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Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae)

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ABSTRACT

Microplastic particles have increasingly been detected in aquatic biota, from zooplankton to fish, raising concern for potential effects on aquatic organisms. In addition, they may potentially influence the toxicity of other contaminants in the marine environment. The aim of this study was to clarify whether polyethylene microspheres (1–5 μm) modulate short-term toxicity of the polycyclic aromatic hydrocarbon pyrene to juveniles (0+ group) of the common goby (*Pomatoschistus microps*). Fish were exposed for 96 h to pyrene (20 and 200 $\mu g\,L^{-1}$) in the absence and presence of microplastics (0, 18.4 and 184 $\mu g\,L^{-1}$). Mortality, bile pyrene metabolites, and biomarkers involved in neurotransmission, aerobic energy production, biotransformation and oxidative stress were quantified. Microplastics delayed pyrene-induced fish mortality and increased the concentration of bile pyrene metabolites. Microplastics, alone or in combination with pyrene, significantly reduced acetylcholinesterase (AChE) activity, an effect also observed for pyrene alone. The mixture also decreased isocitrate dehydrogenase (IDH) activity. No significant effects were found for glutathione *S*-transferase activity or lipid peroxidation. Overall, results show that: (i) microplastics modulate either the bioavailability or biotransformation of pyrene; (ii) simultaneous exposure to microplastics and pyrene decrease the energy available through the aerobic pathway of energy production; and (iii) microplastics inhibit AChE activity. The mechanism for AChE inhibition appeared to be different for pyrene and microplastics, since simultaneous exposure to both did not increase significantly the inhibitory effect. The observed neurotoxic effects of microplastics per se and the effects on IDH activity of the two stressors combined are of concern because they may increase mortality in natural fish populations. More studies need to be carried out on possible combined effects of microplastics and polycyclic aromatic hydrocarbons on fish, particularly juveniles.

1. Introduction

Plastics, synthetic polymers, typically made from non-renewable resources such as crude oil, coal, and natural gas, are indispensable in modern society and are widely used in several types of industries, e.g. construction, medicine, food and drinks storage, cosmetics and personal care products. Due to their low degradation rate, plastics of varying sizes accumulate both in terrestrial and aquatic ecosystems. In the marine environment, plastics have been found to accumulate in coastal sediments and the pelagic zone, from shallow coastal areas to the open ocean, and from polar seas to the tropics (Santos et al., 2009). Their accumulation in ocean gyres together with several other residues, including chemicals, has raised increasing concern. Even if the release of plastics stopped immediately, some of those already present in the aquatic environment may persist for centuries (Barnes et al., 2009), representing a great challenge for remediation (Zarfl et al., 2011). Despite the widespread recognition of the problem, the amount of plastic debris found in the environment is still growing as the result of their increased use. In 2011, global plastics production increased to around 280 million tonnes, continuing a growth pattern of approximately 9% *per annum* since 1950 (Plastics Europe, 2012).

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Despite their persistence, plastic items generally fragment in the aquatic environment, mostly on shoreline (Andrady, 2011). This is a result of prolonged UV exposure and mechanical degradation and is a process that eventually results in very small particles, of micro and nano scales. In addition, micro- and nanoplastics enter the aquatic environment directly as a result of their use as abrasives in industrial and domestic cleaning applications, e.g. shot blasting or scrubbers used in hand cleansers, or spillage of pellets and powders used as a feedstock for the manufacture of most plastic products (Thompson et al., 2009). At some locations microplastics can constitute over 80% of intertidal plastic debris (Browne et al., 2007).

The interactions of large plastic particles with biota are comparatively well known (Moore, 2008), but effects of small plastic particles that become available to a wide range of organisms from planktonic (Moore et al., 2001; von Moos et al., 2012) to benthic (Thompson et al., 2004) have only recently come under investigation. In addition to physical problems that have been described, such as entanglement that may kill marine life through drowning, strangulation, dragging, and reduction of feeding efficiency and ingestion, leading to starvation (Moore, 2008), there is much concern about the potential of micro- and nanoplastics to act as carriers of chemicals, including those intentionally incorporated during manufacture processes as well as environmental contaminants. Such adsorption may allow increased concentrations of contaminants to be taken up in organisms and be incorporated in trophic webs (Teuten et al., 2009). Of special concern are environmental contaminants such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons, organochlorine pesticides, polybrominated diphenylethers, alkylphenols, bisphenol A and metals, some of which are well known carcinogens, and/or endocrine disrupters (Ashton et al., 2010; Bakir et al., 2012; Carpenter and Smith, 1972; Fisner et al., 2013; Rios et al., 2007; Teuten et al., 2007, 2009). Environmental contaminants may adsorb to the surface of plastics, where they may accumulate reaching concentrations considerably higher than those found in the surrounding seawater (Mato et al., 2000). Upon simultaneous exposure to micro/nanoplastics and environmental contaminants, independent uptake may occur and, inside the body, plastic particles may modify the distribution, biotransformation and/or the toxicity of contaminants. Therefore, there is an urgent need to increase the scientific knowledge in all these processes that remain largely unknown. The objective of the present study was therefore to clarify the effects resulting from acute exposure of juveniles (0+ age group) of the common goby (*Pomatoschistus microps*) to polyethylene microspheres and pyrene, singly or in combination.

Common goby juveniles (1.2 cm long) were selected as model in this study because the survival rate of this life-stage is determinant for population fitness. At this life-stage, *P. microps* juveniles are important zooplanktonic feeders, playing a crucial role in controlling this community, and a prey for several higher-level predators in estuaries and other ecosystems where they occur. Furthermore, there are well-established protocols for acclimatization and exposure of *P. microps* juveniles, as well as health-related measurements, e.g. involved in biotransformation and mechanisms of toxicity (Oliveira et al., 2012; Vieira et al., 2008, 2009). Polyethylene microspheres were selected for this study because this polymer corresponds to about one third of the European plastics uses (Plastics Europe, 2012) and is one of the primary synthetic polymers found in oceans (Fries and Zarfl, 2012). Pyrene was selected as model contaminant because it is one of the most common environmental contaminants in estuaries and coastal areas (Xu et al., 2007) and is known to cause toxicity in several aquatic species following short-term and long-term exposure (Almeida et al., 2012; Incardona et al., 2004; Okay et al., 2002; Petersen et al., 2008; Schirmer et al., 1998). Furthermore, baseline information and general data for comparison is available, provided by a previous study on the effects of pyrene on older (2.5–3.0 cm) *P. microps* juveniles (Oliveira et al., 2012). A simultaneous exposure of estuarine biota to this chemical and microplastics is an environmental realistic scenario and the lipophilic nature of pyrene suggests that interactions with plastics are likely to occur. To clarify any effects of exposure to pyrene and/or microplastics, a set of biomarkers including enzymes involved in functions crucial for fish survival and performance was employed, i.e. bile pyrene metabolites as biomarker of exposure to pyrene; levels of lipid peroxidation (LPO) as indicative of oxidative damage in lipids; and the activities of the enzymes acetylcholinesterase (AChE) involved in neuro and neuromuscular transmission in fish, glutathione *S*-transferases (GST) involved in biotransformation and protection against oxidative stress, and $NADP^+$-dependent isocitrate dehydrogenase (IDH), involved in the aerobic pathway of cellular energy production, but also relevant for cellular antioxidant defence. The size of the juvenile common gobies used as well as ethical concerns limited the number of biomarkers that could be included. Biomarkers were selected based on those found to be affected by pyrene in a previous study with older *P. microps* juveniles (Oliveira et al., 2012).

2. Materials and methods

2.1. Tested substances and other chemicals

Pyrene (CAS number 129-00-0) was purchased from Sigma–Aldrich (Germany). All the other chemicals were of analytical grade, obtained from Sigma–Aldrich (Germany), Boehringer (Germany), Bio-Rad (Germany) or Merck (Germany). Red polyethylene microspheres with a size range between 1 and 5 μm were purchased from Cospheric (USA).

2.2. Test organisms

P. microps (1.0–1.2 cm long) were captured using a hand net, at low tide, in a reference area of the Minho River estuary (NW of Portugal 41°53′26.8″ N, 8°49′29.2″ W), that was already used for this purpose in previous studies with this species (Oliveira et al., 2012; Vieira et al., 2008, 2009). After being collected, specimens were immediately transported to the laboratory in oxygenated water from the sampling site. They were acclimated to laboratorial conditions for one week in 80 L aquaria containing aerated and filtered (Eheim filters) artificial saltwater (15 g L^{-1}) in a photoperiod (16 h light:8 h dark) and temperature controlled chamber (20 ± 1 °C). Fish were fed daily with commercial fish food (TetraMin®) and the water in the aquaria was renewed daily.

2.3. Fish bioassay

The general conditions of the bioassay followed the OECD guidelines for fish acute bioassays (OECD, 1992), with slight modifications, especially in the number and type of treatments (since the objective was not to calculate lethal concentrations) and in the exposure conditions (since fish were exposed individually). All glass material was washed with acid (HNO_3 10%) and rinsed with acetone and distilled water before the experiments. Stock solutions of pyrene were prepared every 12 h in acetone due to the low solubility of pyrene in water. Acetone was selected as solvent because it does not contribute to PAH metabolite fluorescence and, at the concentration tested, it is not expected to cause adverse effects on fish (Dissanayake et al., 2008; Oliveira et al., 2012). The pyrene concentrations tested in the present study were selected based on the results of a previous study with older goby juveniles (2.5–3 cm long), where a significant increase of

bile metabolites and reduction of the swimming performance was found at 250 $\mu g\,L^{-1}$ (Oliveira et al., 2012), and on preliminary bioassays with juveniles similar to those used in the present study (1.0–1.2 cm long), that suggested a higher sensitivity to stressors of younger juveniles (unpublished data). Thus, 200 $\mu g\,L^{-1}$ was selected as a concentration of pyrene likely to cause mortality in the tested juveniles, whereas 20 $\mu g\,L^{-1}$ as a concentration not expected to cause mortality. The tested concentrations of microplastics were selected as a high exposure and a low exposure concentration, in both cases not expected to cause death due to microplastic exposure alone but likely interacting with pyrene toxicity as suggested by preliminary bioassays. The test solutions of pyrene (20 and 200 $\mu g\,L^{-1}$) were prepared by dilution of the stock solution in artificial saltwater prepared with distilled water, in final concentration of 15 $g\,L^{-1}$. This water was used as test medium and control water. An additional control with acetone (0.05%) was included in the experimental design. A stock solution of polyethylene microspheres was prepared by dissolution of the microsphere powder in ultrapure water and the working solutions (18.4 and 184 $\mu g\,L^{-1}$), prepared by dilution in artificial saltwater. Thus, the experimental design included the following treatments: control; solvent (acetone) control; 20 $\mu g\,L^{-1}$ pyrene; 200 $\mu g\,L^{-1}$ pyrene; 18.4 $\mu g\,L^{-1}$ microplastics; 184 $\mu g\,L^{-1}$ microplastics; 20 $\mu g\,L^{-1}$ pyrene + 184 $\mu g\,L^{-1}$ microplastics; 200 $\mu g\,L^{-1}$ pyrene + 18.4 $\mu g\,L^{-1}$ microplastics; 200 $\mu g\,L^{-1}$ pyrene + 184 $\mu g\,L^{-1}$ microplastics. The treatment 20 $\mu g\,L^{-1}$ pyrene + 18.4 $\mu g\,L^{-1}$ microplastics was not included due to limitations of fish collected in the field. Fish ($n = 8$ per treatment) were individually exposed to each treatment for 96 h in 1 L glass beakers filled with 500 mL of each test solution, and sealed to avoid evaporation losses. During the test, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period and no food was provided. Test medium was changed every 12 h to prevent significant pyrene degradation and to reduce the build-up of metabolic residues (Almeida et al., 2012; Oliveira et al., 2012) after checking fish mortality and determination of water temperature, salinity, conductivity, pH and dissolved oxygen. Fish were considered dead when neither opercular movement nor response to mechanical stimuli could be detected. After 96 h of exposure, living fish were sacrificed by decapitation and biological material prepared for biomarkers determination as described below.

2.4. Preparation of biological material for biomarkers determination

Immediately after the sacrifice of each fish, the head and gallbladder bile were isolated on ice. The head was homogenized (using an Ystral homogenizer) in ice-cold phosphate buffer (0.1 M, pH 7.2) and centrifuged (Eppendorf 5810R centrifuge) for 3 min at 3300 × g (4 °C). The supernatant was carefully collected and stored at −80 °C until the determination of AChE activity was done. The remaining body portion was homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4). Homogenates were divided in aliquots for lipid peroxidation (LPO) determination and post-mitochondrial supernatant (PMS) preparation. PMS was accomplished by centrifugation (Eppendorf 5810R centrifuge) for 20 min at 13,400 × g (4 °C). PMS aliquots were stored at −80 °C until GST and IDH activities were determined.

2.5. Biomarkers determination

Protein content of samples was determined by the Bradford method (Bradford, 1976) adapted to microplate (Frasco and Guilhermino, 2002) in a BIO-TEK microplate reader, model POWERWAVE. Prior to enzymatic analysis, sample protein content was normalized to 0.5 $mg\,mL^{-1}$. After the enzymatic analysis, all performed in the microplate reader indicated above, the amount of protein in each sample was determined again and this value was used to express enzymatic activities per mg of protein.

Head AChE activity was determined according to the Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996), at 25 °C. The rate of acetylthiocholine degradation was assessed at 412 nm by measuring the increase in the yellow colour due to the binding of thiocholine with 5,5-dithio-bis (2-nitrobenzoic acid) ($\varepsilon = 1.36 \times 10^4\,M^{-1}\,cm^{-1}$). The results were expressed as nmol of substrate (acetylthiocholine) hydrolysed per minute per mg of protein.

IDH activity was determined by measuring the amount of nicotinamide adenine dinucleotide phosphate (reduced form – NADPH) regenerated, according to Ellis and Goldberg (1971) adapted to microplate (Lima et al., 2007), at 25 °C. Absorbance was recorded at 340 nm (25 °C) and expressed as nmol of NADPH regenerated per minute per mg of protein ($\varepsilon = 6.22 \times 10^3\,M^{-1}\,cm^{-1}$).

GST activity was determined in the PMS, following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) by the method of Habig et al. (1974) adapted to microplate (Frasco and Guilhermino, 2002). Absorbance was recorded at 340 nm (25 °C) and expressed as nmol of CDNB conjugate formed per minute per mg of protein ($\varepsilon = 9.6 \times 10^3\,M^{-1}\,cm^{-1}$).

LPO levels were determined by the procedure of Ohkawa et al. (1979) as described by Oliveira et al. (2009). Absorbance was measured at 535 nm and LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein ($\varepsilon = 1.56 \times 105\,M^{-1}\,cm^{-1}$).

Bile samples were diluted in 10 volumes of water for pyrene-type metabolites quantification. Each bile solution was further diluted with methanol (50%). Fluorescent readings were made at 341/383 nm for pyrene and pyrene-metabolites (Aas et al., 1998; Gagnon and Holdway, 2002) using pyrene as a reference standard. Metabolites were expressed as μg per mg of protein.

2.6. Statistical analyses

Median lethal times (LT_{50}) and the lethal time causing 10% of mortality (LT_{10}) were calculated from the mortality curves (probit analysis). For each biomarker, different treatments were compared using one-way analysis of variance (ANOVA), followed by the Dunnett's comparison test whenever applicable (Zar, 1999). Data were previously tested for normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Bartlett's test), and data transformations were done when necessary (Zar, 1999). All statistical analyses were performed using the SPSS 20 software package. The significance level for rejection of H_0: "no difference between treatments" was set at 0.05.

3. Results

In all test beakers, the oxygen concentration in water was always higher than 8.9 $mg\,L^{-1}$, the variation in temperature was less than 1 °C and the pH variation was always less than 0.5 pH unit. No mortality was observed in controls.

In fish exposed to 200 $\mu g\,L^{-1}$ of pyrene, either alone or in combination with microplastics, abnormal swimming and lethargy was observed after 1 h exposure. No such effects were observed in any of the other treatments. Total (100%) mortality was observed after 48 h in fish exposed to pyrene 200 $\mu g\,L^{-1}$ (single exposure) whereas for fish exposed to the mixture containing the same concentration of pyrene, 100% of mortality was found only after 60 h (Table 1). The LT_{50} calculated ranged between 29 and 42 h (Table 2).

Biomarkers were determined in fish from all treatments except in those containing the highest concentration of pyrene

Table 1
Mortality along time in *Pomatoschistus microps* juveniles (0+ group) exposed to the different experimental treatments: pyrene 200 μg L^{-1}; pyrene 200 μg L^{-1} + microplastics 18.4 μg L^{-1}; pyrene 200 μg L^{-1} + microplastics 184 μg L^{-1}. No mortality was recorded during the test in any of the control groups, microplastics treatments without pyrene, pyrene 20 μg L^{-1} nor pyrene 20 μg L^{-1} + microplastics 184 μg L^{-1}. Pyr – pyrene; MP – microplastics (polyethylene) microspheres; "–" – no mortality.

Treatments	Mortality (%) recorded at different times (h)							
	12	24	36	48	60	72	84	96
Pyr 200 μg L^{-1}	–	25	75	100	100	100	100	100
Pyr 200 μg L^{-1} + MP 18.4 μg L^{-1}	–	–	12.5	75	100	100	100	100
Pyr 200 μg L^{-1} + MP 184 μg L^{-1}	–	–	37.5	87.5	100	100	100	100

Table 2
Median lethal time (LT_{50}) and 10% lethal time (LT_{10}) in *Pomatoschistus microps* juveniles (0+ group) exposed to pyrene 200 μg L^{-1}; pyrene 200 μg L^{-1} and microplastics 18.4 or 184 μg L^{-1}. 95%CL – 95% confidence limits. Pyr – pyrene; MP – microplastics (polyethylene) microspheres.

Treatments	Time (h)			
	LT_{10}	95%CL	LT_{50}	95%CL
Pyr 200 μg L^{-1}	16.3	5.53–21.49	29.0	22.47–34.51
Pyr 200 μg L^{-1} + MP 18.4 μg L^{-1}	30.6	16.64-35.90	42.1	35.99-47.22
Pyr 200 μg L^{-1} + MP 184 μg L^{-1}	25.6	10.40-31.28	38.5	31.40-43.93

(200 μg L^{-1}) where all fish died before the end of the bioassay. No significant differences were found between water control and solvent control groups for any biomarker. Thus, for each biomarker, data from the two groups were pooled for further statistical analysis.

The concentration of pyrene metabolites is shown in Fig. 1. For the same concentration of pyrene (20 μg L^{-1}), the bile metabolites concentration was around 3 fold higher in the presence of microplastics relative to the corresponding concentration in fish exposed to pyrene alone. Significant differences in AChE activity among treatments were found ($F_{4,39}$ = 12.291, p < 0.05; Fig. 2), with all the chemical treatments, including microplastics single exposure, showing lower activities (19.8–29.3%) than the control group.

Significant differences in IDH activity were found between treatments ($F_{4,39}$ = 23.218, p < 0.05; Fig. 3), with a significant inhibition (52.3%) of enzymatic activity being observed in fish simultaneously exposed to pyrene and microplastics. No significant differences among treatments were found for GST activity ($F_{4,39}$ = 2.598, p > 0.05; Fig. 4), or LPO levels ($F_{4,39}$ = 1.015, p > 0.05; Fig. 5).

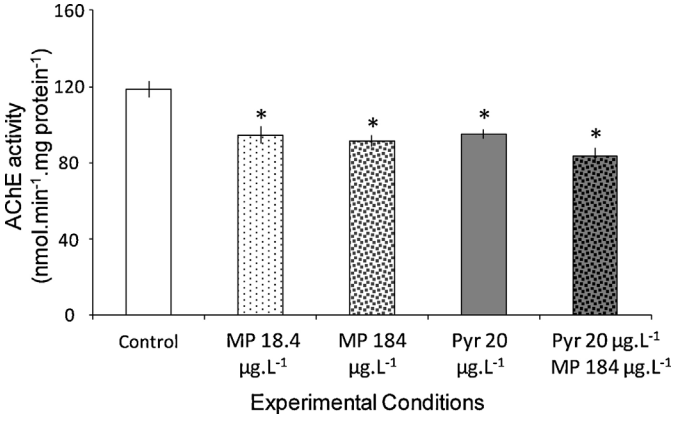

Fig. 2. Acetylcholinesterase activity (AChE) of *P. microps* after 96 h of exposure to microplastics (18.4 and 184 μg L^{-1}), pyrene (20 μg L^{-1}), and combined exposure pyrene and microplastics (20 μg L^{-1} pyrene + 184 μg L^{-1} microplastics). Results are expressed as means ± standard errors (n = 8). MP – microparticles; Pyr – pyrene. *Significantly different from the control group (p < 0.05 Dunnett test).

4. Discussion

The presence of microplastics was found to delay the pyrene-induced mortality: the highest concentration of pyrene (200 μg L^{-1}), in the absence of microplastics, induced 100% mortality after 48 h exposure, whereas in the presence of microplastics, the 100% mortality was only recorded after 60 h of exposure (for both concentrations of plastics). Based on LT_{50}, the microplastic-induced delay in mortality for treatments containing 200 μg L^{-1} of pyrene was 13.1 h for 18.4 μg L^{-1} and 9.5 h for 184 μg L^{-1}. These results suggest toxicologically relevant interactions between microplastics and pyrene. Increased accumulation of pyrene metabolites in the bile of fish simultaneously exposed to

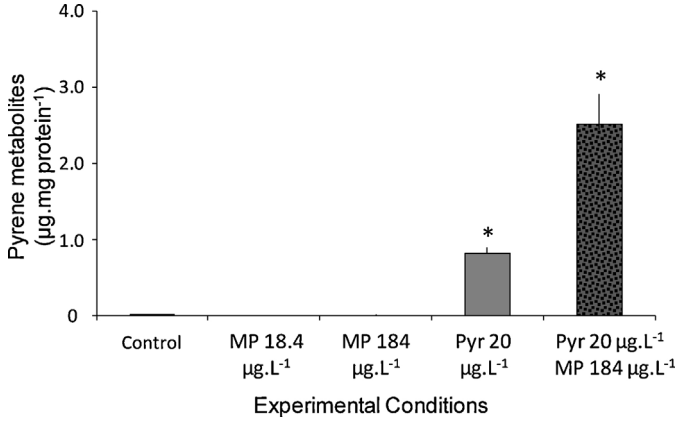

Fig. 1. Pyrene metabolites in the bile of *P. microps* after 96 h of exposure to microplastics (18.4 and 184 μg L^{-1}), pyrene (20 μg L^{-1}), and combined exposure pyrene and microplastics (20 μg L^{-1} pyrene + 184 μg L^{-1} microplastics). Results are expressed as means ± standard errors (n = 8). MP – microparticles; Pyr – pyrene. *Significantly different from the control group (p < 0.05 Dunnett test).

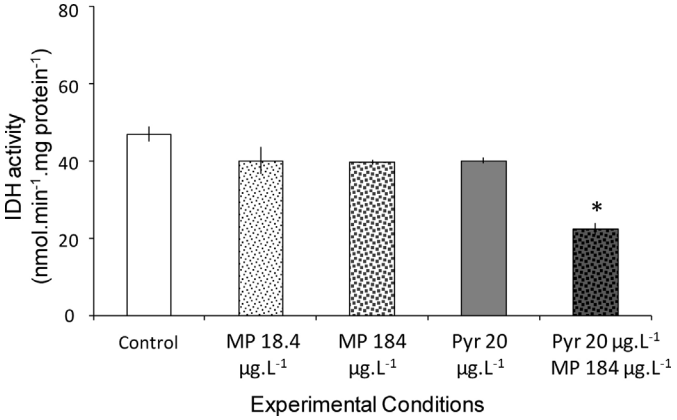

Fig. 3. Isocitrate dehydrogenase (IDH) activity of *P. microps* after 96 h of exposure to microplastics (18.4 and 184 μg L^{-1}), pyrene (20 μg L^{-1}), and combined exposure pyrene and microplastics (20 μg L^{-1} pyrene + 184 μg L^{-1} microplastics). Results are expressed as means ± standard errors (n = 8). MP – microparticles; Pyr – pyrene. *Significantly different from the control group (p < 0.05 Dunnett test).

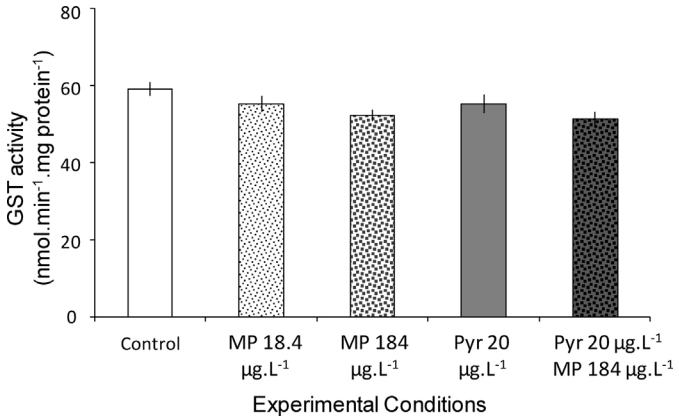

Fig. 4. Glutathione *S*-transferase (GST) activity of *P. microps* after 96 h of exposure to microplastics (18.4 and 184 μg L^{-1}), pyrene (20 μg L^{-1}), and combined exposure pyrene and microplastics (20 μg L^{-1} pyrene + 184 μg L^{-1} microplastics). Results are expressed as means ± standard errors (n = 8). MP – microparticles; Pyr – pyrene.

microplastics and pyrene compared to fish exposed to pyrene alone, seems to support this hypothesis. This may, at least in part, explain the delay of the lethal time observed in fish exposed to the mixture treatment relatively to the pyrene single exposure. The fact that the presence of microplastics delayed the lethal time without protecting fish from death raises at least two hypotheses, not mutually exclusive: (i) the mechanism involved is transitory and can only be maintained for a relatively short time; (ii) protective mechanisms can only cope with a certain level of internal pyrene/metabolites concentrations, with levels higher than this threshold causing severe toxic effects leading to death. Unfortunately, the experimental design in this study does not allow further exploration of this issue. Pyrene and microplastics, either tested individually or in mixture, caused significant reductions in AChE activity, indicating adverse effects in cholinergic neurotransmission and thus potentially in nervous and neuromuscular function. The anticholinesterase properties of pyrene to *P. microps* were reported in a previous study with older juveniles (2.5–3 cm long) where an inhibition of (42%) of the brain AChE activity was found after 96 h exposure to 500 μg L^{-1} of pyrene (Oliveira et al., 2012). In the present study, younger juveniles of the age 0+ group (1.0–1.2 cm long) showed a significant inhibition of head AChE (19.8%) after 96 h exposure to 20 μg L^{-1} of pyrene. To the best of our knowledge, such an ability of microplastics to depress AChE activity has not previously been described. In this study, microplastics alone were

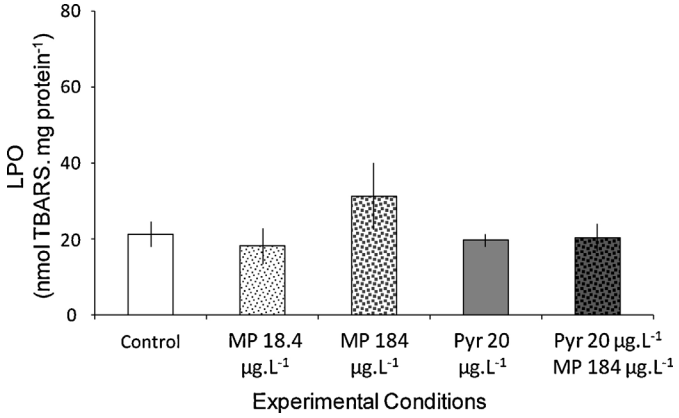

Fig. 5. Lipid peroxidation (LPO) of *P. microps* after 96 h of exposure to microplastics (18.4 and 184 μg L^{-1}), pyrene (20 μg L^{-1}), and combined exposure pyrene and microplastics (20 μg L^{-1} pyrene + 184 μg L^{-1} microplastics). Results are expressed as means ± standard errors (n = 8). MP – microparticles; Pyr – pyrene.

able to significantly inhibit AChE (by an average of 22%), an inhibition rate that has been considered high enough to induce adverse effects in neurofunction (Ludke et al., 1975). The levels of AChE inhibition induced by microplastics and pyrene, as single substances and in combination, were not significantly different, suggesting different mechanisms of enzymatic inhibition. Regardless of the mechanisms causing AChE inhibition, any anticholinesterase effect of microplastics in fish would be alarming taking into consideration the widespread presence of this stressor in the aquatic environment and the pivotal role of this enzyme in neurological function which is crucial to control several physiological (e.g. growth, reproduction) and behavioural (e.g. swimming) processes that directly or indirectly may influence individual and population fitness.

Fish exposed to the mixture 20 μg L^{-1} of pyrene and 184 μg L^{-1} of microplastics showed a significant reduction of IDH activity (52%) relative to the control group, suggesting the occurrence of toxicological interactions in the mechanisms of toxicity responsible for the observed effect since none of the substances alone inhibited IDH activity. This finding justifies further research. IDH is involved in the aerobic pathway of energy production, catalysing the conversion of isocitrate to α-ketoglutarate in the Krebs cycle and regenerating NADH. It has also an important action in the antioxidant system because it regenerates NADPH, a co-factor of antioxidant enzymes, e.g. glutathione reductase (GR). Thus, with IDH activity inhibited, fish are likely to have a reduced ability to acquire energy unless they are able to counterweigh with an increase of the anaerobic pathway. In the wild, the reduction of energy, may limit considerably fish ability to escape from predators and to feed, likely decreasing their individual fitness and increasing the mortality rate in the population. IDH inhibition may also lead to a decrease of NADPH availability thus reducing GR activity and its antioxidant action. In the present study, despite having a significant reduction of IDH activity, fish exposed to the mixture treatment did not display increased LPO levels compared to control fish. Pyrene was found to increase LPO levels in sea bass juveniles (*Dicentrarchus labrax*) at concentrations equal or higher than 150 μg L^{-1} (after 96 h exposure) and in 2.5–3 cm long *P. microps* juveniles at concentrations of 250 and 500 μg L^{-1}, an effect that was not observed in the present study. Exposure to pyrene, in the current study, resulted in no significant effects on GST activity, in agreement with a previous study with older juveniles of this species (Oliveira et al., 2012). Other studies investigating pyrene effects on GST activity in aquatic animals has either observed no effect (Kopecka-Pilarczyk and Correia, 2009; Pathiratne and Hemachandra, 2010) or increased and decreased activities (Almeida et al., 2012; Luis and Guilhermino, 2012; Sun et al., 2008). Microplastics and the combined exposure did not significantly modulate GST activity suggesting no interaction with its functions.

The main aim of the present study was not primarily to assess ecologically relevant scenarios but to investigate mechanisms of any effects of microplastics exposure, alone or combined with pyrene. In this perspective, the exposure concentrations were selected according to the results of a previous study with older juveniles of this species (Oliveira et al., 2012), intending to be the lowest ones likely to induce toxicity. Nevertheless, the concentrations of pyrene used in this study are environmentally relevant (Dissanayake and Bamber, 2010; Dissanayake et al., 2008). Furthermore, pyrene will never be present as the only contaminant in estuarine environments since it will always be present alongside several other polycyclic aromatic hydrocarbons, some of them with similar mode of toxic action and biotransformation. The juveniles of *P. microps* tested in this study (1.0–1.2 cm long) were found to be more sensitive to pyrene than older juveniles (2.5–3.0 cm long) previously tested (Oliveira et al., 2012), with lethal effects being induced after only 12 h of exposure to 200 μg L^{-1}, and with significant inhibition of AChE after 96 h of exposure to 20 μg L^{-1}.

The results therefore highlight the importance of considering early juveniles of fish as models for environmental toxicity since they have crucial direct and indirect roles in aquatic ecosystems. Directly, in the development of their populations and indirectly for the whole ecosystem since they provide top-down control of prey populations. Microplastics are of especial concern because they were found to be able to bind to several other common environmental contaminants, such as PAHs, metals and other very toxic chemicals, in addition to the chemicals that may be incorporated during their manufacture. The present study showed that microplastics either alone or in mixture with pyrene, were able to reduce AChE activity after in vivo exposure and that the mixture was able to significantly reduce IDH activity. Other studies have demonstrated that microplastics may be uptaken by aquatic organisms, including through the digestive system, and induce toxicity in addition to physical effects (e.g. starvation). For example, Browne et al. (2008) found polystyrene microspheres in the circulatory system of mussels (*Mytilus edulis*) for up to 48 days after exposure, although with no adverse effects on the criteria investigated (oxidative status and haemocytes phagocytic ability). In another study also with *M. edulis* (von Moos et al., 2012) industrial polyethylene particles (>0–80 μm) were found in the digestive system tracked inside epithelial cells, resulting in histological changes, strong inflammatory response and lysosomal membrane destabilization. Therefore, more studies on effects of microplastics in aquatic organisms are urgently needed.

5. Conclusions

The presence of microplastics (1–5 μm) was found to delay pyrene-induced death in *P. microps* juveniles (0+ age group, 1.0–1.2 cm long) and to increase the pyrene metabolites concentration in the fish bile. Microplastics when tested alone were able to inhibit AChE activity with an effect similar to pyrene single exposure and to combined exposure to microplastics and pyrene. The pyrene–microplastics combined exposure significantly reduced IDH activity, not observed following exposure to the two stressors separately, suggesting a toxicological interaction of the mixture in the mechanisms leading to enzymatic inhibition. None of the experimental treatments changed GST activity nor LPO levels, suggesting no involvement of GST in their biotransformation or oxidative damage in this species up to 20 $\mu g\,L^{-1}$. These results highlight the importance of carrying out more studies on the mechanisms of toxicity of microplastics either alone or in combination with other environmental stressors.

Conflict of interest

None of the authors have any conflicts of interest regarding the study or financial support for the study.

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