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Short communication

Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: Preliminary results☆

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ABSTRACT

This study investigates, for the first time, the intestinal responses of European sea bass *Dicentrarchus labrax* chronically exposed to microplastics through ingestion. Fish (n = 162) were fed with 3 different treatment diets for 90 days: control, native polyvinyl chloride (PVC) and polluted polyvinyl chloride (PVC) pellets. Intestines were fixed and processed for histological analysis using standard techniques. Histopathological alterations were examined using a score value (from 0 to 4). The distal part of intestine in all samples proved to be the most affected by pathological alterations, showing a gradual change varying from moderate to severe related to exposure times. The histological picture that characterizes both groups especially after 90 days of exposure, suggests that the intestinal functions can be in some cases totally compromised.

The worst condition is increasingly evident in the distal intestine of fish fed with polluted PVC pellets respect to control groups ($p < 0.05$) to different exposure times.

These first results underline the need to assess the impact of increasing microplastics pollution on the marine trophic web.

1. Introduction

In recent years, there has been increasing interest about "*microplastics*" pollution, small plastic debris defined as "any plastic smaller than 5 mm in size" (Andrady, 2011; NOAA, 2014). Microplastics consist of synthetic polymer products, such as exfoliates in cosmetics (Fendall and Sewell, 2009), or particles derived from the fragmentation of several larger plastic debris such as polyester fibers, plastic bags (O'Brine and Thompson, 2010) and polystyrene particles (Davidson, 2012). Their physical and chemical features, as well as the small size facilitate their dispersal in the marine ecosystem, where they are ubiquitous, and make them available within the food-web. In fact, several studies have showed the ingestion of microplastics by marine organisms such as seabirds (Van Franeker et al., 2011), cetaceans (Fossi et al., 2012, 2014), teleosts (Deudero and Alomar, 2015; Romeo et al., 2015), elasmobranches (Fossi et al., 2014), mussels (Browne et al., 2008), and zooplankton (Cole et al., 2013).

Ingested microplastics may cause physical harm (Wright et al., 2013) and, also, could represent a vehicle for the introduction of toxic chemicals in organisms and in the trophic web (Andrady, 2011; Cole et al., 2011; Fossi et al., 2012, 2014; Rochman et al., 2013a; Koelmans et al., 2013), although the "vector effect " as recently defined by Syberg et al. (2015) is still debatable. In particular, toxic chemicals are the result of leaching of additives (such as phthalates and bisphenol A) (Teuten et al., 2009) and of the microplastics propensity to adsorb persistent organic contaminants

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(POPs) (Mato et al., 2001; Rochman et al., 2013b; Teuten et al., 2009).

The information on the biological and ecological impact of this source of pollution is still poor, but is of increasing scientific concern. To the best of our knowledge, Rochman et al., 2013a, b investigated for the first time the hepatic stress and the abnormal proliferation of germ cells in Japanese medaka, *Oryzias latipes*, inducted by polyethylene pellets.

According to Rochman et al., 2013a experiment, this paper assessed, for the first time, the tissutal responses of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) chronically exposed to microplastics through ingestion. The aim of this research, is to study if the microplastics can induce alterations in the intestine of *D. labrax* in relation to different exposure times and plastics typology. Preproduction PVC were used in this experiment: PVC is an important class of plastic, it is widely employed for the manufacture of several products in the world and consequently have a high likelihood of ending up in the ocean environment. The European sea bass, a marine teleost of high commercial interest for aquaculture in the Mediterranean region, was chosen as an experimental model, in order to explore the ingestion phenomenon on a small scale and to give further information on the impact of microplastics on species of high economic value.

2. Material and methods

2.1. Experimental design

The experiment was carried out at Aquaculture Experimental Plant of IAMC U.O.S. Messina.

A total of 162 European sea bass (140 ± 8.42 g) were randomly placed into 9 indoor tanks (1350 L) and after one month of acclimation, three replicate tanks were randomly assigned to each treatment.

The fish were exposed for 90 days to three different treatment diets: control (CTRL), native microplastics (MPV), polluted microplastics (MPI) and were fed daily by hand at 1.4% of body weight supplied in 2 meals.

During the experiment, fish were monitored for any possible signs of impaired health status (i.e. feeding behavior, swimming activity, condition of skin and fins, external lesions) and 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 were applied.

2.2. Microplastics and diet preparation

Samples of native PVC pellets have been deployed for three month in the Milazzo harbor, chosen as Contaminated Site of National Interest (SIN) to obtain a contamination similar to what occurs in the marine environment (polluted PVC pellets).

To permit an uniform distribution of plastic in to the feeds, PVC was previously grinded. In order to avoid loss of chemical contamination due to heat, immediately before grinding, pellets were dipped in liquid nitrogen. The native and polluted PVC pellets (3 mm) were ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass through a 0.5 mm screen. After grinding, before their addition in the respective treatment, plastics were sieved in steel metal sieves to collect fragments lower than 0.3 mm in order to reach the same size of the other ingredients.

The feeds were manufactured in the laboratory at the Institute of Science of Food Production of the Cnr by means of a pelleting process using a 3.5 mm diameter. Diets were prepared utilizing a basal diet formulated to meet the sea bass nutritional requirements. The control treatment diet contained 0% plastic while the native and polluted PVC treatment diets contained 0.1% (w/w) plastic. The ingredients for the control diet (g/kg) consisted of 400 g herring fish meal, 252 g corn gluten meal, 218 g wheat flour, 100 g cod liver oil, 10 g vitamin premix, 10 g mineral premix and 10 g of bentonite (binder). Diets containing plastic consisted of the same ingredients but substituted 1 g of bentonite with native PVC (MPV treatment) and polluted PVC (MPI treatment), respectively.

2.3. Biometric parameters

For each fish the total weight (g) and the total length (cm) were recorded, and the condition factor (CF) was subsequently calculated individually as (total weight in g/(total length in cm)^3)*100 (Table 1). External and internal macroscopic investigations were conducted on each fish.

2.4. Histopathology investigations

Over 162 fish sampled during the experiment, only 54 were considered for histological analysis. The rest of specimens were preserved for further investigations.

Two fishes for each replica (three tanks per treatment) were sampled after 30, 60 and 90 days from each treatment (N = 54). All samples of intestine were immediately dissected into three parts: proximal (PI) mid (MI) and distal (DI) intestine, fixed in Bouin and processed for histological analysis using standard techniques. Sections of 5 μm were stained with hematoxylin and eosin (H&E) and analyzed under a light microscope (Leica DMR). Histopathological alterations of intestine were assessed by assigning a score (from 0 to 4) on the basis of an index scheme proposed by Zimmerli et al., 2007 (0 normal structure; 1 slight structural alterations; 2 moderate structural alterations; 3 pronounced structural alterations; 4 severe structural alterations) (Table 2).

2.5. Statistical analyses

Differences in treatments (CTRL, MPV and MPI) at different exposure times (T30, T60, T90) were analyzed using a two-way nested non-parametric multivariate analysis (PERMANOVA) with fixed-factor “treatments” and random-factor “tank”. The data matrix, based on the score values attributed to histological alterations of DI, was square root transformed and analyzed 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 above analyses were performed using the statistical software PRIMER6 & PERMANOVA+ (Clarke and Warwick, 2001; Anderson et al., 2008).

3. Results and discussion

3.1. General conditions

No mortality was observed during the experimental period for each treatment. Overall, the distal part of intestine of all samples (N = 54) proved to be the most affected by pathological alterations either by the exposure time or by treatment.

The control group showed a normal structure (score 0) or slight structural alterations (score 1) (Fig. 3 a; Fig. 5) compatible with gut health status of farmed sea bass (Saraiva et al., 2015).

Table 1 shows the mean and standard deviations (SD) for biometric parameters of each treatment (CTRL, MPV and MPI) to every exposure time (T30, T60 and T90).

Table 1
Mean and standard deviations for biometric parameters.

Treatment	Time of exposure	Weight (g ± SD)	Length (cm ± SD)	CF	n
CTRL	T30	155.3 ± 21.2	24.6 ± 1.3	1.05 ± 0.07	6
MPV	T30	177.8 ± 44.7	25.4 ± 1.7	1.07 ± 0.13	6
MPI	T30	191.0 ± 39.9	25.7 ± 1.3	1.12 ± 0.09	6
CTRL	T60	163.6 ± 29.5	25.2 ± 1.7	1.01 ± 0.03	6
MPV	T60	194.7 ± 73.4	26.3 ± 2.0	1.03 ± 0.15	6
MPI	T60	230.2 ± 75.1	27.1 ± 2.8	1.13 ± 0.06	6
CTRL	T90	199.2 ± 51.8	26.5 ± 1.9	1.05 ± 0.07	6
MPV	T90	268.8 ± 101.7	29.7 ± 3.6	0.99 ± 0.04	6
MPI	T90	284.1 ± 68	29.5 ± 1.9	1.08 ± 0.07	6

n: sample number; CF: condition factor (weight in g/(length in cm)^3)*100.

Table 2
Histopathological description.

Score	Type of alterations	Description
0	normal structure	No alterations of number and morphology of cells; no inflammatory changes; no architectural and structural changes.
1	slight	Slight alterations of number and morphology of cells; slight inflammatory changes; slight architectural and structural changes.
2	moderate	Hyperplasia and morphology alterations of cells; moderate inflammatory changes; moderate architectural and structural changes.
3	pronounced	Hyperplasia and morphology alterations of cells; pronounced inflammatory changes and circulatory changes; pronounced architectural and structural changes.
4	severe	Hyperplasia and morphology alterations of cells; severe inflammatory changes and circulatory changes; severe architectural and structural changes.

3.2. Histopathological alterations

After 30 days of exposure, an high percentage of individuals (67%) of MPV treatment showed moderate structural alterations (score 2) of the DI consisting in widening of the lamina propria, shortening and swelling of villi, vacuolation of enterocytes and increase of goblet cells especially at the top of villi (Fig. 1b). The other individuals (33%) showed instead slight modifications (score 1) (Fig. 5a). On the contrary in the MPI group, most of individuals (83%) are affected by pronounced alterations (score 3) of the DI (Fig. 5a): the epithelium appears detached by lamina propria, a beginning of fusion, beheading and disepithelization of villi can observed, in addition to apical vacuolation of enterocytes and an increase in rodlet cells (Fig. 1c). In all examined individuals there was also an evident edema of the serosa, muscularis mucose and submucosa/mucosa layers (Fig. 1c). Moderate changes (score 2) were observed in the 17% of the samples (Fig. 5a).

After 60 days of exposure, the sea bass (67%) belonging to the MPV group continued to show moderate alterations (score 2) of DI with an evident detachment of mucosal epithelium from the lamina propria, fusion and beheading of villi and hyperplasia of goblet cells at the top of the villi (Fig. 2b). Pronounced changes (score 3) were observed in the 33% of the samples (Fig. 5b). At the same time, moderate and pronounced changes were observed respectively in 17% and 33% of sea bass belonging to MPI treatment, while half of individuals (50%) showed severe alterations (score 4) (Fig. 2c; Fig. 5b). The serosa, muscularis mucose and submucosa/mucosa layers are edematous, with an evident ectasia of vessels and also leukocyte infiltration. The mucosal epithelium is completely detached (Fig. 4a), the villi are desquamated and beheaded and the lumen appears full of mucus, enterocytes, erythrocytes, goblet cells and whole strips of detached epithelium (Fig. 4b,c). Finally the occurrence of numerous rodlet cells in the intestinal mucosa was observed (Fig. 4b).

After 90 days of exposure, half of individuals (50%) of both MPV and MPI treatments showed severe alterations (score 4) of the DI (Fig. 3b,c; Fig. 5c). As described above (Fig. 2c) their histological picture is the same (Fig. 3b,c), apart from a significant loss of regular structure of serosa, muscularis mucose and submucosa/mucosa and of the structural connections between them in MPI group (Fig. 3c; Fig. 4c). The other individuals (50%) in both groups (MPV and MPI) presented alterations pronounced (score 3) (Fig. 5c).

The results of PERMANOVA applied on the score values data matrix evidenced significant differences only among levels of factor

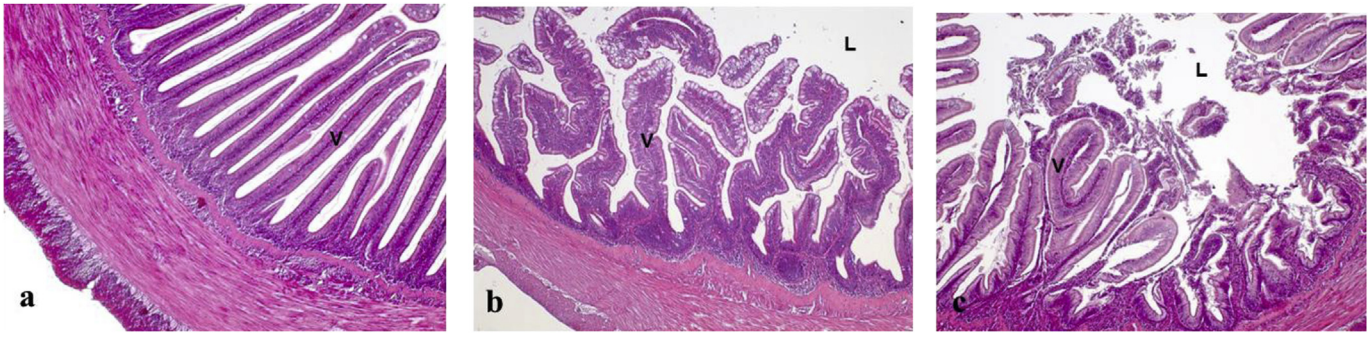

Fig. 1. Transverse section of the distal intestine of *Dicentrarchus labrax* chronically exposed to microplastics for 30 days (a) CTRL treatment (b) MPV treatment (c) MPI treatment. L, lumen; V, villi. (H&E. 10x).

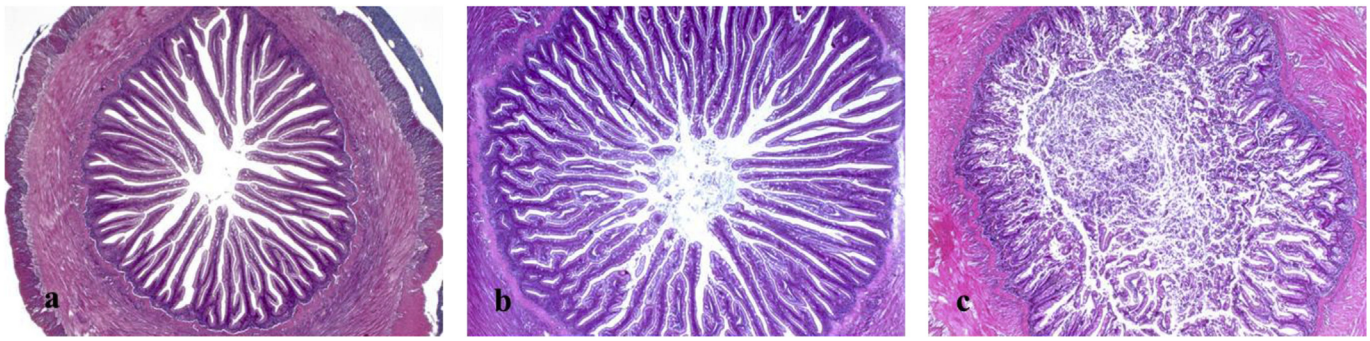

Fig. 2. Transverse section of the distal intestine of *Dicentrarchus labrax* chronically exposed to microplastics for 60 days (a) CTRL treatment (b) MPV treatment (c) MPI treatment. (H&E. 5x).

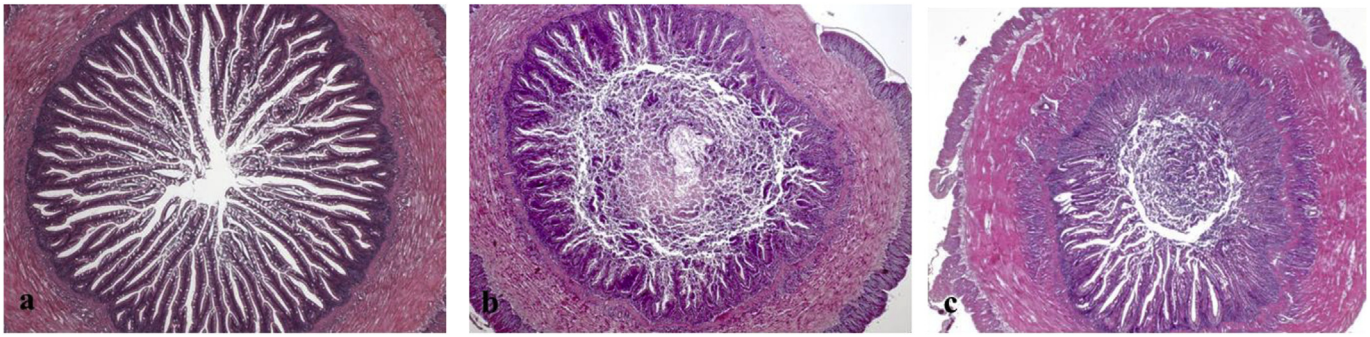

Fig. 3. Transverse section of the distal intestine of *Dicentrarchus labrax* chronically exposed to microplastics for 90 days (a) CTRL treatment (b) MPV treatment (c) MPI treatment. (H&E. 5x).

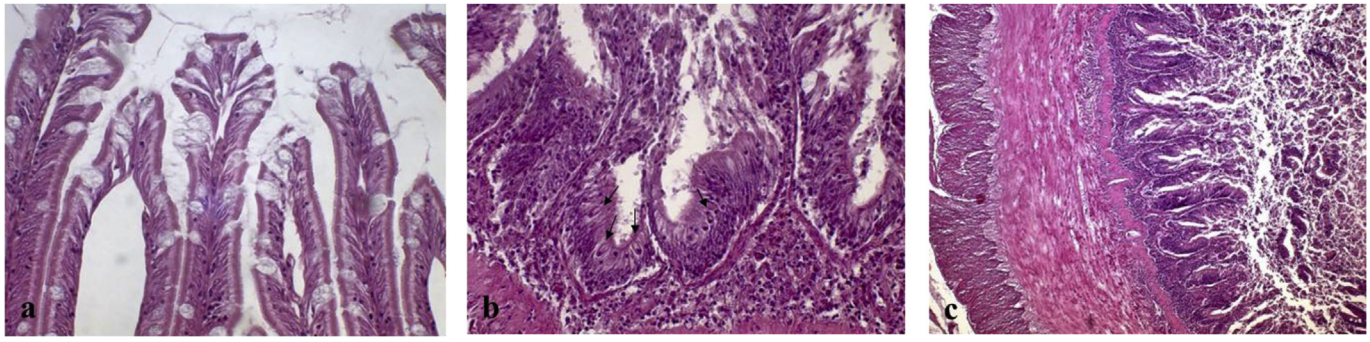

Fig. 4. Alterations observed in sea bass exposed to 60–90 days supply (a) evident detachment of epithelium from lamina propria (b) increase in rodlet cells (arrow) and massive disepithelization (c) significant disepithelization, beheading of villi and edema of the serosa, muscularis mucose and submucosa/mucosa layers. (H&E. a – b 20x, c 10x).

"treatment" in T30 ($p < 0.05$), and highly significant differences in T60 and T90 ($p < 0.01$); no significant differences were detected for factor tank x treatment in all the exposure times. Pairwise comparisons showed significant differences ($p < 0.05$) for CTRL *vs* MPI and MPI *vs* MPV treatments in T30 and T60, and between CTRL and the other two treatments in T90 (Fig. 5).

3.3. Implications and environmental importance

Several study suggest that the constant introduction of plastic debris in nature could be a vector of exposure of toxic substances in wildlife and a hazard for fish health (Cole et al., 2011; Fossi et al., 2012, 2014; Rochman et al., 2013a, 2014). As a matter of fact, Rochman et al., 2013a, 2014 report the hepatic stress and abnormal proliferation of germ cells in Japanese medaka exposed to polyethylene pellets. At the same time, others studies, underline that the microplastics 'organismal-vector' effects may be of limited importance (Koelmans et al., 2013; Khan et al., 2015) because the effects of microplastics to facilitate the uptake of toxic chemicals to aquatic organisms are not so straightforward, and also since the microplastics can alter the bioavailability and uptake route of a contaminants in fish (Khan et al., 2015; Syberg et al., 2015).

In this study, we investigated the interactions between microplastics and intestinal epithelium in marine teleost, being the most important site for the absorption of nutrients, and in particular the distal intestine which is the main site for the endocytosis of proteins (Rombout et al., 1985) but also, one of the routes for the uptake of toxic substances.

Several studies have shown that the intestinal epithelium may be subject to pathological changes when exposed to different

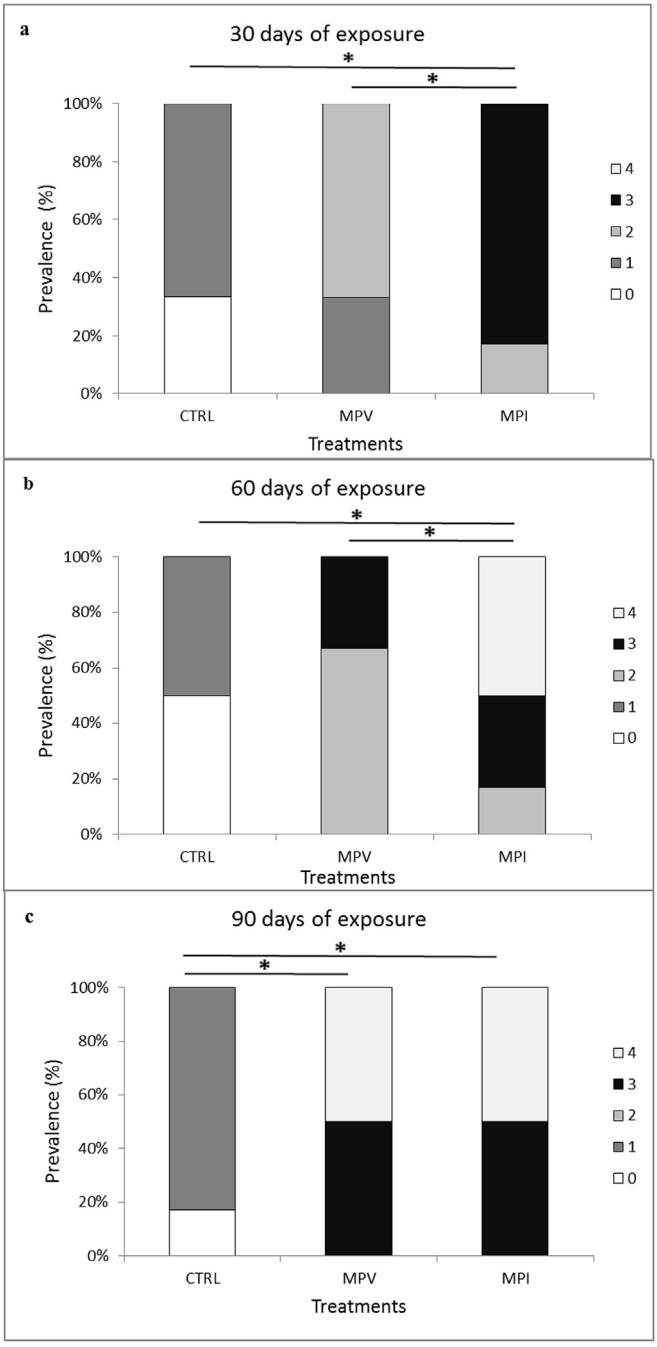

Fig. 5. Bar graph shows prevalence (%) of score value (from 0 to 4) attributed to histological alterations of DI for each treatment (CTRL-MPV-MPI) after 30–60–90 days of exposure. * indicate statistically significant differences ($p < 0.05$) for CTRL vs MPI and MPI vs MPV treatments in T30 and T60, and between CTRL and the other two treatments in T90, (two-way PERMANOVA).

contaminants (Salamat and Zarie, 2012). The results of this study highlight that microplastics represent a cause of structural and functional deterioration of the intestine, especially in the distal part, with a gradual pathological alterations varying from moderate to severe related to exposure times (90 days). After 30 days of exposure, the gut provides the first defense strategy secreting mucus and increasing the number of goblet cells. Subsequently, vacuolation of enterocytes and the coalescence of the villi occur. The severity of damage within the enterocytes (significant vacuolation) increases with the duration of exposure. At 60 days the first circulatory changes and worsening of inflammatory changes are detected (Fig. 5b). The serious histological picture and the reduction of perivisceral fat observed in some individuals of both groups (MPV and MPI) especially after 90 days of exposure (Fig. 5c), suggests that the intestinal functions can be reduced and in some cases totally compromised (Fig. 2c; Fig. 3b,c). Moreover, the presence of numerous leukocyte infiltrations and the increase in population of rodlet cells are typical responses to stress conditions. In particular, an increased number of rodlet cells and cellular alterations in different tissue of teleosts has been recorded in literature, also, in relation to exposure to physical and chemical injuries (Manera and Dezfuli, 2004; Rochman et al., 2013a, 2014). Considering the accumulation of plastics in the gut (Deudero and Alomar, 2015; Romeo et al., 2015) and their capacity to be a direct (plastic additives) and indirect (POPs) vehicle of toxic chemicals (Rochman et al., 2013a, b), this can explain the increase of rodlet cells and the severity of histological changes in the intestines of both treatments observed already after sixty days.

Although the alterations were observed in both groups, the worst condition is increasingly evident in the DI of sea bass fed with polluted PVC pellets (MPI treatment) (Fig. 5). The MPI treatment was deployed in Milazzo harbor, because previous study (Romeo, 2011) reported the presence of environmental contaminants in sediment (16PAHs mean value: 82.96 ng/g d.w; HCHs mean value: 6.63 ng/g d.w; DDTs mean value: 15.06 ng/g d.w; PCBs mean value: 56.32 ng/g d.w.) and in tissue of Comber *Serranus cabrilla* (Linnaeus, 1758) (16PAHs mean value: 41.32 ng/g d.w, muscle) sampled in the same site.

Plastic propensity to adsorb persistent organic contaminants (POPs) in marine environment represents a further hazard, showing that already after 30 days supply may increase damage in the intestine than the native plastic (Fig. 5).

These first results provide a significant contribution to the understanding of the phenomenon of microplastics ingestion in fish, and further studies are needed to measure POPs levels in the native and polluted PVC pellets used in this experiment.

This approach represents an important step to assess the impact of the microplastics on the trophic web and it also suggests that we continue investigating these aspects to evaluate the impact of marine litter.

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