



Research paper

## Chemicals sorbed to environmental microplastics are toxic to early life stages of aquatic organisms



Bettie Cormier<sup>a,b,\*</sup>, Chiara Gambardella<sup>c</sup>, Tania Tato<sup>d</sup>, Quentin Perdriat<sup>a</sup>, Elisa Costa<sup>c</sup>, Cloé Veclin<sup>e</sup>, Florane Le Bihanic<sup>a</sup>, Bruno Grassl<sup>e</sup>, Florian Dubocq<sup>b</sup>, Anna Kärrman<sup>b</sup>, Kim Van Arkel<sup>f</sup>, Soazig Lemoine<sup>g</sup>, Fabienne Lagarde<sup>h</sup>, Bénédicte Morin<sup>a</sup>, Francesca Garaventa<sup>c</sup>, Marco Faimali<sup>c</sup>, Xavier Cousin<sup>i,j</sup>, Marie-Laure Bégout<sup>i</sup>, Ricardo Beiras<sup>d</sup>, Jérôme Cachot<sup>a,\*\*</sup>

<sup>a</sup> Bordeaux University, EPOC, UMR CNRS University of Bordeaux EPHE 5805, Avenue des Facultés, 33400 Talence, France

<sup>b</sup> Man-Technology-Environment Research Centre, School of Science and Technology, Örebro University, Fakultetsgatan 1, 701 82 Örebro, Sweden

<sup>c</sup> Institute for the study of Anthropogenic Impacts and Sustainability in Marine Environment – National Research Council (CNR-IAS), Genova, Italy

<sup>d</sup> Faculty of Marine Sciences, University of Vigo, E-36310 Vigo, Galicia, Spain

<sup>e</sup> CNRS/University of Pau & Pays Adour/E2S UPPA, Institut des Sciences Analytiques et de Physicochimie pour l'Environnement et les Matériaux, UMR 5254, 64000, Pau, France

<sup>f</sup> Race for Water Foundation, Lausanne 1007, Switzerland

<sup>g</sup> Laboratoire de biologie marine, Université des Antilles, French West Indies, Campus de Fouillole, BP 592, 97117, Pointe-à-Pitre, France

<sup>h</sup> Institut des Molécules et Matériaux du Mans (IMMM, UMR CNRS 6283), Université du Maine, Avenu Olivier Messiaen, F-72085 Le Mans, France

<sup>i</sup> MARBEC, University of Montpellier, CNRS, IFREMER, IRD, 34250 Palavas-les-Flots, France

<sup>j</sup> University of Paris-Saclay, AgroParisTech, INRAE, GABI, 78350 Jouy-en-Josas, France

### ARTICLE INFO

Edited by Professor Bing Yan

#### Keywords:

Environmental microplastics  
Leachates  
Early life stages  
Aquatic organisms  
Toxicity

### ABSTRACT

Microplastics are ubiquitous in aquatic ecosystems, but little information is currently available on the dangers and risks to living organisms. In order to assess the ecotoxicity of environmental microplastics (MPs), samples were collected from the beaches of two islands in the Guadeloupe archipelago, Petit-Bourg (PB) located on the main island of Guadeloupe and Marie-Galante (MG) on the second island of the archipelago. These samples have a similar polymer composition with mainly polyethylene (PE) and polypropylene (PP). However, these two samples are very dissimilar with regard to their contamination profile and their toxicity. MPs from MG contain more lead, cadmium and organochlorine compounds while those from PB have higher levels of copper, zinc and hydrocarbons. The leachates of these two samples of MPs induced sublethal effects on the growth of sea urchins and on the pulsation frequency of jellyfish ephyrae but not on the development of zebrafish embryos. The toxic effects are much more marked for samples from the PB site than those from the MG site. This work demonstrates that MPs can contain high levels of potentially bioavailable toxic substances that may represent a significant ecotoxicological risk, particularly for the early life stages of aquatic animals.

### 1. Introduction

Plastic have been a major concern for over 60 years, attracting interest from public, media and scientific community (Law and Thompson, 2014). One of the main reasons behind this interest is the abundance of plastics found in the aquatic environment. Mass production of plastics began in the 1950s, and global production has been rising continuously

since. In 2017, it was estimated that around 348 million tons of plastic were produced worldwide (PlasticsEurope, 2018), with millions of tons of plastic waste being accidentally or intentionally discharged into aquatic ecosystems (Geyer et al., 2017; Jambeck et al., 2015).

Plastics in the environment tend to degrade and fragment into smaller particles through chemical, physical or biological processes (Barnes et al., 2009), leading to debris with a size below 5 mm in

\* Corresponding author at: Bordeaux University, EPOC, UMR CNRS University of Bordeaux EPHE 5805, Avenue des Facultés, 33400 Talence, France.

\*\* Corresponding author.

E-mail addresses: [bettie.cormier@u-bordeaux.fr](mailto:bettie.cormier@u-bordeaux.fr) (B. Cormier), [jerome.cachot@u-bordeaux.fr](mailto:jerome.cachot@u-bordeaux.fr) (J. Cachot).

<https://doi.org/10.1016/j.ecoenv.2020.111665>

Received 2 September 2020; Received in revised form 10 November 2020; Accepted 12 November 2020

Available online 25 November 2020

0147-6513/© 2020 The Author(s).

Published by Elsevier Inc.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

diameter, defined as microplastics (MPs) (Arthur et al., 2009). Estimated quantities of MPs and plastic debris on the ocean surface reach almost 270,000 tons (Eriksen et al., 2014). Due mainly to their slow degradation rate (Cole et al., 2011), plastic debris tend to accumulate in oceanic gyres, in surface slick (Gove et al., 2019) but also in coastal areas (Eriksen et al., 2014; Gove et al., 2019; Law and Thompson, 2014; Lebreton et al., 2018). Beaches are major hotspots for MPs accumulation (Andrady, 2011). The presence of MPs on beaches has been reported in numerous coastal areas around the world, including Caribbean Sea (Acosta-Coley et al., 2019; Acosta-Coley and Olivero-Verbel, 2015; Bosker et al., 2017, 2018).

While plastic contamination in marine ecosystems is now widely documented (Law and Thompson, 2014); few studies have investigated the deleterious effects of MPs on aquatic organisms. However, recent studies have documented both physical and chemical damages induced by several types of MPs, mainly industrial ones. For instance, MPs can lead to blockage of the digestive tract (Cole et al., 2011; Pedà et al., 2016), false satiety (Rochman et al., 2013a, 2013b), growth retardation and death of fish embryos (Pannetier et al., 2020) and impairment of development and swimming activity of oyster larvae (Bringer et al., 2020; Sussarellu et al., 2016). Toxicological effects may also be induced by plastic additives, e.g. bisphenol A, phthalates, UV stabilizer, etc. (Koelmans et al., 2014, 2015; Rochman, 2015) or pollutants sorbed onto plastics (Pannetier et al., 2019a, 2019b; Le Bihanic et al., 2020; Coffin et al., 2020). Pollutants were shown to adsorb on plastic during the process of ageing while certain additives were readily released (Rochman et al., 2013a, 2014; Koelmans et al., 2014; Kedzierski et al., 2018). Chemicals sorbed on plastic are mostly hydrophobic organic compounds, including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbon compounds (PAHs) and organochlorine pesticides (Derraik, 2002; Karapanagioti and Klontza, 2008; Rochman et al., 2013a), but also trace metal elements (Rochman et al., 2014). Toxic effects of artificially spiked MPs with pollutants have been reported, including endocrine perturbation, hepatic damage, oxidative stress induction and enzymatic activity modifications (Browne Mark et al., 2013; Pittura et al., 2018; Teuten et al., 2009).

Exposure to pristine MPs either has no effect or causes slight alterations in exposed organisms (Beiras et al., 2018; Cormier et al., 2019; Le Bihanic et al., 2020; Mazurais et al., 2015). However, toxicity assessment of MPs requires much more in-depth investigation, given that they are widespread, persistent and easily ingested by a wide variety of living organisms (Fossi et al., 2017; Steer et al., 2017). To that end, water or organic extraction protocols have been developed to evaluate the toxicity of industrial or aged MPs (Beiras et al., 2019; Bejgarn et al., 2015; Gandara e Silva et al., 2016; Lithner et al., 2009, 2011; Olivero et al., 2019; Pannetier et al., 2019a, 2019b).

Early life stages of invertebrates and fish are increasingly used in ecotoxicology, mainly due to their high sensitivity to diverse chemicals (Beiras et al., 2018; Embry et al., 2010; Lammer et al., 2009; Olivero et al., 2019). Planktonic larvae are a critical stage in life cycle completion, as well as playing a key role in the recruitment and dissemination of populations. The *Paracentrotus lividus* sea urchin is distributed in European waters and plays an important role in coastal ecosystem maintenance. The larval stage has been reported as the most sensitive stage, and is currently used as a biological model to evaluate toxic effects of contaminants (Bellas et al., 2005; Olivero et al., 2019; Rouchon and Phillips, 2017). The jellyfish *Aurelia* sp. has been reported to be an innovative model organism in ecotoxicology due to its high sensitivity to pollutants, and its key position as basal metazoan (Costa et al., 2015; Faimali et al., 2014). *Aurelia* sp. is essential for the marine food web, both as prey (Cardona et al., 2012; Titelman and Hansson, 2006) and as a consumer of zooplankton. Besides invertebrates, early life stages of zebrafish (*Danio rerio*) have been shown to be as sensitive to chemical pollutants as adult fish (Belanger et al., 2013; Braunbeck et al., 2005, 2015; Lammer et al., 2009; Nagel, 2002).

In 2017, a worldwide sampling campaign was carried out by the

Race for Water Foundation (Odyssey 2017–2021), to collect MPs from the water column, subtidal sediments, beaches and living organisms. The Guadeloupe archipelago in the Caribbean Sea was selected for this study because of its close location to the North Atlantic gyre. The Guadeloupe archipelago includes five main islands; of which two (Basse-Terre and Marie-Galante) were considered in this study.

The purpose of this study was first to characterize MPs stranded on two island beaches in the Guadeloupe archipelago (Basse-Terre and Marie-Galante) in terms of polymer composition and sorbed chemicals. The toxicity of environmental MPs leachates was then investigated for the early life stages of three aquatic organisms, sea urchin (*Paracentrotus lividus*), jellyfish (*Aurelia* sp.) and zebrafish (*Danio rerio*), using leachates.

## 2. Materials and methods

### 2.1. Sample collection

Environmental MPs were collected from Guadeloupe archipelago (France), located in the Caribbean (Fig. SM1), during the Race for Water Odyssey 2017–2021, in October 2017. This study focused on environmental MPs samples collected on two different sandy beaches, Capesterre in Marie-Galante Island (MG), and Petit-Bourg (PB) in Guadeloupe Basse-Terre Island (Table SM1). This campaign took place just after the hurricane Irma that devastated the Caribbean in September 2017. These sites were sampled using the standardized NOAA protocol (Lippiatt et al., 2013) adapted for millimetric plastic debris. In short, debris between 1 and 5 mm were collected along a 100 m shoreline, at the surface of the sand. Sorting was based on a visual assessment. Samples were stored in aluminium tray with an aluminium lid at 4 °C.

### 2.2. MPs composition and chemical characterization

MPs collected from each beach were counted, and then individually weighed. Plastic debris were monitored by a FTIR Vertex 70V Bruker spectrometer (Billerica, Massachusetts, USA) with a deuterated triglycine sulfate detector (DTGS), in ATR mode (attenuated total reflection), to identify polymer of each particles. Results are expressed in % mass. The absorption bands were recorded in the range of 400–4000  $\text{cm}^{-1}$  with 16 scans and a resolution of 2  $\text{cm}^{-1}$ . The data were analyzed using OPUS software. Particles were put into a sample container containing a magnetic bar or impactor and both sides of the container were closed with two stainless steel caps. The container was inserted into a cryogenic mechanical miller (SPEX, 6770 Freezer-mill) filled with liquid nitrogen. Milling consisted in 2 min of pre-cooling followed by 2 cycles of milling. Each cycle lasted 8 min with a 1-min pause between the 2 cycles. The so-obtained powders of particles were then sieved during 12 h on a stain sieve of 100 and 53  $\mu\text{m}$ . The particle-size distribution was measured using a Malvern laser diffraction particle size analyzer.

Qualitative chemical analysis of organic micropollutants was conducted using gas chromatography (GC) hyphenated with Orbitrap mass spectrometry (Q Exactive GC Orbitrap, Thermo scientific, Bremen, Germany) with a non-target analysis (NTA) workflow. Liquid/solid extractions were performed using 0.3 g of mesoplastics in hexane ( $\geq 98\%$ , SupraSolv; Merck, Darmstadt, DE); followed by 30 min of sonication and centrifugation at 8000 revolutions per minute (rpm) for 10 min. Extracts were reduced to 1 mL, using nitrogen flow (6.0) and filtrated through glass wool with sodium sulfate, and finally reduced to 25  $\mu\text{L}$  prior injection. More information about the GC/MS parameters for the Q Exactive GC Orbitrap instrument can be found in supplementary materials (Table SM2).

All measurements were performed in full scan mode with a resolution of 60 000 at  $m/z$  200 between  $m/z$  53.4 and  $m/z$  800. All Q Exactive GC Orbitrap parameters are available in the SM. Plastic samples were analyzed using mass defect-plot. Halogenated compounds were detected using Cl-H/Br-H (mass scale = 34/33.9610 or 78/77.9105) (Jobst et al.,

2013) using HaloSeeker (Nantes, France). Excel software (Microsoft, Redmond, USA) was used for the detection of PAHs and linear hydrocarbons by using C<sub>4</sub>H<sub>2</sub> (mass scale = 50/50.0157) and CH<sub>2</sub> (mass scale = 14/14.0157), respectively.

XCalibur software (Thermo scientific, Bremen, Germany) version 4.1 and TraceFinder EFS software (Thermo scientific, Bremen, Germany) version 4.1 were used to acquire and analyze the data. Fold change values were sorted and the 50 highest fold change features were analysed. Chromatogram peaks were checked and only symmetric peaks with width below 0.5 min were kept. Finally, 26 features were selected for further analysis for elemental composition analysis.

For chemical analysis of trace metals, MPs were weighed using a static eliminator ionizer (Mettler Toledo EN SL LC). Samples were mineralized at 18 °C using nitrogen flow in an ultra wave (Milestone UltraWave, SRC Technology) following Milestone application Note protocol (Milestone, Helping Chemists). Samples were diluted in ultra-pure water to obtain a concentration of acid nitric of 0.2%. Samples were analysed using Inductively Coupled Plasma and Mass Spectrometry (7500 Series, Agilent). Firstly, a qualitative analysis was conducted to screen a large panel of metal trace, then a quantitative analysis was performed by targeting the most important metal detected using the qualitative method. Copper (<sup>63</sup>Cu; <sup>65</sup>Cu), lead (<sup>206</sup>Pb; <sup>207</sup>Pb; <sup>208</sup>Pb), zinc (<sup>66</sup>Zn), cadmium (<sup>111</sup>Cd) and chromium (<sup>52</sup>Cr; <sup>53</sup>Cr) were quantified.

The reference polyethylene and the microplastic powder (200 mg) were solubilized by acid digestion with concentrated HNO<sub>3</sub> (65%) using a standardized procedure with a microwave oven (Milestone UltraWave with internal temperature and pressure control, 1500 W, 230 °C, 35 min). Trace element concentrations were obtained by measurement on a quadrupole ICP-MS (inductively coupled plasma mass spectrometry, Agilent 7500, ®Perkin Elmer) at the IPREM laboratory (Pau, France). The standard ERM-EC 680 and ERM-EC 681 were used to check the validity and the reproducibility of both acid digestion and ICP-MS analyses. Indium was used as internal standards for the ICP-MS measurements. All dilutions were made in 2% (v/v) HNO<sub>3</sub> of purity for trace metal analysis (J.T.Baker™), blank tests indicated that the level of contamination induced by the acid digestion procedure was negligible.

## 2.3. Preparation of leachates

Leachates were prepared according to Beiras et al. (2019), with modifications described below, for all organisms used.

### 2.3.1. Leachates protocol for sea urchin exposure

Sea urchin toxicity test followed the methodology reported in detail in Beiras et al. (2019). MPs samples of PB, MG and a “virgin” polyethylene (PE) resin obtained from Rotogal, Boiro (Spain) were tested. Stock solutions of 10 g/L of MPs particles in artificial seawater (ASW) (Lorenzo et al., 2002) were prepared in 50 mL glass bottles and filled with no head space. The bottles were incubated during 24 h at 20 °C in darkness with rotational shaking (1 rpm) provided by an overhead rotator (GFL 3040, Germany). After extraction, MPs particles were removed from leachates by means of vacuum filtration using glass microfiber filters (Whatman, GF/F) that retain fine particles down to 0.7 µm. Undiluted leachate (10,000 mg/L of MPs) and serial dilutions at 1/3 (3333 mg/L), 1/10 (1000 mg/L) and 1/30 (333 mg/L) in ASW were introduced in 5 mL glass vials with Teflon lined airtight lids (4 replicates per dilution and 8 replicates for blanks of filtered ASW).

### 2.3.2. Leachate protocol for jellyfish exposure

Leachates for jellyfish exposure were obtained using rotary mixing at 20 °C for 24 h in the dark, according to the method described by Beiras et al. (2019), adapted to a solid-to-liquid proportion of 1 g MPs /L in ASW as described for sea urchin exposure, and rotation speed of 2 rpm. Leachates were then filtrated with GF/F filters: undiluted leachate (1 g/L) and serial dilutions at 1/3 (0,33 g/L), 1/10 (0,1 g/L) and 1/30 (0,033 g/L) in ASW were introduced in 5 mL glass vials with Teflon lined

airtight lids (3 replicates per dilution), and stored at 4 °C in darkness for 2 days maximum.

### 2.3.3. Leachate protocol for zebrafish exposure

Leachates for zebrafish were prepared by mixing 400 mg of MPs with 4 mL (100 g/L) of E3 medium (1<sup>-5</sup> mM NaCl; 0.17 mM KCl; 0.33 mM CaCl<sub>2</sub>; 0.33 mM MgSO<sub>4</sub>; 10<sup>-5</sup>% Methylene Blue). Extraction was performed in the dark, with 16 h of shaking on a horizontal mixing table (KS 501 digital IKA-Werke) at 175 rpm. Then, extracts were centrifuged at 4000 rpm for 5 min. Finally, 100 µL of the liquid fraction were sampled with extra-long sterile tip, and re-centrifuged to remove particles. Extracts were kept in darkness at 4 °C for 7 days maximum.

## 2.4. Toxicological analyses

### 2.4.1. Acute toxicity assay using sea urchin early life stages

Sea urchin (*Paracentrotus lividus*) fertilized eggs (identified under the microscope by the presence of the fertilization membrane) were added to vials and randomly assigned to the different treatments at a density of 40 per mL and vials were placed in an incubator (Velp FTC 90E) for 48 h in darkness at 20 °C. After incubation, larvae were fixed with 40% formalin and larval length (maximum linear dimension in n = 35 per vial) was recorded using an inverted microscope (Leica DMI 4000B) and Leica LAS v4.12 image analysis software. The mean size increase from egg to larvae for each treatment corrected by size increase in the controls was calculated as:

$$\Delta Lc = (Lt - E)/(Lc - E)$$

where Lt is the mean larval length in treatment t, Lc is mean larval length in controls and E is the mean egg diameter. Acceptability criteria were the percentage of fertilized eggs >98% and size increase in controls >253 µm (Saco-Álvarez et al., 2010). All experiments conducted in this study met the acceptability criteria of 98%.

### 2.4.2. Acute toxicity assay using jellyfish ephyrae

Jellyfish (*Aurelia* sp.) ephyrae were obtained from polyps through strobilation (Costa et al., 2020). Specifically, after each polyp transformed into multiple ephyra during strobilation (Fuchs et al., 2014), ephyrae were then collected and used for the toxicity test. Two ephyrae were added to each glass vial: for each dilution, 5 vials were prepared, for a total of 10 ephyrae per treatment to avoid interactions among organisms (Faimali et al., 2014). Ephyrae were exposed in semi-dynamic conditions, in a rotatory wheel, to undiluted leachate (1 g/L of MPs) and diluted leachates (0.33, 0.1, 0.033 g/L) of MG, PB MPs and to a positive control (PC) of polyethylene (100–250 µm) MPs in a thermostatic room at 20 °C in darkness for 24 and 48 h. After incubation, two endpoints were measured in ephyrae: the immobility and alternation of frequency of pulsations (AFp). The percentage of immobility (expressed as the ephyrae ability to perform any kind of movement in 5 s; Garaventa et al., 2010) was calculated for each dilution compared to controls. The AFp was calculated by recording the number of pulsations made by the ephyrae in 1 min (Faimali et al., 2014), according to the following formula: %AFp = [(Fp treated - Fp control)/ Fp control]/100.

The median Effective Concentrations (EC<sub>50</sub>: dilution of leachate resulting in 50% Immobility, I% or AFp) effect in the exposed ephyrae and related 95% Confidence Limits (CL) were calculated using Trimmed Spearman–Kärber analysis (Finney, 1978) after 24 and 48 h of leachate exposure and toxic units (TU).

### 2.4.3. Acute toxicity assay using zebrafish embryos

Fish husbandry conditions fully complied with OECD TG 236 (OECD, 2013; Westerfield, 2000) regarding maintenance of adult zebrafish (*Danio rerio*). Zebrafish, TU strain were bred in 10 L tanks at 27 ± 1 °C with a day/night cycle of 14 h/10 h (7.8 ± 0.2, 480 ± 130 µS, for pH and conductivity, respectively). Fish were fed twice-daily ad libitum

with Inicio + 0.5 mm (BioMar, France) and freshly hatched brine shrimp *Artemia* nauplii. Constant filtering or permanent flow-through conditions guaranteed that ammonia, nitrites, and nitrates were kept below detection limits (5, 1 and 140 mg/L, respectively). Zebrafish eggs were collected according to OECD TG 236 (OECD, 2013).

Leachate exposure started after hatching at 72 h post fertilization (hpf). Zebrafish embryos were exposed for 48 h to leachates in E3 medium until the end of the eleutheroembryo phase (96 hpf). Embryos were exposed to two equivalent concentrations of plastics 50,000 and 10,000 mg/L. Positive control was added, using 4 mM of 3,4-dichloroaniline (DCA) for the test validation (OECD, 2013). Glass petri dishes (55 mm diameter) containing 20 mL of E3 were used in semi-static conditions with a daily renewal of 15 mL of exposure medium. Twenty embryos were used for each condition, and each treatment condition was replicated 3 times. To maintain a temperature of  $28 \pm 1$  °C, experiments were performed in temperature-controlled chambers (Snijders Scientific, Tilburg, Netherlands) with a photoperiod set up on 14:10, light:dark. Before hatching, embryos were kept in E3 medium, and then, transferred in glass beakers containing 20 mL of leachate. For the entire exposure period (from 72 to 96 hpf), dissolved oxygen was checked daily (>85%) with a fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburg, Germany).

Individuals were examined following OECD 212 (OECD, 1998). Embryos were checked daily for survival and dead embryos were counted and immediately removed to avoid modification of the medium. Developmental anomalies and lesions were also recorded: edema; hemorrhages, skeletal axis (scoliosis, lordosis) and craniofacial deformations and cardiovascular anomalies (blood circulation) following previously published protocols for Japanese Medaka (Le Bihanic et al., 2014). Leica MZ7.5 stereomicroscope with Leica DFP420C CDD camera and Leica Microsystems software v3.8 were used to perform these measurements (Leica, France).

At 96 hpf, six to ten embryos per replicate were anaesthetized with 200 mg/L of benzocaine. Total length and head length, were measured using a Leica MZ7.5 stereomicroscope with Leica DFP420C CDD camera and Leica Microsystems software v3.8 (Leica, France), and the ratio of head/total length was calculated.

*In vivo* EROD activity was analysed according to published methods with modifications (Jönsson et al., 2002; Le Bihanic et al., 2013). Measurements were conducted on 96 hpf embryos. Five embryos per replicate were placed into individual wells of a 48-well microplate containing 1 µM of 7-ethoxyresorufin (7-ER) in 0.5 mL of E3. After 1 h of incubation in the dark, the 7-ER solution was removed and replaced by freshly prepared 1 µM 7-ER. Fluorescence of 100 µL of the incubation media was measured in duplicate in 96 white well plate at time 0 and after 4 h incubation. Resorufin formed was measured with a BMG Labtech fluorescent microplate reader (Germany) excitation and emission wavelength of 560 and 590 nm respectively. Solution of 7-ER was added to the resorufin standard used, for a final concentration of 1 nM. A batch of five control embryos was exposed to benzo[a]pyrene (70 nM) for 1 h before incubation in 7-ER to obtain a positive control.

Swimming behaviour was investigated following the protocol of Vignet et al. (2014) with a larval photomotor response test (LPMR) to record fish behavioural responses following a light stimulus. Embryos at 96 hpf were transferred into a 48-well plate filled with 500 µL of E3 medium without methylene blue and acclimated for 4 h in the dark prior measurement. Then, embryos were moved to the DanioVision (Noldus, The Netherlands) and acclimated for 20 min in the dark, followed by 5 min light, 5 min dark and 5 min light. Swimming activity was recorded with a video tracking system DanioVision (Noldus, The Netherlands). Swimming behaviour of 10–12 embryos (96 hpf) per replicate was recorded at  $28 \pm 1$  °C (DanioVision Temperature Control Unit, Noldus, The Netherlands). Videos were analyzed with Ethovision software 12.0 (Noldus, The Netherlands).

## 2.5. Statistical analyses

Statistical analyses were conducted using IBM SPSS (Statistical Package for the Social Sciences) statistics software (v. 20 and 24). Normal distribution and homoscedasticity of the data was checked using Shapiro–Wilk's and Levene's tests respectively. ANOVA with Dunnett's post hoc test was applied to determine the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). When data failed to meet the assumption of normality, nonparametric Kruskal Wallis test followed by Mann Whitney test were used to compare individual treatments. Effective median concentrations (EC50) and their 95% confidence limits were calculated using Probit and toxic units (TU) were calculated as  $TU = 1/EC50$ . For Afp, statistical analysis was performed using Frequency pulsation data (SPSS v.20). Data were considered significantly different when  $p < 0.05$ .

For zebrafish assay, three independent runs were performed, and data were tested for normality with the Shapiro–Wilk and Kolmogorov–Smirnov tests. If data were normally distributed, one-way analysis of variance (ANOVA) was run in combination with a post-hoc Dunnett's test; otherwise, a non-parametric Kruskal–Wallis, Mann–Whitney U or Wilcoxon's matched-pairs tests were used for statistical comparison. Data were first analyzed for differences between runs (biological replicates). Since there were no significant differences between independent runs, single data sets were merged for each laboratory, and tests on different exposure groups were performed. In the case of LPMR, a repeated-measure ANOVA was performed to take into account the three successive periods of the test. A p-value of 0.05 was considered statistically significant for all analyses. Graphical illustrations and statistical tests were performed with Sigma Plot 12.5 (Jandel-Systat, Erkrath, Germany).

## 3. Results

### 3.1. Characterization of microplastics

The predominant shape of MPs collected was fragments (>98%) for both sites. Pellets were not found, and only a few particles were fibers, thin films and foams (<2%). Polymer composition was almost the same for the two beaches, with a majority of polyethylene (PE) and polypropylene (PP), few particles of polystyrene (PS) and 2 particles of polyvinyl acetate only at Petit-Bourg beach (Table 1).

Particle size was only measured on the MG site due to the small amount of particles available in PB site. Prior to granulometric analysis, MPs were sieved on 800 µm, and no particles were larger than 800 µm. The first decile (0.1) corresponded to a size of 6.2 µm, the median particle size was 3.7 µm, and the last decile (0.9) was 112.0 µm, while the mean size particles was 87.1 µm.

### 3.2. Pollutant fingerprint on MPs

#### 3.2.1. Organic pollutants

Non-target chemical analyses were performed on samples from both sites. Chromatograms superposition shows that the two chromatograms contain peak similarities and dissimilarities (Fig. SM2). For the chemical analysis, mass defect plot was used to detect chlorinated and brominated compounds. Some halogenated compounds were detected in both

**Table 1**  
Polymer composition of microplastic samples from two different beaches in Guadeloupe archipelago (% in mass).

MPs composition	Marie-Galante	Petit-Bourg
Polyethylene	78.3	74.6
Polypropylene	21.2	24.8
Polystyrene	0.1	0.4
Polyvinyl acetate	0	0.2

samples. Brominated flame retardant as tribromophenol ( $C_6H_3Br_3O$ ) and its metabolite tribromoanisole ( $C_7H_5Br_3O$ ) were found in both samples. Tri- and di-polychlorinated biphenyl were detected as  $C_{12}H_7Cl_3$  and  $C_{12}H_8Cl_2$ . Homologous series of pesticides from pentachlorobenzene ( $C_6HCl_5$ ) and dichlorobenzene ( $C_6H_4Cl_2$ ) were detected but only one chlorinated polycyclic aromatic compound ( $C_{12}H_7Cl$ ), most probably chloroaceneaphthylene. Analysis of PAHs was carried out using a mass defect plot with a PAHs scale, however no homologous series were detected for either MG or PB.

Finally, fold change comparisons of peak surfaces were made between MG and PB samples using TraceFinder, 26 features were selected. The results are presented in supplementary material (Table SM3). The concentrations of halogenated compounds as well as trichlorobenzene, bumetrizole and octabenzene were higher at the MG site.

Phthalates were identified in MG sample as diisobutylphthalate, dibutylphthalate, diethylphthalate, ether di-(2-ethylhexyl) phthalate and di-n-octyl phthalate.

Using mass defect plot  $CH_2$ , more hydrocarbons were detected in the PB sample than from MG, qualitatively, these compounds are mainly  $C_xH_yO_z$ . This could be explained by the location of PB, close to a large industrial zone. Most PB-specific peaks identified, corresponded to alkanes. The three principal peaks identified were  $C_{11}H_{18}O_3^+$  (Confidence level 4),  $C_{20}H_{34}O^+$  (Confidence level 4), seemed to correspond to a phenol  $C_7H_8O$  with an alkane chain and an isomer of octadecanoic acid  $C_{18}H_{36}O_2^+$  (Schymanski et al., 2014).

### 3.2.2. Metallic contamination

The screening analysis of trace metals revealed the presence of various metals for both samples including aluminium (Al), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), gallium (Ga), arsenic (As), selenium (Se), strontium (Sr), silver (Ag), tin (Sn), antimony (Sb), caesium (Cs), cerium (Ce) and uranium (U). A quantitative analysis was then performed, and results are summarized in the Table 2. Experimental blank revealed absence of trace metal with a concentration below the limit of quantification ( $0.01 \mu\text{g/g}$ ). Globally the level of metal contamination of MPs from both sites was high with concentrations ranging from 5 to  $200 \mu\text{g/g}$ . MG sample was characterized by higher concentrations of lead, cadmium and chromium, while copper and zinc were detected at highest concentration in PB sample (Table 2).

## 3.3. Leachate toxicity

### 3.3.1. Sea urchin

No toxicity was found in PE 'virgin' resin and in leachates of MG plastic sample up to a concentration of  $3333 \text{ mg/L}$  equivalent MPs. A moderate toxicity was noted for MG and a slight one for the reference PE for the highest tested concentration of leachate. However, the PB sample was much more toxic for *P. lividus* with a significant reduction in larval growth starting from  $3333 \text{ mg/L}$  equivalent MPs (Fig. 1).

### 3.3.2. Jellyfish

No effects ( $<1\%$ ) were reported in *Aurelia* sp. ephyrae exposed to PC, MG and PB leachates in terms of immobility after the two different exposure times (24 and 48 h) at all dilutions tested. Jellyfish behaviour was not affected after 24 h of exposure to PC and MG samples (Fig. 2A), while a significant effect higher than 20% was observed at  $1/3$  ( $333 \text{ mg MP/L}$ ) and undiluted PB samples.

After 48 h, a significant AFp was observed in ephyrae jellyfish

**Table 2**

Concentration of trace metal in MG and PB samples. Results are expressed in  $\mu\text{g/g}$ , ( $n = 1$ ).

Sample	$^{63}\text{Cu}$	$^{206}\text{Pb}$	$^{208}\text{Pb}$	$^{66}\text{Zn}$	$^{111}\text{Cd}$	$^{52}\text{Cr}$
MG	31	102	102	26	222	47
PB	85	18	18	292	9	4.9

exposed to all undiluted samples (PC, MG, PB) and to diluted MG and PB leachates ( $*p < 0.05$ ). Specifically, about 29–34% of AFp was observed in ephyrae exposed to MG leachates from undiluted leachate up to  $0.1 \text{ g/L}$  (Fig. 2B); PB leachate induced a significant AFp at all dilutions.

### 3.3.3. Zebrafish

Zebrafish (72 hpf) were exposed for 48 h to MPs leachates. No larval mortality was recorded during the experiment except for the PC ( $40 \pm 6\%$ ). No significant developmental anomalies (skeletal and cardiac deformities) were observed except for the PC ( $10 \pm 2.8\%$ ) (Fig. SM3). Average length of negative control larvae was  $2.45 \pm 0.10 \text{ mm}$  with head length of  $0.49 \pm 0.01 \text{ mm}$ . Larval size for individuals exposed to MPs leachates from the two sites at both concentrations were not significantly different from negative control larvae (Fig. SM4). Fish exposed to PC were significantly smaller compared to negative control (total length  $1.54 \pm 0.2 \text{ mm}$ ; head length  $0.23 \pm 0.01 \text{ mm}$ , Kruskal–Wallis,  $p < 0.05$ ).

No significant induction of in vivo EROD activity was observed in larvae exposed to MPs leachates when compared to negative control ( $0.021 \pm 0.003 \text{ pmole/min/larvae}$ ) (Fig. 3), while larvae exposed to  $70 \text{ mM}$  of BaP for 1 h showed a significant induction of EROD activity ( $0.039 \pm 0.003 \text{ pmole/min/larvae}$ ).

The LPMR was performed at 4 dpf to monitor early behavioural disruption. There was no difference in mean velocity of larvae between treatments for a single set of light conditions (ANOVA,  $p > 0.05$ ), (Fig. 4).

### 3.3.4. Sensitivity comparison between species

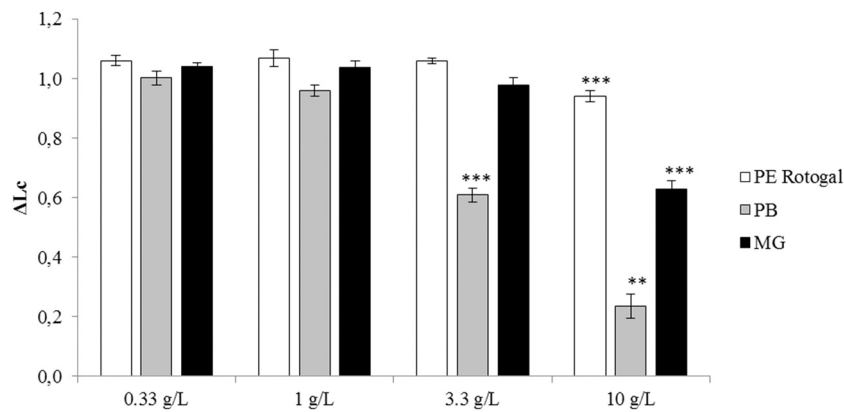
NOEC, LOEC and  $EC_{50}$  were calculated for the different species (Table 3). Regarding jellyfish, results obtained after 48 h exposure to leachates were considered. A striking difference of sensitivity was observed between bioassays. Jellyfish ephyrae was the most sensitive species and frequency of pulsation was significantly altered from  $0.33 \text{ g/L}$  equivalent MPs, after 48 h of exposure. Sea urchin larvae exhibited an intermediate sensitivity after 48 h of exposure while zebrafish embryos were clearly insensitive to MPs leachates whatever the considered endpoints at 72 or 96 hpf.

## 4. Discussion

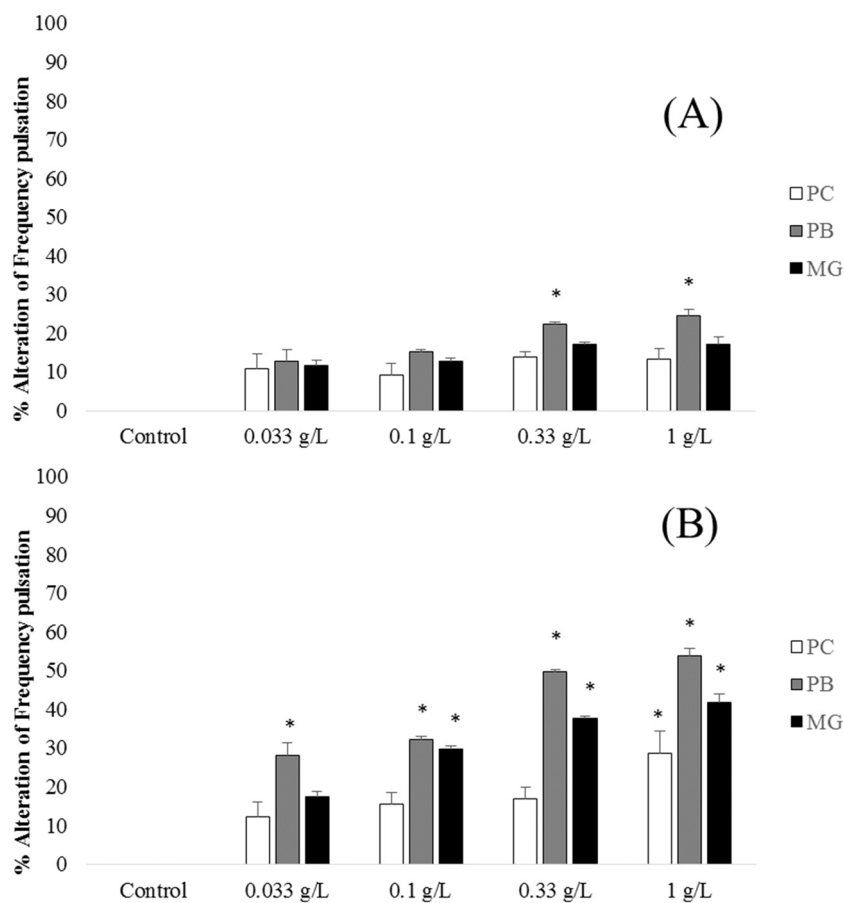
### 4.1. Plastic characterization

In the present study, MPs (1–5 mm) collected from both study beaches were mostly fragments ( $>98\%$ ) composed of 75–80% PE and 20–25% PP. This is in agreement with previous studies reporting that PE is the most abundant polymer found in plastic litter (Cheang et al., 2018; Fossi et al., 2017; Hidalgo-Ruz et al., 2012; Karthik et al., 2018), followed by PP and PS. The density of plastic has an influence on the location of MPs in the water column. MPs with a density below one (e.g., PE, PP), tend to float (Hidalgo-Ruz et al., 2012) and to be brought back to the shore by the tide and waves. As a consequence, PE and to a lesser extent PP are the predominant polymers of MPs collected on beaches over the world (Frias et al., 2010; Pannetier et al., 2019b).

The chemical contamination profile of plastics collected on the two sites demonstrated similarities and dissimilarities. Halogenated compounds including brominated flame retardant, PCBs, chlorinated pesticides and chlorinated PAHs (chloroaceneaphthylene) were found on both sites, while unsubstituted PAHs were not detected. The major differences between the two sites consisted in higher concentrations of halogenated compounds in MG than in PB, along with the detection of phthalates (diisobutylphthalate, dibutylphthalate, diethylphthalate, ether di-(2-ethylhexyl) phthalate and di-n-octyl phthalate). MPs from PB site were characterized by a high variety of hydrocarbons. This result could be explained by the proximity of the industrial area and harbour of Pointe-à-Pitre. It was already shown that environmental samples of MPs often contained PAHs, PCBs and pesticides (Pannetier et al., 2019b;



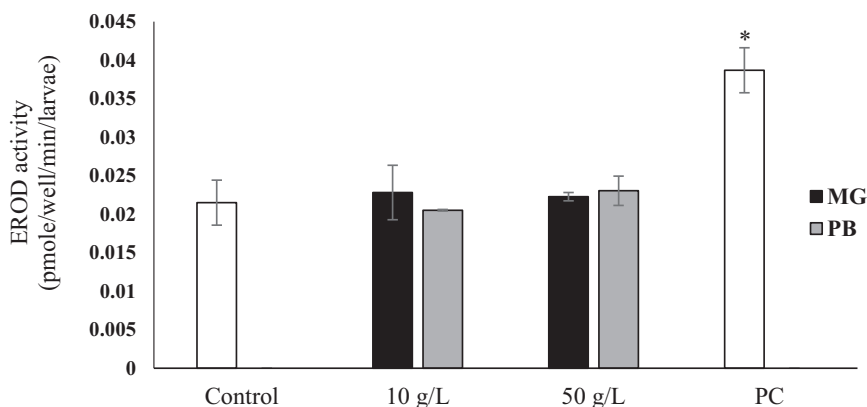
**Fig. 1.** Larvae of *Paracentrotus lividus* length increase with control corrected ( $\Delta Lc$ ) in serial dilutions of leachates from microplastic samples. Virgin polyethylene resin obtained from Rotogal (PE Rotogal), Petit-Bourg (PB) and Marie-Galante (MG). Bars represent mean  $\pm$  SD, N = 4. Asterisks refer to significant differences to the control treatment \*\* p < 0.01 and \*\*\*p < 0.001.



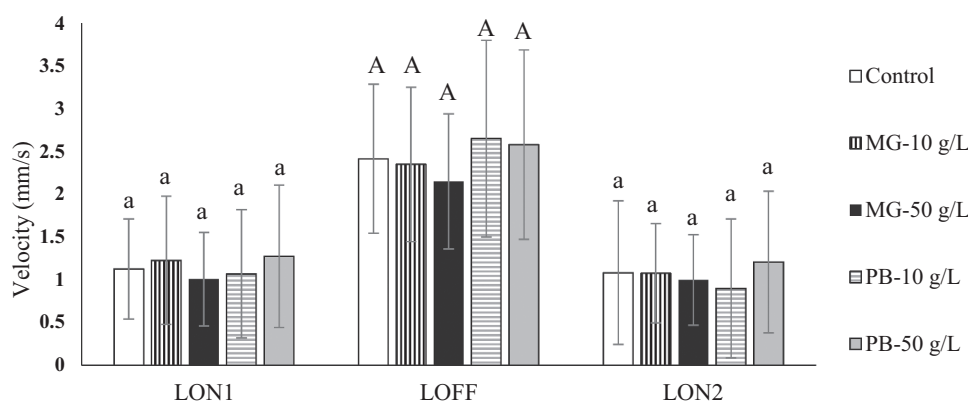
**Fig. 2.** Percentage of alteration of frequency of pulsation (AFp), after 24 h (A) and 48 h (B) of exposure in rotary wheel to PC (white bars), MG (black bars) and PB (grey bars) samples from 0.033 to 1 g/L, using ephyrae of *Aurelia* sp. (\*p < 0.05).

Schönlau et al., 2019). The profile and concentrations of hydrophobic organic pollutants on plastics are linked to the polymer composition and to the chemical exposure of particles throughout their drift history (Rochman, 2015). In the present study, the sampling period was just after the hurricanes Irma and Maria that devastated the Caribbean in September 2017 and, at least some plastic debris collected on beaches came directly from inland, especially in PB due to its proximity with the industrial area and the harbour. In our study, selected metals (Pb, Cd, Cr, Cu and Zn) were quantified at  $\mu\text{g/g}$  range. These metals were commonly detected and quantified on environmental MPs at the same

concentration range (Acosta-Coley et al., 2019; Dobaradaran et al., 2018; Li et al., 2020; Vedolin et al., 2018). The metals composition was different between the two MPs samples with higher concentrations of Pb, Cd and Cr in MG and higher concentrations of Cu and Zn in PB. Variations in metals concentrations might be due to a combination of factors, including residence time at sea leading to differences in weathering and surface erosion, photo-degradation stage but also to some extent of biofouling (Ashton et al., 2010; Holmes et al., 2012; Rochman et al., 2014).



**Fig. 3.** *In vivo* EROD activity in Zebrafish larvae exposed for 48 h to MPs leachates for Marie-Galante (MP) and Petit-Bourg (PG). EROD Activity (pmole per min and per larvae) was measured at 4 dpf zebrafish embryos exposed to negative control, MPs leachates and positive control (PC). E3 medium was used as control. Mean  $\pm$  SD, n = 15, one way ANOVA (\*p < 0.05).



**Fig. 4.** Larval photomotor response in 5-day-old zebrafish (*Danio rerio*) after exposure to negative control, MG and PB samples at 10 and 50 g/L, from hatching time. Average swimming velocity over a 5 min period including two light on periods (LON1 and LON2) with one light-off period (LOFF). Data are given as mean  $\pm$  SD (n = 16–24 larvae per treatment). The letters at the top of the bars indicate no significant differences between conditions within each light on or light off periods (repeated-measure ANOVA).

**Table 3**

Toxicity of leachates from microplastic samples (<250  $\mu$ m) of a “virgin” polyethylene resin obtained from reference PE (PE Rotogal), Petit-Bourg (PB), Marie-Galante(MG) assessed using the sea urchin (48 h), the jellyfish (48 h) and the zebrafish (96 hpf) embryo-larval assays. Concentrations are expressed in equivalent g of MPs per liter. n.c.: not calculable ( $EC_{50} > 1$  g/L).

	MPs sample	NOEC	LOEC	Toxicity units (TU = 1/ $EC_{50}$ )
Sea urchin	PB	1.00	3.33	0.21 (0.18–0.25)
	MG	3.33	10.0	<0.1
Jellyfish	PB	<0.033	<0.033	2 (1–3)
	MG	0.033	0.10	n.c.
Zebrafish	PB	>50.0	>50.0	<0.02
	MG	>50.0	>50.0	<0.02

#### 4.2. Toxicity assessment using early life stages

Embryos and larvae of fish and invertebrates play a fundamental role in the structure and functioning of marine ecosystems. The long-term sustainability of healthy populations depends on the good health and survival of larvae (Steer et al., 2017). In addition, early life stages are particularly sensitive to a wide range of pollutants (Embry et al., 2010; Lammer et al., 2009) including MPs (Bringer et al., 2020; Gambardella et al., 2017; Le Bihanic et al., 2020; Messinetti et al., 2018).

The lack of toxicity of virgin polymers compared to commercial plastics with chemical additives was previously reported. A comparison between leachates of pristine PVC and plastic toys made of PVC showed that the toys had the highest toxicity for *P. lividus* larvae (Oliviero et al., 2019). Leachate analyses have highlighted the presence of phthalates and metals used as plasticizers, stabilizers or colouring agents in plastics

(Oliviero et al., 2019; Omolaoye et al., 2010). Plastic additives including metal stabilizers and heavy-metal pigments are not chemically bound to the polymers and thus can easily leak out of plastics (Guney and Zagury, 2012; Omolaoye et al., 2010).

In the present study, the toxicity of MPs leachates produced from two environmental samples of MPs has been tested on early life stages of sea urchin (*Paracentrotus lividus*), jellyfish (*Aurelia* sp.) and fish (*Danio rerio*). Leachates of plastics collected in the two sites point out no toxicity in terms of survival (mortality, immobility) with the three bioassays. Sub-lethal effects were however observed in sea urchin embryo development and in jellyfish pulsatile behaviour, while no developmental anomalies, growth retardation and behavioural disruption were found in zebrafish larvae. Results of the present study are in agreement with previous results on sub-lethal effects of leachates of environmental MPs such as fishing nets, fishing cages and packaging collected at sea and commercial MPs, on the size and development of *P. lividus* larvae (Oliviero et al., 2019). No data on plastic leachates toxicity in jellyfish and zebrafish are available so far for comparison. Here sub-lethal effects were only observed in sea urchin and jellyfish and not in zebrafish embryo-larval stages. It might suggest that early life stages of invertebrates and more particularly jellyfish ephyrae are particularly sensitive to MPs leachates. This hypothesis is supported by several studies on other emerging contaminants (i.e. MPs, nanoparticles, chlorpyrifos) and traditional ones (i.e. metals, pesticides), demonstrating the high sensitivity of ephyrae jellyfish compared to other zooplankton early life stages, such as crustaceans or mussels (Faimali et al., 2014; Costa et al., 2015, 2020; Gambardella et al., 2015). Regarding MPs, jellyfish ephyrae have been reported to be significantly affected in terms of immobility and frequency of pulsation at concentrations lower by 2–4 orders of magnitude

than those observed in other organisms (Beiras et al., 2018; Costa et al., 2020), thus being proposed as a good bioindicator species for plastic pollution (Macali and Bergami, 2020). Results obtained from sea urchin and jellyfish demonstrated that PB sample was more toxic than MG sample. In this regard, PB leachates affected sea urchin growth and jellyfish behaviour from a concentration of 3333 and 33 mg MPs/L, respectively. Although both samples were similar in polymer composition (>70% of PE and >20% of PP) the differences in toxicity could be due to the differences in chemical contamination between both samples. For instance, MPs from PB sample were characterized by a much higher content of Zn and Cu in comparison with MG sample. Cu is known as one of the most toxic metal for a large range of invertebrate marine species including early life stage of the bivalve *Crassostrea gigas* (Mai et al., 2012), the sea urchin *Paracentrotus lividus* (Fernández and Beiras, 2001) and the polychaete *Hydroides elegans* (Gopalakrishnan et al., 2007). Cu and other metals could be, at least in part, involved in the toxicity of the MPs leachates. Further chemical analyses of leachates are necessary to ascertain this hypothesis. Also, in the same area as PB site, chlordecone has been detected and quantified on environmental MPs at concentration ranging from 0.00036 to 0.00173 µg/µg of microfilter (Sandre et al., 2019). Chlordecone is known for its high toxicity to aquatic organisms with EC50 at a range of µg/L on *Daphnia magna* (immobility at 48 h) or LC50 at µg/L range after 96 h of exposure with different freshwater fish including *Ictalurus punctatus*, *Lepomis macrochirus* and *Anguilla rostrata* (Roberts and Bendl, 1982; US-EPA, 1976). Chlordecone might also participate in the highest toxicity of PB sample compared to MG sample.

Previous studies have highlighted the chemical contamination at the surface of MPs with the presence of phthalates and metals (Oliviero et al., 2019; Omolaoye et al., 2010). Another source of toxicity for environmental MPs are HOCs from the water column accumulated on the hydrophobic polymeric matrix. Gandara e Silva et al. (2016) found that beached pellets showed higher toxicity on bivalve larvae than virgin pellets. Similarly, Pannetier et al. (2019a) did not show toxicity of mixture of virgin MPs on medaka embryos but different degrees of toxicity of beached MPs.

## 5. Conclusion

This study documents the presence of toxic substances on the surface of MPs collected on two beaches in the Guadeloupe archipelago. Substances released by aqueous extraction are toxic to the early life stages of sea urchin and jellyfish. Results using behavioural endpoints are in agreement with previous reports on jellyfish exposure to MPs, chemicals, pesticides and nanoparticles, suggesting them as suitable endpoint to be further considered for the assessment of aquatic pollution by legacy and emergent pollutants, including MPs. This study provides also new evidence of the toxicity of chemicals sorbed to environmental plastics. It would be relevant to go further by characterizing the kinetics of sorption of pollutants and desorption of additives during the aging of plastics and the toxic kinetic and dynamic on different aquatic species.

## Funding

This work was developed under RESPONSE (Towards a risk-based assessment of microplastic pollution in marine ecosystems) and EPHEMARE projects (Ecotoxicological effects of microplastics in marine ecosystems) within the JPI Healthy and Productive Seas and Oceans (JPI Oceans program). In France, it was supported by the National Funding Agency (ANR-15-JOCE-0002-01). In Spain, it was funded by the Spanish Government (MINECO) (PCIN-2015-187-C03-03 and CTM2016-77945-C3) and Ricardo Beiras benefited from the Grant "Program of Consolidation and structuring of competitive research groups in the University system of Galicia" by the Galician Government (ED431C 2017/46). Italy also benefited of PRIN (Research Project of National Interest) EMME (Exploring the fate of Mediterranean microplastics: from distribution

pathways to biological effects) project funded by the Italian Government (code: 2017WERYZP). Bettie Cormier was directly supported by a PhD grant from the University of Bordeaux (IdEx).

## CRediT authorship contribution statement

**Bettie Cormier:** Conceptualization, Methodology, Investigation, Analysis of zebrafish exposure, Writing. **Chiara Gambardella:** Conceptualization, Methodology (Jellyfish exposure), Review, Investigation (Jellyfish exposure). **Tania Tato:** Conceptualization, Methodology, Investigation, Analysis of sea urchin exposure, Review. **Quentin Perdriat:** Investigation (Zebrafish exposure). **Elisa Costa:** Conceptualization, Methodology (Jellyfish exposure), Review, Investigation (jellyfish exposure). **Cloé Veclin:** Investigation (ICP-MS). **Florane Le Bihanic:** Conceptualization, Methodology of zebrafish exposure, Investigation (ICP-MS), Review. **Bruno Grassl:** Resources (ICP-MS), Review. **Florian Dubocq:** Investigation (Analysis of organic compounds, GC MS, Orbitrap), Review. **Anna Kärrman:** Resources (GC MS, Orbitrap). **Kim Van Arkel:** Funding acquisition (Race for Water Foundation). **Soazig Lemoine:** Methodology (Sampling in Guadeloupe). **Fabienne Lagarde:** Resources (FTIR). **Bénédicte Morin:** Methodology (sampling in Guadeloupe), Review. **Francesca Garaventa:** Methodology (sampling in Guadeloupe), Investigation (Jellyfish exposure). **Marco Faimali:** Investigation (Jellyfish exposure). **Xavier Cousin:** Conceptualization (Zebrafish exposure), Resources (Zebrafish eggs), Supervision, Review. **Marie-Laure Bégout:** Conceptualization (Zebrafish exposure), Resources (Zebrafish eggs), Supervision, Review. **Ricardo Beiras:** Conceptualization, Methodology, Investigation, Analysis of sea urchin exposure, Review. **Jérôme Cachot:** Conceptualization (Zebrafish exposure), Resources (Zebrafish eggs), Supervision, Review.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

The authors would like to thank James Emery for providing English proofreading services.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111665.

## References

- Acosta-Coley, I., Mendez-Cuadro, D., Rodriguez-Cavallo, E., de la Rosa, J., Oliviero-Verbel, J., 2019. Trace elements in microplastics in Cartagena: a hotspot for plastic pollution at the Caribbean. *Mar. Pollut. Bull.* 139, 402–411.
- Acosta-Coley, I., Oliviero-Verbel, J., 2015. Microplastic resin pellets on an urban tropical beach in Colombia. *Environ. Monit. Assess.* 187, 435.
- Andrady, A.L., 2011. Microplastics in the marine environment. *Mar. Pollut. Bull.* 62, 1596–1605.
- Arthur, C., Baker, J.E., Bamford, H.A., 2009. Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9–11, 2008. University of Washington Tacoma, Tacoma, WA, USA.
- Ashton, K., Holmes, L., Turner, A., 2010. Association of metals with plastic production pellets in the marine environment. *Mar. Pollut. Bull.* 60, 2050–2055.
- Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. *Philos. Trans. R. Soc. B Biol. Sci.* 364, 1985–1998.
- Beiras, R., Bellas, J., Cachot, J., Cormier, B., Cousin, X., Engwall, M., Gambardella, C., Garaventa, F., Keiter, S., Le Bihanic, F., López-Ibáñez, S., Piazza, V., Rial, D., Tato, T., Vidal-Liñán, L., 2018. Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. *J. Hazard. Mater.* 360, 452–460.
- Beiras, R., Tato, T., López-Ibáñez, S., 2019. A 2-Tier standard method to test the toxicity of microplastics in marine water using *Paracentrotus lividus* and *Acartia clausi* larvae. *Environ. Toxicol. Chem.* 38, 630–637.



- Bejgarn, S., MacLeod, M., Bogdal, C., Breitholtz, M., 2015. Toxicity of leachate from weathering plastics: an exploratory screening study with *Nitocra spinipes*. *Chemosphere* 132, 114–119.
- Belanger, S.E., Rawlings, J.M., Carr, G.J., 2013. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. *Environ. Toxicol. Chem.* 32, 1768–1783.
- Bellas, J., Granmo, Å., Beiras, R., 2005. Embryotoxicity of the antifouling biocide zinc pyrrithione to sea urchin (*Paracentrotus lividus*) and mussel (*Mytilus edulis*). *Mar. Pollut. Bull.* 50, 1382–1385.
- Le Bihanic, F., Clérandeau, C., Cormier, B., Crebassa, J.C., Keiter, S.H., Beiras, R., Morin, B., Bégout, M.-L., Cousin, X., Cachot, J., 2020. Organic contaminants sorbed to microplastics affect marine medaka fish early life stages development. *Mar. Pollut. Bull.* 154, 111059.
- Le Bihanic, F., Clérandeau, C., Le Menach, K., Morin, B., Budzinski, H., Cousin, X., Cachot, J., 2014. Developmental toxicity of PAH mixtures in fish early life stages. Part II: adverse effects in Japanese medaka. *Environ. Sci. Pollut. Res.* 21, 13732–13743.
- Le Bihanic, F., Couillard, C.M., Rigaud, C., Légaré, B., 2013. A simple and reliable in vivo EROD activity measurement in single *Fundulus heteroclitus* embryo and larva. *Mar. Environ. Res.* 84, 17–23.
- Bosker, T., Behrens, P., Vijver, M.G., 2017. Determining global distribution of microplastics by combining citizen science and in-depth case studies. *Integr. Environ. Assess. Manag.* 13, 536–541.
- Bosker, T., Guaita, L., Behrens, P., 2018. Microplastic pollution on Caribbean beaches in the Lesser Antilles. *Mar. Pollut. Bull.* 133, 442–447.
- Braunbeck, T., Böttcher, M., Holler, H., Rudolf, T.K.L.L., Seitz, N., 2005. Towards an alternative for the acute fish LC(50) test in chemical assessment: the fish embryo toxicity test goes multi-species – an update. *ALTEX* 22, 87–102.
- Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., Stengel, D., Strecker, R., 2015. The fish embryo test (FET): origin, applications, and future. *Environ. Sci. Pollut. Res.* 22, 16247–16261.
- Bringer, A., Thomas, H., Prunier, G., Dubillot, E., Bossut, N., Churlaud, C., Clérandeau, C., Le Bihanic, F., Cachot, J., 2020. High density polyethylene (HDPE) microplastics impair development and swimming activity of Pacific oyster *D-larvae*, *Crassostrea gigas*, depending on particle size. *Environ. Pollut.* 260, 113978.
- Browne Mark, A., Niven Stewart, J., Galloway Tamara, S., Rowland Steve, J., Thompson Richard, C., 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Curr. Biol.* 23, 2388–2392.
- Cardona, L., Álvarez de Quevedo, I., Borrell, A., Aguilar, A., 2012. Massive consumption of Gelatinous plankton by Mediterranean apex predators. *PLoS One* 7, e31329.
- Cheang, C.C., Ma, Y., Fok, L., 2018. Occurrence and composition of microplastics in the seabed sediments of the coral communities in proximity of a Metropolitan Area. *Int. J. Environ. Res. Public Health* 15, 2270.
- Coffin, S., Magnuson, J.T., Vliet, S.M.F., Volz, D.C., Schlenk, D., 2020. Effects of short-term exposure to environmentally-relevant concentrations of benzo(a)pyrene-sorbed polystyrene to white seabass (*Atractosteus nobilis*). *Environ. Pollut.* 263, 114617 (Part B).
- Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. *Mar. Pollut. Bull.* 62, 2588–2597.
- Cormier, B., Batel, A., Cachot, J., Bégout, M.-L., Braunbeck, T., Cousin, X., Keiter, S.H., 2019. Multi-laboratory hazard assessment of contaminated microplastic particles by means of enhanced fish embryo test with the zebrafish (*Danio rerio*). *Front. Environ. Sci.* 7, 135.
- Costa, E., Gambardella, C., Piazza, V., Greco, G., Lavorano, S., Beltrandi, M., Bongiovanni, E., Gnone, G., Faimali, M., Garaventa, F., 2015. Effect of neurotoxic compounds on ephyrae of *Aurelia aurita* jellyfish. *Hydrobiologia* 759, 75–84.
- Costa, E., Gambardella, C., Piazza, V., Vassalli, M., Sbrana, F., Lavorano, S., Garaventa, F., Faimali, M., 2020. Microplastics ingestion in the ephyra stage of *Aurelia* sp. triggers acute and behavioral responses. *Ecotoxicol. Environ. Saf.* 189, 109983.
- Derraik, J.G.B., 2002. The pollution of the marine environment by plastic debris: a review. *Mar. Pollut. Bull.* 44, 842–852.
- Dobaradaran, S., Schmidt, T.C., Nabipour, I., Khajeahmadi, N., Tajbakhsh, S., Saeedi, R., Mohammadi, M.J., Keshkar, M., Khorsand, M., Ghasemi, F.F., 2018. Characterization of plastic debris and association of metals with microplastics in coastline sediment along the Persian Gulf. *Waste Manag.* 78, 649–658.
- Embry, M.R., Belanger, S.E., Braunbeck, T.A., Galay-Burgos, M., Halder, M., Hinton, D. E., Léonard, M.A., Lillcrap, A., Norberg-King, T., Whale, G., 2010. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. *Aquat. Toxicol.* 97, 79–87.
- Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borroero, J.C., Galgani, F., Ryan, P.G., Reisser, J., 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PLoS One* 9, e111913.
- Faimali, M., Garaventa, F., Piazza, V., Costa, E., Greco, G., Mazzola, V., Beltrandi, M., Bongiovanni, E., Lavorano, S., Gnone, G., 2014. Ephyra jellyfish as a new model for ecotoxicological bioassays. *Mar. Environ. Res.* 93, 93–101.
- Fernández, N., Beiras, R., 2001. Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. *Ecotoxicology* 10, 263–271.
- Fossi, M.C., Romeo, T., Bainsi, M., Panti, C., Marsili, L., Campani, T., Canese, S., Galgani, F., Druon, J.-N., Airoldi, S., Taddei, S., Fattorini, M., Brandini, C., Lapucci, C., 2017. Plastic Debris occurrence, convergence areas and fin whales feeding ground in the Mediterranean marine protected area Pelagos Sanctuary: a modeling approach. *Front. Mar. Sci.* 4. <https://doi.org/10.3389/fmars.2017.00167>.
- Frias, J.P.G.L., Sobral, P., Ferreira, A.M., 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Mar. Pollut. Bull.* 60, 1988–1992.
- Fuchs, B., Wang, W., Graspeuntner, S., Li, Y., Insua, S., Herbst, E.M., Dirksen, P., Böhm, A.M., Hemmrich, G., Sommer, F., Domazet-Lošo, T., Klostermeier, U.C., Anton-Erxleben, F., Rosenstiel, P., Bosch, T.C., Khalaturin, K., 2014. Regulation of polyop-to-jellyfish transition in *Aurelia aurita*. *Curr. Biol.* 24 (3), 263–273, 3.
- Gambardella, C., Costa, E., Piazza, V., Fabbrocini, A., Magi, E., Faimali, M., Garaventa, F., 2015. Effect of silver nanoparticles on marine organisms belonging to different trophic levels. *Mar. Environ. Res.* 111, 41–49.
- Gambardella, C., Morgana, S., Ferrando, S., Bramini, M., Piazza, V., Costa, E., Garaventa, F., Faimali, M., 2017. Effects of polystyrene microbeads in marine planktonic crustaceans. *Ecotoxicol. Environ. Saf.* 145, 250–257.
- Gandara e Silva, P.P., Nobre, C.R., Resaffe, P., Pereira, C.D.S., Gusmão, F., 2016. Leachate from microplastics impairs larval development in brown mussels. *Water Res.* 106, 364–370.
- Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural endpoint for ecotoxicological surveys. *Ecotoxicology* 19, 512–519.
- Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. *Sci. Adv.* 3, e1700782.
- Gopalakrishnan, S., Thilagam, H., Raja, P.V., 2007. Toxicity of heavy metals on embryogenesis and larvae of the marine sedentary polychaete *Hydroides elegans*. *Arch. Environ. Contam. Toxicol.* 52, 171–178.
- Gove, J.M., Whitney, J.L., McManus, M.A., Lecky, J., Carvalho, F.C., Lynch, J.M., Li, J., Neubauer, P., Smith, K.A., Phipps, J.E., Kobayashi, D.R., Balagso, K.B., Contreras, E. A., Manuel, M.E., Merrifield, M.A., Polovina, J.J., Asner, G.A., Maynard, J.A., Williams, G.J., 2019. Prey-size plastics are invading larval fish nurseries. *Proc. Natl. Acad. Sci.* 116 (48), 24143–24149.
- Guney, M., Zagury, G.J., 2012. Heavy metals in toys and low-cost jewelry: critical review of U.S. and Canadian legislations and recommendations for testing. *Environ. Sci. Technol.* 46, 4265–4274.
- Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microplastics in the marine environment: a review of the methods used for identification and quantification. *Environ. Sci. Technol.* 46, 3060–3075.
- Holmes, L.A., Turner, A., Thompson, R.C., 2012. Adsorption of trace metals to plastic resin pellets in the marine environment. *Environ. Pollut.* 160, 42–48.
- Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. *Science* 347, 768–771.
- Jobst, K.J., Shen, L., Reiner, E.J., Taguchi, V.Y., Helm, P.A., McCrindle, R., Backus, S., 2013. The use of mass defect plots for the identification of (novel) halogenated contaminants in the environment. *Anal. Bioanal. Chem.* 405, 3289–3297.
- Jönsson, E.M., Brandt, I., Brunström, B., 2002. Gill filament-based EROD assay for monitoring waterborne dioxin-like pollutants in fish. *Environ. Sci. Technol.* 36, 3340–3344.
- Karapanagioti, H.K., Klontza, I., 2008. Testing phenanthrene distribution properties of virgin plastic pellets and plastic eroded pellets found on Lesbos island beaches (Greece). *Mar. Environ. Res.* 65, 283–290.
- Karthik, R., Robin, R.S., Purvaja, R., Ganguly, D., Anandavelu, I., Raghuraman, R., Hariharan, G., Ramakrishna, A., Ramesh, R., 2018. Microplastics along the beaches of southeast coast of India. *Sci. Total Environ.* 645, 1388–1399.
- Kedzierski, M., D'Almeida, M., Magueresse, A., Le Grand, A., Duval, H., César, G., Sire, O., Bruzard, S., Le Tilly, V., 2018. Threat of plastic ageing in marine environment. Adsorption/desorption of micropollutants. *Mar. Pollut. Bull.* 127, 684–694.
- Koelmans, A.A., 2015. Modeling the role of microplastics in bioaccumulation of organic chemicals to marine aquatic organisms: a critical review. *Mar. Anthropog. Litter* 309–324.
- Koelmans, A.A., Besseling, E., Foekema, E.M., 2014. Leaching of plastic additives to marine organisms. *Environ. Pollut.* 187, 49–54.
- Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol.* 149, 196–209.
- Law, K.L., Thompson, R.C., 2014. Microplastics in the seas. *Science* 345, 144–145.
- Lebreton, L., Slat, B., Ferrari, F., Sainte-Rose, B., Aitken, J., Marthouse, R., Hajbane, S., Cunsolo, S., Schwarz, A., Levivier, A., Noble, K., Debeljak, P., Maral, H., Schoeneich-Argent, R., Brambini, R., Reisser, J., 2018. Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic. *Sci. Rep.* 8, 4666.
- Lippiatt, S., Opfer, S., Arthur, C., 2013. Marine debris monitoring and assessment. NOAA Tech. Memo. NOS-OR&R-46.
- Lithner, D., Damberg, J., Dave, G., Larsson, Å., 2009. Leachates from plastic consumer products – screening for toxicity with *Daphnia magna*. *Chemosphere* 74, 1195–1200.
- Lithner, D., Larsson, Å., Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Sci. Total Environ.* 409, 3309–3324.
- Li, W., Hoi-Shing, L., Ho-Man, W., Zhou, M., Chun-Yuen, W., Nora, F.T., Siu-Gin, C., 2020. Heavy metals contamination of sedimentary microplastics in Hong Kong. *Mar. Pollut. Bull.* 153, 110977.
- Lorenzo, J.I., Nieto, O., Beiras, R., 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. *Aquat. Toxicol.* 58, 27–41.
- Macali, A., Bergami, E., 2020. Jellyfish as innovative bioindicator for plastic pollution. *Ecol. Indic.* 115, 106375.
- Mai, H., Cachot, J., Brune, J., Geffard, O., Belles, A., Budzinski, H., Morin, B., 2012. Embryotoxic and genotoxic effects of heavy metals and pesticides on early life stages of Pacific oyster (*Crassostrea gigas*). *Mar. Pollut. Bull.* 64, 2663–2670.

- Mazurais, D., Ernande, B., Quazuguel, P., Severe, A., Huelvan, C., Madec, L., Mouchel, O., Soudant, P., Robbens, J., Huvet, A., Zambonino-Infante, J., 2015. Evaluation of the impact of polyethylene microbeads ingestion in European sea bass (*Dicentrarchus labrax*) larvae. *Mar. Environ. Res.* 112, 78–85.
- Messinetti, S., Mercurio, S., Parolini, M., Sugni, M., Pennati, R., 2018. Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. *Environ. Pollut.* 237, 1080–1087.
- Nagel, R., 2002. DarT: the embryo test with the zebrafish *Danio rerio* – a general model in ecotoxicology and toxicology. *ALTEX* 19, 38–48.
- OECD 1998. Test No. 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages. OECD 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test.
- Oliviero, M., Tato, T., Schiavo, S., Fernández, V., Manzo, S., Beiras, R., 2019. Leachates of micronized plastic toys provoke embryotoxic effects upon sea urchin *Paracentrotus lividus*. *Environ. Pollut.* 247, 706–715.
- Omolaoye, J.A., Uzairu, A., Gimba, C.E., 2010. Heavy metal assessment of some soft plastic toys imported into Nigeria from China. *J. Environ. Chem. Ecotoxicol.* 2 (8), 126–130.
- Pannetier, P., Morin, B., Clérandeau, C., Laurent, J., Chapelle, C., Cachot, J., 2019b. Toxicity assessment of pollutants sorbed on environmental microplastics collected on beaches: part II-adverse effects on Japanese medaka early life stages. *Environ. Pollut.* 248, 1098–1107.
- Pannetier, P., Cachot, J., Clérandeau, C., Faure, F., Van Arkel, K., de Alencastro, L.F., Levasseur, C., Sciacca, F., Bourgeois, J.-P., Morin, B., 2019a. Toxicity assessment of pollutants sorbed on environmental sample microplastics collected on beaches: part I-adverse effects on fish cell line. *Environ. Pollut.* 248, 1088–1097.
- Pannetier, P., Morin, B., Le Bihanic, F., Dubreil, L., Clérandeau, C., Chouvellon, F., Van Arkel, K., Danion, M., Cachot, J., 2020. Environmental samples of microplastics induce significant toxic effects in fish larvae. *Environ. Int.* 134, 105047.
- Pedà, C., Caccamo, L., Fossi, M.C., Gai, F., Andaloro, F., Genovese, L., Perdichizzi, A., Romeo, T., Maricchiolo, G., 2016. Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: Preliminary results. *Environ. Pollut.* 212, 251–256.
- Pittura, L., Avio, C.G., Giuliani, M.E., d'Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regoli, F., 2018. Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the Mediterranean mussels, *Mytilus galloprovincialis*. *Front. Mar. Sci.* 5, 103.
- PlasticsEurope2018. *PlasticsEurope: Annual review \_ The Facts 2017–2018*.
- Roberts, M.H., Bendl, R.E., 1982. Acute toxicity of kepone to selected freshwater fishes. *Estuaries* 5, 158–164.
- Rochman, C.M., 2015. The complex mixture, fate and toxicity of chemicals associated with plastic debris in the marine environment. *Marine Anthropogenic Litter*. Springer International Publishing, Cham, pp. 117–140.
- Rochman, C.M., Hentschel, B.T., Teh, S.J., 2014. Long-term sorption of metals is similar among plastic types: implications for plastic debris in aquatic environments. *PLoS One* 9, e85433.
- Rochman, C.M., Hoh, E., Hentschel, B.T., Kaye, S., 2013a. Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. *Environ. Sci. Technol.* 47, 1646–1654.
- Rochman, C.M., Hoh, E., Kurobe, T., Teh, S.J., 2013b. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci. Rep.* 3, 3263.
- Rouchon, A.M., Phillips, N.E., 2017. Short larval exposure to low level of copper has long-lasting latent effects on juvenile performance in the sea urchin *Evechinus chloroticus*. *Mar. Ecol. Prog. Ser.* 579, 67–79.
- Saco-Álvarez, L., Durán, I., Ignacio Lorenzo, J., Beiras, R., 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. *Ecotoxicol. Environ. Saf.* 73, 491–499.
- Sandre, F., Dromard, C.R., Le Menach, K., Bouchon-Navaro, Y., Cordonnier, S., Tapie, N., Budzinski, H., Bouchon, C., 2019. Detection of adsorbed chlordecone on microplastics in marine sediments in Guadeloupe: a preliminary study. *Gulf Caribb. Res.* 30 (1), GCFI 8-GCFI 14.
- Schönlau, C., Larsson, M., Lam, M.M., Engwall, M., Giesy, J.P., Rochman, C., Kärman, A., 2019. Aryl hydrocarbon receptor-mediated potencies in field-deployed plastics vary by type of polymer. *Environ. Sci. Pollut. Res.* 26, 9079–9088.
- Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ. Sci. Technol.* 48, 2097–2098.
- Steer, M., Cole, M., Thompson, R.C., Lindeque, P.K., 2017. Microplastic ingestion in fish larvae in the western English Channel. *Environ. Pollut.* 226, 250–259.
- Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M.E.J., Le Goïc, N., Quillien, V., Mingant, C., Epelboin, Y., Corporeau, C., Guyomarch, J., Robbens, J., Paul-Pont, I., Soudant, P., Huvet, A., 2016. Oyster reproduction is affected by exposure to polystyrene microplastics. *Proc. Natl. Acad. Sci.* 113, 2430–2435.
- Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Björn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C., Viet, P.H., Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkavong, K., Ogata, Y., Hirai, H., Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M., Takada, H., 2009. Transport and release of chemicals from plastics to the environment and to wildlife. *Philos. Trans. R. Soc. B: Biol. Sci.* 364, 2027–2045.
- Titelman, J., Hansson, L.J., 2006. Feeding rates of the jellyfish *Aurelia aurita* on fish larvae. *Marine Biology*. Springer, Berlin Heidelberg, pp. 297–306.
- US-EPA1976. *Semi-Annual Report. April-September 1976*. US EPA, Gulf Breeze, FL.
- Vedolin, M.C., Teophilo, C.Y.S., Turra, A., Figueira, R.C.L., 2018. Spatial variability in the concentrations of metals in beached microplastics. *Mar. Pollut. Bull.* 129–2, 487–493.
- Vignet, C., Le Menach, K., Lyphout, L., Guionnet, T., Frère, L., Leguay, D., Budzinski, H., Cousin, X., Bégout, M.-L., 2014. Chronic dietary exposure to pyrolytic and petrogenic mixtures of PAHs causes physiological disruption in zebrafish – part II: behavior. *Environ. Sci. Pollut. Res.* 21, 13818–13832.
- Westerfield, M., 2000. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, fourth ed. University of Oregon Press, Eugene.