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### ► To cite this version:

Pauline Pannetier, Jérôme Cachot, Christelle Clérandeau, Florian Faure, Kim van Arkel, et al.. Toxicity assessment of pollutants sorbed on environmental sample microplastics collected on beaches: Part I-adverse effects on fish cell line. *Environmental Pollution*, 2019, 248, pp.1088 - 1097. 10.1016/j.envpol.2018.12.091 . hal-03486962

**HAL Id: hal-03486962**

**<https://hal.science/hal-03486962>**

Submitted on 20 Dec 2021

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1 Toxicity assessment of pollutants sorbed on environmental sample microplastics collected on  
2 beaches: Part I-adverse effects on fish cell line.

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12           **Abstract**

13   Microplastics (MPs), are tiny plastic fragments from 1µm to 5mm generally found in the aquatic  
14   environment which can be easily ingested by organisms and may cause chronic physical but also  
15   toxicological effects. Toxicological assays on fish cell lines are commonly used as an alternative tool to  
16   provide fast and reliable assessment of the toxic and ecotoxic properties of chemicals or mixtures.  
17   Rainbow trout liver cell line (RTLW-1) was used to evaluate the toxicity of pollutants sorbed to MPs  
18   sampled in sandy beaches from different islands around the world during the first Race for Water Odyssey  
19   in 2015. The collected MPs were analyzed for polymer composition and **associated persistent organic**  
20   **pollutants: polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and**  
21   **dichlorodiphenyltrichloroethane (DDT).** In addition, DMSO-extracts from virgin MPs, MPs artificially  
22   coated with B[a]P and environmental MPs were analyzed with different bioassays: **MTT reduction assay**  
23   **(MTT), ethoxyresorufin-O-deethylase (EROD) assay** and comet assay. Microplastics from sand beaches  
24   were dominated by polyethylene, followed by polypropylene fragments with variable proportions.  
25   **Organic pollutants found on plastic from beach sampling was PAHs (2-71 ng.g<sup>-1</sup>).** Samples from Bermuda  
26   (Somerset Long Bay) and Hawaii (Makapu'u) showed the highest concentration of PAHs and DDT  
27   respectively. No toxicity was observed for virgin microplastics. No cytotoxicity was observed on cells  
28   exposed to MP extract. However, EROD activity was induced and differently modulated depending on the  
29   MPs locations **suggesting presence of different pollutants or additives in extract.** DNA damage was  
30   observed after exposure to four microplastics samples on the six tested. **Modification of EROD activity**  
31   **level and DNA damage rate highlight MPs extract toxicity on fish cell line.**

32   **Capsule:** **This study demonstrated the toxicity of environmental MP extracts on fish cells.**

33   **Keywords:** sandy beach environmental microplastics, sorbed pollutants, EROD activity, genotoxicity, fish  
34   cell line

35

## 36 **1 Introduction**

37 Marine pollution by plastics, highlighted in the 1970s (Carpenter et al., 1972), has gained  
38 considerable attention in recent years, both among the general public and scientists, as plastic debris  
39 has become more and more present in natural ecosystems, with a worrying impact on aquatic  
40 species. Driven by incessant demand, plastic production has increased exponentially since the 1940s.  
41 Indeed, the numerous properties of this material (light, resistant, inexpensive) make it a product  
42 widely used in industry for all kinds of everyday objects (Laist, 1997). Plastics are now one of the  
43 main types of debris found in the marine environment (Law, 2017). Their persistence in the  
44 environment is estimated to be hundreds or even thousands of years depending on the type of  
45 plastic (Collignon et al., 2012). These materials are found mostly microscopically, with a size between  
46 1  $\mu\text{m}$  and 5 mm, also known as microplastics (Arthur et al., 2009). Microplastics (MPs) come in many  
47 different shapes (fibers, fragments, spherules), colors, sizes, and polymer compositions. In the  
48 environment, two categories of microplastics (MPs) can be identified: primary MPs, produced  
49 directly in a microscopic state and entering the environment via industrial discharges or wastewater  
50 treatment plant waste, and secondary MPs, resulting from macro-waste degradation due to wave  
51 abrasion, UV, marine biological activity and hydrolysis reactions (Andrady, 2011; Cole et al., 2011).  
52 Beaches are hotspots for microplastics resulting from macro debris fragmentation (Andrady, 2011).

53 MPs are of concern because they can be confused with prey by a wide variety of marine organisms  
54 (Kaposi et al., 2014). The small size of microplastics, as well as their color, form and the presence of  
55 biofilm, facilitate and promote their ingestion by different organisms as well as their accumulation,  
56 especially in the digestive tract. Ingesting MPs can impede of digestive function, and create a  
57 sensation of fullness caused by the body being unable to break them down using its natural digestive  
58 fluids (Cole et al., 2011; Rochman et al., 2013, [Lei et al., 2018](#)). Aside from the physical effects of  
59 ingestion, the toxicological effects of MPs are not well documented. These particles have the  
60 particularity of absorbing hydrophobic organic chemicals (HOCs) present in water, such as persistent

61 organic pollutants (POPs), including polychlorinated biphenyls (PCBs), polycyclic aromatic  
62 hydrocarbons (PAHs) and organochlorine pesticides (Derraik, 2002; Karapanagioti et al., 2011;  
63 Koelmans et al., 2016, 2013). High concentrations of these contaminants have previously been  
64 detected in environmental samples of plastics. In some cases, PCB concentration can reach values up  
65 to  $10^5$ - $10^6$  times higher in MPs than in the water column:  $117\text{ng.g.pellet}^{-1}$  and  $499\text{pg.l}^{-1}$  respectively  
66 (Mato et al., 2001). The rate of accumulation of this type of pollutant depends on polymer type,  
67 physical and chemical properties of plastic, particle area, weathering, biofilm formation, and  
68 chemical exposure throughout the particle's drift history (Rochman, 2015). The components of the  
69 plastic itself, particularly additives attached to their surface to improve some of their properties (e.g.  
70 phthalates, bisphenol A, alkylphenols) are also liable to cause effects (Koelmans, 2015; Lusher, 2015;  
71 Rochman, 2015). In addition, mixtures of contaminants absorbed to MPs during their life in the  
72 environment can be toxic to organisms (Koelmans et al., 2016). They can be released from the  
73 surface of MPs during their transport at sea or during transit through the digestive tract, especially  
74 under the action of enzymes (Rochman et al., 2014) and plastics degradation (Cole et al., 2011).  
75 Although MPs alone can be considered biologically inert, pollutants adsorbed into MPs can penetrate  
76 cells, interact with macromolecules, and disrupt biological systems including the endocrine system  
77 (Rochman et al., 2014, Teuten et al. 2009). The resulting biological consequences can compromise  
78 the survival of organisms and cause malformations, particularly in the early life stages of fish that are  
79 particularly sensitive to pollutants (Mazurais et al., 2015, Nobre et al. 2015). Impacts on growth,  
80 mortality, feeding rate or energy metabolism have been observed in different invertebrate species  
81 (Phuong et al., 2016).

82 While the sorption of chemicals from seawater to plastic particles has been clearly demonstrated  
83 (Law, 2017) only a handful of studies have been carried out on the toxic effect of the mixture of  
84 pollutants present on environmental MPs surface on fish. Today, there are a number of economic,  
85 scientific and ethical reasons for supporting efforts to develop and apply *in vitro* assays in aquatic  
86 ecotoxicology as alternative tools to animal testing (Castaño et al., 2003). Utilization of fish cell

87 cultures is being considered as an alternative to whole fish assays in the testing of aquatic pollutants.  
88 Cell lines provide a specific screening tool that is easy, fast, and cost-effective. Useful results can be  
89 obtained from testing substances in small volumes. RTL-W1 (Lee et al., 1993), a cell line from rainbow  
90 trout liver, has been widely used for the measurement of different toxicity endpoints including  
91 cytotoxicity (Dayeh et al., 2005), genotoxicity (Brinkmann et al., 2014; Kienzler et al., 2012), EROD  
92 activity (Hinger et al., 2011; Lee and Steinert, 2003) and reactive oxygen species (ROS) production  
93 (Pietsch et al., 2011).

94 In 2015, the Race For Water foundation launched an environmental expedition around the world  
95 called "Race for Water Odyssey", with the aim of demonstrating the extent of plastic pollution. Their  
96 dedicated boat traveled around world, carrying out a mixture of scientific and publicity-orientated  
97 stops, the latter aimed at raising awareness of the growing seriousness of marine litter  
98 (<https://www.raceforwater.org/en/the-race-for-water-foundation/learn/>). As part of this project,  
99 microplastic samples (MPs) were taken from different beaches of different islands located in or  
100 around the world's 5 major vortexes of plastics.

101 In this study, we characterized MPs collected from 6 remote island beaches during the Odyssey  
102 mission in terms of polymer composition and persistent organic pollutant sorbed to the MPs. We  
103 also investigated the toxicity of those environmental microplastics on liver trout cell lines (RTL-W1).

## 104 **2 Materials and methods**

### 105 **2.1 Cell line**

106 The cell line used in this study was a fibroblast-like non transformed permanent cell line derived from  
107 the liver of adult of rainbow trout, *Oncorhynchus mykiss* : RTL-W1 (Lee et al., 1993). This sensitive  
108 tool is used for assessing the toxic potentials of chemicals due to its high biotransformation capacity.  
109 Cells were cultured routinely in 75 cm<sup>2</sup> culture flasks (Cell start<sup>®</sup> cell culture Flask Greiner) at 20°C in  
110 CO<sub>2</sub> free incubators. Growth medium was composed of Leibovitz's (L-15) medium supplemented with  
111 5% fetal bovine serum (FBS) and with mixture of Penicillin (100 IU/mL) and streptomycin (100 lg/mL).

112 Cells were trypsinized once a two week with TrypLE<sup>TM</sup> Express (Invitrogen). Experiments were  
113 carried out with cells aged from passage 62–75.

## 114 **2.2 Plastic samples**

### 115 *2.2.1 Sample collection*

116 Plastic debris were collected from sandy beaches during the Race for Water Odyssey in 2015, 2 to 5  
117 different shorelines were sampled following the standardized NOAA's protocol (Lippiatt et al., 2013).  
118 A total of 30 beaches were selected to identify, score and weight the debris above 2.5 cm  
119 (macrodebris), debris between 2.5 cm and 5 mm (mesoplastics) and debris less of 5 mm  
120 (microplastics). Environmental MPs analyzed in this study were collected in Azores (Az), Bermuda  
121 (Be), Easter island (Ea), Hawaii (O'ahu (HaM) and Big island (HaK)), and Guam (Gu). These beaches  
122 have specific characteristics recorded in Table 1. **A sampling map is provided in supplementary**  
123 **material (SI 1). These data were obtained during beach sampling by Race for Water foundation.**

Table 1: Location and characteristics of the sampled beaches

	Sample	GPS Coordinates	Place	Main use	Cleaning frequency
<b>North Atlantic</b>	<b>Az</b>	N 38°31,407 W 02°83,753	Porto Pim beach, Faial Island, Portugal	Touristic	Between 1 time/day to 1time/week
	<b>Be</b>	N 32°18,147 W64°52,189	Somerset, Long Bay Beach, Bermuda	Recreational/ Fishing activities	1 time/month
<b>South Pacific</b>	<b>Ea</b>	S 27°04,421 W 09°19,394	Anakena, Easter Island, Chile	Touristic	Between 1 time/day to 1time/week
<b>North Pacific</b>	<b>Gu</b>	N 13°25,258 E 144°47,028	Pago Bay, Guam, USA	Recreational activities	1 time/month
	<b>HaM</b>	N 21°17,465. W 57°39,791	Makapu'u beach, Oahu, Hawaii, USA	Surf spot	1 time/month
	<b>HaK</b>	N 19°07,719 W155°30,639	Kawa Bay, Big Island, Hawaii, USA	Wild	1 time/month

125

126 Microplastics (<5mm) were collected on a sandy area of 50cm x 50cm x 10cm deep. The first step in  
 127 extracting debris was to mix and shake 0,025m<sup>3</sup> of sand with 1L of sea water, based on the  
 128 Microplastic Sampling Protocol of the National Oceanic and Atmospheric Administration (Lippiatt et  
 129 al., 2013). The floating microparticles on the water surface were recovered with a 300 µm sieve.  
 130 Samples were stored in a 180 mL plastic buckets (PP) until analysis at ambient temperature.

### 131 2.2.2 Control samples

132 A virgin plastic control (C-) was made with newly purchased laboratory plastic items grinded with a  
 133 mortar or electric crusher (10 min) and sieved on 600 µm sieve. This control was made up of Low  
 134 Density PolyEthylene (LDPE, 40%), High Density PolyEthylene (HDPE, 25%), PolyPropylene (PP, 25%)



135 and PolyStyrene (PS, 10%) according to environmental sample of MPs from Swiss lakes (Faure et al.,  
136 2015).

137 A positive plastic control (C+) was prepared from virgin microplastic control coated with  
138 Benzo[a]pyrene (B[a]P, CAS Number: 50-32-8). In an amber flask, 100mg of control microplastic was  
139 placed with 1mL of 100 $\mu$ M (0.025 mg.mL<sup>-1</sup>) of B[a]P in methanol. After 16h of shaken (175rpm)  
140 methanol was evaporated under flux of azote (2h, 37°C). Final theoretical concentration of B[a]P on  
141 MP particles was 250 $\mu$ g.g<sup>-1</sup>. Positive and virgin control samples were conserved at 20°C in amber  
142 flask until analysis.

### 143 *2.2.3 Preparation of organic extracts of MPs*

144 Before extraction, environmental MP samples were grinded with mortar (10 minutes) and sieved to  
145 obtain microparticles measuring under 600  $\mu$ m **in the same way than virgin plastic control.**  
146 Environmental or control MPs (100 mg) were added to an amber flask with 1mL of dimethyl sulfoxide  
147 (DMSO). After 16h of shaking (175rpm), the solid part was separated from the liquid phase (extract)  
148 with extra-long sterile tip. The extract was used in toxicological assays and conserved between 1 and  
149 7 days at 4°C in dark.

### 150 **2.3 Microplastic composition**

151 **Before to be grind and sieve for toxicological analysis, type and composition of MPs were analyzed.**

152 In the laboratory, the samples were sieved to separate debris into fractions of different sizes, > 1 mm  
153 (large microplastics) and > 300  $\mu$ m (small microplastics). Large microplastics were identified visually  
154 and sorted manually according to their appearance, characteristics and possible origin. Small  
155 microplastics were sorted using a stereomicroscope (Leica EZ4W, Germany). All sorted particles were  
156 then dried for 24h at ambient temperature. Plastic fractions were listed in seven categories: foams,  
157 thin films, textile fibers, lines as fishing lines, microbeads, preproduction pellets and hard fragments  
158 from the degradation of larger plastic debris. After being counted and weighed by categories, a

159 representative part of each sample of MPs, the proportion of each kind of plastic in the same as for  
160 the full samples, was analyzed through Fourier-transform infrared spectroscopy (FT-IR) using a  
161 spectrum Two (Perkin Elmer, USA) to identify their polymeric structure according to Faure et al.,  
162 (2015). Results are expressed in % mass.

## 163 **2.4 Chemical analysis**

### 164 *2.4.1 Beach fingerprint*

165 Due to limited amount of MPs collected in beaches and provided for toxicological analysis (<1 g),  
166 chemical analysis was performed on mesoplastics (2.5cm-5mm), sampled on the same beach  
167 following the NOAA's protocol (Lippiatt et al., 2013). These chemical analyses were carried out in  
168 order to obtain the chemical fingerprint of the plastics present on the beach. Plastics were stored in a  
169 polypropylene bucket (180mL), in the dark and at 4°C until analysis. Plastic samples were analyzed  
170 for PCBs, PAHs and DDT.

171 In this project, to avoid contamination between samples, every tool and each piece of glassware was  
172 cleaned with acetone and high quality dichloromethane. Gloves were changed regularly. Each plastic  
173 sample was ground finely in a laboratory mill to obtain a homogenous sample. Two grams of sample  
174 were weighted because this mass was initially estimated by the limit of detection. Each sample was  
175 stirred in 100 ml of hexane for 30 minutes. Plastics were filtered, and the solvent was evaporated  
176 until less than 10ml remained. The remaining liquid was then transferred to a vial using three  
177 portions of 10ml of methanol, the alcohol was used to precipitate any matrices dissolved in the  
178 hexane. The solution was cooled down to 0°C for 2h to improve the precipitation. Then, the residues  
179 were filtrated using a Millipore filter. The solution was transferred quantitatively into another vial to  
180 be evaporated under a nitrogen flow. The internal standard (chlorodiphenylmethane) was added and  
181 the residue was recovered with 1 ml acetone (solvent used for standard solution). The solution was  
182 finally injected in the GC-MS (Thermo Fisher Trace 1300, USA). The GC-MS was operated in split

183 mode with a solvent delay of 2 min, with an ion-chamber temperature of 280°C. Three different  
184 temperature programs were used for the pollutant:

185 1) PCB: the injector temperature 270°C and transfer line was 300°C. The column temperature  
186 was programmed from 140°C (1 min) to 240°C (1 min), at a rate of 6°C min<sup>-1</sup>.

187 2) PAH: the injector temperature 300°C and transfer line was 300°C. The column temperature  
188 was programmed from 40°C (2 min) to 100°C (2 min), at a rate of 30°C min<sup>-1</sup> and from 100°C  
189 to 300°C (3 min), at a rate of 10°C min<sup>-1</sup>.

190 3) DDT: the injector temperature 260°C and transfer line was 300°C. The column temperature  
191 was programmed from 160°C (1 min) to 240°C (2 min), at a rate of 6°C min<sup>-1</sup>. The carrier gas  
192 was helium (ultra-high purity), with a constant flow rate of 1.2 mL min<sup>-1</sup>.

193 Results were expressed as pollutant concentration (ng.g<sup>-1</sup>) ± Instrumental incertitude.

#### 194 2.4.2 Quantification of organic pollutants in extract

195 Extracts contamination was assessed through analysis of 19 polycyclic aromatic hydrocarbons (PAHs),  
196 13 polychlorinated biphenyls (PCBs) and 26 pesticides compounds by stir bar sorptive extraction-  
197 thermal desorption-gas chromatography-tandem mass spectrometry (SBSE-TD-GC-MS/MS). For  
198 environmental sample, 500 µL of DMSO extract were mixed to an ethanolic solution containing 5 ng  
199 of 8 deuterated internal standards (naphthalene d8, biphenyl d10, phenanthrene d10,  
200 ethylparathion d10, pyrene d10, benzo(a)anthracene d12, benzo(a)pyrene d12, benzo(g,h,i)perylene  
201 d12) before addition of 100 mL of reverse-osmosis water. For positive control, DMSO extract was  
202 diluted by 2500 before ethanolic solution addition. Analyses was realized by gas chromatography  
203 system coupled to an triple quadrupole mass spectrometer following same protocol and conditions  
204 described in previous studies (Lacroix et al., 2014; Pannetier et al., 2018b). Results are expressed as  
205 ng analytes.g<sup>-1</sup> plastic.

#### 206 2.5 Toxicological analysis

207 2.5.1 *Cell line exposure conditions*

208 Cells were seeded in 24- (Comet assay) or 96-well plates (MTT test and EROD activity) at  $2 \times 10^5$   
209 cells.mL<sup>-1</sup>. After 24h of culture in total medium, cells were exposed in triplicate to different  
210 concentrations of organic MP organic extract. For the EROD and MTT assays, cell lines were exposed  
211 to a serial ½ dilutions from 1% to  $3 \times 10^{-5}$  % of MPs organic extracts. For the comet assay, only two  
212 concentrations were tested 0.01% and 0.1% of MPs extract. Two controls were performed for each  
213 assay: one with the maximum DMSO concentration used in the test (1%) and a control without  
214 DMSO.

215 2.5.2 *Cytotoxicity*

216 Cell cytotoxicity was measured using the MTT (3(4,5-dimethyl-2thiazohlyl)-2,5-diphenyl-2H-  
217 tetrazolium bromide)) assay. After cell exposure, the medium was replaced by a serum free medium  
218 containing 10% MTT solution at 5mg.mL<sup>-1</sup> in ultrapure water (Thiazolyl Blue Tetrazolium Bromide,  
219 Sigma-Aldrich) for 1h in the dark. The medium was discarded prior to the addition of acidified  
220 isopropanol solution (4% 1N HCl, 15min of dark incubation) to dissolve the formazan. The  
221 absorbance was read at 540 nm and was expressed as the percentage of mean absorbance (n = 3)  
222 relative to the negative controls.

223 2.5.3 *EROD activity*

224 Induction of 7-ethoxyresorufin-*O*-deethylase was measured in the fish liver cell line RTL-W1 with  
225 slight modifications to a method previously published (Behrens et al., 1998; Hinger et al., 2011). After  
226 exposure, medium was replaced by complete L-15 medium with 2µM of 7-ethoxyresorufin (Sigma-  
227 aldrich). After 15 min incubation in the dark, production of resorufin was recorded using a microplate  
228 fluorimeter (Fluostar optima) at 538nm/590nm ( $\lambda_{excitation}/\lambda_{emission}$ ). Total protein content was  
229 determined using a fluorescamine assay according to Lorenzen and Kennedy, (1993). Cells were  
230 washed twice with PBS and frozen 1h at -80°C. Cells were since thawed at room temperature, and  
231 100µL of PBS and 50µL of fluorescamine solution at 0.3 mg.mL<sup>-1</sup> in acetone (Sigma aldrich) were

232 added in each well. Fluorescence was recorder at 390nm/460nm ( $\lambda_{excitation}/\lambda_{emission}$ ) after 15min  
233 incubation in the dark. Data is expressed as picomoles of resorufin produced per minute and per  
234 milligram of protein (pmol/min/mg of protein). To express EROD activity, the following formula was  
235 used to compare variation of sample to basal EROD activity:

$$EROD \text{ activity variation} = \frac{x \times 100}{T}$$

236 With:

237 x= Sample EROD activity in pmol/min/mg of protein

238 T= Mean of control activity (T or TDMSO) activity in pmol/min/mg of protein

#### 239 2.5.4 Comet assay

240 The comet assay was only carried out for treatments allowing cell viability greater than 80%. The  
241 formamidopyrimidine glycosylase(Fpg)-modified comet assay was performed in alkaline conditions as  
242 described in details in Pannetier et al. (2018a). Utilization of Fpg enzyme increase the panel of lesions  
243 detected, including oxidized guanine, and the sensitivity of the comet assay. DNA damage was  
244 expressed as percentage of tail DNA (DNA break (%)), which is the percentage of DNA which has  
245 migrated from the head (Collins, 2004).

#### 246 2.6 Statistical analysis

247 Results were expressed as the mean  $\pm$  standard error (SD) of three independent experiments with  
248 three replicates. Differences between sample and control were tested using the R software.  
249 Normality (Shapiro-Wilk's test) and homoscedasticity of variances were verified (Levene's test) and  
250 statistical analysis was performed by the nonparametric Kruskal-Wallis tests or parametric ANOVA  
251 according to Levene's and Shapiro-Wilk's results. In addition, Tukey's HSD post hoc test was used for  
252 pairing comparison. Significance difference was accepted when  $p < 0.05$ .

### 253 3 Results

#### 254 3.1 Abundance and characterization of microplastics

255 The microplastic abundance is shown in Table 2. High variability of microplastic both by number and  
 256 by weight was observed along the different beaches. The highest accumulation of MP occurred in  
 257 Azores and Easter Island beaches (4043 particles; 54.0 g/m<sup>2</sup> and 4816 particles; 54.7 g/m<sup>2</sup>  
 258 respectively). Long Bay Beach in Bermuda and Pago Bay in Guam showed a concentration of 5.6 g/m<sup>2</sup>  
 259 and 5.9 g/m<sup>2</sup>, respectively. The two sampled beaches in Hawaii were surf spot and wild and have the  
 260 lowest amount of microplastics.

261 Four polymers were identified from the six microplastic samples: the most abundant was  
 262 polyethylene (PE) followed by polypropylene (PP) except for HaK which is mainly composed of PP.  
 263 Small proportion of polystyrene (PS) and Ethylene Vinyl Acetate (EVA) were also observed in HaK and  
 264 Az respectively (Table 2). Two main shapes (i.e. fragments and pellets) were identified. The  
 265 predominant microplastic shape in the samples was fragments. The percentage abundance of  
 266 fragments in the sampled beaches ranged between 56% at Easter island to 99% at Bermuda site  
 267 (Table 2). High proportion of pellets was found in Ea beach samples (47.75 %). Samples from Hawaii  
 268 were only composed by fragments and pellets. The other microplastic types found in lower  
 269 proportions were lines, fibers, thin films and foams.

270 *Table 2: Characteristics of microplastics in sampled beaches*

Sample	North Atlantic		South Pacific	North Pacific			
	Az	Be	Ea	Gu	HaM	HaK	
<b>Number of particles (pieces/m<sup>2</sup>)</b>	4043	1050	4816	1140	644	280	
<b>Weight (g/m<sup>2</sup>)</b>	54.0	5.6	54.7	5.9	9.2	4.0	
<b>Polymer type (% mass)</b>	PE	97.38	98.97	94.23	59.01	87.98	27.38
	PP	2.31	1.03	5.77	36.55	12.02	71.96
	PS	-	-	-	-	-	0.66
	EVA	0.31	-	-	-	-	-
	Not identified	-	-	-	4.44	-	-
<b>Shape (% mass)</b>	Fragments	93.38	99.44	56.13	93.59	96.70	85.96
	Pellets	6.56	-	47.75	4.64	3.30	14.04
	Beads	-	-	-	-	-	-
	Lines	-	0.55	0.06	0.28	-	-
	Fibers	0.01	0.01	-	-	-	-
	Thin films	0.05	-	0.05	1.18	-	-

Foams	-	-	-	0.31	-	-
Others	-	-	0.03	-	-	-

PE: Polyethylene, PP: Polypropylene, PS: Polystyrene, EVA: ethylene vinyl acetate and not identified particle.

271

### 272 3.2 Chemical analysis

273 Chemical analysis was carried out on mesoplastics collected from five islands (Bermuda, Easter  
 274 Island, two Hawaii islands and Guam). Polycyclic aromatic hydrocarbons (PAHs), polychlorinated  
 275 biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) were analyzed in plastic fragments  
 276 collected from every island (Table 3). Detail of PAHs composition was described in supplementary  
 277 material (SI 2). Concentrations of PAHs and DDT varied among the sampling sites. The highest  
 278 concentration of PAHs was found in Long Bay Beach in Bermuda (Be) with  $70 \pm 14 \text{ ng.g}^{-1}$ . PAHs  
 279 contamination gradient among the beaches was  $\text{Be} \gg \text{HaK} > \text{Gu} = \text{HaM} > \text{Ea}$ . The concentration of total  
 280 PCBs was under quantification limits for every samples. The measured DDT was under quantification  
 281 limits excepted for HaM. On this beach, plastic DDT concentration was  $10 \pm 3 \text{ ng.g}^{-1}$ .

282

Table 3: Persistent organic pollutants on plastic samples from beaches.

	Sample	PAHs ( $\text{ng.g}^{-1}$ )	PCBs ( $\text{ng.g}^{-1}$ )	DDT p,p' ( $\text{ng.g}^{-1}$ )
North Atlantic	Az	ND	ND	ND
	Be	$71.0 \pm 14.0$	<5.0	<4.0
South Pacific	Ea	$2.0 \pm 1.0$	<5.0	<4.0
	Gu	$6.0 \pm 2.0$	<5.0	<4.0
North Pacific	HaM	$6.0 \pm 2.0$	<5.0	$10.0 \pm 3.0$
	HaK	$18.0 \pm 4.0$	<5.0	<4.0

ND: No data

283

284 Due to limited amount of MPs collected in beaches (<1g), chemical analysis of MP extracts was not  
 285 performed. However, chemical analysis of positive control extract was done. Expected concentration  
 286 of B(a)P in the positive control was  $250 \mu\text{g.g}^{-1}$  of MP. Measured concentration of B(a)P was  $172 \mu\text{g.g}^{-1}$   
 287 of MP (data not shown) which represents 68.8% recovery compared to nominal concentration. No  
 288 other organic pollutant was detected.

### 289 **3.3 Toxicological analysis**

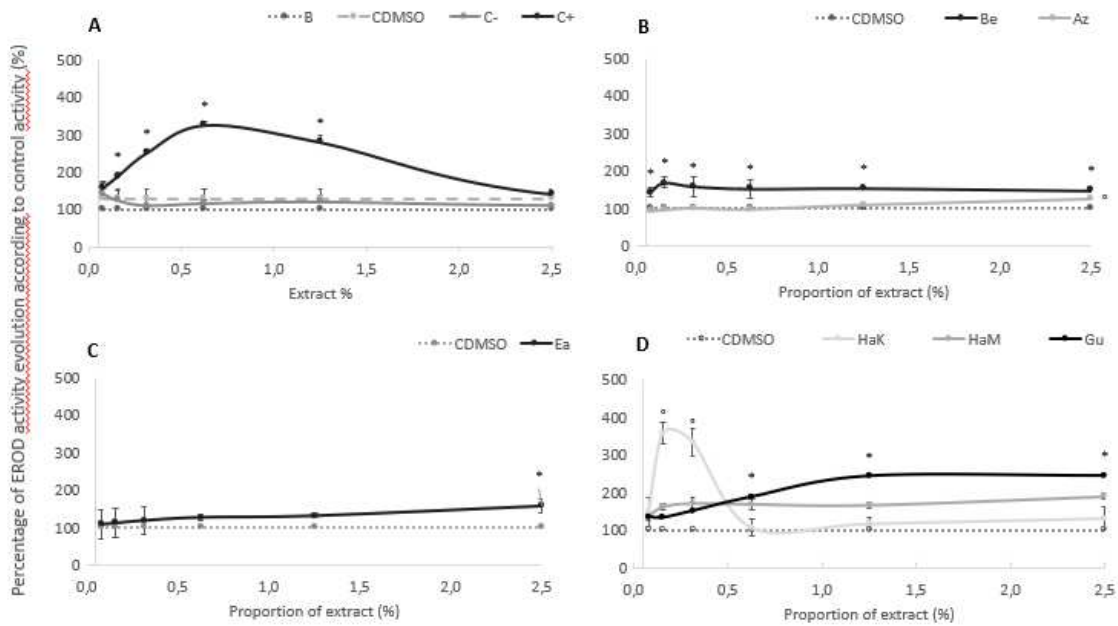
#### 290 *3.3.1 EROD activity*

291 Before measuring EROD activity measurement, cytotoxicity was checked. Using the MTT assay, no  
292 cytotoxicity was observed on RTL-W1 cells exposed to different amount up to 1% of MPs extracts  
293 (data not shown).

294 Figure 1A presents the development of EROD activity for various control samples: cells only (B:  
295 blank), cells with 1% DMSO ( $C_{DMSO}$ ), cells with virgin microplastics organic extracts (C-) and cells with  
296 B[a]P coated microplastic extracts (C+). No significant difference was observed between the three  
297 negative controls: Blank,  $C_{DMSO}$  and C-. Basal EROD activity was considered for the cell line in  
298 presence of 1% DMSO only. Positive control C+ induced significant EROD activity from 0.15% of  
299 extract to 1.25% of extract compared to control sample. For samples from the North Atlantic (Fig.  
300 1B), EROD activity induced by exposure to MP extracts (125-160% versus  $C_{DMSO}$ ) was generally lower  
301 than that induced by North Pacific MP extracts (150-375% versus  $C_{DMSO}$ ). Az MP extracts did not  
302 induce a variation in EROD activity, except at the highest concentration (2.5% of Extract). Be MP  
303 extracts showed significant induction from the first concentration but no change as the  
304 concentration was increased. Samples from South Pacific islands (Fig. 1C) did not exhibit significant  
305 EROD activity excepted a weak induction for the highest proportion of Ea extract. Finally, for  
306 environmental samples from North Pacific gyre (Fig. 1D), EROD activity was not induced for HaM  
307 extract. However, Gu extract induces a dose-dependent induction of EROD activity from 0.6% of  
308 extract. After exposure to HaK, a peak of EROD activity was observed from 0.15% to 0.5% of extracts.  
309 This induction is constant with no concentration-induced changes. Maximum EROD activity (+375%  
310 versus  $C_{DMSO}$ ) was reached for HaK sample but only for diluted extract at 0.15% and 0.5%. EROD  
311 values are provided in supplementary material (SI 3).

312





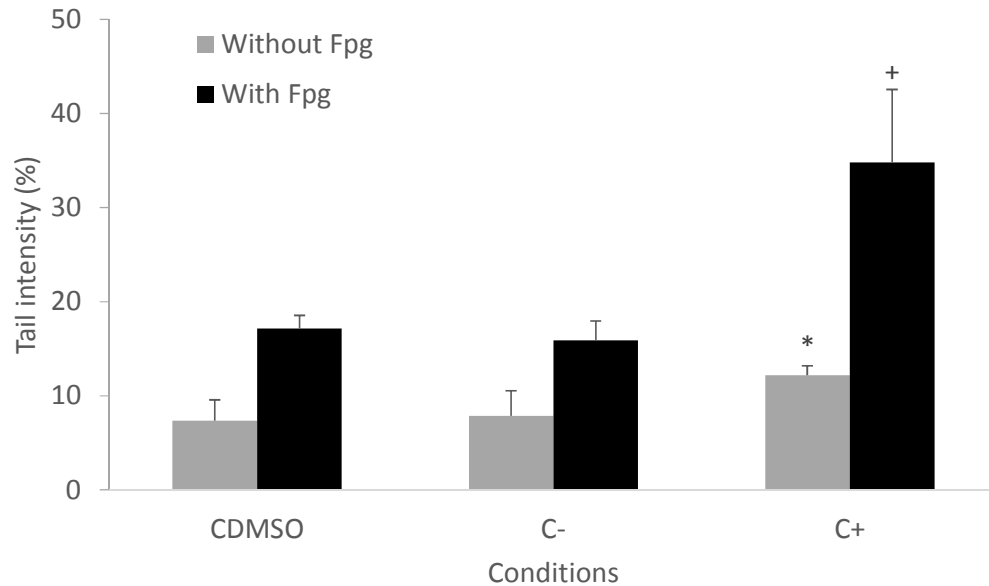
313

314 *Figure 1: Evolution of EROD activity according to basal EROD activity (B: Blank or DMSO control (CDMSO)*  
 315 *of control cell (n=3, mean±SD) for A-DMSO control (CDMSO), B(a)P coated microplastics extract (C+)*  
 316 *and virgin microplastics extracts (C-); B- for environmental microplastics extracts from North Atlantic beaches*  
 317 *(Be and Az); C- for environmental microplastics extracts from South Pacific beaches (Ea) ; D- for*  
 318 *environmental microplastics extracts from North Pacific beaches (HaK, HaM and Gu). \* and ° indicate*  
 319 *significant difference between conditions and basal EROD activity (Kruskall-Wallis, p<0,05).*

320

### 321 3.3.2 Comet assay

322 No significant variations in DNA damage were observed for non-exposed cells and cells exposed to  
 323 extracts of virgin MPs according to control DMSO (Fig. 2). After exposure to extract of B(a)P coated  
 324 MPs, a significant increase in DNA damage was detected in RTL-W1 cells both with and without Fpg.

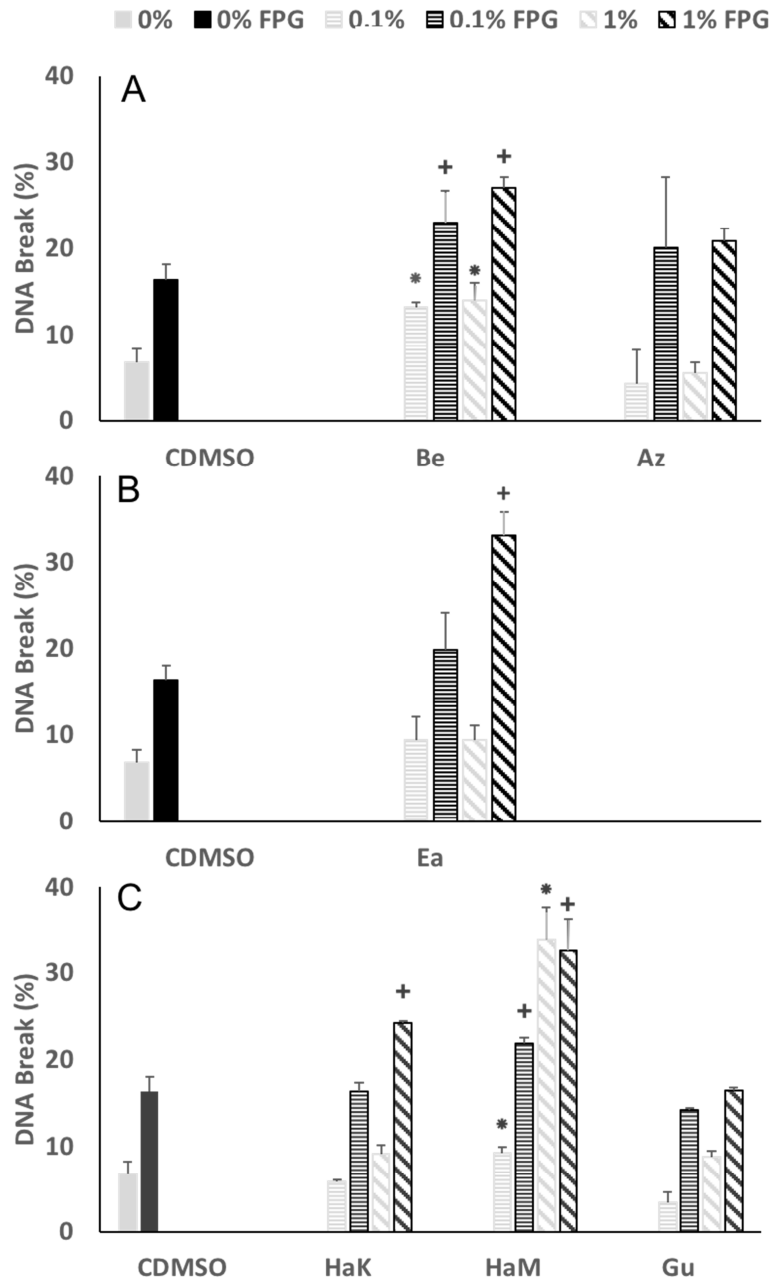


325

326 *Figure 2: DNA damage on RTL-W1 cells after exposure to organic extract of virgin microplastics (C-) and*  
 327 *B[a]P coated microplastics (C+) or to DMSO only (C<sub>DMSO</sub>) with and without addition of the repair enzyme*  
 328 *Fpg (mean ± SD; n=3). Significant differences compared to control \* for classic comet assay or + for Fpg*  
 329 *modified assay (p<0.05).*

330

331 For North Atlantic MP samples (Fig. 3A), no genotoxicity was detected with Azores MP extracts, but a  
 332 significant increase in DNA damage was observed with Bermudes MPs at any proportion of extract  
 333 tested with and without Fpg treatment. After exposure to MP extracts from Easter Island (Fig.3B),  
 334 DNA damage was observed but only at the highest concentrations tested (1%) in presence of Fpg.  
 335 When using the Fpg digestion step, DNA strand breaks reached 34.1% compared to 16.3% in the  
 336 control. For North Pacific samples (Fig. 3C), no significant variation of DNA breaks was detected after  
 337 exposure to extract of MPs from Guam. For MPs collected in Hawaii (HaK), a statistically significant  
 338 increase in DNA damage was only highlighted for 1% of MPs extract in presence of the repair enzyme  
 339 Fpg. However, higher genotoxicity of MPs from Hawaii (HaM) was detected at any of the extract  
 340 concentrations tested, both with and without the use of Fpg.



341

342 Figure 3: DNA damage on RTL-W1 after exposure to organic extract of environmental microplastics from  
 343 A-North Atlantic beaches (Be and Az); B- South Pacific beaches (Ea); C- from North Pacific beaches (HaK,  
 344 HaM and Gu); with and without addition of the repair enzyme Fpg (mean  $\pm$  SD; n=3). Significant difference  
 345 between treatment and control (CDMSO) are highlighting by \* for classic comet assay and + for Fpg  
 346 modified assay ( $p < 0.05$ ).

347 **4 Discussion**

348 **4.1 Plastic characterization**

349

350 Microplastic loads vary widely between islands and are influenced by many factors such as the  
351 tourism and population of the island, shore use, hydrodynamics and maritime activities.  
352 Interpretation of temporal trends is complicated by seasonal changes in the flow rate of rivers,  
353 currents, wave action, winds, tides, etc. For example, the high abundance of microplastics on Easter  
354 Island, previously reported in a recent study (Hidalgo-Ruz et Thiel, 2013), likely results from the  
355 transport of plastic particles through the oceanic current system to the South Pacific Subtropical  
356 Gyre, a region of accumulation of debris and microplastics (Lebreton et al, 2012). All sampled  
357 beaches present microplastics comprising both fragments and pellets. Most items are fragments  
358 except in Easter Island where a high abundance of pellets (47%) was found. This type of plastic pellet  
359 is known to be abundant on beaches in areas near plastic manufacturing factories and cargo loading  
360 docks. However, to our knowledge, there is no plastic production plant next to the study site and the  
361 contribution of South America to primary microplastics release to the world ocean is low (9.1%)  
362 (Bouchet et Friot, 2017). One can think of a spontaneous accidental spillage due to close shipping  
363 routes.

364 MPs collected on beaches were mostly made up of polyethylene (PE) in the North Atlantic and South  
365 Pacific islands. In the North Pacific islands, PE and PP were both represented. In fact, PE was reported  
366 to be the most abundant polymer in plastic litter worldwide (Hidalgo-Ruz et al., 2012; Karthik et al.,  
367 2018; Cheang et al., 2018), followed by PP and PS. Nylon, polyester, acrylic, and other polymers were  
368 found less frequently (Ziccardi et al., 2016). Plastic density influence also localization of microplastics  
369 in the water column. Indeed, some plastics have a density above 1 (e.g., PS, PVC,...) and therefore  
370 sink near point sources and are found mostly in sediment. Other plastics have a density below 1 (e.g.,  
371 PE, PP), float in water column (Hidalgo-Ruz et al., 2012), and are consequently the most abundant  
372 polymer reported in beached MPs.

373 In our study, the PAH concentrations in mesoplastics ranged from 2 ng.g<sup>-1</sup> to 70 ng.g<sup>-1</sup>. Phenantrene,  
374 pyrene and fluoranthene were the most abundant PAHs. The rate of accumulation of this kind of

375 pollutant depends on different factors, such as polymer type, particle surface area, physical and  
376 chemical properties of plastic, weathering, and biofilm formation. Concentration and occurrence of  
377 hydrophobic organics chemicals (HOCs) were also linked with chemical exposure throughout the  
378 particle's drift history (Rochman, 2015). The age of plastics can also influence the accumulation of  
379 chemicals. Indeed, the majority of HOCs will be at equilibrium after 2 years and more in sea. Since 80  
380 to 90 % of the plastics of marine litter are older than 2 to 4 years, they will be at or close to sorption  
381 equilibrium for all HOCs including additives and plasticizers (Koelmans et al., 2016). In addition to  
382 beach characteristics, pollutants sorption could explain difference of toxicity between beaches.  
383 Measured chemical concentrations on **plastic debris** from beaches in this study are in the same range  
384 as hydrophobic organic chemicals (HOCs) sorbed to plastics from other studies: from less than  
385 nanograms per gram to milligrams per gram (Ziccardi et al., 2016). On PE particles from the North  
386 Pacific Gyre, Rios et al (2010) found concentration of  $\Sigma$ PCBs from detection limit ( $0.15 \text{ ng.g}^{-1} \text{ dw}$ ) to  
387  $2058 \text{ ng.g}^{-1} \text{ dw}$  and  $\Sigma$ PAHs from detection limit ( $0.8 \text{ ng.g}^{-1} \text{ dw}$ ) to  $1728 \text{ ng.g}^{-1} \text{ dw}$ . These MPs were  
388 collected in surface seawater inside the "eastern garbage patch" (Rios et al., 2010). Ogata et al,  
389 (2009) found PCBs concentration (sum of 13 PCB congeners (CB66, 101, 110, 149, 118, 105, 153, 138,  
390 128, 187, 180, 170, 206) between 5 and  $453 \text{ ng.g}^{-1}$  of pellet according to world different beaches.

391 The same polymer composition and chemical contamination of MPs has previously been observed on  
392 Portuguese beaches with presence of PAHs, PCBs and DDT at the surface of MPs mostly composed of  
393 PP and PE (Frias et al., 2010). **The sorption and desorption of pollutants from MPs depend on  
394 multiple factors, including plastic composition and properties, plastic aging and degradation,  
395 molecular interactions between polymer and chemicals or competition between chemicals, but also  
396 external condition as temperature, pH, water or gut fluids composition or biofouling (Endo and  
397 Koelmans, 2016). Consequently, all of these parameters can influence MP contamination and hence  
398 MP toxicity.**

399 **4.2 MP extract toxicity assessments**

400 The marked difference in toxicity in cells between beached and virgin plastics was likely caused by  
401 additives or/and pollutants contained in the different microplastic samples and extracted by DMSO  
402 treatment. No cytotoxicity was observed on RTL-W1 exposed to MP extracts suggesting no acute  
403 toxicity of these chemical compounds.

404 The virgin plastics (C-) extract, a negative control, caused no effect on RTL-W1 cells. All marketed  
405 plastics contain polymer and several plastic additives (Wright et al., 2013) with the aim of modifying  
406 color or properties, to provide better resistance to heat, light degradation, flame and aging, and to  
407 improve performance of polymer (Murphy, 2001). These additives could be organic compounds as  
408 phthalates and phenol (bisphenol, nonylphenol) but also inorganic as barium (Ba) or zinc (Zn) (Fries  
409 et al., 2013; Koelmans et al., 2014). The majority of these plastic additives (PAs) are known to have  
410 toxic effects including endocrine disruption (Andrady, 2011). It has been documented that  
411 concentration levels of additive chemicals are relatively high in new plastics compared to aged ones  
412 found in marine litter. This imply the potential leaching or degradation of additives during use or  
413 after disposal of plastic (Rani et al., 2017). In the present study, the absence of virgin microplastic  
414 toxicity on RTL-W1 cells could be explained either by the additive composition of the virgin plastics,  
415 or by a low extraction of plastic additives depending on the protocol used. It is also possible that the  
416 extract concentration in PAs was too low to cause toxic effects on RTL-W1 cells. **Recent publications**  
417 **have documented low or little acute toxicity of virgin microplastics on marine organisms (Beiras et**  
418 **al., 2018; Jovanovic et al., 2018) and especially on fish cells (Espinosa et al., 2018) but those studies**  
419 **mainly investigated the direct contact with virgin MP particles and not with the MP extracts.**

420 MPs coated with B[a]P (C+), exhibited high toxicity on rainbow trout cells. Similar values of EROD  
421 activity and DNA damage were observed after exposure of RTL-W1 to B[a]P alone (Kienzler et al.,  
422 2012; Pannetier et al., 2017, 2018a). These results suggest a good desorption of B[a]P artificially  
423 coated on virgin MPs as determined with chemical analysis (176 µg B(a)P/g microplastic). The use of

424 negative and positive controls was useful to validate extraction protocols and conditions of the  
425 bioassay.

426 The marked difference in toxicity between environmental MPs and virgin plastics was likely caused  
427 by different contaminants and concentrations from both types of plastics. Overall, environmental  
428 samples of MPs showed very contrasted toxicity responses. Little or no toxicity was detected after  
429 exposure to Az and Gu samples. Conversely, HaK, HaM, Be extracts exhibited a high toxicity.  
430 Intermediate toxicity was observed for Ea extracts. HaK, HaM, Be and Ea extracts seemed to cause  
431 oxidative damages. This was suggested by high DNA breaks increased in the presence of the repair  
432 enzyme, Fpg, which is known to highlight DNA breaks cause by oxidative stress (Kienzler et al., 2012).  
433 Oxidative stress have been observed in previous study after exposure to MPs of worms (Browne et  
434 al., 2013) and mussel (Avio et al., 2015) but not on fish (Oliveira et al., 2013). In 2016, Gandara e Silva  
435 et al showed the effects of leachate from both virgin and beached pellets on brown mussel embryos.  
436 In this study, toxicity of beached pellets was much higher than that of virgin pellets.

437 We can hypothesize that contaminants adsorbed onto the surface of beached pellets were  
438 responsible for the high toxicity of leachate from beached pellets, while the toxicity of leachate from  
439 virgin pellets was mainly due to plastic additives.

440 MP toxicity can be associated with hydrophobic organic compounds (HOC) sorbed into plastics and  
441 plastic additives. Physical and chemical properties of different types of plastics leads to different  
442 affinities with contaminants present in the environment (Fries and Zarfl, 2011; Rochman et al., 2013).  
443 According to Rochman et al. (2013), PAHs and PCBs are mostly sorbed on PE and PP surface. PE in  
444 marine litter is known to sorb greater amounts of PAHs and PCBs than other plastics including PP and  
445 polyvinyl chloride (PVC) (Claessens et al., 2015; Koelmans et al., 2014). Apart from HOCs, plastic  
446 additives, could have harmful effects on aquatic organisms because they are labile in the plastic and  
447 therefore leak out into the environment during MP aging. However, depending on the plastic  
448 manufacturer, identical plastic polymers may result in drastically different levels of potential toxicity,

449 as the plastic additives incorporated in different types of plastic are different, their exact chemical  
450 make-up is generally unknown (Hamlin et al., 2015). The extract from beached debris made of  
451 different types of plastic polymers may therefore result in a very complex mixture of contaminants.  
452 **In this study, even if organic pollutants in environmental MP extracts have not be quantified, results**  
453 **obtained on positive control suggests a high efficiency of the extraction method.** MP toxicity on RTL-  
454 W1 is also related to geographical area. MPs collected in North Pacific beaches were more toxic than  
455 those sampled on North Atlantic beaches, followed by those from the South Pacific. This toxicity  
456 gradient can be correlated with the greater amounts of macro and microplastics collected on the  
457 beaches in each zones. Indeed, beaches in the North Pacific were the most heavily contaminated  
458 with plastics. This could be explained by the effects of localization. The North Pacific has experienced  
459 a dramatic increase in meso and macro-plastics since the 1990s (Barnes et al., 2009). Particularly high  
460 levels of microplastic contamination were recorded on sandy beaches in Korea (Lee et al., 2015). Law  
461 et al., (2014) observed an accumulation zone (25 to 41°N, 130 to 180°W) in the North Pacific  
462 subtropical gyre where maximum plastic concentrations from individual surface exceeded  $10^6$   
463 pieces/km<sup>-2</sup>. They highlighted the direct influence of surface currents and winds on plastic  
464 distributions but also on plastic drift history and transportation time. Both of these factors can also  
465 influence chemical sorption on MP surfaces. Density of microplastics is another factor determining  
466 the type and amount of MP particles arriving on beaches (Law et al., 2010). Seasonal and temporal  
467 trends in MP deposits on beaches were also highlighted (Barnes et al., 2009). Overall, the abundance  
468 and distribution of microplastics on beaches were influenced by local wind and currents conditions,  
469 coastline geography and point of entry of plastic into the system as urban area, and trade routes  
470 (Barnes et al., 2009). All of these parameters can influence chemical sorption and desorption on  
471 microplastics, therefore also affecting MP toxicity.

472

## 473 **5 Conclusion**



474 This study demonstrates that extracts of MPs from Pacific and Atlantic islands can be toxic depending  
475 on their location. Results obtained here support that fish cell lines such as RTL-W1 can be an  
476 interesting tool to assess the potential chemical toxicity of MPs. **Indeed**, marine plastic debris is  
477 associated with a cocktail of chemicals. **Even if**, the role of plastics in the transport of HOCs to  
478 organisms may be relatively low compared with other pathways, additional work is needed to  
479 understand the relative impact of microplastics in various environments (Ziccardi et al., 2016). The  
480 cell-line approach would appear to be a relevant alternative tool, making it possible to study a large  
481 number of samples at different concentrations to obtain information about the toxicity of  
482 environmental samples of MPs. This type of prescreening on cell-lines could be combined with a  
483 toxicity assay on whole organisms, including fish at different life stages (Pannetier et al., 2018b). This  
484 study demonstrated the presence of organic pollutants at the surface of microplastics from different  
485 Pacific and Atlantic island, as well as demonstrating the toxicity of those pollutants to fish cells.

## 486 **6 Acknowledgements**

487 The authors are very grateful to Dr. L.E.J. Lee and Dr. N.C. Bols (University Wilfrid Laurier, Waterloo,  
488 Canada) for allowing us to hold the RTL-W1 cell line for research purposes. We gratefully  
489 acknowledge Dr. Camille Lacroix (Cedre, Brest, France) for the chemical analysis of the plastic  
490 positive control. Pauline Pannetier received a Ph.D fellowship from the French Ministère de  
491 l'Enseignement Supérieur et de la Recherche. The authors would like to thank James Emery for  
492 providing English proofreading services.

493

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701

**Big Island,  
Hawaii (HaK)**

EROD: +++  
DNA breaks: +

**Oahu,  
Hawaii (HaM)**

EROD:  $\emptyset$   
DNA breaks: +++

**Bermuda (Be)**  
EROD: +  
DNA break: ++

**Azores (Az)**  
EROD: +  
DNA breaks:  $\emptyset$

**Guam (Gu)**  
EROD: ++  
DNA breaks:  $\emptyset$

**Easter Island (Ea)**  
EROD: +  
DNA breaks: +

