Juveniles at risk: behaviour and colour changes in sole juveniles (*Solea solea*) after exposure to estuarine ragworms (*Hediste diversicolor*) contaminated with microplastics

Pauline Pannetier[1,2], Caroline Vignet[1,2,3], Messika Revel[4,5], Florane Le Bihanic[1,6], Christelle Clérandeau[1], Catherine Mouneyrac[4], Thibaut Larcher[8], Bruno Grassl[7], Johnny Gaspéri[8], Jérôme Cachot[1], Xavier Cousin[2], Marie-Laure Bégout[2].

1. Univ. Bordeaux, CNRS, Bordeaux INP, EPOC, UMR 5805, F-33600 Pessac, France

2. MARBEC, Univ. Montpellier, CNRS, Ifremer, IRD, INRAE, Palavas-les-flots 34250, France

3. Biochimie et Toxicologie des Substances Bioactives (BTSB), EA 7417, Université de Toulouse, INU Champollion, Albi, France ;

4. Université Catholique de L'Ouest, Laboratoire BIOSSE UCO, F-49000, Angers, France

5. UniLaSalle - Ecole des Métiers de L'Environnement, CYCLANN, Campus de Ker Lann, F-35170, Bruz, France

6. APEX, cellules d'expertises en anatomie pathologique pour la recherche, Inra, Nantes, France ;

7. IPREM, UMR 5254, UPPA/CNRS, Technopole Helioparc, Pau, France

8. Laboratoire Eau et Environnement (LEE), Université Gustave Eiffel, Nantes, France

Corresponding author : caroline.vignet@univ-jfc.fr

Abstract

Due to strong anthropogenic pressures and their location at the interface between continental and oceanic environments, estuarine areas are affected by significant microplastic (MPs) pollution and species that live in these areas are particularly exposed. The objective of this study, is to evaluate the effects of environmental MPs on an emblematic and common species of the European coasts, the common sole (*Solea solea*). Sole juveniles were fed with estuarine ragworms (*Hediste diversicolor*) previously exposed to MPs via the sediment. The MPs used were either a mixture of micronized plastics collected from the Seine Estuary (used as environmental MPs at two concentrations: 1 or 100 mg MP/kg sediment) or PVC particles (100-250 µm) at 1 g/kg of sediment, either uncontaminated or contaminated with Benzo(a)Pyrene (BaP, 11.5 µg/g MPs) or benzophenone-3 (BP3, 66 ng/g MPs). Several indicators of health status such as survival, growth, behaviour, energy metabolism, and histopathology of liver and intestine were studied. Individuals fed ragworms exposed to environmental MPs or PVC, displayed a change in behaviour (place preference between black or white background). The colour of the soles changed with an increase in intensity of a colour (chroma value) when exposed to the highest concentration of the Seine Estuary MPs and a change in the frequency of appearance of different colours (value/chroma) in soles exposed to BP3-PVC MPs. These observations are indicative of physiological stress. Exposure to environmental MPs did not reveal any changes in energy reserves (glycogen and lipid concentration), TBARS levels, histopathological lesions or DNA damage. In contrast, exposure to BP3-PVC MPs increased DNA damage and lipid content in muscle.

Keywords: Microplastic, behaviour, metabolism, skin color change, fish, estuarine invertebrate, food chain exposure,

Highlights:

- Trophic contamination of sole juveniles by pollutant-spiked and environmental microplastics
- No effects of MPs on survival, growth and energy reserves of sole
- Benzophenone-spiked and environmental MPs affect skin colour and behaviour of sole
- Benzophenone-spiked MPs increase DNA damage and lipid content of sole

1 Introduction

Plastics mainly have terrestrial origin and rivers are recognized as a major conveyor for the transport of plastics to the oceans (Gola et al., 2021; Lebreton and Andrady, 2019; Mai et al., 2020). Consequently, estuaries and nearby coastal areas, which are also the most inhabited areas worldwide, are characterised by large amount of plastics and MPs, defining estuaries as hot spots for macro and MPs pollution (Arias et al., 2019; Booth and Sørensen, 2020; Browne et al., 2011; Frias et al., 2010; Tong et al., 2023; Tramoy et al., 2021; Wright et al., 2003). Indeed, some studies have reported widespread plastic contamination of estuaries, intertidal sediments, water surface microlayer, aquatic organisms including fish, all over the world for example and more particularly in China (Han et al., 2020; Yan et al., 2019; Zhao et al., 2015), the United States (Gray et al., 2018), South America (Alves and Figueiredo, 2019; Arias et al., 2019; Pegado et al., 2018; Vendel et al., 2017), Australia (Hitchcock and Mitrovic, 2019; Willis et al., 2017), Portugal (Bessa et al., 2018; Rodrigues et al., 2019) and France (Tramoy et al., 2021). This is substantiated by the fact that in some estuaries, the number of MPs equal or exceed the number of fish larvae (Lima et al., 2014; Rodrigues et al., 2019). Sometimes very high concentrations of MPs found in sediments of urbanized estuaries suggest a high risk of contamination of biota especially in benthic organisms and demersal fish (Alves and Figueiredo, 2019). The presence of MPs in aquatic environments is of particular concern for their potential impact on food webs because they can enter by accident or by direct ingestion in a wide range of species and be transferred throughout the food webs (Avio et al., 2020; Bradney et al., 2019; Rochman et al., 2013; Yang et al., 2021). Savoca et al in 2021 reported ingestion of plastic debris by 386 species of marine fish, including 210 commercially important species (Savoca et al., 2021). Furthemore, in a meta-analysis of published studies up to 2020, Wooton et al, showed that 49% of the fish studied (laboratory and *in situ* samples) have ingested microplastics with an average occurrence of 3.5 pieces per individual (Wootton et al., 2021). These fish samples came from a variety of habitats including freshwater, estuarine, pelagic, reef, demersal, benthic, and deep-water and the frequency of occurrence of MPs ranged from 2% to 100% in the sampled fish (Arias et al.,

2019; Bajt, 2021; Chan et al., 2019; Foekema et al., 2013; Lusher et al., 2016). This frequency of plastic ingestion has doubled over the past decade and is increasing every year (Savoca et al., 2021). Indeed, MPs covers a wide range of sizes, and overlaps the size of plankton and some sediments which poses a serious ingestion risk to various types of organisms including suspension feeders, detritus feeders and planktivores (Wright et al., 2013). Ingestion can occur through uptake of MPs directly from the natural environment, or by confusing it with prey, or indirectly through trophic transfer from contaminated preys.

Due to increasing anthropogenic pressures and their location at the interface between continental and marine environments, estuarine areas are facing growing MPs pollution and aquatic species inhabiting these areas are particularly exposed (Collard et al., 2019). Fish from freshwater and estuarine environments had more than twice the plastic load of those of marine environments (Wootton et al., 2021). At the same time, estuaries are nurseries hosting early life stages and juveniles for many fish species and thus are of major ecological importance (Elliott et al., 2007). The early life stages, including embryos and pelagic larvae and the juvenile stage of fish are particularly sensitive to variations of physico-chemical conditions of their environment. In addition, these fish stages are essential for species renewal and are of major ecological importance for the food web functioning.

Common sole (*Solea solea*) is an important commercial species with a distribution range from the north-western coast of Africa in the Mediterranean Sea to the southern coast of Norway in the Atlantic Ocean. After metamorphosis, juveniles settle in coastal nursery grounds, feeding on epi and endobenthic preys (Zambonino-Infante et al., 2013). Cumulative anthropogenic pressures, including climate change, makes common sole particularly at risk (Sardi et al., 2021). A study conducted in the Adriatic Sea on this species revealed the presence of at least one microplastic particle in 95% of the digestive tract of fish sampled. The most frequently found polymers were PVC, Polyamide, PP, PE and polyester, with fragments accounting for 72% of the particles and fibers 28% (Pellini et al., 2018).

97 The risks associated with MPs come from the material itself with additives and the chemical
98 pollutants that bind to it from surrounding waters (Rochman et al., 2013). Ingestion of MPs can cause
99 tissue abrasions, inflammatory responses, and gastrointestinal tract blockage that can lead to false
100 satiety (Limonta et al., 2019; Peda et al., 2016; Welden and Cowie, 2016; Yang et al., 2020). Ingested
101 MPs can also provoke, histopathological damages (Limonta et al., 2019; Yang et al., 2020; Yin et al.,
102 2018), growth retardation (Le Bihanic et al., 2020; Mbugani et al., 2022; Pannetier et al., 2020), and
103 reproduction defects (Cormier et al., 2021b; Yang et al., 2020). A least part of these effects may be
104 related to changes in energy reserves management (Capriotti et al., 2021; Yin et al., 2018) e.g.
105 changes in lipid metabolism (Limonta et al., 2019).
106 The objective of this study was to investigate the effects of MPs on common sole juveniles
107 contaminated through trophic exposure. To get closer to environmental situations, juvenile soles
108 were fed estuarine ragworms (*Hediste diversicolor*) previously exposed to spiked MP sediment. MPs
109 produced from plastic items collected in the Seine Estuary were used as well as PVC MPs
110 contaminated with well-known model pollutants: benzo(a)pyrene (BaP) and benzophenone-3
111 (oxybenzone, BP3), used as artificial positive controls. BaP is a polyaromatic hydrocarbon (PAH)
112 ubiquitously distributed in coastal and offshore marine environment (Antunes et al., 2013) which
113 was shown to adsorb to MPs and have wide range of effects on fish including genotoxic and
114 carcinogenic, immunotoxic and endocrine disrupting effects. BP3 is commonly used as a UV filter in
115 cosmetics such as sunscreen, but also as a light stabilizer in plastics such as PE. This compound has
116 been repeatedly reported to act as an endocrine disrupting chemical in fish and mammals (Kim and
117 Choi, 2014; Kinnberg et al., 2015; Rodríguez-Fuentes et al., 2015). Several indicators of health status
118 such as survival, growth, behaviour, energy metabolism, immune system and digestive system were
119 studied after 20 days of exposure to MPs contaminated ragworms.

2 Materiel and methods

2.1 Microplastics sample

2.1.1 Environmental microplastics

Macroplastic debris were collected at Villequier on the shores of the Seine estuary in spring 2018 and identified by Raman spectroscopy. The macroplastic debris was separated by polymer type, and cut into pieces of about 1 cm^2 and cryo-ground during two consecutive 4 min cycles (Freezer/Mill 6770, Spex Sampleprep, NJ, USA). The resulting powder was then sieved during 10 min (Sieve shaker AS 200, Retsch) to retain only the fraction below 100 µm. The particle size distribution (d10, d50 and d90) was performed with a laser particle size analyzers (Malvern Panalytical, UK). From these micronized MPs, a mixture was made by mixing PE and PP in majority proportion (40% both) and PVC and PET in smaller proportion (10% both). This Seine estuary's MP mixture (eMPs) represents an average polymer composition of MPs found in sediments (Leslie et al., 2017; Phuong et al., 2018). PVC MPs were purchased from Fainplast (Ascoli Piceno AP, Italia) for a given size range of 125–250 µm. They were spiked with BaP (BaP-MPs; benzo[*a*]pyrene; CAS 50–32–8; purity ≥96%; Sigma Aldrich Stockholm, SE) or BP3 (BP3-MPs; (2-hydroxy-4-methoxyphenyl)-phenylmethanone; CAS 131–57–7; purity ≥98%, Sigma Aldrich Stockholm, SE) as described in detail by Cormier et al. (2021a). The final concentrations of BaP and BP3 on coated PVC MPs were measured at 11.5 ± 1.4 µg/g and 107.0 ± 1.5 ng/g, respectively.

2.1.2 Chemical analysis of environmental microplastics.

Five trace metals were analysed in the eMPs: lead (Pb), zinc (Zn), chromium (Cr), cadmium (Cd) and copper (Cu). Mineralization was carried out in the presence of 10% nitric acid, under nitrogen flow, using a Milestone Ultrawave mineralizing oven following the Milestone application note (Milestone, Helping Chemists). The residues were diluted in ultra-pure water to obtain a 2% HNO_3 concentration. The metal content was then determined by ICP-MS (7500 Series, Agilent).

Some organic micropollutants including polycyclic aromatic hydrocarbons (PAH, n=16), alkylphenols (AP, n=7) and bisphenol A (BPA), as well as phthalate acid esters (PAE, n=15) were analysed on the

146 eMPs. Three successive solid-liquid extractions with 40 mL of a methanol/dichloromethane/hexane
147 mixture (1, 1, 1, v/v) were first conducted. The three fractions were then pooled. Two sub-samples
148 were considered, respectively for the analysis of AP, BPA and PAE (sub-sample 1) and PAH (sub-
149 sample 2). Sub-sample 1 was evaporated using a rotary evaporator and purified on alumina
150 cartridge. After elution with 5 mL of ethyl acetate, PAEs were collected and this fraction was
151 evaporated to dryness under nitrogen flow then recovered in 50 µL of heptane for analysis by gas
152 chromatography coupled to mass spectrometry (GC-MS). APs and BPA were recovered with 10 mL
153 of ethanol. For this fraction, the fraction was evaporated to dryness under nitrogen flow then taken
154 back in the mobile phase (Methanol) for analysis by high-pressure liquid chromatography coupled
155 to tandem mass spectrometry (UPLC-MS/MS). Sub-sample 2 was partially evaporated with a rotary
156 evaporator and then purified on a silica column. After recovery of the PAH fraction, fraction was
157 evaporated to dryness under nitrogen flow then recovered in 50 µL of heptane for GC-MS analysis.
158 Analytical protocols are described in the literature (Bressy et al., 2012; Cladière et al., 2013).

159 ### *2.2 Biological material*

160 All the experimentations followed the EU directive 2010/63 regarding animal treatment and took
161 place in the Laboratoire Ressources Halieutiques, Ifremer (facility authorization A171901; ethic
162 project authorization APAFIS#17351-2018101914592899v3).
163 Juveniles of common sole, *Solea solea* (Linnaeus, 1758) were collected during two autumn samplings
164 in Bay of Aiguillon (Atlantic coast, France) with a trawl net (2 m wide). Common soles were placed
165 in 400L tanks with sea water at 34.3±0.5 PSU, 17.7±0.6°C, oxygen 99.9±1.7% (7.7±0.3mg/L) for four
166 weeks of adaptation. Juveniles were fed with specific fish food (Larviva Inicio 500, BIOMAR, France),
167 live artemia (SepArt INVE, OceanNutrition, Belgium) and red bloodworms (*Chironomus sp.*, Europrix,
168 France) according to biomass with a 2% wet weight ratio.
169 For the first experiment, ragworms, *Hediste diversicolor* (Müller, 1776) and sediments were
170 collected in mudflats located in Bay of Bourgneuf (Port du Collet, French Atlantic coast) which is
171 considered to be in a reference site due to very low domestic and industrial activities. Four hundred

ragworms were carefully sampled by hand and 60 kg of sediment were collected. Ragworms were kept in sediment with aerated Artificial SeaWater (ASW) prepared with reconstituted salt (Tropic Marin, Germany) at 15 °C in the dark. Sampled ragworms were depurated in ASW for 24 h to eliminate their gut content before MPs exposure was carried out. The sediment was sieved at 1 mm and an evaluation of MPs contamination based on ZnCl density separation method (Zobkov and Esiukova, 2017) was conducted and revealed a relatively low number of microparticles (30 ± 7 particles/kg of sediment, size > 20 µm). For the second experiment, ragworms and sediment were collected during two samplings (autumn and winter) in mudflats located in Bay of Aiguillon (Point Saint-Clément, French Atlantic coast). A total of 1000 ragworms were carefully sampled by hand and 60 kg of sediment were collected. Ragworms were kept in sediment with aerated naturel filtered seawater (NSW) at 15 °C in the dark. Sampled ragworms were sorted from sediment and placed in fresh ASW for depuration during 24 h to eliminate their gut content before MPs exposure was carried out.

2.3 Ragworms exposure

Ragworms were separately exposed to the two different types of MPs presented above for 96 h through sediment (Port du Collet or Pointe Saint-Clément) according to the protocol of ASTM (ASTM, 2013) with minor adaptations.

For the first experiment, three experimental conditions were set up: Control, Seine estuary mix MPs at 1 mg/kg of sediment (eMPs-1), Seine estuary mix MPs at 100 mg/kg of sediment (eMPs-100). This was performed in triplicate with 20 to 22 ragworms per replicate. For the second experiment, three experimental conditions were set up: Control (MPs), coated PVC with BaP at 1 g/kg of sediment (BaP-MPs) and coated PVC with BP3 at 1g/kg of sediment (BP3-MPs). This was performed in triplicate with 30 ragworms per replicate (90 per condition). Glass aquariums of 4 L were filled with 3 kg of sediment and each MPs type was weighted and directly added and mixed with sediments to all aquariums except the controls. Sediment was then covered with 300 mL of ASW or NSW (Mouneyrac et al., 2014). Considering the reduced duration of the exposure and the fact that natural sediment was

198 used, ragworms were not fed during the experiment. Aeration was applied to oxygenate the water.
199 The organisms were left in dark room with a controlled temperature at 15°C ± 1°C. Contamination
200 of the ragworms was checked by analyzing the MPs content of the ragworms after digestion of 18-
201 19 ragworms per condition according to the protocol described in Revel et al (Revel et al., 2020). No
202 particles were identified (observation limit 20 µm) in the controls and an average of 0.242 ± 0.348
203 and 0.875 ± 1.05 MPs/ragworms were found in the ragworms exposed to 1 and 100 mg MPs/kg of
204 sediment respectively.

205 *2.4 Fish exposure*

206 Ten juveniles of sole (G0, stage confirmed by otolith analyses) by condition were selected to form
207 size-match groups (Experimentation 1 ≈ 8.2±0.9 cm / 7.1±2.3 g and experimentation 2 ≈ 10.2±1.1 cm
208 / 12.0±4.2 g). Each juvenile was individually placed in 3L tank and fed one ragworm (≈0.22±0.09 g)
209 each 48h during the 20 days of the exposure. Temperature (Exp. 1: 17.7±1.4°C; Exp. 2: 18.1±1.1°C),
210 salinity (34.1±0.6 PSU) and oxygen (100.3±1.2 %) were checked every day. The water circulation was
211 continuous resulting in renewal of half of the water volume every 5 days. The photoperiod was
212 12h/12h. At the end of the 20 days of exposure, the fish were euthanized and blood was collected
213 and cryopreserved for DNA damage analysis. In addition, the liver was collected for EROD analysis,
214 RT-qPCR and histopathology, the intestines for histopathological analyses and measurement of
215 TBARS. Muscles were used for the determination of energy reserves (glycogen and lipids). Organs
216 collected for histopathological analysis were preserved in formalin at 4% and the others stored at -
217 20°C or -80°C until analysis.

218 *2.5 End point*

219 2.5.1 Biometric parameters, skin colour and sampling

220 At day 0 and day 20, standard length (mm) and fresh weight (mg) were determined for each
221 individual. Skin colour was determined using Munsell colour system according to Davenport and
222 Bradshaw protocol to obtain value and chroma data (Davenport and Bradshaw, 1995). The Munsell
223 chart used in this study was hue 2.5YR which corresponds to one yellow-red dimension. In the

Munsell chart dimension, a higher value indicates a darker colour, while a higher chroma, in this specific chart, represents a brighter red colour. The fish were dissected and the organs removed and weighed. The Fulton index (Weight/Length^3) was calculated as well as the hepatosomatic index (liver weight/total weight; HSI) and splenosomatic index (spleen weight/total weight; SSI).

2.5.2 Behaviour

To investigate behavioural changes, two assays were conducted in a dedicated room with the same photoperiod and temperature conditions as the exposure room: background colour choice to evaluate place preference and spontaneous swimming activity. For the first test, each sole juveniles were placed in 10L tank whose background has been divided into 2 areas of equal size: one with a white background and one with a black background. After 30 min of acclimation post transfer, the fish were filmed for 2 hours. After 2 hours, the black backgrounds were removed and the fish were filmed for an additional 16 hours to monitor their spontaneous behaviour. The videos were analysed with Ethovision XT software (Noldus, The Netherlands). Variables of interest used were the time spent in the black zone and in the white zone (in minutes), the number of transitions between the two zones, the average distance moved (cm).

2.5.3 Histopathology

Liver samples and intestinal section fixed in formalin were embedded in paraffin. For all samples, a 4 µm section was cut and then stained with hematoxylin-eosin-saffron (Larcher et al., 2014). In the liver, the slides were examined for the presence of parenchymal lysis foci (Fig. 1, A), glycogen overload of the liver parenchyma (Fig. 1, B) and melanoma-macrophage centres (Fig. 1, C). Finally, the digestive tract was examined in order to highlight diffuse oedemas in the mucosa (Fig. 1, D)

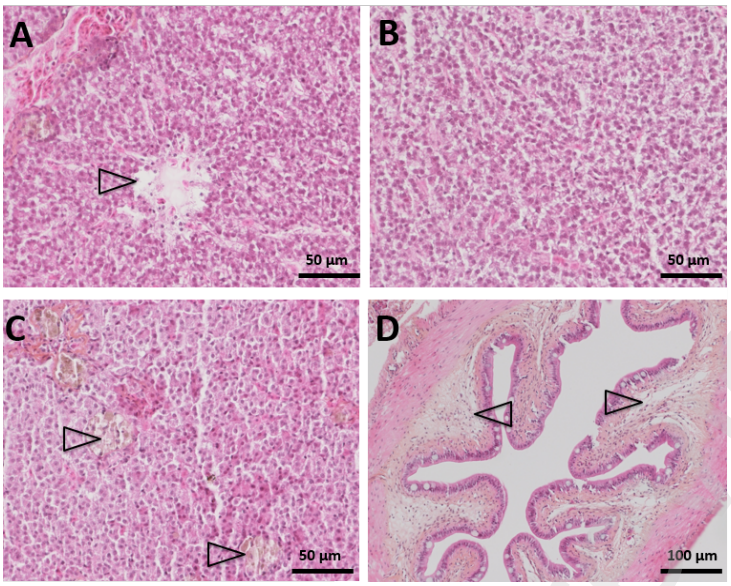


Figure 1: Liver (A-C) and intestine (D) were analysed for histological defects revealing foci of lysis (arrowhead in A), Glycogen storage revealed by clear cytoplasm (B), Melano-macrophage centres (arrowheads in C) and oedema of the sub-mucosa (arrowheads in D).

2.5.4 Other biomarkers

Part of the liver was stored in RNALater (Qiagen, Les Ulis, France) prior to be extract with RNeasy Plus Universal Mini Kit (Qiagen). The expression of reference genes *apr*, *eef1* and *ubiquitin* and target genes *fasn, cyp1a, pparα, pck 1, hexokinase* and *gsto* was then determined by RT-qPCR. The detailed protocol was described in supplementary data S1. The energy reserves (glycogen and lipids), TBARS, EROD activity and the DNA break rate were also determined in liver tissue samples following the protocol described in supplementary data S1.

2.6 Statistics

Statistical tests were carried out using R studio (ver. 1.3.1093 with R ver. 3.6.3). Normality was checked using Shapiro-Wilks' test. Variance heterogeneity was tested with Levene's test. When all the conditions of use were met, a one-way ANOVA with Tukey's multiple comparison test was performed. When the values were not normally distributed, the analyses were carried out with a Kruskal-Wallis test. Concerning behaviour data, linear mixed model with restricted maximum likelihood of the R-package “lme4” (Bates et al., 2015) was applied. Model checking was performed by visual assessment of QQ-plots and residual plots. A post-hoc pair-wise comparison by T-test

(Tukey contrasts) was performed (Hothorn et al., 2008). For each analysis, a significance threshold of 5 % was applied.

3 Results

3.1 Environmental microplastics from Seine estuary (eMPs)

3.1.1 Size distribution

Collected macroplastics were mainly composed of PE (62%) and PP (28%), the three other polymers being much less abundant (PET, 4%; PVC, 5%; PS, 1%). The median size (d(50) µm) for all micronized plastics ranged from 52 to 77 µm depending on the polymer type (Tab. 1). For each polymer, 2.4 to 4.0% are below 5 µm and 90% are below 116 to 145 µm.

Table 1: Granulometry of the different plastic particles after micronization according to size distributions (d10, d50 and d90) and % of particles < 5 µm

Polymer	< 5 µm (%)	d (10) µm	d (50) µm	d (90) µm
PE	3.6	14	52	121
PET	2.4	28	77	145
PP	4.0	12	52	116
PVC	3.0	19	62	121

3.1.2 Trace metals content

The concentrations of the different metals in MPs were very variable from one polymer to another and were particularly high in PP and PVC (Tab. 2). The zinc (Zn) content was particularly high for PP and rather low in PE. The cadmium (Cd) concentration was high in PP and very low in PET and PVC. The lead (Pb) content was higher in PVC and of the same order of magnitude in the other polymers. Finally, for copper (Cu) and chromium (Cr), the concentrations were the highest in PET.

Table 2: Contents of Lead (Pb), Zinc (Zn), Chromium (Cr), Cadmium (Cd) and Copper (Cu) (µg/g of plastic) according to the different polymer types. LOQ: Limit of quantification

	Concentration (µg/g of plastic)				
Polymer	Cu	Pb	Zn	Cd	Cr
PP	3.5	9.0	4169.8	123.2	77.9
PET	13.8	19.5	52.6	<LOQ	359.5
PVC	4.7	786.0	432.3	<LOQ	23.1
PE	2.0	4.9	9.0	6.0	<LOQ

283 3.1.3 Organic chemicals content

284 Total PAH concentrations ranged from 3.5 to 7 µg/g according to the polymer type (Tab. 4). Heavy
285 PAHs (4 to 5 rings) were the most abundant representing more than 75% of total PAHs compared to
286 light PAHs (2-3 rings). The main compound was a 5-rings PAH, benzo(ghi)perylene which represented
287 more than 30% of the total PAHs analysed. The concentration of phthalates was high (>200 µg/g)
288 and dibutylphthalate represented almost 60% of the total amount of analysed phthalates (Tab. 3).
289 For alkylphenols, the total concentration was around 3 µg/g and 4-nonylphenol (NP) represented
290 more than 78% of alkylphenols (Tab. 3).

291 *Table 3: Concentration (mg/kg) of alkylphenols, Phthalates and PAHs (2-3 ring PAHs are given above the line while 4 and*
292 *more rings PAHs are below) in eMPs. <LD : below limit of detection.*

293

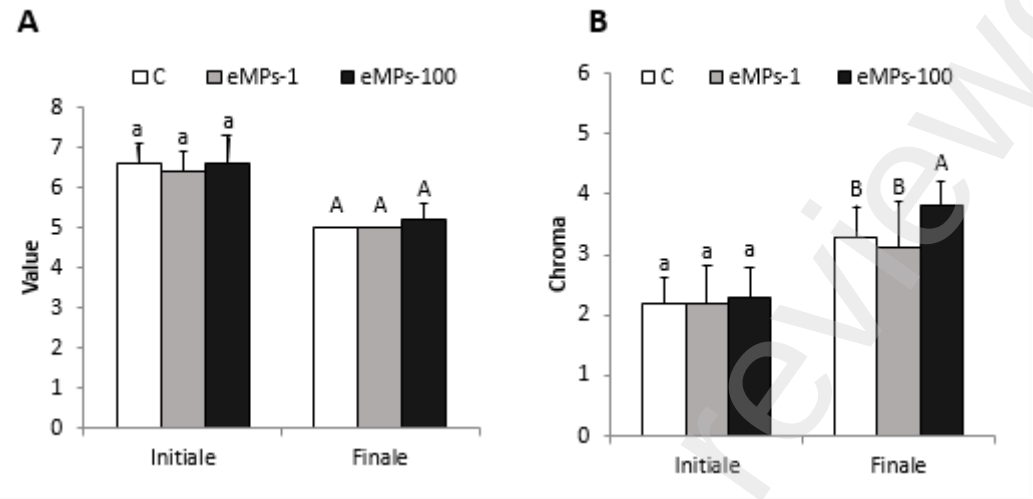
	Compound	Abbreviation	Concentration (µg/g)
Alkylphenols (AP)	Bisphenol A	BPA	0.013
	4-nonylphenol	4-NP	2.349
	Nonylphenol monoethoxyl	NP1EO	0.451
	Nonylphenol diethoxyl	NP2EO	0.093
	Acide nonyphenol carboxylic	NP1EC	< LD
	Octylphenol	OP	0.012
	Octylphenol monoethoxyl	OP1EO	0.062
	Octylphenol diethoxyl	OP2EO	0.008
Phthalates (PAE)	Dimethyl phtalate	DMP	2.508
	Diethyl phtalate	DEP	7.527
	Diisobutyl phtalate	DiBP	13.657
	Dibutyl phtalate	DBP	126.612
	Dimethoxyethyl phtalate	DMEP	5.706
	Di-4-methyl-2-pentyl phtalate	DMPP	0.633
	Diethoxyethyl phtalate	DEEP	2.080
	Di-n-pentyl phtalate	DnPP	0.060
	Butylbenzyl phtalate	BBP	6.905
	Di-n-hexyl phtalate	DNHP	5.698
	Di-2-butoxyethyl phtalate	DBEP	2.365
	Dicyclohexyl phtalate	DCHP	0.900
	Di-2-ethylhexyl phtalate	DEHP	34.766
	Di-n-octyl-phtalate	DnOP	0.694
	Dinitrophenol	DNP	1.251
Polycyclic aromatic hydrocarbons (PAH)	Naphtalene	N	< LD
	Acenaphtylene	Acyl	0.178
	Acenaphtene	Acen	0.066
	Fluorene	F	0.255
	Phenanthrene	P	0.659
	Anthrancene	An	0.537
	Fluoranthene	Fluo	0.471
	Pyrene	Pyr	0.835
	Benzo(a)anthracene	B(a)A	0.041
	Chrysene	Chry	0.053
	Benzo(b)fluoranthene	B(b)F	0.358
	Benzo(k)fluoranthene	B(k)F	0.090
	Benzo(a)pyrene	B(a)P	0.356
	Indo(123)pyrene	IP	0.695
	Dibenzo(ah)anthracene	D(ah)A	0.233
	Benzo(ghi)perylene	B(ghi)P	2.097

294 3.1.4 Effects on sole juveniles

295 No significant change in survival, weight, length, Fulton index, HIS and SSI were observed whatever

296 the treatment considered (Supp Data S2). In contrast, the chroma number (Fig.2) of the skin colour

297 significantly increased after consumption of ragworms exposed to the highest concentration of
298 eMPS (3.8±0.48) compared to control fish (3.3±0.63).



299

300 *Figure 2: Value (A) and chroma (B) of skin colour of sole juveniles after consumption of ragworms exposed to 0 (control),*
301 *1 (eMPs-1) or 100 (eMPs-100) mg/kg of eMPs (mean ± SD). Different letters at the top of the bars indicate significant*
302 *differences between conditions (ANOVA, p-value <0.05, n=9-10 per condition).*

303 Sole juveniles also showed modification of bottom colour preferences after consumption of
304 ragworms exposed to the eMPs (Fig. 3). Indeed, soles fed ragworms exposed to eMPs-1 mix travelled
305 significantly higher distances (1324.2±704.6 cm), displayed higher transition number (39.0±39.5) and
306 spent more time on white bottom (82.2±34.8 min) compared to control fish (496.4±308.2 cm;
307 7.0±6.1; 45.9±47.3 min and 74.1±47.3 min respectively). Soles fed ragworms exposed to eMPs-100
308 showed a significant increase in transition number (16.7±6.4) and time spent on white bottom
309 (72.0±36.2 min) compared to control.

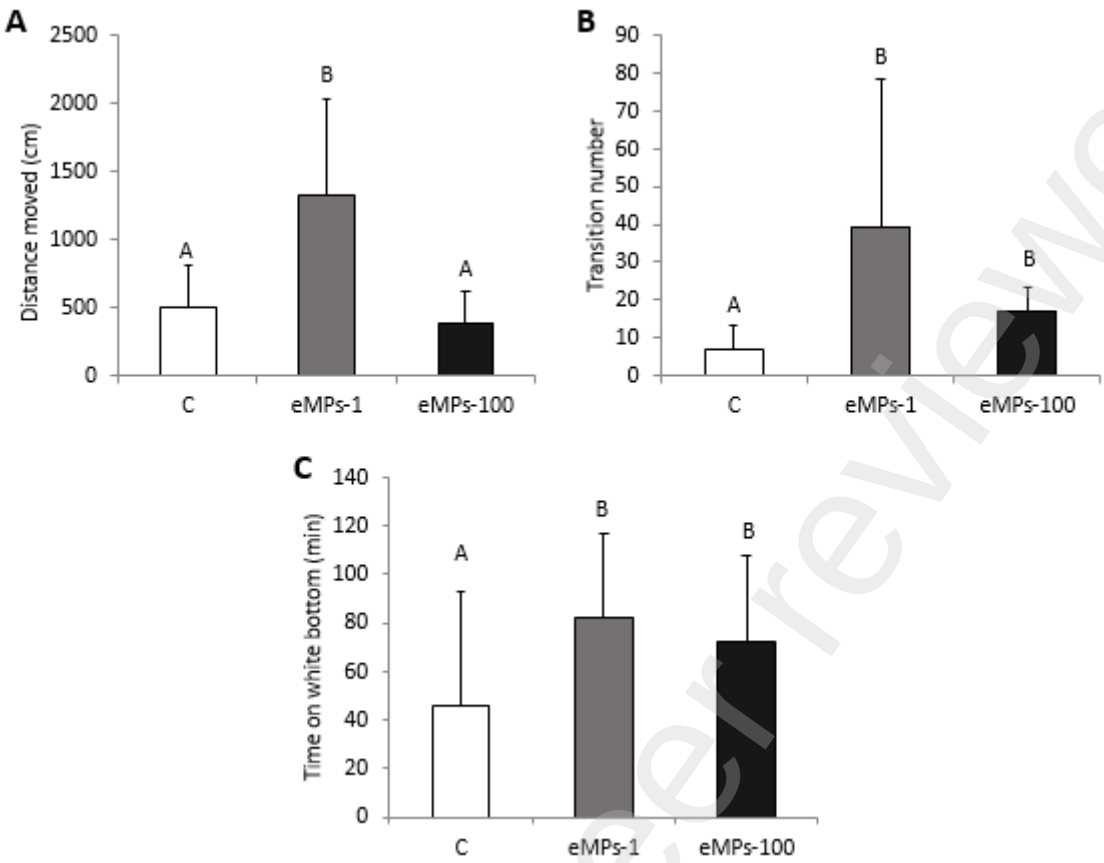


Figure 3: Distance moved (A), transition number (B) and time on white bottom (c) of sole juveniles after 20 days of consumption of ragworms exposed to 0 (control), 1 (eMPs-1) or 100 (eMPs-100) mg/kg of eMPs (mean ± SD). Different letters at the top of the bars indicate significant differences between conditions (ANOVA, p-value <0.05, n=9-10)

Analysis of genes expression revealed a significant but weak increase in *fasn* gene transcription (sup data, S4). No significant differences between treatments were observed for glycogen and lipids concentrations, TBARS, DNA breaks and EROD activity (sup data S5-S8).

Histopathology analyses showed no impact on liver or digestive tract of sole juveniles after consumption of ragworms exposed to eMPs mix whatever the concentration tested (Tab.5). For parenchymal lysis foci, the absence of inflammation and tissue remodelling indicate a very recent lesion, probably related to the capture of fish with no differences between concentrations. Cytoplasmic glycogen content is moderate to normal and demonstrates a good glycogen status of hepatocytes. Depending on the physiological stage, this glycogen overload maybe related to the animal's overweight (reserves). Regarding the melanoma-macrophagic centers, this is a common observation of immune stimulation. Some individuals had diffuse oedemas under the mucosa of intestine but without difference between conditions.

Table 5: Histopathological observation of liver and intestinal tract of sole juveniles after consumption of unexposed ragworms (Control) or exposed to 1 or 100 mg MPs /kg eMPs

	Apparition frequencies (%)						
	Oedema of the sub-mucosa		Glycogen storage		Foci of lysis	Melano-macrophage centers	
	Marked	Moderate	Marked	Moderate		Numerous	Large
C	0.0	20.0	40.0	10.0	30.0	0.0	0.0
eMPs-1	11.1	11.1	44.4	0.0	33.3	11.1	0.0
eMPs-100	30.0	10.0	60.0	0.0	40.0	0.0	0.0

3.2 Spiked microplastics (BaP-MPs and BP3-MPs)

As we observed for soles exposed to the eMPs, no change in survival, weight, length, Fulton index, HSI and SSI was observed (Fig. S3). No significant changes in skin colour for value numbers were observed (Fig 4, A). However, the mean chroma value of soles exposed to BP3-MPs was significantly higher (Fig 4, B) and they displayed a wider range of colours compared to control group (Fig. 4, C)

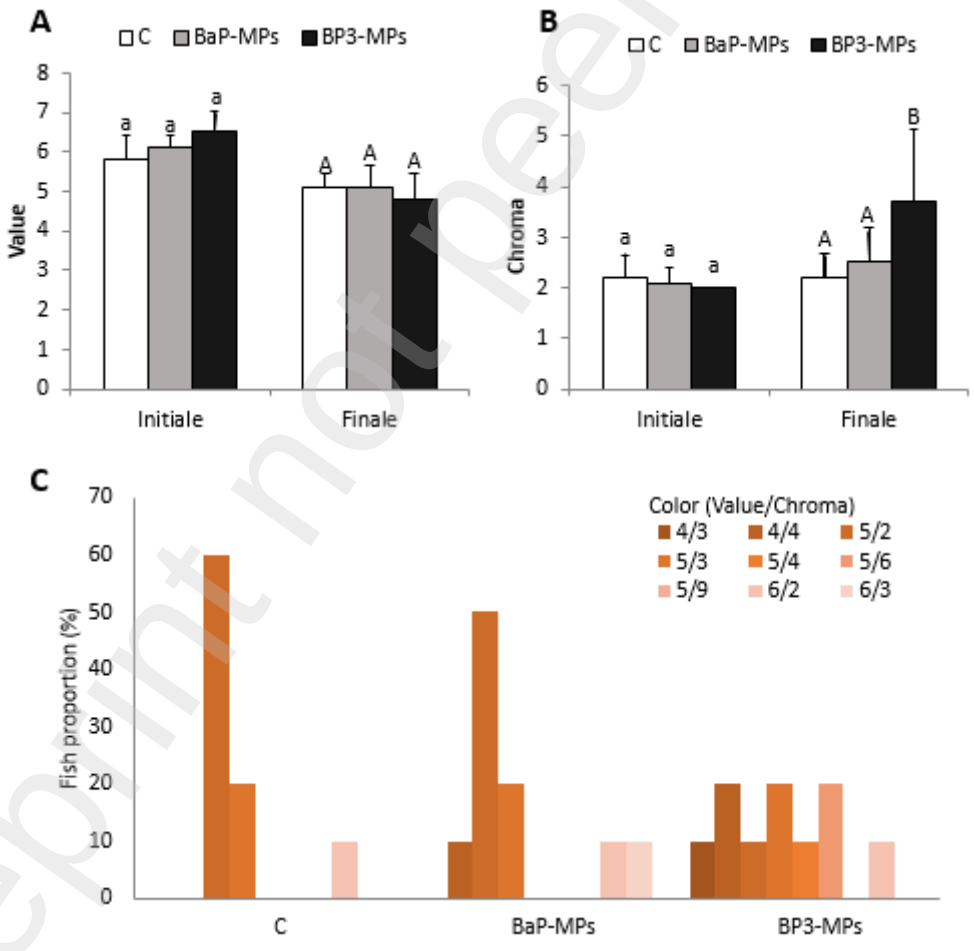


Figure 4: Value number (A), chroma number (B) and frequency of colour apparition (value/chroma; C) of sole juveniles after 20 days of consumption of ragworms exposed to 0 (control), BaP-MPs or BP3-MPs (mean ± SD, n=10) Different letters at the top of the bars indicate significant differences between conditions (ANOVA, p<0.05, n=10).

Sole juveniles showed behavioural modification after consumption of ragworms exposed to MPs (Fig. 5). Soles fed ragworms exposed to BaP-MPs spent significant less time on the white background (36.11±36.40 min) compared to control fish (89.39±33.95). On the contrary, soles fed ragworms exposed to BP3-MPs showed a significant increase in time spent on the white background (110.38±15.57 min).

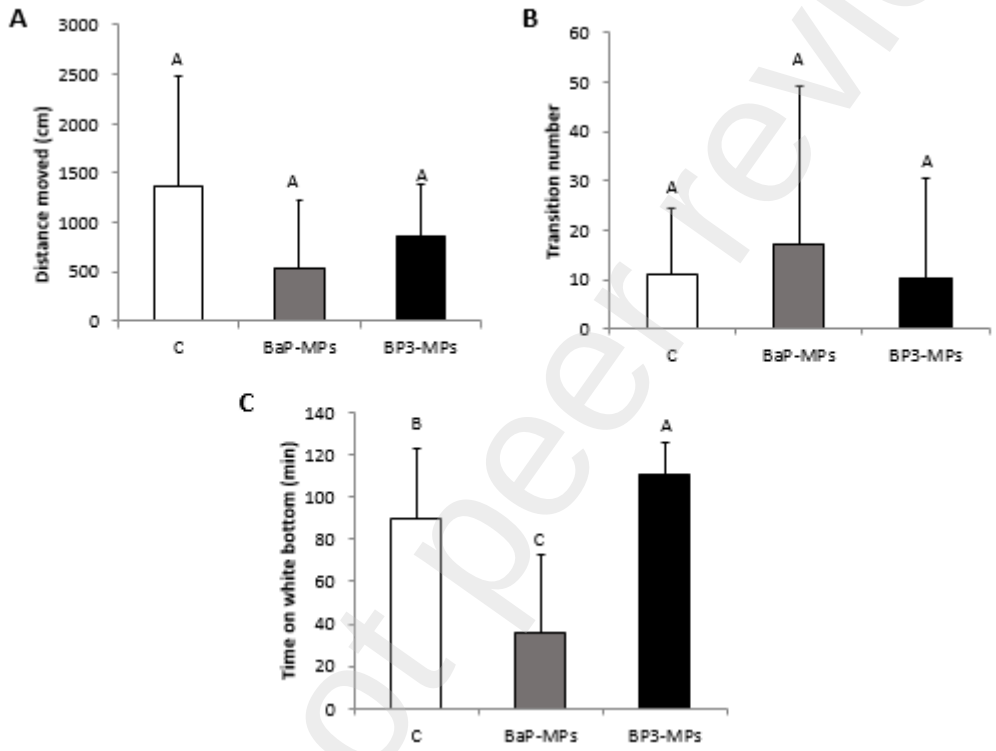


Figure 5: Distance moved (A), transition number (B) and time on white bottom (c) of sole juveniles after 20 days of consumption of ragworms exposed to 0 (control), BaP-MPs and BP3-MPs (mean ± SD). Different letters at the top of the bars indicate significant differences between conditions (ANOVA, p value <0.05, n=9-10 per condition)

Analysis of targeted genes expression revealed a significant but weak increase in expression of *gsto* gene transcription in liver of soles fed ragworms exposed to BP3-MPs (sup data S4).

Regarding energetic metabolism (Fig. 6), sole juveniles fed ragworms exposed to BP3-MPs showed a significant increase in lipids concentration in muscle (4.86±2.01 mg/g) compare to control fish (1.59±0.90 mg/g).

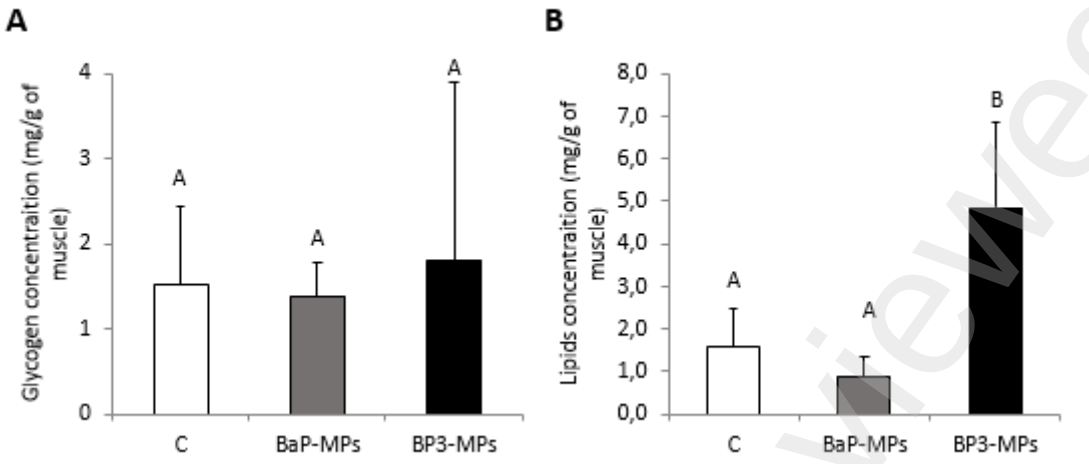


Figure 6: Glycogen (A) and lipids (B) concentration (mg/g) in muscle of sole juveniles after 20 days of consumption of ragworms exposed to 0 (control), BaP-MPs and BP3-MPs (mean±SD). Different letters at the top of the bars indicate significant differences between conditions (ANOVA, p<0.05, n=10).

No significant differences were observed for TBARS content, EROD activity and histopathology in both liver and intestine of exposed in comparison to control fish (sup data, S7-S9).

A significant increase in DNA damage (Fig. 7) was observed for sole juveniles fed ragworms exposed to BP3-MPs (42.69±9.18%) compared to control fish (26.68±10.32%).

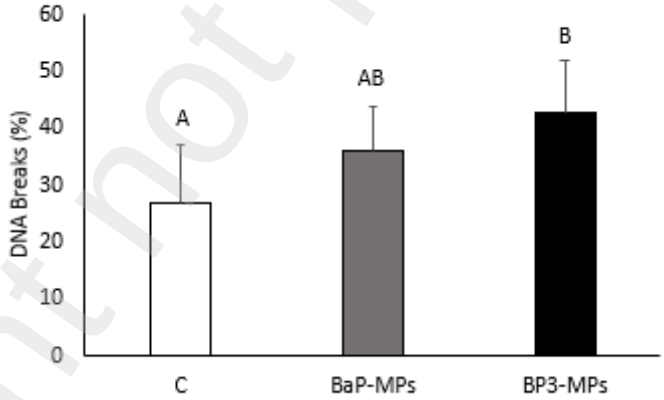


Figure 7: DNA damage in sole juvenile blood after 20 days of consumption of ragworms exposed to 0 (control), BaP-MPs and BP3-MPs (mean±SD). Different letters at the top of the bars indicate significant differences between conditions (ANOVA, p<0.05, n=10).

4 Discussion

MPs characterization

Macroplastics collected on the banks of the Seine estuary as part of this study were mainly composed of PE (62%) then PP (28%). The other three polymers, PET (4%), PVC (5%), PS (1%) were very poorly

represented. The composition of macroplastic debris collected was similar to what is found in aquatic environment, i.e. a majority of PE and PP. On a large scale, the main microplastics in sublittoral sediment from Guanabara Bay were translucent PE microfibers and secondary microplastics from washing machines wastes (Alves and Figueiredo, 2019). In Arcachon bay and ocean beaches nearby 69% of PE, 17% of PP and 10% PS were found for 1-5mm MPs (Lefebvre et al., 2021) and similar distribution where found on the Guadeloupe archipelago beaches (Cormier et al., 2022). Similar proportions are found in fish digestive tract as shown for fish (Bessa et al., 2018)(Wang et al., 2021). Pellets can represent up to 97.4% of ingested microplastics in various fish species (Pegado et al., 2018). Benthic species exhibited the highest variety of ingested polymers, including dense and light plastics, confirming that the original density can change due to weathering and biofouling processes and does not necessarily limit the distribution and bioavailability of MPs in the water column (Bour et al., 2018). In the Seine estuary, polycaprolactam (Nylon6), polyethylene terephthalate, and polyurethane MPs were found in the stomach of encaged and wild European flounders (Kazour et al., 2020). In the present study, for *S. solea* exposure, a mixture of polymers was made according to the average polymer composition of MPs in marine sediments (Leslie et al., 2017; Phuong et al., 2018) with PE and PP in majority (40% each) and PVC and PET in smaller proportion (10% each).

The trace metal concentrations found in this study are of the same order of magnitude as those reported in MPs from surface sediments of the Bejiang River (Wang et al., 2017). These authors hypothesize that most of the metals are initially present in the plastic and probably added during manufacturing. Recent findings suggest that plastics may play a key role as vectors for Cu and Zn ions in the marine system (Brennecke et al., 2016). Naqash et al. (2020), reported that trace metals concentrations in microplastics particles of PS and PVC can be 800 times higher than in the surrounding environment. Pannetier et al, found similar composition on PAHs content in microplastics but with different concentration (∑13PAHs 2-71 ng/g) compare to the concentration measured in this study (∑16PAHs 6920ng/g) on environmental plastic from various Pacific islands

(Pannetier et al., 2019). This can be explained by the location where microplastics were collected indeed Seine estuary is known as an area with high PAHs concentrations (Burgeot et al., 2017; Fernandes et al., 1997; Uher et al., 2016). In the same way, the PAHs levels measured on plastics from the Seine estuary are significantly higher (6x) than those measured on plastic pellets in a sediment core on the beach of Santos Bay in Brazil (Fisner et al., 2013). Some studies, such as this one, show much higher concentrations: 818 ± 874 ng/g on MPs collected e.g. on Southwest coast of Taiwan (Chen et al., 2020) or 3400−120,000 ng/g on MPS of surface water of Bahai and Huanghai seas in china (Mai et al., 2018). In river and estuary, PAH concentrations measured at the surface of MPS can also be high as from 11.2 to 7710 ng/g in Pearl river and estuary (Eastern Guangdong) sediment linked to spatial distribution and sources (Shi et al., 2020) or between 337 and 75400 ng/g (∑16PAHs) depending on polymer type (He et al., 2023). Capriotti et al also found the similar composition of organic pollutant at the surface of their collected plastic in Adriatic Sea Italian coastal water (Capriotti et al., 2021). Among phthalates (∑15PAE 211.36 µg/g) and alkylphenols (∑8AP 2.99 µg/g) detected on MPs samples there are some additives added to plastics during manufacturing to shape their physicochemical properties. The PAE detected in our samples are mainly dibutyl phtalate then di-2-ethylhexyl phtalate, diisobutyl phtalate, diethyl phtalate, butylbenzyl phtalate, dimethoxyethyl phtalate and di-n-hexyl phtalate (ranked by concentration). The other PAE are detected in smaller proportions. On Yangtze estuary the concentration found are between 26.8–4241.8 µg/g with also a domination of diisobutyl phthalate and dibutyl phthalate but also dioctyl phthalate (Deng et al., 2021). According to Cao et al, microplastics can be a major source of phthalate esters in aquatic environments (Cao et al., 2022). In terms of AP, the main one measured was 4-nonylphenol. In marine condition, Phuong et al shown a high affinity between nonylphenol and PE (Phuong et al., 2023) which may explain it. In Estuaries of Northern Taiwan, high levels of PAE, bisphenol-A, nonylphenol, and MPs are detected in fish (Lu et al., 2021).

Microplastic exposure effects:

In this study, no mortality and morphometric effects were observed in sole juveniles whatever the condition. In 2020, Le Bihanic et al. (2020) showed a reduction in growth and an increase in developmental abnormalities in Japanese medaka larvae exposed to BaP-MPs- or BP3-MPs. Other studies showed reduced growth after exposure to MPs but on younger life stages of zebrafish, marine medaka or marine jacopever, with longer exposure time and, often, at higher concentrations (Cormier et al., 2021b; Yin et al., 2018). Microplastics ingestion rate was in some studies independent of fish size and functional groups (Vendel et al., 2017) and in other ones positively correlated with fish body size (Pegado et al., 2018).

After feeding ragworms exposed to ePMs-100 or BP3-MPs, soles showed body colour changes. Colour changes are dependent on the dispersion or concentration of melanin in melanophores in skin cells (Davenport and Bradshaw, 1995). These colour changes can be explained by stress as revealed by behavioural modifications. Indeed, in fish, skin pigmentation is controlled by hormones, the main hormone involved in skin darkening being the α-melanocyte-stimulating hormone (αMSH), which is also involved in the regulation of the response to stressors (Van der Salm et al., 2004). Disruption of thyroid signalling was also shown to interfere with proper pigmentation in fish (Guillot et al., 2016; McMenamin et al., 2014; Prazdnikov and Shkil, 2023). BP3 is an organic UV filter and an endocrine disruptor (Kim et al., 2014; Kinnberg et al., 2015). One could hypothesize that it could have a direct impact on melanin production or on melanophores functioning but there are no studies on this subject to date. However, there are evidences that benzophenones, including BP3 interfere with thyroid which may support the observed phenotype (Lee et al., 2018; Zucchi et al., 2011).

Behavioural changes were also observed in sole juveniles fed MPs exposed ragworms. Fish from the eMPs-1 condition spent more time on the white coloured bottom. Similarly, soles from the BP3-MPs condition spent more time on the white coloured bottom. Conversely, soles from the BaP-MPs condition had preference for the black coloured bottom. Some studies have already tested the colour and texture preferendum of different sole species, i.e. the common sole and the Senegalese sole, *Solea senegalensis* (Almansa et al., 2017; Fatsini et al., 2017; Liu et al., 2016). However, the

444 results obtained in these studies do not support a specific colour preference although soles appeared
445 to slightly prefer the bright substrate (Ellis et al., 1997; Reig et al., 2010).
446 An increase in distance moved was observed for sole fed ragworms exposed to eMPs-1 but not for
447 the other conditions. Cormier et al (2021a) and Le Bihanic et al (2020), reported that BP3 coated
448 MPs produced behavioural disruption in medaka and zebrafish larvae with an increase in distance
449 moved. This discrepancy can probably be explained by the higher concentration of microplastics
450 used and the longer exposure time in the two latter studies. Pannetier et al (2020) also showed an
451 increase in distance moved after exposure to Hawaii environmental MPs after direct trophic
452 exposure.
453 Regarding energetic reserves (glycogen/lipid), no change was observed except an increase in lipid
454 concentrations in fish muscle after BP3-MPs exposure. Alterations of energy reserves including lipid
455 content or lipid metabolisms were already observed after MPs exposure of some fish species
456 (Capriotti et al., 2021; Limonta et al., 2019; Yin et al., 2018). An *in vitro* screening on 3T3-L1 murine
457 pre-adipocytes cell line of MP extracts indicated potential metabolic effects resulting in both
458 adipogenesis and lipid uptake/storage (Capriotti et al., 2021).
459 No change of TBARs, protein content or EROD activity in sole liver were observed in this study as in
460 Cormier et al (2021b) with similar MPs. However, a significant increase in DNA breaks was observed
461 after BP3-MPs exposure. Pannetier et al (2020) have also reported an increase in DNA damage after
462 trophic exposure of 2-month-old medaka to environmental MPs.
463 The few toxic effects observed in our sole juveniles could be linked to the low number of microplastic
464 ingested by ragworms, between 0.2 and 0.9 MP/ragworm. Indeed, with the same exposure protocol
465 and similar concentration of MPs in the sediment, Revel et al (2020) found less than 1 MP/ragworm
466 (Revel et al., 2020). This could explain the absence of histopathologic effect on soles for this
467 experiment. At higher concentration some studies have shown histopathological changes in
468 zebrafish's gallbladder and liver (Yin et al., 2018) or tissue alterations on intestinal mucosa (epithelial
469 detachment/mucus hypersecretion) and gills (adhesion and partial fusion of secondary lamellae/

mucus hypersecretion) as well as higher occurrence of neutrophils observed in gills and intestinal epithelium of Marine jacopever (Limonta et al., 2019). However, Wang et al, (2021) found similar MPs concentration in sand ragworms (0.88±1.04 items/individual) with higher concentrations in the higher trophic levels (0.83-1.58 items/individual in clam; 1.33 items/individual in crustacean and 2.67-3.87 items/individual in fish) in the Liaohe estuary (China). If the concentration of microplastic increases as a function of trophic level, this suggests that the effects could also be more marked.

In conclusion, BaP or BP3-MPs but also environmental MPs induce effects on skin colour and might have long term impact on the common sole, particularly on its behaviour. By showing slight but significant effects on sole juveniles fed ragworms exposed to microplastics, this study confirms (i) the transfer of MPs or associated chemicals through the trophic chain and (ii) their adverse effects even with a relatively low quantity of ingested particles.

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