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

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Abstract

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

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

1 Introduction

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

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

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

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

2 Materials and methods

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 cm^2 culture flasks (Cell start ® cell culture Flask Greiner) at 20°C in CO_2 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.

Table 1: Location and characteristics of the sampled beaches

	Sample	GPS Coordinates	Place	Main use	Cleaning frequency
North Atlantic	**Az**	N 38°31,407 W 02°83,753	Porto Pim beach, Faial Island, Portugal	Touristic	Between 1 time/day to 1time/week
	Be	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
North Pacific	**Gu**	N 13°25,258 E 144°47,028	Pago Bay, Guam, USA	Recreational activities	1 time/month
	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 0,025m^3 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.

2.2.2 Control samples

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%)

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 250$\mu g.g^{-1}$. Positive and virgin control samples were conserved at 20°C in amber flask until analysis.

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.

2.3 Microplastic composition

Before to be grind and sieve for toxicological analysis, type and composition of MPs were analyzed. In the laboratory, the samples were sieved to separate debris into fractions of different sizes, > 1 mm (large microplastics) and > 300 µm (small microplastics). Large microplastics were identified visually and sorted manually according to their appearance, characteristics and possible origin. Small microplastics were sorted using a stereomicroscope (Leica EZ4W, Germany). All sorted particles were then dried for 24h at ambient temperature. Plastic fractions were listed in seven categories: foams, thin films, textile fibers, lines as fishing lines, microbeads, preproduction pellets and hard fragments 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.

2.4 Chemical analysis

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.

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

1) 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}.
2) 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}.
3) 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}.

Results were expressed as pollutant concentration ($ng.g^{-1}$) ± Instrumental incertitude.

2.4.2 Quantification of organic pollutants in extract

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

2.5 Toxicological analysis

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.

2.5.2 *Cytotoxicity*

Cell cytotoxicity was measured using the MTT (3(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2H-tetrazolium 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.

2.5.3 *EROD activity*

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

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).

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 $p<0.05$.

3 Results

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^2 and 4816 particles; 54.7 g/m^2
258 respectively). Long Bay Beach in Bermuda and Pago Bay in Guam showed a concentration of 5.6 g/m^2
259 and 5.9 g/m^2, 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*

		North Atlantic		South Pacific	North Pacific		
	Sample	**Az**	**Be**	**Ea**	**Gu**	**HaM**	**HaK**
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
Polymer type (% mass)	**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	-	-
Shape (% mass)	**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.

3.2 Chemical analysis

Chemical analysis was carried out on mesoplastics collected from five islands (Bermuda, Easter Island, two Hawaii islands and Guam). Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) were analyzed in plastic fragments collected from every island (Table 3). Detail of PAHs composition was described in supplementary 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 contamination gradient among the beaches was Be>>HaK>Gu=HaM>Ea. The concentration of total 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 $ng.g^{-1}$.

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

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

ND: No data

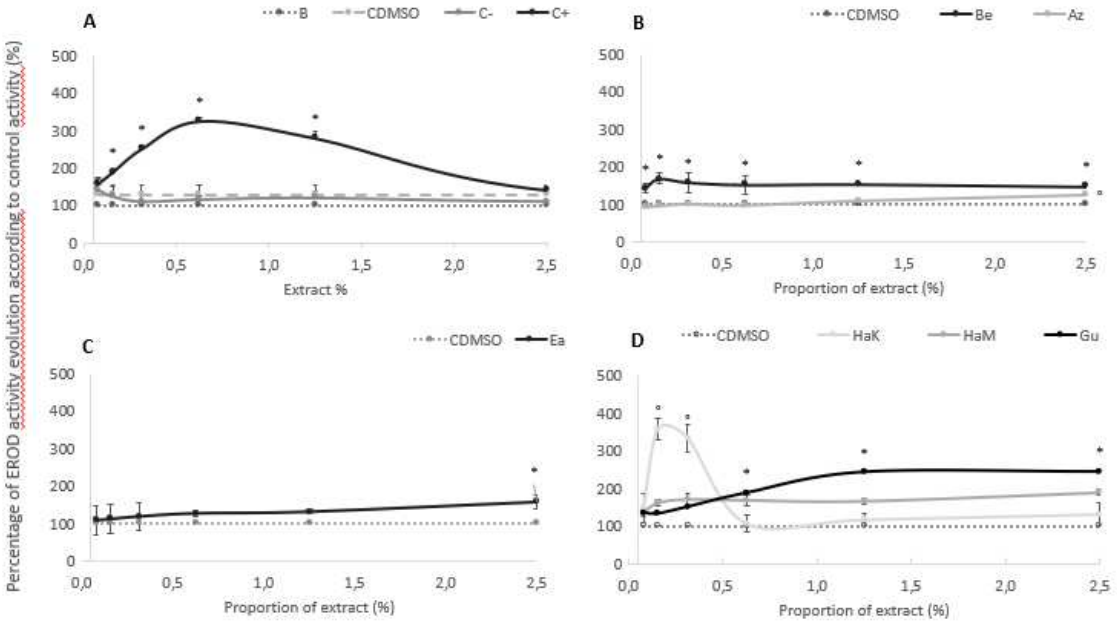
Due to limited amount of MPs collected in beaches (<1g), chemical analysis of MP extracts was not performed. However, chemical analysis of positive control extract was done. Expected concentration of B(a)P in the positive control was 250 $\mu g.g^{-1}$ of MP. Measured concentration of B(a)P was 172 $\mu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

3.3 Toxicological analysis

3.3.1 EROD activity

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

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



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+) and*
316 *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.

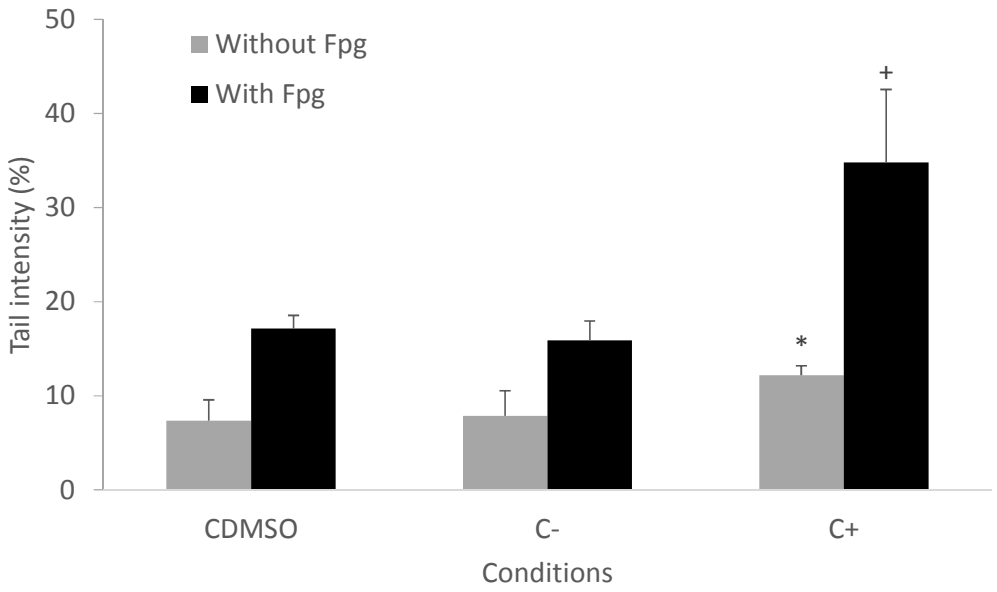


*Figure 2: DNA damage on RTL-W1 cells after exposure to organic extract of virgin microplastics (C-) and B[a]P coated microplastics (C+) or to DMSO only (C_{DMSO}) with and without addition of the repair enzyme Fpg (mean ± SD; n=3). Significant differences compared to control * for classic comet assay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

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

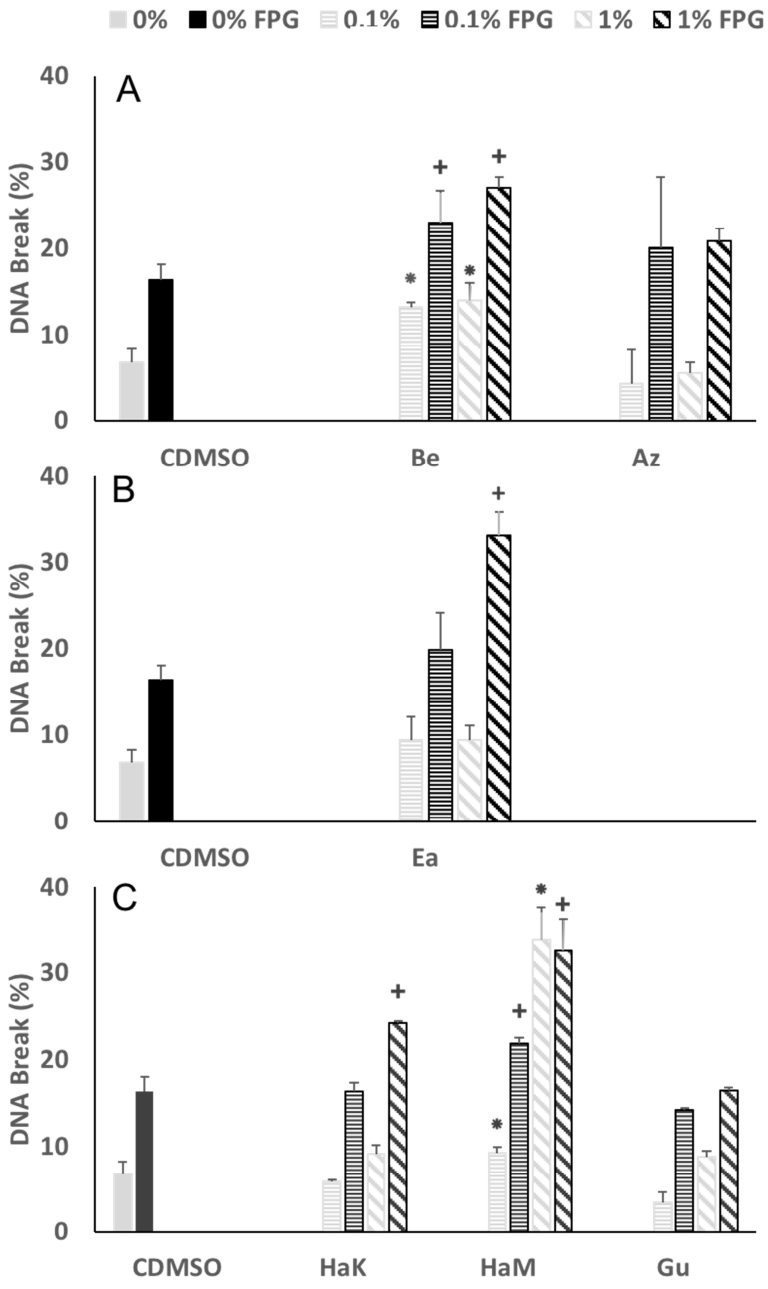


*Figure 3: DNA damage on RTL-W1 after exposure to organic extract of environmental microplastics from **A**-North Atlantic beaches (Be and Az); **B**- South Pacific beaches (Ea); **C**- from North Pacific beaches (HaK, HaM and Gu); with and without addition of the repair enzyme Fpg (mean ± SD; n=3).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

4 Discussion

4.1 Plastic characterization

Microplastic loads vary widely between islands and are influenced by many factors such as the tourism and population of the island, shore use, hydrodynamics and maritime activities. Interpretation of temporal trends is complicated by seasonal changes in the flow rate of rivers, currents, wave action, winds, tides, etc. For example, the high abundance of microplastics on Easter Island, previously reported in a recent study (Hidalgo-Ruz et Thiel, 2013), likely results from the transport of plastic particles through the oceanic current system to the South Pacific Subtropical Gyre, a region of accumulation of debris and microplastics (Lebreton et al, 2012). All sampled beaches present microplastics comprising both fragments and pellets. Most items are fragments except in Easter Island where a high abundance of pellets (47%) was found. This type of plastic pellet is known to be abundant on beaches in areas near plastic manufacturing factories and cargo loading 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%) (Bouchet et Friot, 2017). One can think of a spontaneous accidental spillage due to close shipping routes.

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

pollutant depends on different factors, such as polymer type, particle surface area, physical and chemical properties of plastic, weathering, and biofilm formation. Concentration and occurrence of hydrophobic organics chemicals (HOCs) were also linked with chemical exposure throughout the particle's drift history (Rochman, 2015). The age of plastics can also influence the accumulation of chemicals. Indeed, the majority of HOCs will be at equilibrium after 2 years and more in sea. Since 80 to 90 % of the plastics of marine litter are older than 2 to 4 years, they will be at or close to sorption equilibrium for all HOCs including additives and plasticizers (Koelmans et al., 2016). In addition to beach characteristics, pollutants sorption could explain difference of toxicity between beaches. Measured chemical concentrations on plastic debris from beaches in this study are in the same range as hydrophobic organic chemicals (HOCs) sorbed to plastics from other studies: from less than 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 ΣPCBs from detection limit (0.15 $ng.g^{-1}$ dw) to 2058 $ng.g^{-1}$ dw and ΣPAHs from detection limit (0.8 $ng.g^{-1}$ dw) to 1728 $ng.g^{-1}$ dw. These MPs were collected in surface seawater inside the "eastern garbage patch" (Rios et al., 2010). Ogata et al, (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.

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

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,

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

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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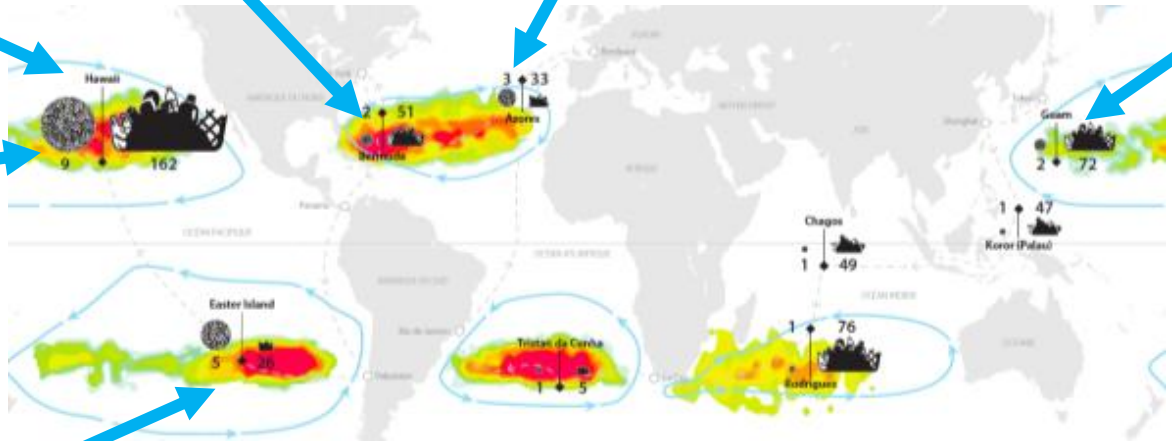
Big Island, Hawaii (HaK) EROD: +++ DNA breaks: +

Oahu, Hawaii (HaM) EROD: ⌀ DNA breaks: +++

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

Azores (Az) EROD: + DNA breaks: ⌀

Guam (Gu) EROD: ++ DNA breaks: ⌀



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

