise comparisons were computed when significant differences ($p < 0.05$) among factors levels were detected. The analysis was performed using the statistical software PRIMER6 & PERMANOVA+ (Clarke et al., 2014; Gorley and Clarke, 2008).

Principal component analysis (PCA) was applied to the biomarker's matrices including previously published data on intestinal alterations detected in specimens from the same trial (Pedà et al., 2016). The PCA on the biomarker matrix was used to evaluate the relative contribution of each biomarker to the treatment's differences (CTRL, MPV and MPI) at each exposure time. Missing data in the dataset were imputed using the R package missMDA ver. 1.10, (Josse and Husson, 2016) and the results were visualized in biplots. PCAs were generated using the R package FactoMiner ver. 2.3 (Lê et al., 2008).

Results and Discussion

Seabass health status

No mortality and signs of impaired health status were observed during the experimental period for each treatment. Similar results are reported in previous studies of short-term exposure to PVC in fish (Espinosa et al., 2019, 2017). Table S3 shows seabass biometric parameters for each treatment (CTRL, MPV, MPI) to every exposure time.

A first screening to assess the potential impact of MPs exposure was carried out using somatic indices (HSI and CF) shown in Table S4. HSI and CF values measured in the present study ranged from 1.26 to 2.34 and 0.98 to 1.12 g/cm^3 respectively, in all the examined groups. HSI data are similar to those reported by Peres and Oliva-Teles (1999) in farmed seabass (1.64 - 2.8) while the CF values remain within the threshold of 1, a value used as a benchmark for healthy fish (Lloret et al., 2013).

No significant differences of HSI were observed at T30 and T60, whereas HSI decreases significantly ($p < 0.01$) both in MPV and MPI compared with the CTRL group at T90 (Table S7, S8). CF was significantly higher in MPI than CTRL at T60 ($p < 0.05$) and was significantly lower for MPV compared to CTRL at T90 ($p < 0.05$), no significant alterations of the condition index were detected at T30 (Table S7, S8). The ecological indices results are consistent with those of previous studies (Critchell and Hoogenboom, 2018) suggesting that after 90 days both fish fed with incubate and virgin PVC may be subject to hepatic stress. Additionally, the MPV group may also show signs of alteration of the physiological status.

Contaminants levels

PVC-MPs and CTRL feed matrices

Organochlorine compounds (HCB, PCBs, DDTs) and phthalates (DEPH) were detected in both PVC-MPs and CTRL pellet (Table S5). Incubated PVC showed higher levels of total PCBs, almost twice than virgin PVC sample, and the same result was found for HCB compounds, albeit to a lesser extent. Conversely, the values for total DDTs and DEHP were higher in virgin compared to incubated PVC sample.

These results confirm the ability of PVC to adsorb concentrations of persistent organic pollutants (POPs) and mostly, to leach plastic additives such as phthalates (Lambert et al., 2014; Rochman et al., 2013a). The deployment of virgin PVC for 3 months in this contaminated site (D'Alessandro et al., 2016) assured the increase of PCBs and HCBs levels on incubated PVC samples, even if unintelligibly, there was no adsorption of DDTs.

DEHP values were found three times lower in the incubated than virgin PVC showing the leachability of great concentrations of plasticizer from the PVC sample surface in marine environment during the three deployed months. Indeed, DEHP is the most frequently plasticizer used to soften PVC products and, as well all phthalates, it is not chemically bound to the polymer matrix but easily can migrate to the products surface and leaches from it (Lambert et al., 2014).

All contaminants investigated are present in the control feed (Table S5). This result shows as the pellets can be a source of contamination. The PCB and DDTs levels are probably due to fish meal and fish oil used as ingredient in the commercial feeds (Ginés et al., 2018; Schnitzler et al., 2008). Moreover, the presence of corn gluten meal may have affected the control feed for DDTs concentrations. Indeed, the partial or total replacement of fish meal with plant-derived alternatives such as soybeans, wheat gluten, and corn gluten on the one hand could adversely impact the environment with increasing fertilizers and pesticides use (Karbalaei et al., 2020) and on the other hand could contribute to the contamination of feed used in aquaculture industry. The detection of DEHP levels in the CTRL pellet, confirm its abundance and ubiquity in the environment (Lambert et al., 2014).

Seabass muscle tissues

Total PCBs and the eight PAEs (MBZP, MBP, MEPH, DNHP, BBzP, DEPH, DIOIP, DNDP) were quantified in muscle tissue of seabass sampled at each time of exposure and from each treatment groups (Table S6). Our data show similar values of PCBs concentration in the muscles of all groups (CTRL, MPV, MPI) at each exposure time, in the range 0.07 – 0.12 μg/g d.w. Concerning PAEs results, DIOIP and DNDP levels were below the limit of detection in all samples analyzed. Muscle of fish fed with MPI treatment show higher PAEs concentration than MPV and CTRL. PAEs levels in muscle decrease with increasing of the exposure time. Among the investigated PAEs, DEHP is the most frequently detected in all experimental groups, with concentrations ranging from 1.10 to 3.56 μg/g d.w. The decrease of PAEs concentration may be due to the histopathological alterations of the intestine previously described by Pedà et al. (2016) in specimens from the same trial. In this instance, despite the long-term exposure, PAEs are not assimilated in the intestine and neither moved into the muscle.

The levels of PCBs detected in muscle tissues of fish from each treatment group, may be attributed to the higher lipid content and the physiological condition of farmed fish (Antunes and Gil, 2004; Lo Turco et al., 2007). The muscle as well as the liver are considered major sites of lipid storage in fish species (Pérez et al., 2007), where PCBs may be biologically concentrated and stored. Also, considering the ubiquity of PCBs in the environment, it's difficult to distinguish the contribution of PCBs attributable to MPs exposure than that of feed ingredients such as the fish meal and cod liver oil (Rochman et al., 2013b). In addition, although PCB levels were much higher in the incubated PVC samples than in the virgin ones, the same trend was not detected in the muscle tissues of the PVC-MPs feed-fed seabass. This result could be related to the biological mechanism under which MPs would absorb POPs from the organism's tissues, acting as a cleaner of POPs (Koelmans, 2015) but also as in the case of phthalates, it is possible that intestinal inflammations (Pedà et al., 2016) may have interfered with their assimilation.

Finally, the results of this chronic exposure suggest as the PVC microparticles does not effectively transfer POPs and PAEs contaminants to the fish muscles. Indeed, several factors such as the type of polymer and chemical pollutant, the exposure time and the digestion, assimilation and metabolization processes can significantly influence the levels of bio accumulation in organisms exposed to a complex mixture of plastics and to their vehicled pollutants (additives, POPs) (Herrera et al., 2022).

Biomarker responses

EROD values were similar among T30 and T90 treatments, with no significant differences. This result shows that at the concentration of PVC MPs (0.1% w/w), no variations in EROD activity occurred. (Fig. 1a). Significant decrease of EROD activity was observed at T60 for MPI *vs* CTRL ($p < 0.001$) and for MPI *vs* MPV ($p < 0.05$; Table S7, S8). It is possible that some contaminants conveyed by PVC may act as inhibitors when present at high concentrations and in long-term exposures, which is consistent with our results at 60 days of exposure (Rochman et al., 2013b).

LPO levels decreased in MPV and MPI groups at T30, compared to the CTRL, whereas an increase was observed in MPV and MPI groups at T60 and in MPI at T90 (Fig. 1b). Statistically significant differences were observed for MPV *vs* CTRL at T30 ($p < 0.01$) and for MPI *vs* MPV at T90 ($p < 0.01$; Table S7, S8). Furthermore, CAT activity increased for MPI group at T60 compared to CTRL and decreased in both groups (MPV and MPI) at T90 (Fig. 1c). Significant differences were evidenced only at T60 for MPI *vs* MPV ($p < 0.05$; Table S7, S8). Given the propensity for microplastic to interfere with redox homeostasis, the use of antioxidant enzymes (e.g. catalase) and quantification of oxidation levels of lipids and proteins (lipid peroxidation) is widespread in ecotoxicological studies.(Benedetti et al., 2015; Hook et al., 2014; Trestrail et al., 2020) In fact, an increase of antioxidant enzyme or lipid products can highlighted an excess of ROS production. In this study CAT activity and LPO levels were induced after chronic exposure to PVC-MPs only at T60 and T90 for MPI group (for CAT and LPO, respectively). The results of this study indicate a low or absent oxidative cell damage in seabass treated with MPV, while treatment with MPI indicated the presence of oxidative stress, although only related to specific endpoints and treatment times. It is also possible that, to cope with the oxidative stress induced by PVC-MPs, the seabass have activated other components of the antioxidant defense systems, that have not been investigated in this study.(Ding et al., 2018) For these reasons, it cannot be excluded that in the treated seabass the defence from oxidative damage was indeed fully activated as demonstrated by Espinosa et al. (2019).

For the first time, ENA test was used to assess the potential genotoxicity of chronic exposure to PVC-MPs in seabass, showing an increase in ENA frequencies in the MPV and MPI treatments compared to the CTRL group at all exposure times, with the higher value in the MPI treatments (Fig. 1d). The exposure to MPI induced a significant DNA damage at T90 ($p < 0.01$), (Table S7, S8). Although, the ENA frequency observed are lower than to those reported in the literature for fish species exposed to contaminants, we can hypothesize that the MPI treatment in seabass caused a time-dependent response compared to the MVP group especially after 90 days of exposure. Results of MPV group at 90 days are also in line with those found for lipid peroxidation.

TNF receptor-associated factor 3 (TRAF3) is a member of the TNF receptor-associated factor protein family important for the regulation of immune responses (Hildebrand et al., 2011; Zhang et al., 2018). The relative expression of TRAF3 seem to be downregulated in the MPV and MPI treatment compared to the CTRL at any exposure time, with the lowest value in the MPI treatments (Fig. 1e). However, no significant differences were observed in TRAF3 mRNA levels (Table S7, S8). The down-regulation of TRAF3 gene could be related to the presence of pollutants carried by MPs, which act as inhibitors of the TRAF3 antitumor activities (Williams and Hubberstey, 2014). The exposure to nanoplastics and microplastics has been demonstrated to induce the overexpression or inhibition of tumor necrosis factor related genes in seabream and catfish (Balasch et al., 2021; Li'ang Li et al., 2021).

PPAR-α mRNA levels increased in the MPV and MPI treatments compared to the CTRL at T30 and T60, whereas it decreased in both groups at time 90 (Fig. 1f). Significant differences ($p < 0.05$) for MPI *vs* CTRL and for MPI *vs* MPV treatments were detected at T30 (Table S7, S8). PPAR-γ mRNA expression levels seem to have an opposite pattern of expression than PPAR-α. An increase in expression levels was observed in both treatments (MPV and MPI) compared to CTRL at T30 and T60, with a highest value in the MPV treatments. After 90 days of exposure, the PPAR-γ expression decreases in the MPV and MPI treatment compared to the CTRL (Fig. 1g). Significant down-regulation of PPAR-γ expression compared to CTRL were observed in the MPI ($p < 0.01$) and MPV ($p < 0.05$) treatments at T90 (Table S7, S8). Peroxisome proliferator-activated (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor family that regulate the expression of target genes involved in cellular proliferation, differentiation and apoptosis, in lipid and lipoprotein metabolism, in glucose homeostasis and immune and inflammation responses (la Cour Poulsen et al., 2012). Changes of the expression of the peroxisome proliferator-activated receptors could be attributed to plastic additives present in the PVC-MPs administered to seabass as previously demonstrated in humans (Kannan and Vimalkumar, 2021).

The significant up-regulation of PPAR-α in MPV and MPI treatment groups at 30 days of exposure and the higher expression of PPAR-γ in the MPV treatments at T30 and T60 could be correlated with phthalates' presence and their leaching from PVC-MPs. Interestingly, the significant down-regulation of PPAR-γ in both MPV and MPI treatment groups at T90 can be partially correlated with the reduced intake of contaminants, in accordance with the decrease of PAEs concentration in seabass muscle at T90, but may also indicate the development of chronic inflammation (Heming et al., 2018; Straus and Glass, 2007). Finally, ER-α expression was used to assess the potential endocrine disruption due to virgin and incubated MPs. ER-α mRNA levels appear to be lower in the MPV and MPI treatments than in the CTRL at all exposure times, with the lowest value in the MPI treatment. Up-regulation of ER-α compared to the CTRL, although not significantly, was observed only in the MPV group (Fig. 1h). PERMANOVA analysis showed significant differences ($p< 0.05$) for MPI *vs* CTRL and MPI *vs* MPV at T60 and for MPI *vs* CTRL treatments at T90 ($p< 0.01$), (Table S7, S8). The down-regulation of ER-α in both experimental treatments suggest that chemicals associated with PVC-MPs act as anti-estrogenic and/or antagonize the binding of endogenous estrogens (Rochman et al., 2014b). On the other hand, the higher expression of this gene in the CTRL may also be due to the higher contaminant's concentration in control feed. It is worth mentioning that the ERα level of expressions does not correlate with the contaminants load detected in the food pellets, this can be explained by the fact that endocrine disruptors have shown evidence of a nonlinear or nonmonotonic dose-response relationship, meaning that low doses may have larger effects than mid-level doses.

At tissue level, the liver was chosen as the target organ to evaluate the effects of PVC-MPs exposure because it plays an important role in the detoxification and biotransformation processes of toxic compounds in organisms. All liver samples in the different experimental times analyzed during the histological investigation were characterized by strongly lipid accumulation (steatosis). This liver para-physiological condition is reported in the literature for farmed fish, because of a diet based on commercial feed (Saraiva et al., 2015). Therefore, the CTRL group at each exposure time showed slight to pronounced alterations (Fig. 1i), hyperemia, lipid accumulation and hepatocyte vacuolization were observed (Fig. S3a). The animals treated with PVC-MPs presented worse histological conditions than in the CTRL at each exposure time, presenting slight to markedly severe alterations ranged from 1.5 to 5 score values (Fig. 1i), other than the MPI group at T60. Only the latter, in fact, presented similar conditions to the control group. The liver of MPV and MPI treatments showed abnormal cell morphology, hypertrophy, vacuolation and increase of lipid in hepatocytes (Fig. S3c). Consequently, an alteration of tissue architecture was observed, with a loss of parenchymal organization, with hepatocytes organized as irregular cord-like structures and nuclei placed in a lateral position, often irregular in shape and smaller in size (Fig. S3b, c, d, e). In addition, circulatory disorders such as oedematous areas and vessels congestion were also detected (Fig. S3d, e). PERMANOVA test applied on the score values data matrix showed significant differences ($p < 0.01$) for CTRL *vs* MPV and MPI treatments at T30 and for CTRL *vs* MPI in T90, and significant differences ($p< 0.05$) between CTRL and MPV group at T90 (Table S7, S8). Our histological results show that after just 30 days of exposure, ingested MPs may already be able to affect the liver health and functioning in fish and that these changes may persist during the chronic exposure (90 days) in accordance with the HSI results presented. Moreover, similar effects in fish fed with both virgin and incubated MPs were previously reported by Rochman et al. (2013b). Other recent studies on fish species fed with PVC-MPs (Espinosa et al., 2019; Iheanacho and Odo, 2020; Rochman et al., 2017; Xia et al., 2020) or PE and PS-MPs (Rochman et al., 2013b) observed the same hepatic alterations. In addition, Rochman et al. (2017) found the most severe histological alterations in freshwater organisms exposed to PVC and PCBs rather than to polymers such as PET and PE.

Principal Component Analysis on biochemical, molecular and histopathological biomarkers

In order to gain a better understanding of the integrated biomarker response, the variability among samples was analyzed through a Principal Component Analysis (PCA).

The PCA analysis of the CTRL groups data during the experimental period, showed a substantial overlap between the three CTRLs indicating a higher variability within groups than between different groups (Fig.2a).

At T30 the highest variability was observed between CTRL and the MPI treatment, the biomarkers that strongly contributed to the variation were liver and intestine histology, PPAR- α and ER- α, while LPO and PPAR-γ variability was associated with the MPV treatment (Fig.2b). At T60, the most of the variability is explained by the PC1 (43.26%), where the MPI treatment is clearly separated from CTRL, with the strongest contribution from EROD, ER- α, PPAR-α and liver and intestine histology. Compared to T30, the contribution of EROD, which seems correlated with ER- α expression, became preponderant, suggesting that this biomarker may not be suitable for the detection of acute PVC-MPs effects (Fig.2c). At T90, PCA analyses showed clear differences among CTRL and both PVC-MPs treatments, which present a more evident overlap between them. At 90 days of exposure, ENA, PPAR-γ contribution is stronger than at T30 and T60, according to this PVC-MPs may be able to exert genotoxic effects after a chronic exposure (Fig.2c).

Neoplasms

On coelomic cavity macroscopic examination, neoplasm-like structures were observed in 27.8% of specimens belonging to the MPI group after 60 and 90 days of exposure and in 16.7 % of individuals in the MPV treatment at T90. The same structures were not found in the CTRL group at any exposure time. The masses were associated to perivisceral adipose tissue and located in the coelomic cavity between the pyloric cecum, between intestine and spleen and at the mesenteric level. In some specimens, the presence of multiple neoplasm-like structures was also found in different parts of the coelomic cavity. The neoplasm-like structures appeared pedunculated, vascularized, well delimited and circumscribed, nodular or elongated shaped, sometime flattened and variable in size (not exceed 2 cm). These structures were also variable in color from yellowish to cherry red, sometimes mottled, and in consistency from soft to lardaceous (Fig. 3a, b, c, d). Histologically, these structures are attributable to different phases of the visceral fat degenerative processes that in some cases have evolved into lipomas. The neoplastic structures diagnosed as lipoma were characterized by grouped of well-differentiated mature adipocytes relatively homogeneous in shape and size (Fig. 3e). Lipoma is a rare benign tumor observed in marine fish and their occurrence increases with age (Çiğdem, 2021). It was also reported in farmed seabass but with different localization and features than to this study (Çiğdem, 2021; Marino et al., 2011). Generally, the aetiology is unknown, but it is not excluded that alterations in fat metabolism, dysmetabolic disturbance, viral infections, endocrine or neurological disorders and chemical contaminants exposure may induce their development (Çiğdem, 2021; Marino et al., 2011; Volpatti et al., 1998). In this study, we could hypothesize that the development of these structures and particularly of neoplasm tissue (lipomas), could be related to the MPs exposure. As shown in Figure S4, comparing the biomarker responses in *D. labrax* specimens with and without neoplasms at T60 and T90 times, statistical analysis shows a significant difference in the MPI group with neoplasms for EROD activity at T60 and for ENA frequencies and PPAR-γ expression at T90. No statistical differences were found in specimens with and without neoplasms of MPV group at T90 (Table S9, S10).

General conclusion and future developments

Exposure to MPs may induce complex responses and their potential environmental and ecological consequences are a challenging task. Although several laboratory studies investigated the toxicological effects of different polymer-type MPs on marine biota, there is still a knowledge gap on the of MPs effects at environmentally relevant concentrations and the role of MPs contaminants.

This work, represents one of the few studies, that investigates the toxicity effects of PVC-MPs exposure in seabass under controlled laboratory condition:

- during 90 exposure days (a long-term experiment);
- using an environmentally realistic MPs concentration (0.1% w/w) (Caruso et al., 2018);
- examining biomarker responses at different levels of biological organization;
- analyzing the chemical contaminants of MPs (additives and POPs levels) and their role during the chronic exposure.

The results showed that virgin and incubated PVC-MPs ingestion after 90 days of exposure, does not cause irreversible alterations in *D. labrax*. However, the chronic exposure at low PVC-MPs concentrations, may cause mechanical-physical injury at intestinal level (Pedà et al., 2016). Furthermore, early warning signs of toxicological harm in liver were observed, as supported by the changes of somatic indices, the presence of oxidative stress, the histopathological alteration and also by the modulation of the PPARs and Er-α genes expression. The gradual increase of DNA alterations and the observation of some neoformations attributable to lipomas indicates genotoxic and cancerogenic effects of PVC, which can affect the metabolism, altering some physiological processes. Further research is necessary to better clarify neoplastic masses development and their relationship to PVC ingestion.

These results could provide an important contribution to better understand the regulatory biological processes affected by MPs ingestion in marine organisms and may also support the interpretation of results provided by studies on wild species. Furthermore, long-term studies are needed, also considering the inter-relationship of plastic polymers, additives, and other contaminants as inescapable parameters for reliable MPs toxicity assessment in fish.

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References

Aebi, H., 1984. [13] Catalase in vitro, in: Methods in Enzymology. Elsevier, pp. 121–126.

Amelia, T.S.M., Khalik, W.M.A.W.M., Ong, M.C., Shao, Y.T., Pan, H.-J., Bhubalan, K., 2021. Marine microplastics as vectors of major ocean pollutants and its hazards to the marine ecosystem and humans. Prog. Earth Planet. Sci. 8, 1–26.

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605.

Antunes, P., Gil, O., 2004. PCB and DDT contamination in cultivated and wild sea bass from Ria de Aveiro, Portugal. Chemosphere 54, 1503–1507.

Baini, M., Martellini, T., Cincinelli, A., Campani, T., Minutoli, R., Panti, C., Finoia, M.G., Fossi, M.C., 2017. First detection of seven phthalate esters (PAEs) as plastic tracers in superficial neustonic/planktonic samples and cetacean blubber. Anal. Methods 9, 1512–1520.

Balasch, J.C., Brandts, I., Barría, C., Martins, M.A., Tvarijonaviciute, A., Tort, L., Oliveira, M., Teles, M., 2021. Short-term exposure to polymethylmethacrylate nanoplastics alters muscle antioxidant response, development and growth in Sparus aurata. Mar. Pollut. Bull. 172, 112918.

Benedetti, M., Giuliani, M.E., Regoli, F., 2015. Oxidative metabolism of chemical pollutants in marine organisms: molecular and biochemical biomarkers in environmental toxicology. Ann. N. Y. Acad. Sci. 1340, 8–19.

Bird, R.P., Draper, H.H., 1984. [35] Comparative studies on different methods of malonaldehyde determination, in: Methods in Enzymology. Elsevier, pp. 299–305.

Boyle, D., Catarino, A.I., Clark, N.J., Henry, T.B., 2020. Polyvinyl chloride (PVC) plastic fragments release Pb additives that are bioavailable in zebrafish. Environ. Pollut. 263, 114422.

Bucci, K., Tulio, M., Rochman, C.M., 2020. What is known and unknown about the effects of plastic pollution: A meta- analysis and systematic review. Ecol. Appl. 30, e02044.

Caliani, I., Poggioni, L., D'Agostino, A., Fossi, M.C., Casini, S., 2019. An immune response-based approach to evaluate physiological stress in rehabilitating loggerhead sea turtle. Vet. Immunol. Immunopathol. 207, 18–24. https://doi.org/10.1016/j.vetimm.2018.11.013

Caruso, G., Pedà, C., Cappello, S., Leonardi, M., La Ferla, R., Lo Giudice, A., Maricchiolo, G., Rizzo, C., Maimone, G., Rappazzo, A.C., 2018. Effects of microplastics on trophic parameters, abundance and metabolic activities of seawater and fish gut bacteria in mesocosm conditions. Environ. Sci. Pollut. Res. 25, 30067–30083. https://doi.org/10.1007/s11356-018-2926-x

Çiğdem, Ü., 2021. Subcutaneous Infiltrative Lipoma in a Cultured European Seabass (Dicentrarchus labrax). Aquat. Sci. Eng. 36, 34–37.

Clarke, K.R., Gorley, R.N., Somerfield, P.J., Warwick, R.M., 2014. Change in marine communities: an approach to statistical analysis and interpretation.

Cormier, B., Le Bihanic, F., Cabar, M., Crebassa, J.-C., Blanc, M., Larsson, M., Dubocq, F., Yeung, L., Clérandeau, C., Keiter, S.H., 2021. Chronic feeding exposure to virgin and spiked microplastics disrupts essential biological functions in teleost fish. J. Hazard. Mater. 415, 125626. https://doi.org/10.1016/j.jhazmat.2021.125626

Cózar, A., Echevarría, F., González-Gordillo, J.I., Irigoien, X., Úbeda, B., Hernández-León, S., Palma, Á.T., Navarro, S., García-de-Lomas, J., Ruiz, A., 2014. Plastic debris in the open ocean. Proc. Natl. Acad. Sci. 111, 10239–10244. https://doi.org/10.1073/pnas.1314705111

Critchell, K., Hoogenboom, M.O., 2018. Effects of microplastic exposure on the body condition and behaviour of planktivorous reef fish (Acanthochromis polyacanthus). PLoS One 13, e0193308.

D'Alessandro, M., Esposito, V., Giacobbe, S., Renzi, M., Mangano, M.C., Vivona, P., Consoli, P., Scotti, G., Andaloro, F., Romeo, T., 2016. Ecological assessment of a heavily human-stressed area in the Gulf of Milazzo, Central Mediterranean Sea: an integrated study of biological, physical and chemical indicators. Mar. Pollut. Bull. 106, 260–273.

De Sá, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? Sci. Total Environ. 645, 1029–1039.

Di Bella, G., Saitta, M., La Pera, L., Alfa, M., Dugo, G., 2004. Pesticide and plasticizer residues in bergamot essential oils from Calabria (Italy). Chemosphere 56, 777–782. https://doi.org/10.1016/j.chemosphere.2004.04.024

Ding, J., Zhang, S., Razanajatovo, R.M., Zou, H., Zhu, W., 2018. Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (Oreochromis niloticus). Environ. Pollut. 238, 1–9. https://doi.org/10.1016/j.envpol.2018.03.001

Ebrahimpour, K., Baradaran, A., Darabi, H., 2021. Subacute toxic effects of polyvinyl chloride microplastics (PVC-MPs) in juvenile common carp (Cyprinus carpio).

Espinosa, C., Cuesta, A., Esteban, M.Á., 2017. Effects of dietary polyvinylchloride microparticles on general health, immune status and expression of several genes related to stress in gilthead seabream (Sparus aurata L.). Fish Shellfish Immunol. 68, 251–259. https://doi.org/10.1016/j.fsi.2017.07.006

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

Ferreira, M., Caetano, M., Antunes, P., Costa, J., Gil, O., Bandarra, N., Pousão-Ferreira, P., Vale, C., Reis-Henriques, M.A., 2010. Assessment of contaminants and biomarkers of exposure in wild and farmed seabass. Ecotoxicol. Environ. Saf. 73, 579–588.

Foley, C.J., Feiner, Z.S., Malinich, T.D., Höök, T.O., 2018. A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates. Sci. Total Environ. 631, 550–559.

Fossi, M.C., Pedà, C., Compa, M., Tsangaris, C., Alomar, C., Claro, F., Ioakeimidis, C., Galgani, F., Hema, T., Deudero, S., Romeo, T., Battaglia, P., Andaloro, F., Caliani, I., Casini, S., Panti, C., Baini, M., 2018. Bioindicators for monitoring marine litter ingestion and its impacts on Mediterranean biodiversity. Environ. Pollut. 237, 1023–1040. https://doi.org/10.1016/j.envpol.2017.11.019

Ginés, R., Camacho, M., Henríquez-Hernández, L.A., Izquierdo, M., Boada, L.D., Montero, D., Robaina, L., Zumbado, M., Luzardo, O.P., 2018. Reduction of persistent and semi-persistent organic pollutants in fillets of farmed European seabass (Dicentrarchus labrax) fed low fish oil diets. Sci. Total Environ. 643, 1239–1247.

Gola, D., Tyagi, P.K., Arya, A., Chauhan, N., Agarwal, M., Singh, S.K., Gola, S., 2021. The impact of microplastics on marine environment: A review. Environ. Nanotechnology, Monit. Manag. 16, 100552.

Gorley, A.M., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: guide to software and statistical methods. Prim. Plymouth, UK.

Heming, M., Gran, S., Jauch, S.-L., Fischer-Riepe, L., Russo, A., Klotz, L., Hermann, S., Schäfers, M., Roth, J., Barczyk-Kahlert, K., 2018. Peroxisome proliferator-activated receptor-γ modulates the response of macrophages to lipopolysaccharide and glucocorticoids. Front. Immunol. 9, 893. https://doi.org/10.3389/fimmu.2018.00893

Herrera, A., Acosta-Dacal, A., Luzardo, O.P., Martínez, I., Rapp, J., Reinold, S., Montesdeoca-Esponda, S., Montero, D., Gómez, M., 2022. Bioaccumulation of additives and chemical contaminants from environmental microplastics in European seabass (Dicentrarchus labrax). Sci. Total Environ. 153396. https://doi.org/10.1016/j.scitotenv.2022.153396

Hildebrand, J.M., Yi, Z., Buchta, C.M., Poovassery, J., Stunz, L.L., Bishop, G.A., 2011. Roles of tumor necrosis factor receptor associated factor 3 (TRAF3) and TRAF5 in immune cell functions. Immunol. Rev. 244, 55–74. https://doi.org/10.1111/j.1600-065X.2011.01055.x

Hook, S.E., Gallagher, E.P., Batley, G.E., 2014. The role of biomarkers in the assessment of aquatic ecosystem health. Integr. Environ. Assess. Manag. 10, 327–341.

Iheanacho, S.C., Igberi, C., Amadi-Eke, A., Chinonyerem, D., Iheanacho, A., Avwemoya, F., 2020. Biomarkers of neurotoxicity, oxidative stress, hepatotoxicity and lipid peroxidation in Clarias gariepinus exposed to melamine and polyvinyl chloride. Biomarkers 25, 603–610. https://doi.org/10.1080/1354750X.2020.1821777

Iheanacho, S.C., Odo, G.E., 2020. Dietary exposure to polyvinyl chloride microparticles induced oxidative stress and hepatic damage in Clarias gariepinus (Burchell, 1822). Environ. Sci. Pollut. Res. 27, 21159–21173.

Josse, J., Husson, F., 2016. missMDA: a package for handling missing values in multivariate data analysis. J. Stat. Softw. 70, 1–31.

Jovanović, B., Gökdağ, K., Güven, O., Emre, Y., Whitley, E.M., Kideys, A.E., 2018. Virgin microplastics are not causing imminent harm to fish after dietary exposure. Mar. Pollut. Bull. 130, 123–131. https://doi.org/10.1016/j.marpolbul.2018.03.016

Kannan, K., Vimalkumar, K., 2021. A review of human exposure to microplastics and insights into microplastics as obesogens. Front. Endocrinol. (Lausanne). 978.

Karbalaei, S., Golieskardi, A., Watt, D.U., Boiret, M., Hanachi, P., Walker, T.R., Karami, A., 2020. Analysis and inorganic composition of microplastics in commercial Malaysian fish meals. Mar. Pollut. Bull. 150, 110687.

Koelmans, A.A., 2015. Modeling the role of microplastics in bioaccumulation of organic chemicals to marine aquatic organisms. A critical review. Mar. Anthropog. litter 309–324.

Koelmans, A.A., Bakir, A., Burton, G.A., Janssen, C.R., 2016. Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. Environ. Sci. Technol. 50, 3315–3326. https://doi.org/10.1021/acs.est.5b06069

Kühn, S., Van Franeker, J.A., 2020. Quantitative overview of marine debris ingested by marine megafauna. Mar. Pollut. Bull. 151, 110858. https://doi.org/10.1016/j.marpolbul.2019.110858

la Cour Poulsen, L., Siersbæk, M., Mandrup, S., 2012. PPARs: fatty acid sensors controlling metabolism, in: Seminars in Cell & Developmental Biology. Elsevier, pp. 631–639.

Lambert, S., Sinclair, C., Boxall, A., 2014. Occurrence, degradation, and effect of polymer-based materials in the environment. Rev. Environ. Contam. Toxicol. Vol. 227 1–53.

Lê, S., Josse, J., Husson, F., 2008. FactoMineR: an R package for multivariate analysis. J. Stat. Softw. 25, 1–18.

Lee, H., Shim, W.J., Kwon, J.-H., 2014. Sorption capacity of plastic debris for hydrophobic organic chemicals. Sci. Total Environ. 470, 1545–1552.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018. Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. Sci. Total Environ. 619, 1–8. https://doi.org/10.1016/j.scitotenv.2017.11.103

Li'ang Li, R.X., Jiang, L., Xu, E.G., Wang, M., Wang, J., Li, B., Hu, M., Zhang, L., Wang, Y., 2021. Effects of Microplastics on Immune Responses of the Yellow Catfish Pelteobagrus fulvidraco Under Hypoxia. Front. Physiol. 12.

Limonta, G., Mancia, A., Benkhalqui, A., Bertolucci, C., Abelli, L., Fossi, M.C., Panti, C., 2019. Microplastics induce transcriptional changes, immune response and behavioral alterations in adult zebrafish. Sci. Rep. 9, 1–11. https://doi.org/10.1038/s41598-019-52292-5

Lithner, D., Larsson, Å., Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Sci. Total Environ. 409, 3309–3324.

Liu, X., Shi, H., Xie, B., Dionysiou, D.D., Zhao, Y., 2019. Microplastics as both a sink and a source of bisphenol A in the marine environment. Environ. Sci. Technol. 53, 10188–10196.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods 25, 402–408.

Lloret, J., Shulman, G., Love, R.M., 2013. Condition and health indicators of exploited marine fishes. John Wiley & Sons.

Lo Turco, V., Di Bella, G., La Pera, L., Conte, F., Macrí, B., 2007. Organochlorine pesticides and polychlorinated biphenyl residues in reared and wild Dicentrarchus labrax from the

Mediterranean Sea (Sicily, Italy). Environ. Monit. Assess. 132, 411–417.

Lubet, R.A., Nims, R.W., Mayer, R.T., Cameron, J.W., Schechtman, L.M., 1985. Measurement of cytochrome P-450 dependent dealkylation of alkoxy-phenoxazones in hepatic S9s and hepatocyte homogenates: effects of dicumarol. Mutat. Res. 142, 127–131.

Lusher, A.L., Tirelli, V., O'Connor, I., Officer, R., 2015. Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples. Sci. Rep. 5, 1–9. https://doi.org/10.1038/srep14947

Marino, F., Chiofalo, B., Mazzullo, G., Panebianco, A., 2011. Multicentric infiltrative lipoma in a farmed Mediterranean seabass Dicentrarchus labrax: a pathological and biochemical case study. Dis. Aquat. Organ. 96, 259–264.

Marsili, L., Focardi, S., 1997. Chlorinated hydrocarbon (HCB, DDTs and PCBs levels in cetaceans stranded along the Italian coasts: an overview. Environ. Monit. Assess. 45, 129–180.

Mohamed Nor, N.H., Koelmans, A.A., 2019. Transfer of PCBs from microplastics under simulated gut fluid conditions is biphasic and reversible. Environ. Sci. Technol. 53, 1874–1883.

NOAA, 2014. National Oceanic and Atmospheric Administration.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Pacheco, M., Santos, M.A., 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (Anguilla anguilla L.). Ecotoxicol. Environ. Saf. 53, 331–347.

Palmer, J., Herat, S., 2021. Ecotoxicity of microplastic pollutants to marine organisms: A systematic review. Water, Air, Soil Pollut. 232, 1–21.

Pedà, C., Caccamo, L., Fossi, M.C., Gai, F., Andaloro, F., Genovese, L., Perdichizzi, A., Romeo, T., Maricchiolo, G., 2016. Intestinal alterations in European sea bass Dicentrarchus labrax (Linnaeus, 1758) exposed to microplastics: preliminary results. Environ. Pollut. 212, 251–256. https://doi.org/10.1016/j.envpol.2016.01.083

Peres, H., Oliva-Teles, A., 1999. Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (Dicentrarchus labrax). Aquaculture 179, 325–334.

Pérez, M.J., Rodríguez, C., Cejas, J.R., Martín, M. V, Jerez, S., Lorenzo, A., 2007. Lipid and fatty acid content in wild white seabream (Diplodus sargus) broodstock at different stages of the reproductive cycle. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 146, 187–196.

Plastics Europe. Plastics - The fact 2020, 2020. An Analysis of European Plastics Production, Demand and Waste Data. Brussels, Belgium.

Prata, J.C., da Costa, J.P., Lopes, I., Duarte, A.C., Rocha-Santos, T., 2020. Environmental exposure to microplastics: An overview on possible human health effects. Sci. Total Environ. 702, 134455.

Rochman, C.M., Hentschel, B.T., Teh, S.J., 2014a. Long-term sorption of metals is similar among plastic types: implications for plastic debris in aquatic environments. PLoS One 9, e85433. https://doi.org/10.1371/journal.pone.0085433

Rochman, C.M., Hoh, E., Hentschel, B.T., Kaye, S., 2013a. Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. Environ. Sci. Technol. 47, 1646–1654. https://doi.org/10.1021/es303700s

Rochman, C.M., Hoh, E., Kurobe, T., Teh, S.J., 2013b. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Sci. Rep. 3, 1–7. https://doi.org/10.1038/srep03263

Rochman, C.M., Kurobe, T., Flores, I., Teh, S.J., 2014b. 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. 493, 656–661.

Rochman, C.M., Parnis, J.M., Browne, M.A., Serrato, S., Reiner, E.J., Robson, M., Young, T., Diamond, M.L., Teh, S.J., 2017. Direct and indirect effects of different types of microplastics on freshwater prey (Corbicula fluminea) and their predator (Acipenser transmontanus). PLoS One 12, e0187664.

Romano, N., Ashikin, M., Teh, J.C., Syukri, F., Karami, A., 2018. Effects of pristine polyvinyl chloride fragments on whole body histology and protease activity in silver barb Barbodes gonionotus fry. Environ. Pollut. 237, 1106–1111.

Saraiva, A., Costa, J., Serrão, J., Cruz, C., Eiras, J.C., 2015. A histology-based fish health assessment of farmed seabass (Dicentrarchus labrax L.). Aquaculture 448, 375–381.

Savoca, M.S., McInturf, A.G., Hazen, E.L., 2021. Plastic ingestion by marine fish is widespread and increasing. Glob. Chang. Biol. 27, 2188–2199. https://doi.org/10.1016/j.aquaculture.2015.06.028

Schnitzler, J.G., Koutrakis, E., Siebert, U., Thomé, J.P., Das, K., 2008. Effects of persistent organic pollutants on the thyroid function of the European sea bass (Dicentrarchus labrax) from the Aegean sea, is it an endocrine disruption? Mar. Pollut. Bull. 56, 1755–1764.

Slooff, W., Van Kreijl, C.F., Baars, A.J., 1983. Relative liver weights and xenobiotic-metabolizing enzymes of fish from polluted surface waters in the Netherlands. Aquat. Toxicol. 4, 1–14.

Straus, D.S., Glass, C.K., 2007. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. Trends Immunol. 28, 551–558.

Syberg, K., Khan, F.R., Selck, H., Palmqvist, A., Banta, G.T., Daley, J., Sano, L., Duhaime, M.B., 2015. Microplastics: addressing ecological risk through lessons learned. Environ. Toxicol. Chem. 34, 945–953. https://doi.org/10.1002/etc.2914

Tanaka, K., Takada, H., Yamashita, R., Mizukawa, K., Fukuwaka, M., Watanuki, Y., 2015. Facilitated leaching of additive-derived PBDEs from plastic by seabirds' stomach oil and accumulation in tissues. Environ. Sci. Technol. 49, 11799–11807.

Trestrail, C., Nugegoda, D., Shimeta, J., 2020. Invertebrate responses to microplastic ingestion: Reviewing the role of the antioxidant system. Sci. Total Environ. 734, 138559.

Vijayaraghavan, G., Neethu, K.V., Aneesh, B.P., Suresh, A., Saranya, K.S., Bijoy Nandan, S., Sharma, K.V., 2022. Evaluation of toxicological impacts of Polyvinyl Chloride (PVC) microplastics on fish, Etroplus suratensis (Bloch, 1790), Cochin estuary, India. Toxicol. Environ. Health Sci. 1–10.

Volpatti, D., Patarnello, P., Novelli, A., D’Angelo, L., Musetti, R., Galeotti, M., 1998. Lipoma, fibrolipoma, liposarcoma in mormore, Lithognatus mormyrus (L.) allevate: osservazioni istologiche e ultrastrutturali. V convegno, Soc. Ital. di Pathol. Ittica (SIPI), Rome.

Waller, C.L., Griffiths, H.J., Waluda, C.M., Thorpe, S.E., Loaiza, I., Moreno, B., Pacherres, C.O., Hughes, K.A., 2017. Microplastics in the Antarctic marine system: an emerging area of research. Sci. Total Environ. 598, 220–227.

Wang, L.-C., Chun-Te Lin, J., Dong, C.-D., Chen, C.-W., Liu, T.-K., 2021. The sorption of persistent organic pollutants in microplastics from the coastal environment. J. Hazard. Mater. 420, 126658.

Wang, W., Ge, J., Yu, X., 2020. Bioavailability and toxicity of microplastics to fish species: a review. Ecotoxicol. Environ. Saf. 189, 109913. https://doi.org/10.1016/j.ecoenv.2019.109913

Williams, R., Hubberstey, A. V, 2014. Benzo (a) pyrene exposure causes adaptive changes in p53 and CYP1A gene expression in Brown bullhead (Ameiurus nebulosus). Aquat. Toxicol. 156, 201–210.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492.

Xia, B., Sui, Q., Du, Y., Wang, L., Jing, J., Zhu, L., Zhao, X., Sun, X., Booth, A.M., Chen, B., 2022. Secondary PVC microplastics are more toxic than primary PVC microplastics to Oryzias melastigma embryos. J. Hazard. Mater. 424, 127421. https://doi.org/10.1016/j.jhazmat.2021.127421

Xia, X., Sun, M., Zhou, M., Chang, Z., Li, L., 2020. Polyvinyl chloride microplastics induce growth inhibition and oxidative stress in Cyprinus carpio var. larvae. Sci. Total Environ. 716, 136479.

Xu, S., Ma, J., Ji, R., Pan, K., Miao, A.-J., 2020. Microplastics in aquatic environments: occurrence, accumulation, and biological effects. Sci. Total Environ. 703, 134699.

Zhang, W., Jia, P., Liu, W., Li, Y., Yi, M., Jia, K., 2018. Functional characterization of tumor necrosis factor receptor-associated factor 3 of sea perch (Lateolabrax japonicas) in innate immune. Fish Shellfish Immunol. 75, 1–7. https://doi.org/10.1016/j.fsi.2018.01.039

Figure 1

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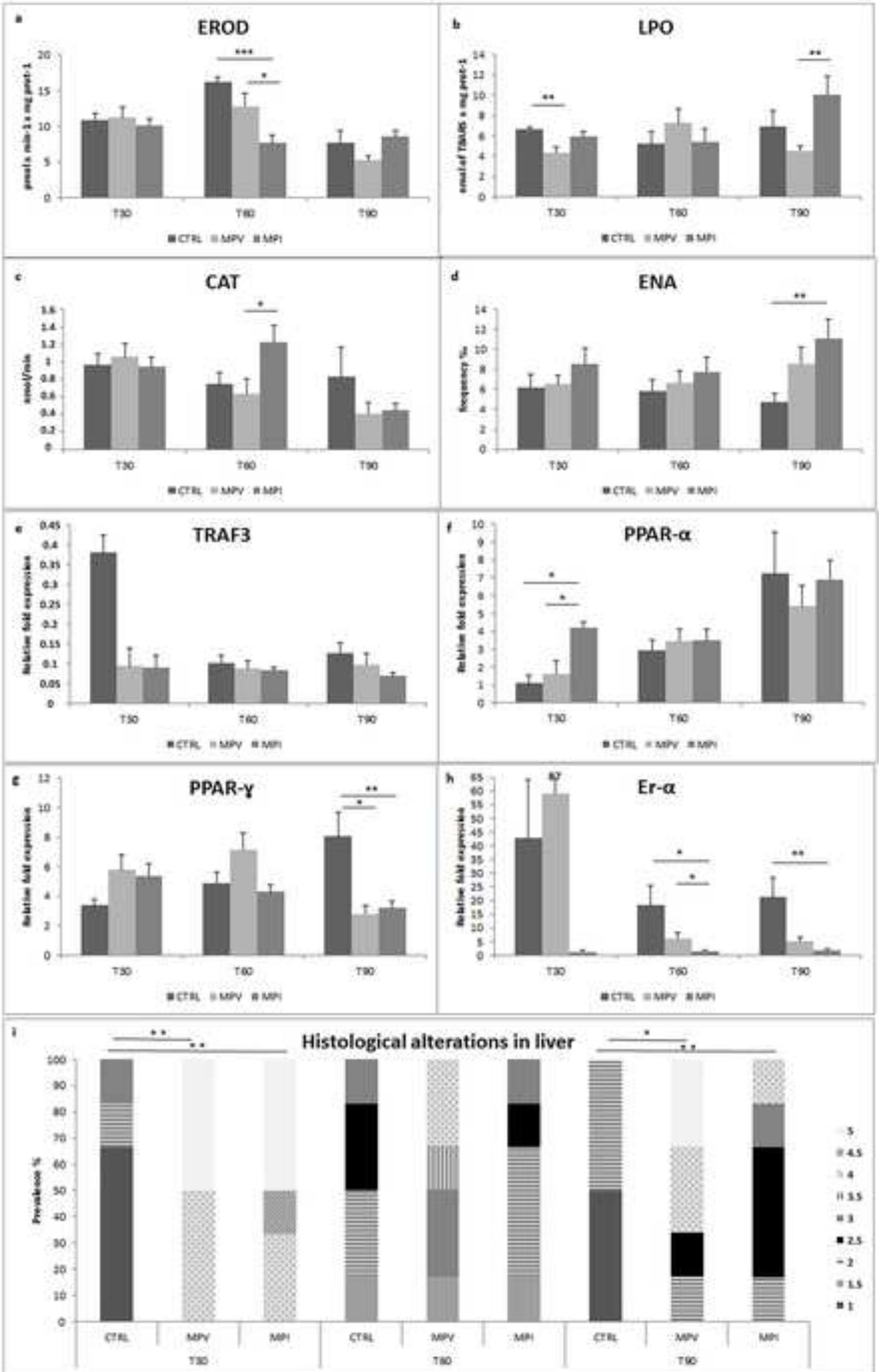

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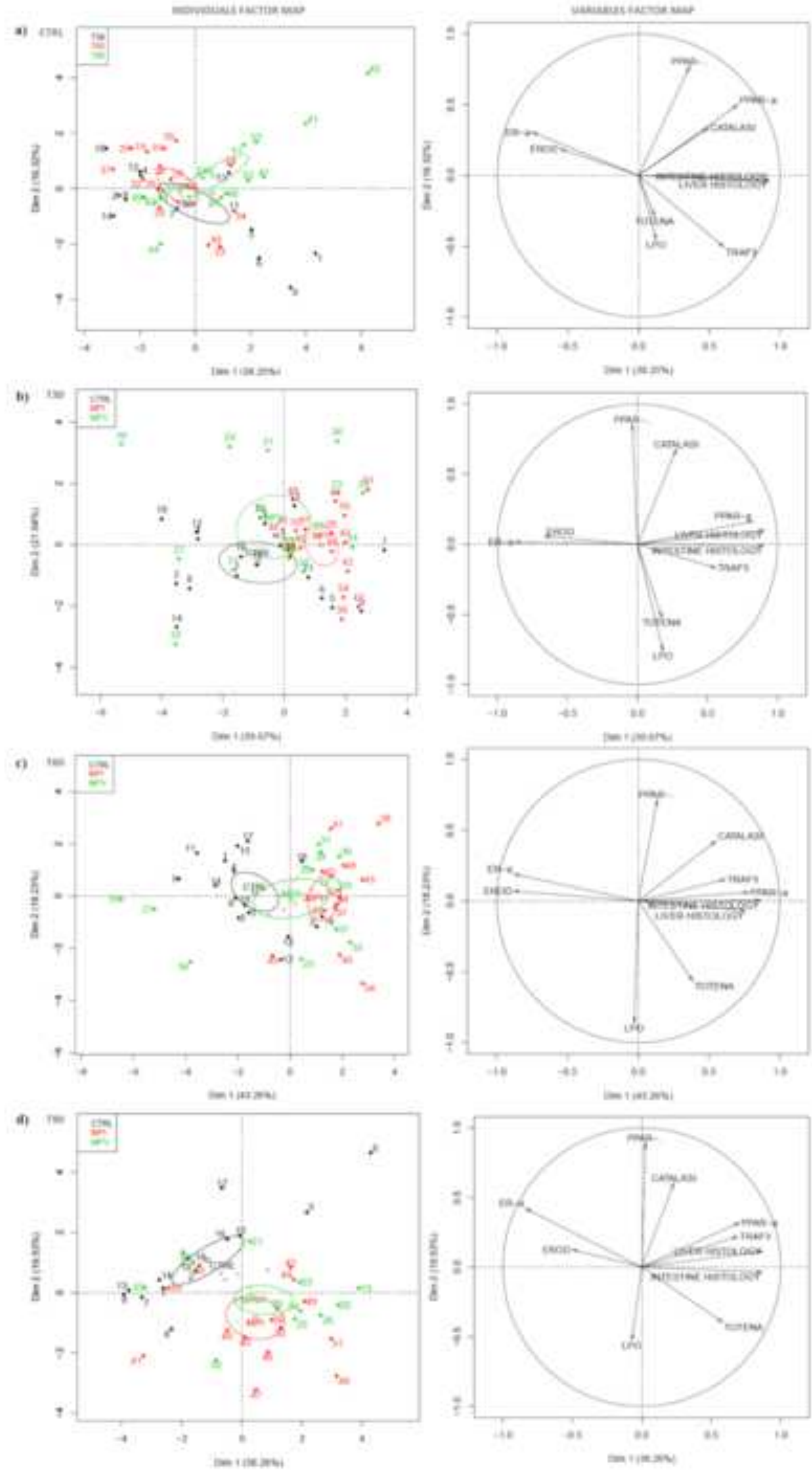

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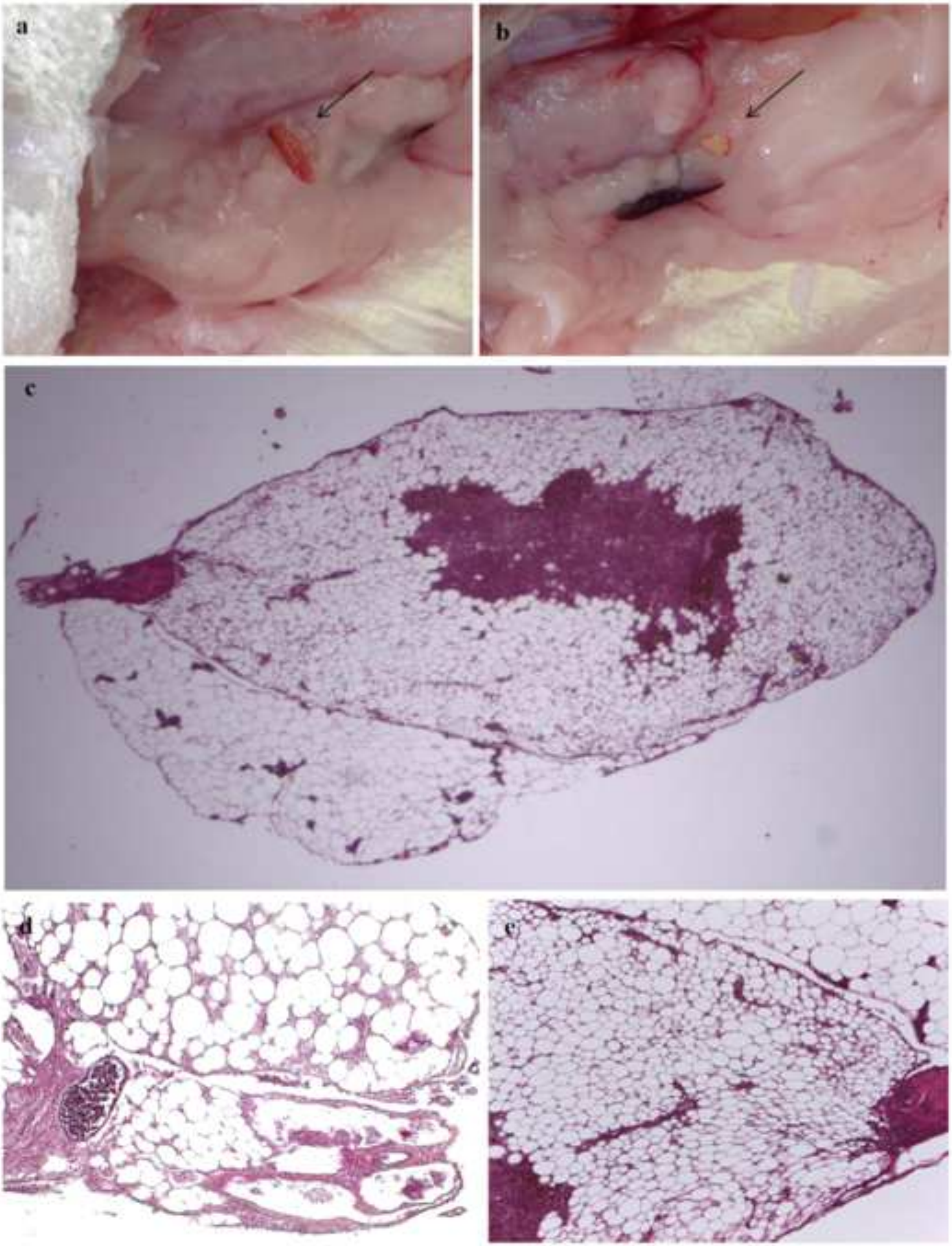

FIGURE CAPTIONS

Figure 1. Biomarker responses in *D. labrax* liver and blood: a) EROD activity, b) LPO values, c) CAT activity, d) ENA frequency, e) gene expression levels of TRAF3, f) PPAR-α, g) PPAR-γ, h) ER-α. i) Prevalence (%) of score value (from 0 to 5) assigned to histological alterations in liver of *D. labrax* chronically exposed to PVC-MPs treatments. Values are expressed as the mean ± SEM. Significant statistical differences between exposed treatments: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 2. Principal Components Analysis (PCA) of biomarker responses in liver, blood and intestine of *D. labrax* chronically exposed to PVC-MPs treatments. PCA was performed for: a) CTRL treatment (black: T30; red: T60; green: T90) and b, c, d) three exposure time (black: CTRL group; red: MPI group; green: MPI group).

Figure 3. Neoplasms sections (H&E): a, b) Macroscopic appearance of pedunculated and well delimitated structures (arrows) located in the coelomic cavity of *D. labrax* specimens exposed to PVC-MPs treatments. c) Longitudinal section of neoplasm, it appears well circumscribed and highly vascularized (2.5x). d) Detail of peduncle (40x). e) Histological section of Lipoma (10x).

Research ethics approval

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Authorship contribution

Cristina Pedà: Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing - Original Draft.

Teresa Romeo: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Cristina Panti: Investigation, Visualization, Writing - Review & Editing.

Ilaria Caliani: Investigation, Visualization, Writing - Review & Editing.

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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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: