

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

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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⁻¹). 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.

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

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 µm 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 to $10^5 \cdot 10^6$ times higher in MPs than in the water column: 117ng.g-pellet⁻¹ and 499pg.l⁻¹ respectively 65 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).

While the sorption of chemicals from seawater to plastic particles has been clearly demonstrated (Law, 2017) only a handful of studies have been carried out on the toxic effect of the mixture of pollutants present on environmental MPs surface on fish. Today, there are a number of economic, scientific and ethical reasons for supporting efforts to develop and apply *in vitro* assays in aquatic ecotoxicology as alternative tools to animal testing (Castaño et al., 2003). Utilization of fish cell cultures is being considered as an alternative to whole fish assays in the testing of aquatic pollutants.
Cell lines provide a specific screening tool that is easy, fast, and cost-effective. Useful results can be
obtained from testing substances in small volumes. RTL-W1 (Lee et al., 1993), a cell line from rainbow
trout liver, has been widely used for the measurement of different toxicity endpoints including
cytotoxicity (Dayeh et al., 2005), genotoxicity (Brinkmann et al., 2014; Kienzler et al., 2012), EROD
activity (Hinger et al., 2011; Lee and Steinert, 2003) and reactive oxygen species (ROS) production
(Pietsch et al., 2011).

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

The cell line used in this study was a fibroblast-like non transformed permanent cell line derived from the liver of adult of rainbow trout, *Oncorhynchus mykiss* : RTL-W1 (Lee et al., 1993). This sensitive tool is used for assessing the toxic potentials of chemicals due to its high biotransformation capacity. Cells were cultured routinely in 75 cm² culture flasks (Cell start $^{\circ}$ cell culture Flask Greiner) at 20°C in CO₂ free incubators. Growth medium was composed of Leibovitz's (L-15) medium supplemented with 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 TrypLETM 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.

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

Microplastics (<5mm) were collected on a sandy area of 50cm x 50cm x 10cm deep. The first step in extracting debris was to mix and shake 0,025m³ of sand with 1L of sea water, based on the Microplastic Sampling Protocol of the National Oceanic and Atmospheric Administration (Lippiatt et al., 2013). The floating microparticles on the water surface were recovered with a 300 µm sieve. Samples were stored in a 180 mL plastic buckets (PP) until analysis at ambient temperature.

131 2.2.2 Control samples

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    A virgin plastic control (C-) was made with newly purchased laboratory plastic items grinded with a
    mortar or electric crusher (10 min) and sieved on 600 μm sieve. This control was made up of Low
    Density PolyEthylene (LDPE, 40%), High Density PolyEthylene (HDPE, 25%), PolyPropylene (PP, 25%)
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and PolyStyrene (PS, 10%) according to environmental sample of MPs from Swiss lakes (Faure et al.,2015).

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

143 2.2.3 Preparation of organic extracts of MPs

Before extraction, environmental MP samples were grinded with mortar (10 minutes) and sieved to obtain microparticles measuring under 600 µm in the same way than virgin plastic control. Environmental or control MPs (100 mg) were added to an amber flask with 1mL of dimethyl sulfoxide (DMSO). After 16h of shaking (175rpm), the solid part was separated from the liquid phase (extract) with extra-long sterile tip. The extract was used in toxicological assays and conserved between 1 and 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 μ 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

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

163 2.4 Chemical analysis

164 2.4.1 Beach fingerprint

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

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

- PCB: the injector temperature 270°C and transfer line was 300°C. The column temperature
 was programmed from 140°C (1 min) to 240°C (1 min), at a rate of 6°C min⁻¹.
- PAH: the injector temperature 300°C and transfer line was 300°C. The column temperature
 was programmed from 40°C (2 min) to 100°C (2 min), at a rate of 30°C min⁻¹ and from 100°C
 to 300°C (3 min), at a rate of 10°C min⁻¹.
- DDT: the injector temperature 260°C and transfer line was 300°C. The column temperature
 was programmed from 160°C (1 min) to 240°C (2 min), at a rate of 6°C min⁻¹. The carrier gas
 was helium (ultra-high purity), with a constant flow rate of 1.2 mL min⁻¹.
- 193 Results were expressed as pollutant concentration $(ng.g^{-1}) \pm Instrumental incertitude.$
- 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 (naphathalene 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 ng analytes.g⁻¹ plastic. 205

206 2.5 Toxicological analysis

207 2.5.1 Cell line exposure conditions

Cells were seeded in 24- (Comet assay) or 96-well plates (MTT test and EROD activity) at $2x10^5$ cells.mL⁻¹. After 24h of culture in total medium, cells were exposed in triplicate to different concentrations of organic MP organic extract. For the EROD and MTT assays, cell lines were exposed to a serial ½ dilutions from 1% to $3x10^{-5}$ % of MPs organic extracts. For the comet assay, only two concentrations were tested 0.01% and 0.1% of MPs extract. Two controls were performed for each assay: one with the maximum DMSO concentration used in the test (1%) and a control without DMSO.

215 2.5.2 Cytotoxicity

Cell cytotoxicity was measured using the MTT (3(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2Htetrazolium bromide)) assay. After cell exposure, the medium was replaced by a serum free medium containing 10% MTT solution at 5mg.mL⁻¹ in ultrapure water (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich) for 1h in the dark. The medium was discarded prior to the addition of acidified isopropanol solution (4% 1N HCl, 15min of dark incubation) to dissolve the formazan. The absorbance was read at 540 nm and was expressed as the percentage of mean absorbance (n = 3) 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 ($\lambda_{\text{excitation}}/\lambda_{\text{emission}}$). 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⁻¹ in acetone (Sigma aldrich) were

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

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

With:
x= Sample EROD activity in pmol/min/mg of protein
T= Mean of control activity (T or TDMSO) activity in pmol/min/mg of protein

239 2.5.4 *Comet assay*

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

246 2.6 Statistical analysis

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

253 **3 Results**

254 3.1 Abundance and characterization of microplastics

The microplastic abundance is shown in Table 2. High variability of microplastic both by number and by weight was observed along the different beaches. The highest accumulation of MP occurred in Azores and Easter Island beaches (4043 particles; 54.0 g/m² and 4816 particles; 54.7 g/m² respectively). Long Bay Beach in Bermuda and Pago Bay in Guam showed a concentration of 5.6 g/m² and 5.9 g/m², respectively. The two sampled beaches in Hawaii were surf spot and wild and have the 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

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

5 8 1 11 1	22 2 4						
	Others	-	-	0.03	-	-	-
	Foams	-	-	-	0.31	-	-

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 concentration of PAHs was found in Long Bay Beach in Bermuda (Be) with 70±14 ng.g⁻¹. PAHs 278 279 contamination gradient among the beaches was Be>>HaK>Gu=HaM>Ea. The concentration of total 280 PCBs was under quantification limits for every samples. The measured DDT was under quantification limits excepted for HaM. On this beach, plastic DDT concentration was 10±3 ng.g⁻¹. 281

282

283

	Sample	PAHs (ng.g ⁻¹)	PCBs (ng.g ⁻¹)	DDT p,p' (ng.g ⁻¹)
North Atlantic	Az	ND	ND	ND
NOT LI ALIAILIC	Ве	71.0 ± 14.0	<5.0	<4.0
South Pacific	Ea	2.0 ± 1.0	<5.0	<4.0
	Gu	6.0 ± 2.0	<5.0	<4.0
North Pacific	HaM	6.0 ± 2.0	<5.0	10.0 ± 3.0
	НаК	18.0 ± 4.0	<5.0	<4.0

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

ND: No data

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 μ g.g⁻¹ of MP. Measured concentration of B(a)P was 172 μ 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

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

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.



Figure 2: DNA damage on RTL-W1 cells after exposure to organic extract of virgin microplastics (C-) and B[a]P coated microplastics (C+) or to DMSO only (C_{DMSO}) with and without addition of the repair enzyme Fpg (mean ± SD; n=3). Significant differences compared to control * for classic comet assay or + for Fpg 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.



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

347 4 Discussion

348 4.1 Plastic characterization

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 contribution of South America to primary microplastics release to the world ocean is low (9.1%) 361 362 (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.

In our study, the PAH concentrations in mesoplastics ranged from 2 ng.g⁻¹ to 70 ng.g⁻¹. Phenantrene,
 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 Pacific Gyre, Rios et al (2010) found concentration of Σ PCBs from detection limit (0.15 ng.g⁻¹ dw) to 386 2058 ng.g⁻¹ dw and Σ PAHs from detection limit (0.8 ng.g⁻¹ dw) to 1728 ng.g⁻¹ dw. These MPs were 387 collected in surface seawater inside the "eastern garbage patch" (Rios et al., 2010). Ogata et al, 388 389 (2009) found PCBs concentration (sum of 13 PCB congeners (CB66, 101, 110, 149, 118, 105, 153, 138, 128, 187, 180, 170, 206) between 5 and 453 ng.g⁻¹ of pellet according to world different beaches. 390

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

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

MPs coated with B[a]P (C+), exhibited high toxicity on rainbow trout cells. Similar values of EROD
activity and DNA damage were observed after exposure of RTL-W1 to B[a]P alone (Kienzler et al.,
2012; Pannetier et al., 2017, 2018a). These results suggest a good desorption of B[a]P artificially
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 the425 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.

We can hypothesize that contaminants adsorbed onto the surface of beached pellets were
responsible for the high toxicity of leachate from beached pellets, while the toxicity of leachate from
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 10⁶ pieces/km⁻². They highlighted the direct influence of surface currents and winds on plastic 463 464 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.

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