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Dietary administration of PVC and PE microplastics produces histological damage, oxidative stress and immunoregulation in European sea bass (*Dicentrarchus labrax* L.)

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ABSTRACT

Worldwide, plastic waste is increasingly being discharged into the oceans, where it breaks down into smaller particles. Of these particles, the ingestion of microplastics (MPs; particles smaller than 5 mm) have been documented in some aquatic animals, including fish, whose health and welfare suffer as a consequence. However, their precise effects are not completely understood. To shed light on this issue, European sea bass (*Dicentrarchus labrax* L.) specimens were fed diets containing 0 (control), 100 or 500 mg polyvinylchloride (PVC) or polyethylene (PE) MPs kg^{-1} diet for three weeks, after which samples of liver, intestine, skin mucus and head kidney (HK) were obtained. A histological study of the liver and intestine revealed important alterations in the fish fed the MP diets, compared with control fish. At a functional level, PE-MPs, but not PVC-MPs, decreased the activity of antioxidant enzymes, suggesting a certain level of oxidative stress. As regards immunity, the intake of PVC-MPs increased the phagocytic and respiratory burst activities of HK leucocytes whilst the intake of PE-MPs increased skin mucus immunoglobulin M levels and the respiratory burst activity of leucocytes. The results suggest that the short-medium term intake of PVC- or PE-MPs by fish slightly depresses their immunity and produces oxidative stress. However, based on the histological alterations found, it seems that longer exposure times might lead to irreversible damage that could compromise fish health and welfare.

1. Introduction

It is an unfortunate fact that most of the plastic produced worldwide (more than 300 million tonnes annually) is discarded and not reused [1]. Among all the plastic produced, 90% is composed of polyethylene terephthalate (PET), polystyrene (PS), polyvinyl chloride (PVC), polypropylene (PP) and polyethylene (PE), the most common being PE, PP and PVC [2]. Polyethylene is composed of high molecular weight hydrocarbons, saturated and nonpolar $(C_2H_4)_n$, while PVC is produced by the polymerization of vinyl chloride monomers [2]. Plastics are durable, strong, lightweight, corrosion resistant, and thermally and electrically insulative [3]; due to which properties, great amounts of plastics reach rivers, coasts, seas and oceans around the world where they accumulate [4–9], PE being the most common polymer found in aquatic environments (*Plastics Europe,* 2006) [10]. In water, due to a mixture of biological and mechanical forces, plastic pieces are fragmented into smaller particles known as microplastics (MPs; < 5 mm) and nanoplastics (NPs; < 100 nm) [11,12]. These MPs may not only negatively affect marine organisms, but could also increase exposure to the chemical compounds associated with them, including persistent organic pollutants (POPs) and plastic additives [13–16].

Although many studies have documented the impact of MPs on different marine organisms, the mechanisms involved are not well understood. The intake of plastic debris has been documented in fish [17,18], and several plastic items have been found in the digestive system of pelagic fish [19,20] as well as in benthic specimens [21,22] although such findings depend on the species and sampling region. For example, the occurrence of MPs in demersal fish has been reported both in wild-caught and marine cultured species: more than 54% of sampled fish in Hong Kong [23], more than 78% of *Lepidopus caudatus* from the Tyrrhenian Sea [24] and up to 100% in fish species from the Adriatic and Mediterranean Seas contained MPs in their stomach [25,26]. In addition, the number, shape and chemical nature of the microplastics in the digestive tract vary widely: i) while most studies show a low number of items per individual (1–2) others have found quite high numbers (22.21 ± 1.70 items/individual or 11.19 ± 1.28 items/g

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tissue), ii) the shape of MPs vary, too, although most are fragments or lines, and iii) the most common MPs are of PE or nylon [23–27]. Blockage and damage of the digestive system has been suggested as a direct consequence of MPs ingestion, leading to starvation and even death [28]. Different effects of MPs and NPs on aquatic organisms and their wellbeing have also been reported. Some authors [29–33] showed that ingested NPs could interact with the immune system, and it has recently been reported that MPs, both *in vivo* [34] and *in vitro* [35], are able to modulate some cellular innate immune parameters (such as phagocytosis and respiratory burst) in fish. Also, PVC-MPs provided in the diet were able to affect the expression of *prdx*5, *coxIV, ucp1* and *hsp90* genes [34], whereas the expression of *nrf2* gene was up-regulated in isolated leucocytes exposed to MPs [35], suggesting oxidative stress. In line with these observations exposure to MPs increased lipid oxidation in brain of European sea bass, causing oxidative stress and lipid damage [36]. Similarly, other studies have reported that MPs could produce negative effects on the antioxidant defence of some invertebrates [37,38]. All these findings point to oxidative stress as being one of the main mechanisms involved in the toxicity of MPs. Subsequently, disorder in the redox status may entail several biological responses, including oxidative stress-induced signalling pathways, apoptosis, and inflammation [39,40].

Considering all these data, the present study was designed to analyse the effects caused by the dietary administration of PVC-MPs and PE-MPs on the oxidative status and immune parameters of European sea bass (*Dicentrarchus labrax* L.), which was selected as a marine fish model.

2. Materials and methods

2.1. Animals

Twenty-five specimens of the seawater European sea bass (*Dicentrarchus labrax* L.), with a mean body-weight of 8.57 ± 0.72 g, were purchased from a local farm (Murcia, Spain). The fish were randomly divided among five aquaria (250 L; flow rate 900 L h-1; 22‰ salinity, 20 °C, and 12 L:12D photoperiod) in the Marine Fish Facilities at the University of Murcia. After a 4-week quarantine period, the fish were fed a commercial pellet diet (Skretting) at a rate of 1.5% body weight day^{-1}. The experiments comply with the Guidelines of the European Union Council (2010/63/UE) and were approved by the Committee on the Ethics of Animal Experiments of the University of Murcia.

2.2. Diet

In each aquarium, the fish (n = 5) received a commercial diet (control) alone or containing 100 or 500 mg of virgin PVC-MPs or PE-MPs per kg diet. MPs were donated by the *Centro Tecnológico del Calzado y del Plástico* (Murcia, Spain) and ranged in size from 40 to 150 μm (the mean diameter was 104.1 ± 36.2 μm for PVC-MPs and 77.5 ± 18.3 μm for PE-MPs). All the MPs used in the present experiment were considered virgin (without additives), had an irregular shape (Supplementary Fig. S1) and had been previously tested in *in vivo* and *in vitro* studies [34,35]. The diets were prepared daily by adding the MPs to cod oil, which facilitated the attachment of the MPs to the pellet. Cod oil (control) and cod oil containing MPs was sprayed on the commercial pellets before feeding the animals, at a rate never exceeding 1% of cod oil per kg of diet. The oil with the MPs was vigorously shaken for 10 min to allow the uniform distribution of the MPs in the food. Fish were closely observed during feeding to ensure they ate all the feed provided.

2.3. Sampling

After 3 weeks of feeding, all the animals from each dietary group were sacrificed by an overdose of MS222 (100 mg L^{-1}; Sandoz) and skin mucus, head-kidney (HK), liver and intestine were obtained and analysed separately. The skin mucus was centrifuged and stored at −20 °C [41] until used for humoral immunity analysis. HK leucocytes (HKLs) were isolated [42] and adjusted to 10^7 cells mL^{-1} for cellular innate immune parameter determinations. Liver samples were stored at −80 °C for antioxidant status analysis. Samples of liver and intestine were also stored in TRIzol Reagent (Invitrogen) at −80 °C for gene expression analysis or processed for routine light microscopy. All analyses were carried out in individual fish specimens, which were never pooled.

2.4. Growth performance

The body weight of animals was monitored throughout the trial, and the growth parameters weight gain (WG%) and specific growth rate (SGR%) were calculated [43]:

$$WG\ \% = [(\text{final weight} - \text{initial weight})/\text{initial weight}] \times 100$$

$$SGR\ \% = [(\text{Ln(final weight)} - \text{Ln(initial weight)})/\text{days}] \times 100$$

2.5. Histopathological and morphometric study of intestine and liver

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

2.6. Antioxidant enzyme activities

Liver samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0) for 30–60 s in ice, vortexed and centrifuged (12,000 g, 10 min, 4 °C). Enzymatic activities were determined according to standard methods: superoxide dismutase (SOD) [44], catalase (CAT) [45] and glutathione reductase (GR) [46]. SOD activity was determined by the inhibition of the reduction of cytochrome *c* at 550 nm during 1 min. CAT activity was determined by the decrease in absorbance at 240 nm due to the decomposition of hydrogen peroxide. GR activity was measured using NADPH and glutathione oxidized (GSSG) solution and absorbance was monitored at 340 nm for 3 min. In all cases the enzymatic activities were expressed as units/mg protein. For normalization, the protein concentration in samples was also determined [47]. All analyses were made in triplicate.

2.7. Skin mucus immune parameters

The peroxidase activity of skin mucus was determined colorimetrically [48], with one unit of peroxidase activity being considered

that which produced a change of 1 OD. Total IgM levels of skin mucus were determined by enzyme-linked immunosorbent assay (ELISA) using commercial antibodies [49] and expressed as optical density at 450 nm. All analyses were made in triplicate.

2.8. Cellular innate immune parameters

The cellular innate immune parameters of sea bass HKLs of phagocytosis, respiratory burst and peroxidase activities were determined as detailed elsewhere. Phagocytosis against *Saccharomyces cerevisiae* yeast cells was evaluated by flow cytometry [48], whereby the phagocytic ability is represented by the percentage of HKLs with ingested yeast cells and the phagocytic capacity by the relative amount of ingested yeast cells per leucocyte. Respiratory burst upon stimulation by phorbol myristate acetate (PMA) was determined by a chemiluminescent method [50]. Finally, HKLs were lysed and peroxidase activity was determined as in the mucus samples. All analyses were made in triplicate.

2.9. Gene expression

Total RNA was extracted from the liver and intestine using TRIzol Reagent [51] and the expression of selected genes [Nuclear factor (erythroid-derived 2)-like 2 (*nrf2*), superoxide dismutase *(sod),* catalase *(cat),* peroxiredoxin 1 *(prdx1),* peroxiredoxin 2 *(prdx2*), heat-shock protein 70 *(hsp70),* interleukin 1β (*il1β*), interleukin 8 (*il8*) and caspase 3 (*casp3*)] was analysed by real-time PCR as elsewhere [34]. Supplementary Table 1 shows the primers used in this study. The relative expression of all genes was calculated by the $2^{-\Delta CT}$ method [52], using *18S* and *ef1a* as housekeeping genes.

2.10. Statistical analyses

Comparison among groups was performed by analysis of variance (one-way ANOVA), followed by the Bonferroni or Games Howell test ($P < 0.05$), after checking assumptions of normality and homoscedasticity. Shapiro–Wilk and Levene tests were performed to confirm normality and homogeneity of variance, respectively. All the data were analysed using SPSS Statistics 15.0 (SPSS, Chicago, IL) package programs.

3. Results

3.1. Growth

In the course of the experiment, animals did not show alterations in feeding behaviour, willingly consuming all the supplemented diets. No significant difference was observed in growth performance (21 days) and no mortalities were registered (Supplementary Table S2).

3.2. Histopathological study

Potential histopathological damage produced in the intestine (Fig. 1) and liver (Fig. 2) of European sea bass as a result of dietary exposure to PVC- or PE-MPs was evaluated by light microscopy followed by morphometrical analysis. The intestine from animals exposed to MPs showed multiple signs of damage. Surprisingly, the anterior intestine of fish fed the diet supplemented with 100 mg PVC-MPs kg^{-1} (Fig. 1B) showed similar morphological features than the control fish (Fig. 1A), although incipient signs of leucocyte infiltration were observed in their mucosa. At the same time, different signs of intestinal injury were noted in animals fed the 500 mg PVC-MPs kg^{-1} diet (Fig. 1D), including hyperplasia and a significant increase in the number of goblet cells ($P < 0.001$), increased villus thickness ($P = 0.017$), as well as a dissociation of the mucosal epithelium from the lamina propria (Table 1). On the other hand, intestine micrographs from fish fed the diets supplemented with PE-MPs (Fig. 1C, E) showed areas with high levels of enterocyte vacuolization, especially in the apical parts of the villus. The morphometric analyses of sea bass fed the PE-MP diets showed a significant decrease in the number of goblet cells compared with the control group as well as a decrease in villus height, which was correlated with a higher intestinal diameter:villus height ratio (Table 1).

In liver, healthy hepatocytes were distributed in cords between blood microvessels (sinusoids) (Fig. 2A), whose organization and distribution was altered in specimens fed the different MPs diets compared with the control (Fig. 2B–E). Alterations included changes in the hepatocyte morphology, hypertrophy and signs of blood sinusoid congestion. The morphometric analyses showed a significant increase in hepatocyte vacuolation, accompanied by congestion of blood vessel in animals fed each of the diets containing MPs (Table 1).

3.3. Liver antioxidant enzymes

The activity of SOD and CAT enzymes was significantly decreased in the liver of animals fed the PE-MPs diets (both 100 and 500 mg kg^{-1}) compared with the control fish ($P < 0.001$ in both cases) (Fig. 3A and B). Nevertheless, exposure to MPs did not affect GR activity (Fig. 3C). The activity of antioxidant enzymes evaluated in fish fed the PVC-MPs diets for 21 days was not significantly affected (Fig. 3).

3.4. Skin mucus immune parameters

Different parameters related to humoral immunity were evaluated in the skin mucus from fish fed the experimental diets. The peroxidase activity of fish fed the 100 mg kg^{-1} PVC-MPs diet was significantly higher than the activity observed in the mucus of animals fed 500 mg kg^{-1}, although there were no differences from the controls (Fig. 4A). On the other hand, IgM levels measured in mucus from animals fed both doses of PVC-MPs and the lower dose of PE-MPs slightly increased, while the same parameter increased significantly in the skin mucus of animals fed the higher dose of PE-MPs (500 mg kg^{-1}) with respect to the level measured in the mucus from control fish ($P < 0.05$) (Fig. 4B).

3.5. Head-kidney leucocyte innate immune parameters

The leucocyte phagocytic ability was evaluated in fish fed each experimental diet, a significant increase with respect to the control group only being observed in fish fed the PVC-MPs diets ($P < 0.05$) (Fig. 5A). However, no significant change was observed in the leucocyte phagocytic capacity of fish fed either the PVC- or PE-MPs diets (Fig. 5B). On the other hand, the intake of 100 mg PVC-MPs kg^{-1} or 500 mg PE-MPs kg^{-1} in the diet for 21 days significantly increased the respiratory burst of leucocytes ($P < 0.001$) (Fig. 5C). Finally, the peroxidase activity of HKLs from sea bass was not affected by the intake of MPs (Fig. 5D).

3.6. Gene expression

The expression of different genes related to immunity and stress was analysed in the liver and intestine from European sea bass fed diets containing MPs (Fig. 6). In the liver, the expression of *sod* in the specimens fed the diet with the highest dose of PE-MPs was significantly down-regulated with respect to the values shown by fish fed the control diet, while the expression of *il1β* in specimens fed the diet with the lowest dose of PE-MPs was significantly up-regulated with respect to the values found in fish fed the control diet ($P < 0.05$) (Fig. 6A). Relative gene expression evaluated in the intestine revealed a significant increase of *nrf2* in fish fed the 500 mg PVC-MPs kg^{-1} diet with respect to the control ($P < 0.05$). In addition, the expression of *prdx1* in fish fed 100 mg PE-MPs kg^{-1} and the expression of *hsp70* in fish fed 100 and

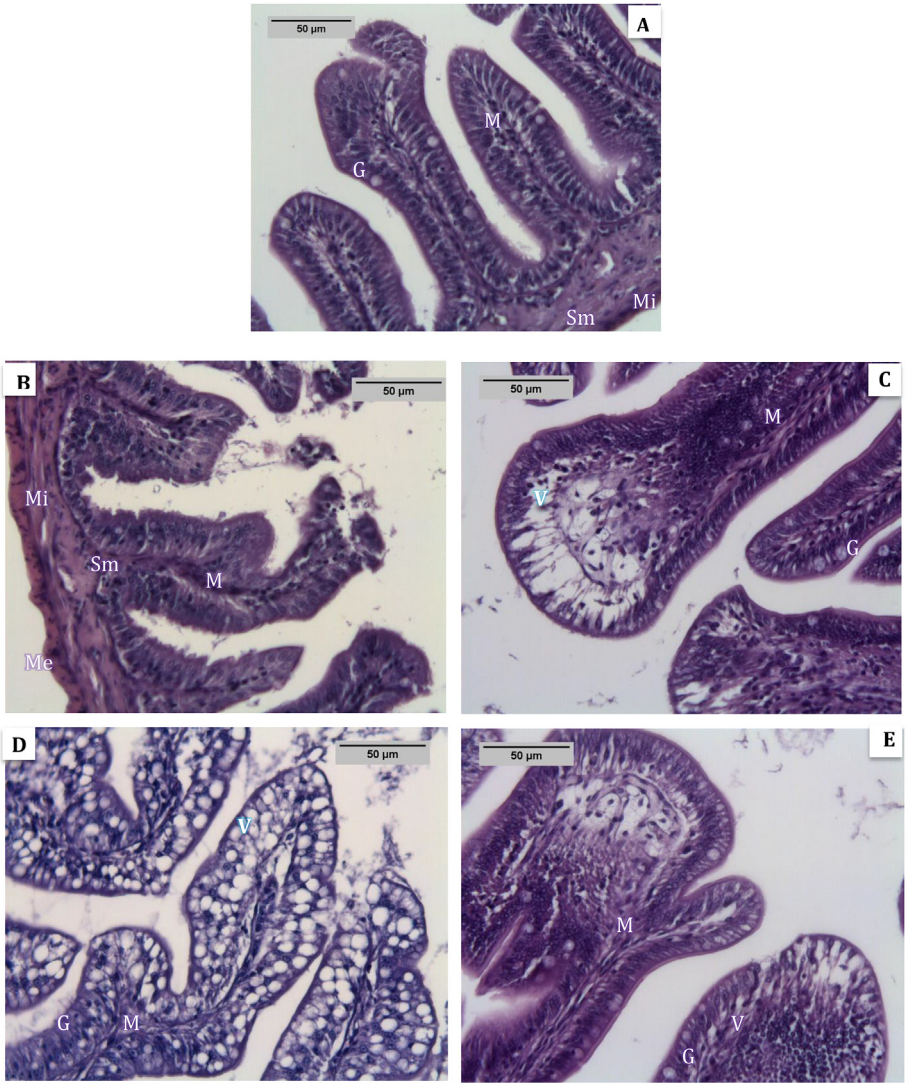

Fig. 1. Histopathological analysis of intestine from European sea bass fed different diets: (A) control diet, (B) 100 mg PVC-MPs kg^{-1} diet, (C) 100 mg PE-MPs kg^{-1} diet, (D) 500 mg PVC-MPs kg^{-1} diet, (E) 500 mg PE-MPs kg^{-1} diet, stained with haematoxylin-eosin. M, mucosa; SM, submucosa; Mi, muscularis interna; Me, muscularis externa; G, goblet cells; V, vacuolization.

500 mg PE-MPs kg^{-1} was significantly lower than in fish from the control group ($P < 0.001$ and $P < 0.05$; respectively) (Fig. 6B). Both PVC- and PE-MPs diets failed to affect the relative gene expression of *cat, prdx2, il8* and *casp3* genes (Fig. 6B).

4. Discussion

PE and PVC are the most commonly produced plastics and also the most frequently found in marine environments [8], where their presence is correlated with various negative effects on marine biota [53]. However, the mechanisms behind these effects remain unclear. With the aim of shedding light on this issue, we selected two MPs of different compositions (PCV and PE). Their chemical and physical properties lead to them being differently placed in the water column (densities of 1.16–1.30 and 0.95 $g\,cm^{-3}$, respectively, for PVC and PE) and consequently integrated into the trophic chain at different levels. Indeed, MPs have been found both in the water column and in the sediment fraction, with concentrations that are determined by the proximity to polluted areas [0.16–0.62 particles per m^2 [54,55] or 2.5 particles per m^3 in the Atlantic Ocean [56], or more than 81.43 mg per kg of sediment in the Indian Ocean [57]]. Based on our previous report [34], we suggest that a diet containing 100 mg of MPs per kg of diet may be taken an analogy of a polluted area, whilst 500 mg of MPs per kg of diet could be considered as reflecting extreme exposure for marine organisms. Interestingly, it has been reported that the great egestion capacity of European sea bass may significantly limit the physiological effects of diets containing MPs [58], although the number of studies in controlled conditions on this issue is very limited.

Relevant histopathological effects were noted in the two analysed tissues (liver and intestine) of European sea bass fed the MPs diets, the effects increasing with the dose and differing according to the type of plastic. In the intestine of fish fed the PE-MPs diet several alterations in the enterocytes and goblet cells from the mucosa were evident, as well as a decrease in villus height, which could be related with mechanical abrasion produced by the MPs. On the other hand, fish fed the PVC-MPs diet showed signs of infiltration in the submucosa, and a significant decrease in the number of goblet cells and a villus height, which may have been related with a degree of chemical injury, as has been described previously in sea bass [59]. Concomitantly, both he PVC- and PE-MPs diets had similar effects in the liver, such as a clear increase in hepatocyte vacuolation, loss of organization of parenchyma and congestion of blood sinusoids. These results are consistent with other reports that showed that the intake of MPs or NPs may affect the morphology and functions of the liver. In this respect, it has been reported

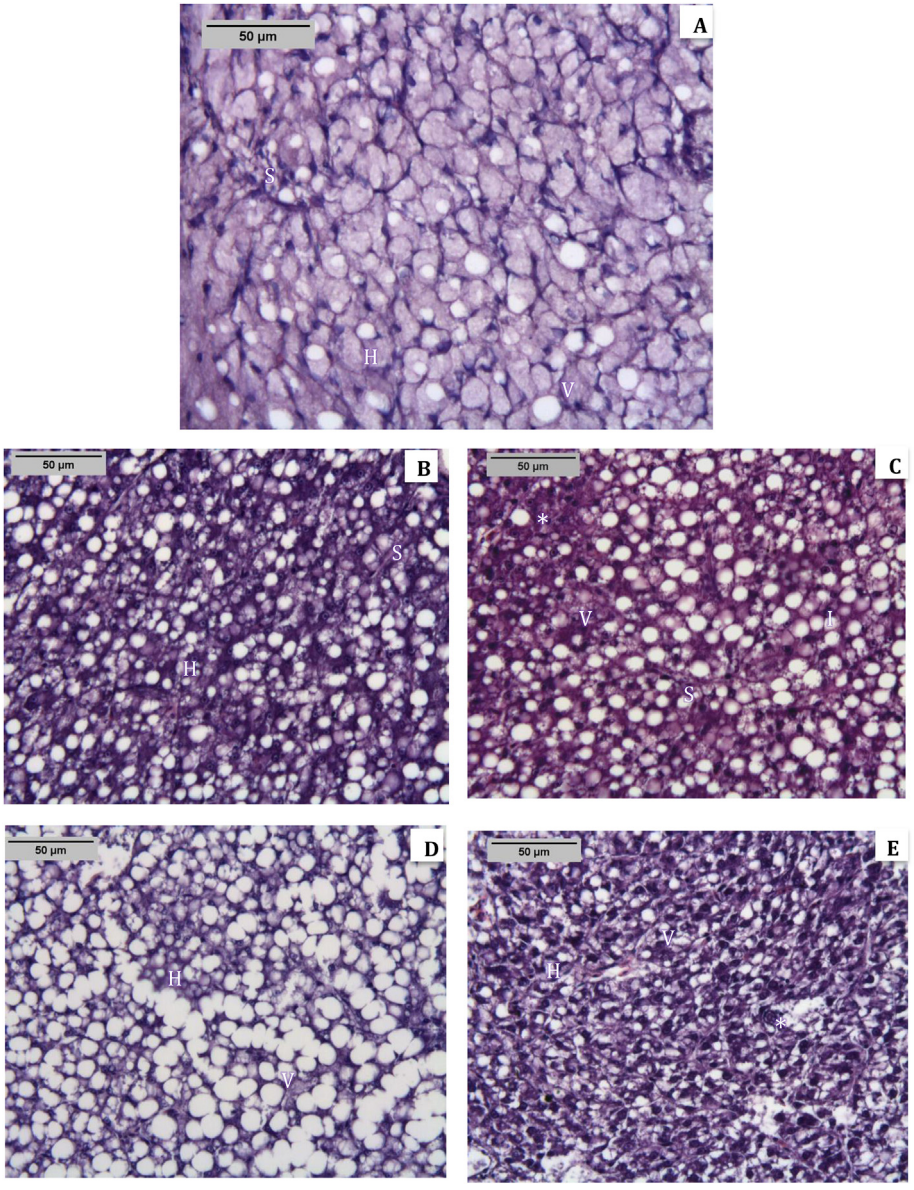

Fig. 2. Histopathological analysis of the liver from European sea bass fed different diets: (A) control diet, (B) 100 mg PVC-MPs kg^{-1} diet, (C) 100 mg PE-MPs kg^{-1} diet, (D) 500 mg PVC-MPs kg^{-1} diet, (E) 500 mg PE-MPs kg^{-1} diet, stained with haematoxylin-eosin. H, hepatocytes; V, vacuolization; I, infiltration; *, focal necrosis.

that NP ingestion may affect the metabolism of fish through alteration of triglycerides and cholesterol in blood serum, as well as their distribution in muscle and liver tissues [60]. For example, exposure to polymethylmethacrylate NPs for 96 h was seen to affect different parameters related with fatty acid metabolism, such as the expression of *ppar-α*, *ppar-γ*, and NADH dehydrogenase subunit 5 in sea bass liver as well as arylesterase activity in serum [61]. Similarly, the exposure of mussels to NPs seemed to affect esterase activity, aspartate aminotransferase and alanine transaminase in gills [62]. In addition, metabolic alterations have been described in fish exposed to MPs and NPs,

Table 1
Morphometric analysis in intestine and liver from European sea bass fed commercial diet (0, control) or the diet supplemented with 100 or 500 mg kg^{-1} diet of PVC-MPs or PE-MPs for 21 days. Values are the mean ± SEM (n = 5). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

Tissue	Histological character	Control diet	PVC-MPs		PE-MPs		P-value
			100 mg kg^{-1}	500 mg kg^{-1}	100 mg kg^{-1}	500 mg kg^{-1}	
Intestine	Goblet cells per 100,000 μm^2	66.2 ± 10.0^{a}	61.8 ± 10.9^{a}	139.5 ± 21.6^{b}	40.0 ± 14.1^{c}	55.1 ± 10.3^{c}	< 0.001
	Villus thickness (μm)	49.6 ± 1.5^{a}	39.0 ± 2.5^{ab}	58.36 ± 1.9^{b}	44.4 ± 1.9^{a}	33.27 ± 1.7^{a}	0.017
	Villus height (μm)	396.5 ± 25.5^{a}	259.2 ± 20.2^{ab}	300.6 ± 11.5^{ab}	249.6 ± 14.3^{b}	259.9 ± 12.7^{b}	< 0.001
	Intestinal diameter (μm)	1,179.9 ± 69.1	1,188.7 ± 27.0	1,106.2 ± 90.3	1,103.2 ± 20.2	1,108.2 ± 21.1	0.324
	Intestinal diameter: Villus height ratio	3.2 ± 0.2^{a}	4.0 ± 0.2^{ab}	3.8 ± 0.2^{a}	4.8 ± 0.3^{b}	4.5 ± 0.2^{b}	0.001
Liver	Vacuole presence (%)	4.7 ± 0.8^{a}	20.2 ± 1.0^{b}	33.7 ± 1.3^{c}	35.1 ± 2.5^{b}	18.5 ± 1.9^{c}	< 0.001
	Congestion (%)	0.06 ± 0.01^{a}	0.25 ± 0.03^{b}	0.26 ± 0.01^{b}	1.3 ± 0.3^{c}	1.6 ± 0.1^{c}	< 0.001

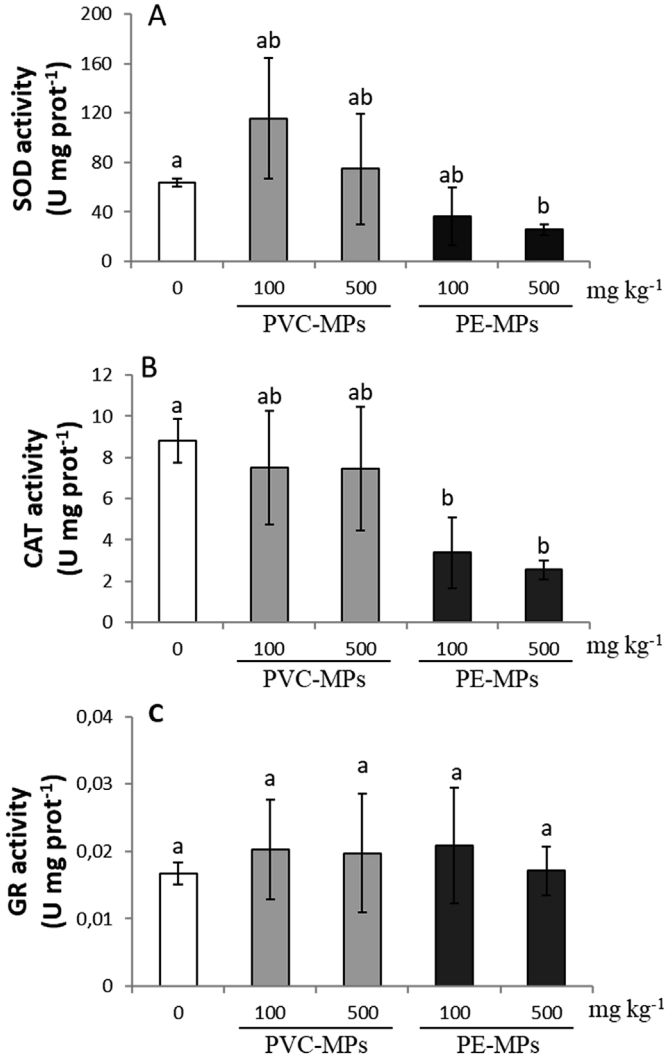

Fig. 3. Antioxidant enzyme activity in liver homogenates. Superoxide dismutase (SOD; A), catalase (CAT; B) and glutathione reductase (GR; C) activities were determined in the liver from European sea bass fed commercial diet (0, control) or the diet supplemented with 100 or 500 mg kg^{-1} diet of PVC- or PE-MPs for 21 days. Values are the mean ± SEM (n = 5). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

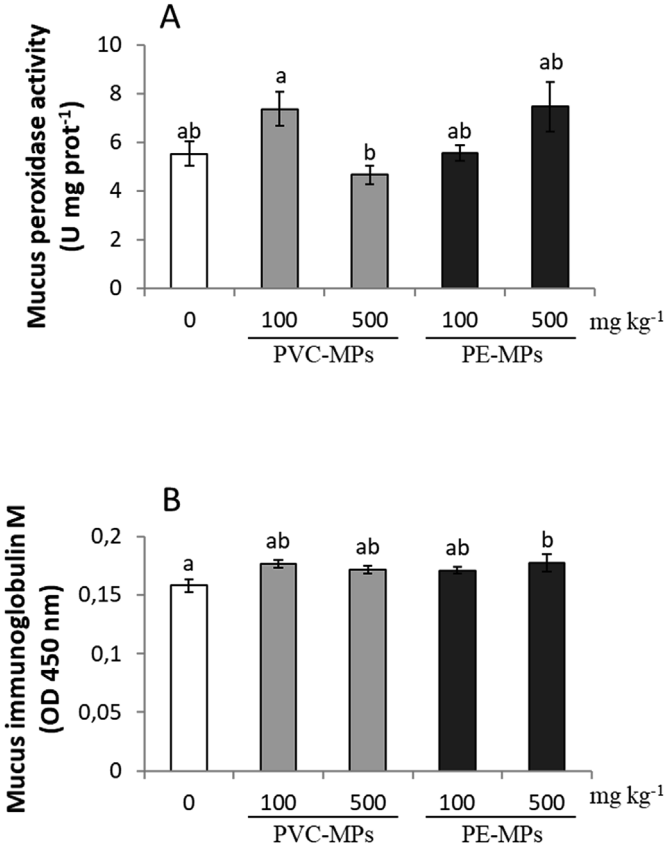

Fig. 4. Humoral immune parameters in the skin mucus. Peroxidase activity (A) and immunoglobulin M levels (B) were determined in the skin mucus from European sea bass fed commercial diet (0, control) or the diet supplemented with 100 or 500 mg kg^{-1} diet of PVC- or PE-MPs for 21 days. Values are the mean ± SEM (n = 5). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

such as increased levels of fatty acids and decreased levels of amino acids [63], while several signs of stress have been reported on fish exposed to MPs (decreased glycogen, vacuolation and necrosis) [64]. Also, in zebrafish exposed to MPs and NPs, liver necrosis, infiltration and vacuolation (signs of inflammation and lipid accumulation) were reported [63]. Taken together, all the histological parameters analysed in our experiment point to an effect on intestinal functions, promoting alterations in the liver. Nevertheless, this hypothesis is not sustained by the growth parameters, which remained unaltered, perhaps because these alterations only occur at morphological level, but not at functional level during the exposure time of the study. The possibility that MPs or NPs could be translocated from intestine to the liver in fish is still under intense debate with both supporting and non-supporting studies [63,65–67]. Although all possibilities need to be considered, it is a fact that, in our experiment at least, the possibility that the tested MPs could be translocated seems to be remote. Since we failed to detect this phenomenon in sea bass, the first hypothesis concerning metabolic alterations is reasonably well supported, although more research is needed to shed light on in this issue.

In general, the direct or indirect production of reactive oxygen species (ROS) has been suggested as one of the most mechanisms through which MPs may impair marine organisms [37,62,68,69]. Although we failed to detect alterations in the activity of GR as a result of MPs ingestion at 21 days, both SOD and CAT activities decreased in fish fed the PE-MPs diets. It is true that ROS was not evaluated and neither oxidative damage endpoint was assessed, the decrease in the activity of these enzymes could have induced oxidative stress. In this sense, our results are consistent with those of other authors that reported MPs could induce oxidative stress both *in vivo* [in invertebrates [37,68,70,71] and vertebrates [34,63]] or *in vitro* [cell lines from fish [72], rats [30] or human [73]]. At the same time, the expression of *nrf2* decreased in fish fed the PCV-MPs diet, although we failed to detect any significant change in *cat* or *sod* mRNA expression in either intestine or liver. These results are consistent with our previous report in seabream fed PVC-MPs, where no change in *cat* and *sod* expression was observed [34], and with the results of other authors who described how exposure to MPs failed to produce any oxidative damage in *Pomatoschistus microps* [74]. Interestingly, Nrf2 can be activated by other mechanisms in addition to oxidative stress (such as growth factors, increases in unfolded and misfolded proteins, endoplasmic reticulum stress as well as by compounds with the capacity to produce the separation of Nrf2 from Keap1 (its inhibitor) [75,76]. Since PVC-MPs intake failed to affect the expression and activity of catalase and superoxide dismutase enzymes, we hypothesized that PVC-MPs could modulate t*nrf2* expression not only as a result of oxidative stress. Additionally, peroxiredoxins play an important role role in the oxidative stress defence. In our study, the expression of peroxiredoxins was unaltered by PVC-MPs intake, although *prdx1* was down-regulated in the intestine by the PE-MPs diet,

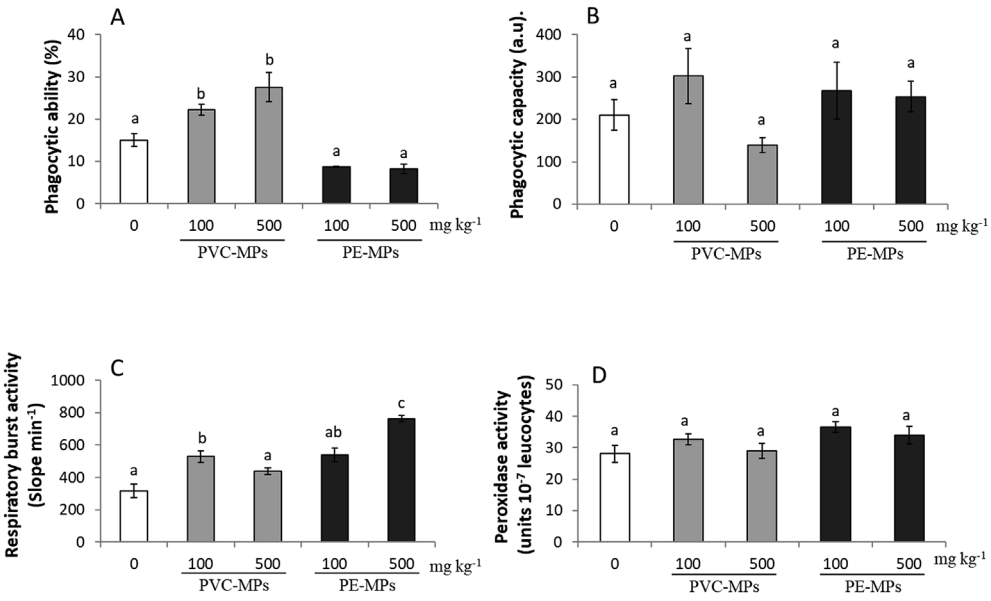

Fig. 5. Cellular innate immune parameters in head kidney leucocytes. Phagocytic ability (A), phagocytic capacity (B), respiratory burst (C) and peroxidase (D) activities determined in the head kidney leucocytes isolated from European sea bass fed commercial diet (0, control) or the diet supplemented with 100 or 500 mg kg^{-1} diet of PVC-MPs or PE-MPs for 21 days. Values are the mean ± SEM (n = 5). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

which may have led to the accumulation of ROS and consequently oxidative stress. In fish, the expression of peroxiredoxins changes in oxidative stress situations [77]. Consequently, the expression of *sod* and *prdx1*, as well as the activity of SOD, was only affected by the ingestion of PE-MPs, suggesting that MPs composed of different polymers may affect fish through a variety of mechanisms and/or by different degrees, reinforcing the idea that oxidative stress could be one of the mechanisms through which MPs damage marine organisms.

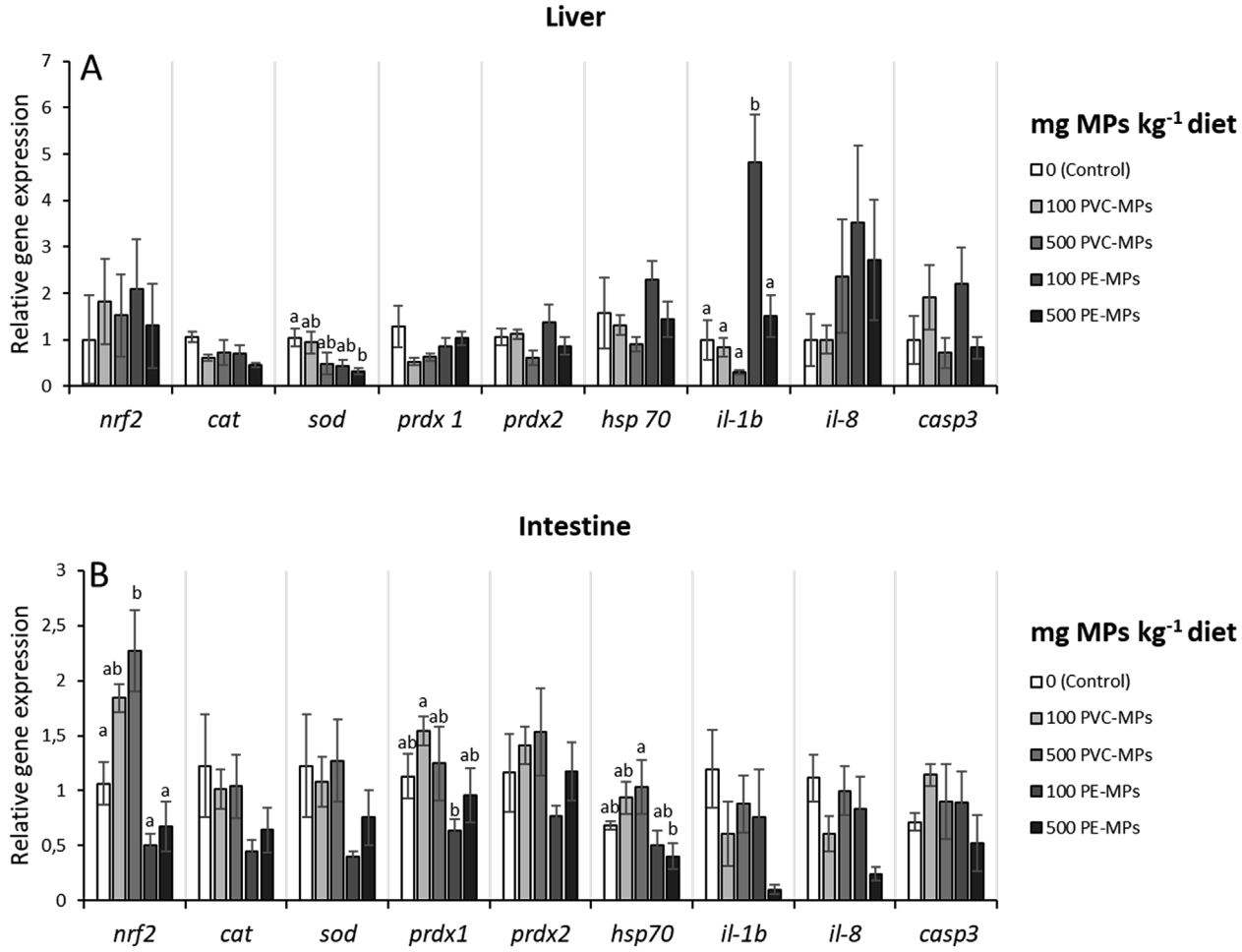

Fig. 6. Expression of selected genes in liver and intestine. The expression of relevant genes was determined in the liver and intestine from European sea bass fed commercial diet (0, control) or the diet supplemented with 100 or 500 mg kg^{-1} diet of PVC-MPs or PE-MPs for 21 days. Values are the mean ± SEM (n = 5). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

On the other hand, the observed alterations (both morphological and oxidative stress) could be the result of a decreased cell repair capacity. In this respect, heat-shock proteins are involved in repairing cell proteins after different kinds of damage [78]. PVC-MPs intake by sea bass, failed to affect the expression of *hsp70* mRNA, which is consistent with the results previously reported in seabream fed PVC-MPs [34] and *Parvocalanus crassirostris* exposed to PET-MPs [79]. The expression of *hsp70* gene evaluated in sea bass fed PE-MPs was down-regulated after 21 days, which is consistent with the observations of other authors in *Daphnia magna* exposed to MPs, in which the expression of *hps60* gene was up-regulated and *hsp70* gene was down-regulated [80]. In the face of a stress situation, the expression of heat shock-proteins is expected to be primed: *hsp70* up-regulation is a common response to restore the cell's status [81] and a normal response after stressor exposure [82]. For this reason, a decrease in the expression of the proteins responsible for cell repair in fish fed PE-MPs may partially explain the morphological alterations and the oxidative damage observed.

The effects of MPs and NPs on the immune system of marine organisms has been little studied, although in a previous study, we demonstrated that PVC-MPs were able to partially affect the immune system of seabream [34], which is consistent with the results of the present experiment. It is well known that IgM is an important player in the humoral immune system of teleost, where it is found in blood, skin mucus and other secretions, acting as immune effector [83]. The results obtained in our present study are consistent with those previously reported in gilthead seabream, where the levels of peroxidase and IgM were significantly increased after 30 days of ingesting PVC-MPs in the diet [34]. The secretion of IgM by B lymphocytes may be stimulated by the intake of MPs, affecting the response produced by the immune system, as has been suggested by others authors [29,30,32]. At the same time, MP ingestion affected the cellular innate response of HKLs. The process of phagocytosis, considered as one of the most important activities in innate immunity, increased in fish fed PVC-MPs for 21 days, suggesting that MPs have several effects on cellular immunity. At the same time, the intake of both PVC and PE-MPs affected respiratory burst to different degrees. In a previous work, we reported that PVC-MP intake slightly affected HKLs from seabream [34], which, in general, is consistent with our observations in seabass. The impairment of leucocyte oxidative burst by MPs has been reported *in vitro* in fathead minnow [72], sea bass and seabream [35]. However, neither the intake of PVC-MPs nor of PE-MPs affected the peroxidase activity of HKLs from sea bass, which is consistent with our previous reports [34,35]. Nevertheless, taken together, our data suggest that PE-MPs affected sea bass more strongly than PVC-MPs. For this reason, further studies should be developed using different types of MPs with the aim of determining both the relationships and interactions between a particular MP and the immune system, although ROS production might also be involved [37,68,69].

In conclusion, the dietary intake for 21 days of two types of microplastics (PVC-MPs or PE-MPs; ranging from 40 to 150 μm) affected European sea bass, promoting histopathological alterations in the intestine and liver, while changing the immune parameters analysed and the redox status. Such alterations could be classified as ranging from moderate to severe, depending on the concentration and kind of MP. Additional experiments are needed to evaluate the possible effect of different types of MPs on fish homeostasis and the mechanisms involved.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.072.

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