



## Are whale sharks exposed to persistent organic pollutants and plastic pollution in the Gulf of California (Mexico)? First ecotoxicological investigation using skin biopsies

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(Article begins on next page)

1 **Are whale sharks exposed to persistent organic pollutants and plastic pollution in the Gulf of**  
2 **California (Mexico)? First ecotoxicological investigation using skin biopsies.**

3

4 Maria Cristina Fossi<sup>1</sup>, Matteo Baini<sup>1</sup>, Cristina Panti<sup>1</sup>, Matteo Galli<sup>1</sup>, Begoña Jiménez<sup>2</sup>, Juan Muñoz-  
5 Arnanz<sup>2</sup>, Letizia Marsili<sup>1</sup>, Maria Grazia Finoia<sup>3</sup>, Dení Ramírez-Macías<sup>4</sup>

6 <sup>1</sup>Department of Physical, Earth and Environmental Sciences, University of Siena, Siena, Italy,

7 <sup>2</sup>Department of Instrumental Analysis and Environmental Chemistry. Institute of Organic Chemistry  
8 (IQOG-CSIC). Juan de la Cierva 3, 28006 Madrid, Spain

9 <sup>3</sup>ISPRA, Institute for Environmental Protection and Research, Via V. Brancati 48, 00144, Rome, Italy

10 <sup>4</sup>Tiburón Ballena Mexico proyecto de ConCiencia Mexico AC, La Paz, BCS, Mexico

11

12 **Corresponding author:**

13 **Matteo Baini, e-mail: [matteo.baini@unisi.it](mailto:matteo.baini@unisi.it)**

14

15

16 **ABSTRACT**

17 The whale shark (*Rhincodon typus*) is an endangered species that may be exposed to micro- and  
18 macro-plastic ingestion as a result of their filter-feeding activity, particularly on the sea surface. In  
19 this pilot project we perform the first ecotoxicological investigation on whale sharks sampled in the  
20 Gulf of California exploring the potential interaction of this species with plastic debris (macro-,  
21 micro-plastics and related sorbed contaminants). Due to the difficulty in obtaining stranded  
22 specimens of this endangered species, an indirect approach, by skin biopsies was used for the  
23 evaluation of the whale shark ecotoxicological status. The levels of organochlorine compounds  
24 (PCBs, DDTs), polybrominated diphenylethers (PBDEs) plastic additives, and related biomarkers  
25 responses (CYP1A) were investigated for the first time in the whale shark. Twelve whale shark skin  
26 biopsy samples were collected in January 2014 in La Paz Bay (BCS, Mexico) and a preliminary  
27 investigation on microplastic concentration and polymer composition was also carried out in  
28 seawater samples from the same area. The average abundance pattern for the target contaminants  
29 was PCBs>DDTs>PBDEs>HCB. Mean concentration values of 8.42 ng/g w.w. were found for PCBs,  
30 1.31 ng/g w.w. for DDTs, 0.29 ng/g w.w. for PBDEs and 0.19 ng/g w.w. for HCB. CYP1A-like protein  
31 was detected, for the first time, in whale shark skin samples. First data on the average density of  
32 microplastics in the superficial zooplankton/microplastic samples showed values ranging from 0.00

33 items/m<sup>3</sup> to 0.14 items/m<sup>3</sup>. A Focused PCA analysis was performed to evaluate a possible correlation  
34 among the size of the whale sharks, contaminants and CYP1A responses. Further ecotoxicological  
35 investigation on whale shark skin biopsies will be carried out for a worldwide ecotoxicological risk  
36 assessment of this endangered species.

37

38 **Keywords:** whale shark, plastic pollution, OCs, PBDEs, CYP1A, Gulf of California

## 39 **1 Introduction**

40 The whale shark (*Rhincodon typus*) has a circum-equatorial distribution in all tropical and warm  
41 temperate seas (Colman, 1997; Compagno, 1984). This species is epipelagic, oceanic, and coastal,  
42 forming seasonal near-shore aggregations in many areas that are related to local seasonal  
43 productivity (Rowat and Brooks, 2012; Sequeira et al., 2013). The presence and movements of whale  
44 sharks have been linked to the spawning of corals and fishes, upwelling, plankton abundance, and  
45 changes in the temperature of water masses (Heyman et al., 2001; Motta et al., 2010; Robinson et  
46 al., 2013; Wilson et al., 2001). In the late 90s, some whale shark populations declined drastically  
47 (Norman, 2005; Rowat and Brooks, 2012) and, in 2000, the species was listed as vulnerable on the  
48 IUCN Red List (Norman, 2000). In 2016, the conservation status was assessed as endangered (Pierce  
49 and Norman, 2016). This species has a k-selected life history that makes them vulnerable to  
50 exploitation such as large size, slow growth, late maturation, production of few offspring and  
51 extended longevity (Colman, 1997; Rowat and Brooks, 2012). Major threats to this species include  
52 interaction with fishing activity (direct catches and bycatch), vessel strikes, inappropriate tourism  
53 and climate change (Pierce and Norman, 2016). Furthermore, the increasing human activity in whale  
54 shark grounds gives rise to chemical pollution from urban wastewaters, vessels, agriculture and  
55 waste including plastic debris. During surface ram filter feeding, sharks swim at an average velocity  
56 of 1.1 m/s with 85% of their mouth open below the water's surface, as reported by Motta and  
57 collaborators (Motta et al., 2010). Whale sharks spend, on average, approximately 7.5 h/day feeding  
58 at the surface on dense plankton dominated by calanoid, copepods, sergestids, chaetognaths and  
59 fish larvae (Motta et al., 2010). During the feeding, the whale shark could be exposed to the  
60 ingestion of pollutants floating on the sea surface and associated to sea surface microlayer,  
61 including floating plastic debris. However, these impacts on filter feeder sharks are largely unknown  
62 (Fossi et al., 2014). Juvenile whale sharks (total length <9 m) aggregate seasonally in different areas  
63 of the Gulf of California, specifically in coastal waters of "Bahía de Los Angeles", off the north-central  
64 coast of the Baja California Peninsula (Mexico) and "La Paz Bay" off the south-eastern coast of the

65 peninsula (Ramírez-Macías et al., 2012b). Several studies have shown that most sighted  
66 aggregations are composed of juvenile male whale sharks (Meekan et al., 2006; Ramírez-Macías et  
67 al., 2012b, 2012a; Rowat and Brooks, 2012). In La Paz Bay, a high number of whale sharks aggregate  
68 to feed in a predictable manner and for long periods. In this area, the juvenile sharks have showed  
69 fidelity to the area remaining in the Bay during the season for up to 135 days and returning during  
70 the years, in a season up to 38% of the sharks can be re-sighted from previous years. This shows the  
71 importance of this habitat for juvenile sharks (Ramírez-Macías et al., 2012b). La Paz city is one of  
72 the most highly populated coastal areas in the Gulf of California and has the highest growth rate  
73 (2.6%) in the state. Boat traffic is increasing in the whale shark aggregation area with new marinas,  
74 new tourist companies and fisherman's boats. Whale shark tourist activity has also increased, with  
75 the government authorizing 109 boats in 2014. Whale sharks represent an important part of the  
76 tourist attraction, but their presence imposes also a challenge to protect them. The increasing  
77 human impact in whale shark feeding grounds in this area gives rise to urban and industrial waste  
78 waters, including macro and micro-litter.

79 Marine litter represents a serious concern for the marine environment (Eriksen et al., 2014; Kühn et  
80 al., 2015). Presence and distribution of plastic debris in the marine environment has been  
81 documented and, it is widely known, that marine debris originates from land; however, the quantity  
82 of plastic entering the ocean from mismanaged waste on land is unknown. Jambeck and  
83 collaborators calculated that out of the 275 million MT produced by 192 coastal countries in 2010,  
84 4.8 to 12.7 million metric tons (MT) entering the ocean (Jambeck et al., 2015). Along with the land  
85 based sources, other inputs from ocean-based sources include maritime traffic, fishing activities  
86 (both commercial and recreational) and aquaculture sites (Galvani et al., 2015). Among marine  
87 litter, microplastics, generally defined as fragments less than 5mm in dimension (Arthur et al., 2009)  
88 represents an emerging world-wide concern for marine organisms as a wide range of organisms,  
89 from plankton to larger vertebrates such as turtles or whales, may ingest them (Wright et al., 2013).  
90 Plastic particles can harm marine organisms, causing physical damages (Wright et al., 2013) and/or  
91 transporting POPs and partitioning plastic additives (Rochman, 2015). Due to high sorption capacity  
92 of plastics for hydrophobic organic chemicals, the chemicals can be transported by microplastics  
93 and macroplastics traveling long distances (Lee et al., 2013). Therefore, plastic debris can serve as  
94 carrier of persistent organic pollutants (POPs) in marine ecosystems (Besseling et al., 2013;  
95 Rochman et al., 2013). In addition, several plastic additives (e.g. flame retardants, stabilizers, and  
96 plasticizers) may leach out and become bioavailable to marine organisms (Rochman, 2015).

97 Despite the growing scientific attention on this issue, little scientific investigation has focused on  
98 the potential impact of micro- and macroplastics on large filter feeding marine organisms such as  
99 baleen whale and planktivorous sharks (Fossi et al., 2014; Besseling et al., 2015; Fossi et al., 2016).  
100 In particular, we lack information about inputs, spatial and temporal distributions and interactions  
101 with biota in semi-closed basins, such as the Gulf of California.  
102 In this paper, we perform the first ecotoxicological investigation on whale sharks sampled in the  
103 Gulf of California exploring the potential interaction of this species with plastic debris (macro- and  
104 micro-plastics), the levels of PBDEs and OCs and related biomarkers responses ( CYP1A) using skin  
105 biopsies as target tissue due to the lack of stranded organisms and the protected status of the whale  
106 shark. Skin biopsy samples were collected from twelve whale sharks in La Paz Bay and a preliminary  
107 investigation on microplastic concentration and polymer composition was also carried out in  
108 samples collected in the whale shark ground.

109

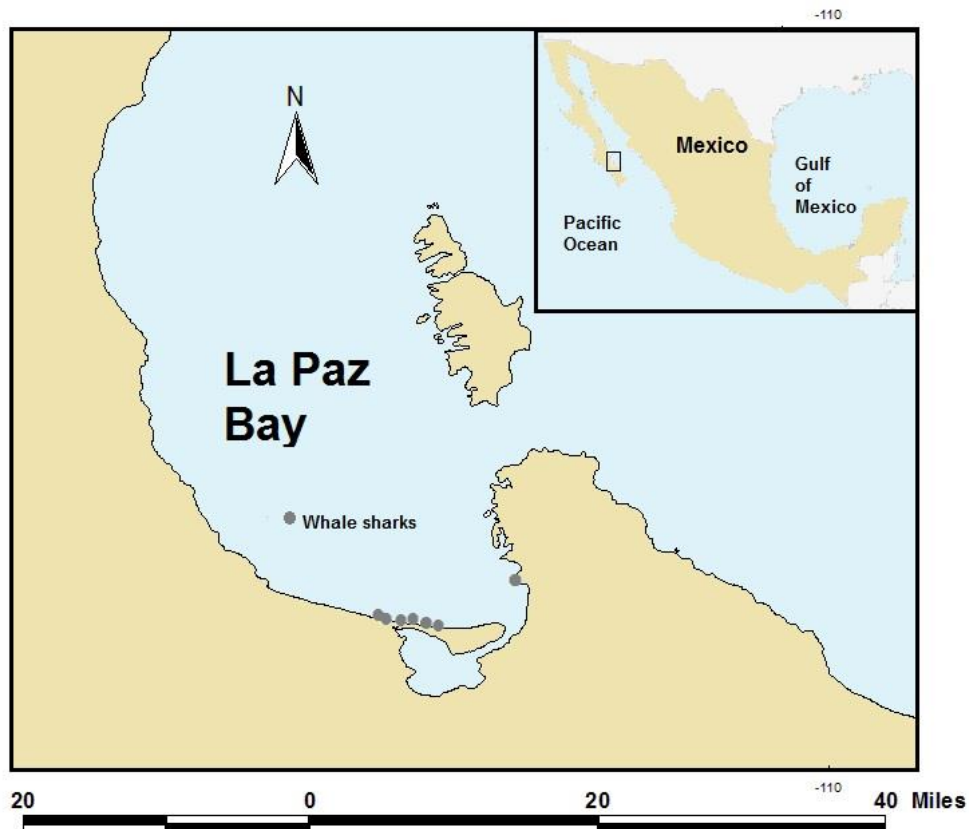
## 110 **2 Material and Methods**

### 111 **2.1 Study area and collected samples**

112 La Paz Bay is located in the south of the Gulf of California (BCS, Mexico), with shallow coastal (< 50  
113 m) and deep oceanic (>200 m) areas. Juvenile sharks aggregate to feed in the coastal waters of the  
114 bay, near to the city. Skin biopsy sample from 12 whale sharks (11 males and 1 female), ranging  
115 from 3.5 to 8 m total length, were collected on January and February of 2014, in inshore waters of  
116 La Paz Bay (Fig.1). Biopsies were sampled using biopsy tips mounted on a pole and immediately  
117 placed in liquid nitrogen in order to prevent any degradation for biomarker analysis (Ramírez-Macías  
118 et al., 2007, 2012b).

119 Each shark was geo-referenced using a Global Positioning System, and were photographed with an  
120 underwater camera for future identification. The pattern of lateral markings behind the five gill slits  
121 on the left side is unique to each individual and is an effective marker for capture-mark-recapture  
122 studies (Taylor, 1994). Scars and other present markings were also recorded. Gender was  
123 determined by the presence or absence of claspers. Total length was measured while swimming and  
124 using a metric measuring tape. The Table 1 shows the characteristics of each shark collected.

125



126  
 127 **Figure 1.** Gulf of California and La Paz Bay (BCS, Mexico), with grey spots representing juveniles whale shark  
 128 (*Rhincodon typus*) sampled.

129 **Table 1.** Size and sex of each whale shark (WS) collected in La Paz Bay (BCS, Mexico) in January and  
 130 February 2014.

Sample	Date	Sex	Size
WS 1	30/01/2014	M	5.5
WS 2	30/01/2014	M	5
WS 3	30/01/2014	M	4.5
WS 4	30/01/2014	M	4
WS 5	30/01/2014	F	5
WS 6	31/01/2014	M	3.5
WS 7	31/01/2014	M	4
WS 8	31/01/2014	M	7
WS 9	01/02/2014	M	4
WS 10	01/02/2014	M	6
WS 11	01/02/2014	M	4
WS 12	01/02/2014	M	8

## 132 **2.2 POPs determination**

### 133 **2.2.1 Sample treatment**

134 The analysis of HCB (hexachlorobenzene), DDTs (sum of dichloro-diphenyltrichloroethane (DDT) and  
135 its main metabolites), PCBs (polychlorinated biphenyls) and PBDEs (polybrominated diphenyl  
136 ethers) was carried out in the subcutaneous tissues of freeze-dried skin biopsy samples (n=12).  
137 Initially, samples were spiked with isotopic labeled surrogates of HCB, DDTs, PCBs and PBDEs  
138 (detailed list to be found at QA/QC) prior to soxhlet extraction for 24 h with a mixture of n-  
139 hexane:dichloromethane (9:1, v:v). A subsequent clean-up process was achieved by using open  
140 columns packed with neutral and acidic-modified silica gel and the same mixture of solvents as  
141 eluting agent. Final extracts were evaporated using a TurboVap® system until ~1 mL, transferred to  
142 vials, and dried under a gentle nitrogen steam. Samples were reconstituted in 20 µL of a solution of  
143 <sup>13</sup>C<sub>12</sub>-p, p'-DDT, <sup>13</sup>C<sub>12</sub>-PCB-111, 170, 178 and <sup>13</sup>C<sub>12</sub>-BDE-139 in nonane as injection/internal  
144 standards for instrumental analysis.

### 145 **2.2.2 Instrumental analysis**

146 Whale sharks biopsy samples were screened for the following compounds: HCB, six DDTs (p,p'- and  
147 o,p'-isomers of DDE, DDD and DDT), twenty ortho and mono-ortho PCB congeners (# 28, 52, 95,  
148 101, 105, 114, 118, 123, 132, 138, 149, 153, 156, 157, 167, 170, 180, 183, 189, 194) and 14 PBDEs  
149 (# 28, 47, 66, 85, 99, 100, 153, 154, 183, 184, 191, 196, 197, 209). HCB, DDTs and PCBs were  
150 quantified by gas chromatography coupled to low resolution mass spectrometry (GC-LRMS) using a  
151 7890N series gas chromatograph coupled with a 5975C quadrupole mass spectrometer (Agilent,  
152 Palo Alto, CA, USA) operated in selected ion monitoring mode with electronic impact (EI) ionization  
153 at an electron voltage of 70 eV. Quantification of the target analytes was based on the isotope  
154 dilution technique. PBDEs were quantified by GC-LRMS using a 6890N gas chromatograph coupled  
155 with a 5975 quadrupole mass spectrometer (Agilent, Palo Alto, CA, USA) with electron capture  
156 negative ionization (ECNI). Comprehensive details about instrumental methods of quantification for  
157 each group of target compounds can be found in (Muñoz-Arnanz et al., 2016).

### 158 **2.2.3 QA/QC in POPs determination**

159 Quality criteria were based on the application of quality control and quality assurance measures,  
160 which included the analysis of blank samples covering the complete analytical procedure (one  
161 procedural blank in each set of four or five samples). Accordingly, reported values for POPs were  
162 blank corrected. Special care was taken to minimize exposure to UV light throughout the whole  
163 analytical procedure. Quantification of all target analytes was carried out according to the following  
164 criteria: (a) ratio between the two monitored ions within ±15% of the theoretical value, and (b)

165 limits of quantification (LOQs) corresponding to S/N of 10. Calibration curves were checked daily.  
166 Average recoveries values (%  $\pm$  SD) for the used surrogates were: 13C6-HCB (53 $\pm$ 10), 2H8-p,p'-DDE  
167 (112 $\pm$ 13), 2H8-o,p'-DDT (107 $\pm$ 12), 2H8-p,p'-DDT (115 $\pm$ 14), 13C12-PCB-28 (78 $\pm$ 12), 13C12-PCB-52  
168 (87 $\pm$ 13), 13C12-PCB-101 (106 $\pm$ 13), 13C12-PCB-138 (109 $\pm$ 13), 13C12-PCB-153 (114 $\pm$ 13), 13C12-PCB-  
169 180 (103 $\pm$ 12), 13C12-PCB-209 (85 $\pm$ 9), 13C12-BDE-138 (92 $\pm$ 12).

170

### 171 **2.3 Cytochrome P450 1A protein determination**

172 Cytochrome P450 1A (CYP1A) protein, used in this study as marker of POPs exposure, was analyzed  
173 in the dermal part of the skin biopsies of 8 out of 12 specimens of whale shark using western-blotting  
174 (WB) techniques. Four samples were not analyzed due to their small size that could not allow  
175 performing the analysis. Semi-quantitative analysis was performed for each WB (in triplicate) with  
176 Quantity One software (Bio-Rad, 1-D Analysis Software) using Adjusted Volume (Intensity \*mm<sup>2</sup>) as  
177 quantitative parameter. Homogeneous sub-samples of biopsies were homogenized in aryl-  
178 hydrocarbon-receptor (AhR) buffer (Wilson et al., 2007) using a Tissue Lyser (Qiagen). The  
179 homogenate was centrifuged twice and the supernatant (S9) was analyzed for total proteins and  
180 then by WB. For WB analysis, S9 tissue homogenates (in duplicate) were separated by SDS-PAGE  
181 (10% polyacrylamide gels) and blotted onto nitrocellulose; the membranes were saturated with  
182 blocking solution for 1 h. Primary polyclonal antibodies were used from Biosense Laboratories AS  
183 (Norway). There are no specific antibodies for this species, for this reason a Rabbit anti-fish CYP1A  
184 peptide Polyclonal antibody (CP-226) from Biosense Laboratories AS (Norway) has been used. This  
185 product consists of rabbit polyclonal antibodies (affinity-purified IgG fraction) against peptides 190-  
186 204 and 282-296 of rainbow trout (*Oncorhynchus mykiss*) cytochrome P450 1A (CYP1A). Due the  
187 detection of CYP1A with a heterologous antibody, the protein detected has been named hereafter  
188 as CYP1A-like protein. The antibody was diluted 1:500 in TTBS-1% gelatin and it was incubated with  
189 shark proteins overnight. Incubation with goat anti-rabbit IgG HRP-labelled secondary antibody (Bio-  
190 Rad) (1:3000) was performed (1.30 h) and detected according to the Bio-Rad Immun-Star-HRP-  
191 Chemiluminescent-Kit booklet. Semi-quantitative analysis was performed for each WB with  
192 Quantity-One software (Bio-Rad) using Adjusted Volume (Intensity\*mm<sup>2</sup>) as quantitative  
193 parameter. The lane-based functions have been used to calculate molecular weights for CYP1A-like  
194 peptide with multiple regression models using as a Precision Plus Protein™ Standards (Bio-Rad).  
195 Precision Plus Protein All Blue Standards are a mixture of ten blue-stained recombinant proteins  
196 (10–250 kDa), including three reference bands (25, 50, and 75 kDa).



197 **2.4 Sampling and characterization of microplastics**

198 **2.4.1 Sampling of microplastics**

199 Microplastics samples (n=4) were collected in January and February 2014, in inshore waters of La  
200 Paz Bay, in the whale sharks feeding ground. All zooplankton/microplastic samples were collected  
201 during daylight hours and under calm weather and sea conditions. The samples were collected with  
202 a manta trawl equipped with a flowmeter to measure the volume of filtered water (m<sup>3</sup>).

203 The net was towed horizontally in surface waters at a speed of approximately 1.5 knots for 20 min.  
204 The net was washed on board and the collected sample preserved in a 4% formaldehyde-seawater  
205 buffered solution for subsequent analyses of plastic particles.

206 **2.4.2 Microplastics analysis**

207 For the analysis of plastic particles, the samples were observed under a stereomicroscope (Stereo  
208 Zoom NBS, mod. NBS-STMDLX-T) with a LED light and micrometer ocular lens for measuring the  
209 fragments of plastic. During the laboratory procedure, particular care was taken to prevent airborne  
210 contamination of samples by performing sample analysis in a clean air flow room. Microplastic  
211 collected with the manta trawl (number of items) were normalized to the total water surface filtered  
212 (S), calculated from the following formula and expressed as items/m<sup>3</sup>:  $S = N \times A \times C$ ; in which N =the  
213 number of propeller revolutions measured by the flowmeter; A is the mouth area of the net inside  
214 the water; C = a constant value, typical of each flow meter.

215  
216 **2.4.3 Polymer identification: Fourier Transform Infrared Spectroscopy**

217 The polymer composition was identified using Fourier transformed infrared (FT-IR) spectroscopy  
218 technique (Hummel, 2002). Agilent Micro Lab FTIR software was used for the output spectra  
219 elaboration. For each plastic fragment, depending on its heterogeneity, three measurements were  
220 carried out. The samples were compressed in a diamond anvil compression cell and infrared spectra  
221 were acquired using an Agilent Cary 630 spectrophotometer. Spectra were collected in transmission  
222 mode in 16 scans, with a resolution of 4 cm<sup>-1</sup>. For the identification of polymers, a similarity  
223 algorithm was used searching in three different Agilent polymer spectral databases, followed by a  
224 visual comparison analysis of characteristic bands in the reference spectrum. Only spectra matching  
225 more than 80% with reference polymers were accepted, being this minimum hit quality greater than  
226 the one adopted by (Lusher et al., 2013).

227

## 228 **2.5 Statistics on biomarker responses and contaminants**

229 Focused Principal Component Analysis (FPCA) was used to analyzed biomarker responses and  
230 contaminant levels in whale shark. This analysis allowed to show simultaneously both the  
231 correlations between the set of variables in relation to a particular variable of interest, and also the  
232 correlations within all set of variables. The graphical output of the FPCA analysis shows the  
233 correlations in graphical format as concentric circles in which those with the lowest radius,  
234 represented the highest correlations. The center of these circles (target variables) contains the  
235 variable of interest on which the analysis is "focused." The interpretation of the position of the  
236 variables within the correlation circle coincides with the interpretation of the PCA. In a specific way,  
237 if a variable of the set is closest to the center of the circle it is most correlated to the target variable  
238 in the correlation circle. The correlations among the variables contained in the set and the target  
239 variable are plotted with different colors negative (yellow) and positive (green). The correlation is  
240 considered to be significant at the level of  $p = 0.05$  when the variable is placed inside the red circle.  
241 Moreover, the FPCA can give information regarding the relation between two variables according  
242 to their reciprocal positions in the graph regardless of the color: i) a positive correlation if the  
243 variables are close, ii) a negative correlation if they are in opposite position and iii) independent if  
244 they are perpendicular to each other. In addition, a hierarchical cluster analysis by the minimum  
245 energy (E) distance method was also used to define clusters on the basis of variables, and canonical  
246 discriminant analysis on PCA factors was performed to reveal clustering variables. The significance  
247 of the analysis was tested using the Monte-Carlo test (a non-parametric version of Pillai's test) on  
248 coinertia analysis with 999 permutations (Dray et al., 2003). All statistical analyses were performed  
249 using the "ade4" (Dray and Dufour, 2007) and "energy" (Rizzo and Székely, 2010) packages of R  
250 software (R Core Team, 2015).

251

## 252 **3 Results**

### 253 **3.1 POP concentrations**

254 Table 2 summarizes POP concentrations detected in whale shark skin biopsies expressed in ng/g wet  
255 weight (w.w.) basis. It is noticeable the wide variability among the values measured (up to two  
256 orders of magnitude for PCBs and PBDEs), partially explained by unknown dissimilarities on sex, age  
257 and reproductive status of the specimens studied. The average abundance pattern for the target  
258 contaminants in skin biopsies was PCBs>DDTs>PBDEs>HCB. Mean concentration values of 8.42 ng/g  
259 w.w. were found for PCBs, 1.31 ng/g w.w. for DDTs, 0.294 ng/g w.w. for PBDEs and 0.192 ng/g w.w.

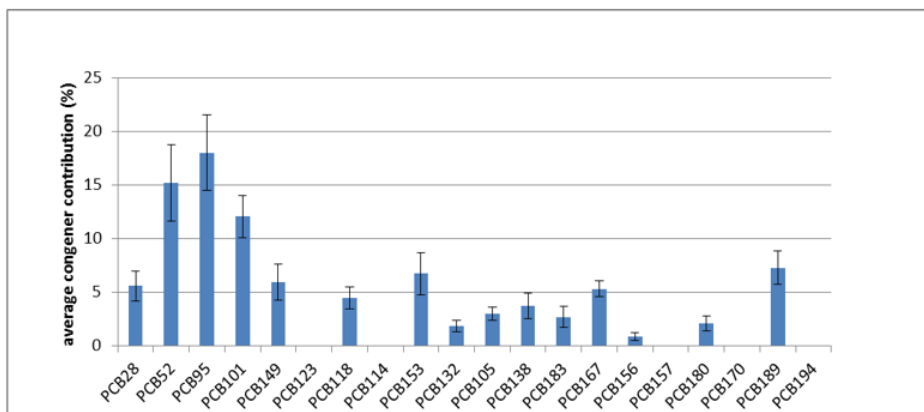
260 for HCB (Table 2). The PCB content was mostly dominated by congeners with medium-low chlorine  
 261 content such as PCB 95, 101 and 52 with contributions >10% (Fig. 2A). Other relevant contributions  
 262 (>5%) were presented by congeners 189>153>149>28>167. It is worth highlighting how this pattern  
 263 of abundance differs from what is usually reported in biotic matrices, where the most recalcitrant  
 264 PCB congeners (153, 138 and 180) made up the bulk for most PCB burdens. The relative contribution  
 265 to the total DDT content was: *p,p'*-DDT (~33%) > *p,p'*-DDE (~30%) > *o,p'*-DDT (~26%) > *p,p'*-DDD  
 266 (~7%) > *o,p'*-DDD (~3.15%) > *o,p'*-DDE (~0.06%) (Table 2). Ratios about different isomeric forms  
 267 might yield information concerning the age and origin of this pesticide (Muñoz-Arnanz and Jiménez,  
 268 2011). The average value of 1.47 obtained for ratio  $R_{p,p'/p,p'}(=[p,p'\text{-DDE} + p,p'\text{-DDD}]/[p,p'\text{-DDT}])$   
 269 seems to indicate a relative recent input of DDT in this area.

270

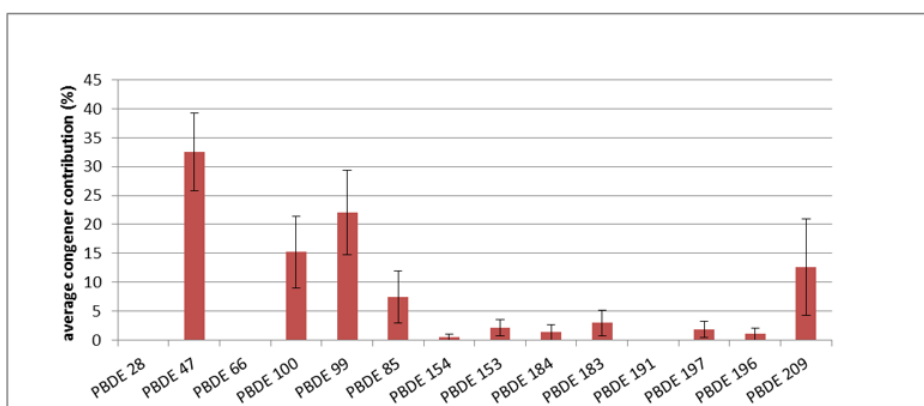
271 **Table 2.** Average, median, range for target POP contaminants in whale shark skin biopsies. Values expressed  
 272 in ng/g w.w. Cytochrome P450 1A-like (CYP1A) was expressed as Adjusted Volume Intensity\*mm<sup>2</sup>/μg  
 273 protein).

	Average	Median	Range
HCB	0.192	0.104	0.018 – 0.659
DDTs (6 isomers)	1.31	0.545	0.201 – 6.36
<i>ortho</i> PCBs (20 congeners)	8.42	4.39	0.270 – 41.4
PBDEs (14 congeners)	0.294	0.253	0.028 – 1.14
CYP1A-like	1397.25	1365.05	439.85 – 2273.39

A



B



274

275 **Figure 2. A)** Average PCB congener profile skin biopsies of whale sharks (n=12). Error bars represent standard  
276 errors (SE). **B)** Average PBDE congener profile in skin biopsies of whale sharks (n=12). Error bars represent  
277 standard errors (SE).

278 As with PCBs, the PBDE content was dominated by lower-medium brominated congeners such as  
279 47 > 99 > 100. Not surprisingly, these are examples of predominant congeners found in aquatic food  
280 webs. Unexpected, however, was the important contribution found for BDE-209 accounting for an  
281 average of 12.6% of the total PBDE burden (Fig. 2B).

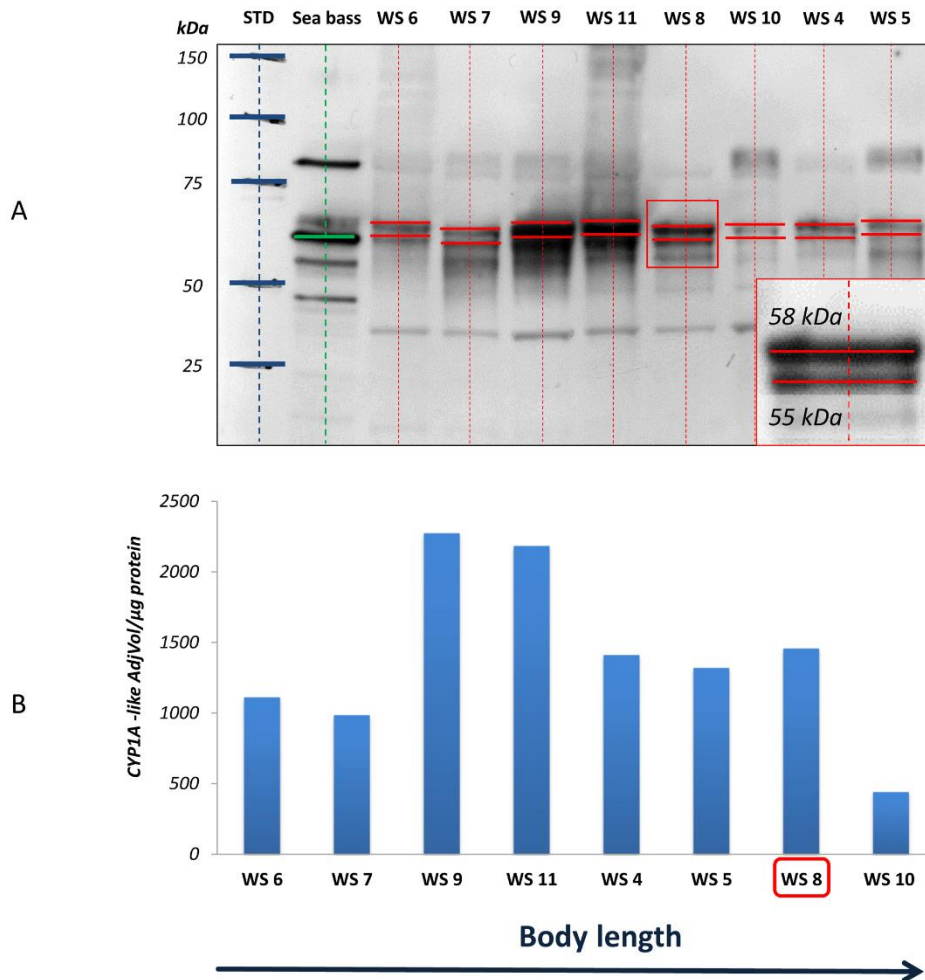
282

### 283 3.2 Western blot analysis of CYP1A-like protein

284 CYP1A-like protein was also detected for the first time, by WB techniques, in whale shark skin  
285 samples and used as biomarker of POPs exposure. The European seabass liver was used as a positive  
286 control.

287 The lane-based functions have been used to calculate molecular weights for CYP1A-like peptides,  
288 with multiple regression models using as a Precision Plus Protein™ Standards (Bio-Rad), and two  
289 possible isoforms at 58 kDa and 55 kDa were detected in whale shark skin biopsies (Fig. 3 A).

290 The semi-quantitative analysis of whale shark's CYP1A-like protein was performed with the Quantity  
 291 One software (Bio-Rad, 1-D Analysis Software) using Adjusted Volume (Intensity \*mm<sup>2</sup>) as  
 292 quantitative parameter. The Adjusted Volume ranged from 439.85 (intensity\*mm<sup>2</sup>/μg protein) in  
 293 WS10 to 2273.39 (intensity\*mm<sup>2</sup>/μg protein) in WS9 (Tab.2 and Fig 3 B).



294 **Figure 3. A)** Western blot analysis of CYP1A-like protein in skin biopsy of whale sharks (WS) (red) and  
 295 European seabass liver (green). Precision Plus Protein™ Standards (Bio-Rad) (blue). **B)** Semi-quantitative  
 296 analysis of WS's CYP1A-like protein performed with Quantity One software (Bio-Rad, 1-D Analysis Software)  
 297 using Adjusted Volume (Intensity \*mm<sup>2</sup>) in skin biopsies of males and one female (red square).  
 298  
 299

### 300 3.3 Microplastic abundance and polymer identification

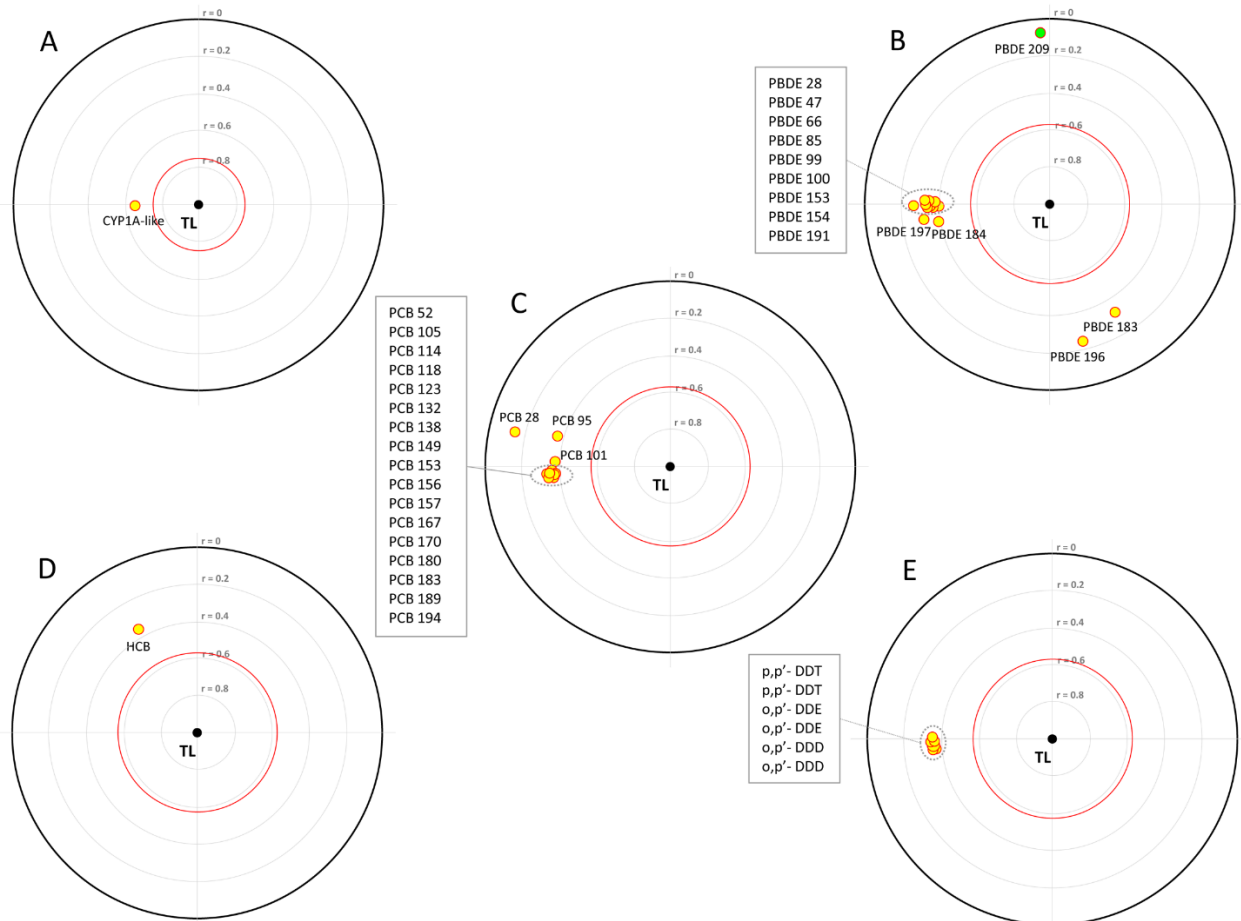
301 Preliminary investigations on the average of microplastic concentration in the superficial  
 302 zooplankton/microplastic samples collected from La Paz Bay during the whale shark sampling show  
 303 values ranging from 0.00 items/m<sup>3</sup> to 0.14 items/m<sup>3</sup>.

304 Polymer identification, revealed that the most abundant polymer detected in the samples was  
 305 polyethylene (35%) (Fig. S1).

306

307 **3.4 Focused Principal Component Analysis on CYP1A-like protein, contaminants and whale**  
 308 **shark size**

309 The data were analyzed considering the size of the specimens as a variable. In Figure 4 we report  
 310 the Focused Principal Component Analysis (FPCA) related to the whale shark's size (total length) and  
 311 the contaminant and biomarker variables. The correlation circle at top left (A) indicated that the size  
 312 appears to be negatively correlated with CYP1A-like protein responses.



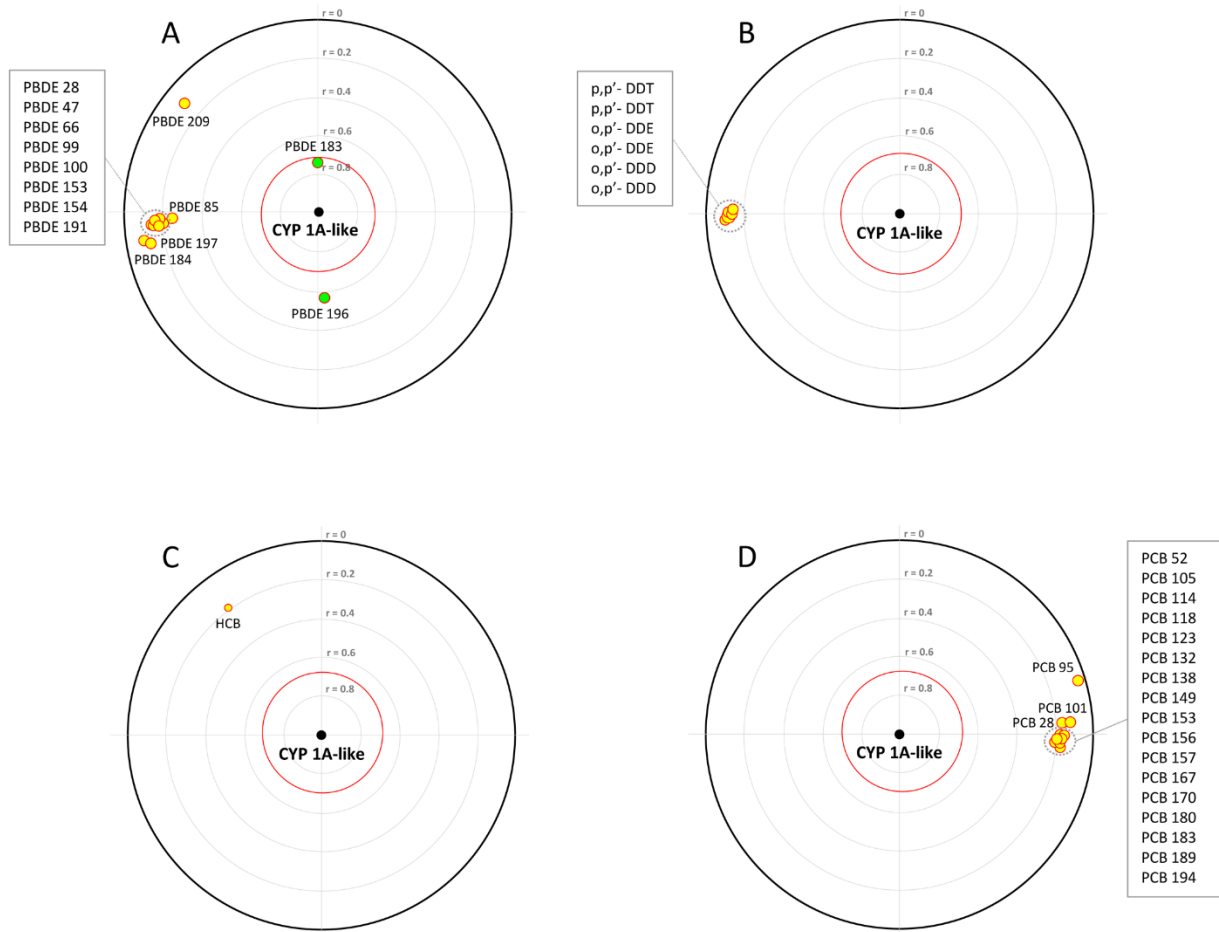
313

314 **Figure 4.** Focused Principal Component Analysis (FPCA). Whale sharks total length (TL) as variable of interest  
 315 in relation to contaminant and biomarker set variables: **A)** CYP1A-like protein; **B)** PBDEs; **C)** PCBs; **D)** HCB; **E)**  
 316 DDTs. Red circles indicate the significant level of  $p = 0.05$ .

317 The correlation circle at the top right (B) indicated that none of the PBDE congeners is significantly  
 318 correlated with the size probably due to low sample size, except for congener PBDE 209 that show  
 319 a positive correlation with whale shark's size. Other PBDE congeners are negatively correlated with  
 320 PBDE 209. None of the other parameters (POPs) (C, D, E) are correlated in a statistically significant  
 321 manner with the size, probably due to the low sample size (see the other circles).

322 In Figure 5, we report the Focused Principal Component Analysis (FPCA) related to whale shark's  
 323 biomarker responses (CYP1A-like protein) and the contaminants variables. The correlation circle at

324 top (A) left indicated that the biomarker CYP1A-like protein is correlated positively and statistically  
 325 significantly ( $p < 0.05$ ) with PBDE 183, which is negatively correlated with PBDE congener 196. The  
 326 other congeners are negatively correlated to biomarker responses except for PBDE 209. None of the  
 327 other parameters (POPs) is correlated in a statistically significant manner with the CYP1A-like  
 328 responses probably due to the low sample size (see the other circles).  
 329



330

331 **Figure 5.** Focused Principal Component Analysis (FPCA). Whale sharks biomarkers response (CYP1A-like  
 332 protein) as variable of interest in relation to contaminants set variables: **A)** PBDEs; **B)** DDTs; **C)** HCB; **D)**  
 333 PCBs. Red circles indicate the significant level of  $p = 0.05$ .

334

#### 335 **4 Discussion**

336 This study has generated data for the first time, to the best of our knowledge, about the  
 337 contamination status of POPs, CYP1A-like protein responses and the potential impact of plastic  
 338 pollution in this endangered shark species.

339 Establishing comparative analysis among, either the abundance and pattern of chemicals  
340 contaminants, becomes not feasible due to the absence of any data regarding POP presence in  
341 whale sharks or any other filter-feeder shark species in the investigated area. Bibliographic research  
342 on POP presence and concentrations in other shark species worldwide (Table 3), mainly top  
343 predator species, showed levels of PCBs, DDTs, PBDEs and HCB, orders of magnitude higher than  
344 those found in the planktivorous species investigated in this paper. The very short length of the  
345 whale shark's food chain may partially explain the lack of a heightened biomagnification of the  
346 higher chlorinated congeners. On the other hand, the major abundance of lower chlorinated PCBs  
347 is consistent with the fact that surface oceans are enriched in this type of congeners (Jurado et al.,  
348 2004). The comparative analyses on POP levels cannot be easily performed also due to the lack of  
349 information on the same tissues analyzed in this study (skin biopsy). Some of the bioaccumulation  
350 patterns found in the whale shark analyzed seem to indicate recent inputs for the studied  
351 contaminants (e.g. DDT) in the Gulf of California. Specifically, the value obtained for the ratio  $R_{p,p'/p,p'}$   
352 might be due to the use of the pesticide dicofol, still allowed in Mexico, and which contains traces  
353 of DDT in its technical formulation, based on the average value of 0.97 calculated for  $R_{o,p'/p,p'} (= [o,p'-$   
354  $DDT]/[p,p'-DDT])$  (Muñoz-Arnanz and Jiménez, 2011). Dicofol is an organochlorine pesticide  
355 (miticide) chemically related to DDT, very effective against red spider mite. Whale sharks sightseeing  
356 reported that sharks aggregates to feed in La Paz Bay. Photo-identification has shown that they can  
357 stay up to 135 days to feed in this area (Ramírez-Macías et al., 2012b) in front of gulf camps, Marinas  
358 and hotels; the use of pesticides on these tourist facilities could be one of the main origin of dicofol  
359 in the WS's feeding ground. Although whale sharks migrate seasonally in area with agricultural  
360 development such as Sonora and Sinaloa.

361 Interestingly, the unusual level of BDE-209, which distribution through the water column decrease  
362 with depth (Salvadó et al., 2016), may result from the superficial filter-feeding activities of this  
363 species. BDE-209 constitutes the 97-98% of the Deca-BDE formulation and it is used as flame  
364 retardant in several type of plastic polymers (Alaee, 2003). This congener is rarely reported in  
365 aquatic food webs; therefore, it seems plausible to link its presence in whale shark tissues with the  
366 possible ingestion of plastics debris by this species. Previous research suggested that  
367 bioaccumulation of higher brominated PBDEs is indicative of plastic ingestion (Gassel et al., 2013;  
368 Tanaka et al., 2013). Rochman and coauthors (Rochman et al., 2014) observed a relationship between  
369 the concentration of PBDEs in myctophid and plastic densities. In that study, the authors suggest  
370 that BDE#s 183–209 were present in myctophids fish as a consequence of living in regions with



371 larger plastic densities. The same authors concluded that higher brominated PBDEs might be  
 372 associated with plastic debris as an additive ingredient and not sorbed from ambient seawater,  
 373 suggesting that BDE#s 183–209 contaminants in myctophid sampled may be indicative, and  
 374 consequential, of plastic pollution in their habitat. A similar interpretation can be done for the whale  
 375 sharks in La Paz Bay in which relevant concentrations of BDE-209 can be attributed to plastic  
 376 pollution in their feeding ground.

377 The mean abundance of microplastics detected in the area (0.07 items/m<sup>3</sup>) confirms previous data  
 378 from the same area (Fossi et al., 2016) and the low levels of accumulation in the area of the Gulf of  
 379 California within the Eastern Pacific Ocean (Cózar et al., 2014; Law et al., 2014). The polyethylene is  
 380 the most abundant polymer found in the samples, as it is also the most abundant polymer in plastic  
 381 litter worldwide (Hidalgo-Ruz et al., 2012). This finding suggests the potential origin of microplastic  
 382 particles by degradation of packaging items in the investigated area (see Supplementary  
 383 information; Fig1 SI).

384 Based on calculated flow speed and underwater mouth area, proposed by (Motta et al., 2010), it  
 385 was estimated that a whale shark of 443 cm total length (TL) filters 326m<sup>3</sup>/h, and a 622 cm TL shark  
 386 614m<sup>3</sup>/h. With an average plankton biomass of 4.5 g/m<sup>3</sup> at their feeding site, the two sizes of sharks  
 387 (similar in size of the WS investigated in this study) would ingest on average 1467 and 2763 g of  
 388 plankton per hour (Motta et al., 2010). Using these data, a theoretical number of daily ingested  
 389 microplastic items for whale sharks in La Paz Bay feeding ground can be calculated (Table 4). The  
 390 microplastic intake per day per shark is calculated between one and two orders of magnitude lower  
 391 than those calculated for other large filter feeders such as basking sharks and fin whales (Fossi et  
 392 al., 2014) feeding in microplastics polluted areas of the Mediterranean sea.

393

394 **Table 4.** Total volume filtered daily, total plankton daily consumption and theoretical number of microplastic  
 395 items ingested by whale sharks in La Paz Bay.

<b>Average juvenile by length</b>	<b>443 cm total length (TL)</b>
<b>Filtration rate</b>	<b>326 m<sup>3</sup>/h</b>
<b>Total volume daily filtered</b>	<b>2445 m<sup>3</sup></b>
<b>Total plankton hourly consumed</b>	<b>1467 g/h</b>
<b>Total plankton daily consumed</b>	<b>11002 g</b>
<b>Theoretical number of MP items ingested daily</b>	<b>171</b>

396

397 An additional hypothesis of potential plastic impact on this endangered shark can be established  
398 focusing on the macroplastics presence in La Paz Bay feeding ground (Fig 2 SI). Whale sharks spend  
399 there, as already mentioned, approximately 7.5 h/day feeding at the surface on dense plankton  
400 dominated by a large amount of macro-plastic debris (see Supplementary Information; Fig 3 SI).  
401 Several images were obtained of whale sharks ingesting macroplastics in the study areas (see  
402 Supplementary Information; Fig. 3a SI). The impact of macroplastics ingestion, as a main cause of  
403 plastic debris intake during the continuous surface feeding activities, needs to be further explored  
404 in the study areas, calculating with a dedicated survey the abundance of macroplastic in the shark  
405 feeding ground. In addition to direct intake, whale sharks may also indirectly ingest microplastics  
406 through consumption of large quantities of copepods, calanoid, chaetognaths and fish larvae  
407 potentially contaminated with microplastics as, in La Paz Bay, juveniles sharks feed mainly on  
408 copepods (Hacohen-Domenè et al., 2006). Fossi and coauthors (2016) reported concentrations of  
409 mono-(2-ethylhexyl) phthalate (MEHP), ranged from 13.08 ng/g to 13.69 ng/g in  
410 zooplankton/microplastic samples collected in the same areas (whale shark feeding ground). These  
411 data suggested a potential direct (through plastic debris ingestion) and indirect (microplastic ingested  
412 by copepods) input of plastic additive during the in whale shark feeding in La Paz Bay. Regarding  
413 CYP1A-like protein responses, this research has generated data for the first time about the  
414 identification of CYP1A-like isoforms and a semi-quantification of these proteins in this endangered  
415 shark species. The usefulness of dermal CYP1A-like protein expression as a biomarker of POPs  
416 exposure in sharks is further supported by studies showing the presence of CYP1A-like protein levels  
417 in white sharks by Marsili et al. (2016). Interestingly, as reported in Figure 5, Focused Principal  
418 Component Analysis indicated that the size of the sharks analyzed appears to be negatively  
419 correlated with CYP1A-like protein levels, suggesting a variation of the protein expression related  
420 to the shark development. Moreover, it is interesting to underline that FPCA (Fig. 6) reported that  
421 CYP1A-like protein responses are positively correlated ( $p < 0.05$ ) with PBDE 183 and PBDE 209 levels,  
422 suggesting, the signal of this biomarker induction as a potential warning of POPs and plastic  
423 additives exposure in this endangered species. Further investigation need to be done to support  
424 these findings.

## 425 **5 Conclusions**

426 In conclusion, this pilot project has generated the first data on organochlorine compounds (PCBs,  
427 DDTs), plastic additives (PBDEs) and CYP1A-like protein in the whale shark. The first data on  
428 microplastic abundance and characterization in Gulf of California whale shark feeding grounds were

429 also showed, suggesting the potential impact of plastics pollution on this endangered shark species.  
430 Some of the bioaccumulation patterns found seem to indicate recent inputs in the Gulf of California  
431 for the study contaminants, such as DDT, related to the recent use of dicofol in the areas.

432 Moreover, the higher contribution of PBDE-209 may result from the superficial filter-feeding  
433 activities of this species, responsible for plastics ingested both of micro- and macro-plastic in the  
434 whale shark feeding ground. This finding it is also confirmed by a positive correlation between the  
435 size of the sharks and the level of the congeners PBDE-209 in the tissues. Model-supported analysis  
436 can be used in further investigation in order to define the flux of POPs and plastic additives in this  
437 species.

438 Adverse physiological effects to marine organisms exposed to organochlorine pesticide and plastic  
439 debris can pose a severe chemical hazard. DDTs are recognized as endocrine disrupting chemicals  
440 (EDCs), on the other hand, plastic debris may have toxic components in their matrix, like persistent  
441 brominated flame retardants and other chemicals (e.g. phthalates) with endocrine disruptor activity  
442 which may leach into the environment when the plastic weathers or by ingestion. Particular  
443 attention should be given to the role of these EDCs on species already seriously threatened by  
444 human impacts such as the whale shark.

445 In conclusion, the presence and impact of marine litter in Gulf of California marine organisms  
446 represent an issue that requires a series of mitigation actions in order to reduce future negative  
447 effects on the extraordinary biodiversity of this region. Further ecotoxicological investigation on  
448 whale shark skin biopsies and in other large filter feeder species inhabiting this area, such as baleen  
449 whales and manta ray, should be carried out for an ecotoxicological risk assessment of these  
450 endangered species.

451

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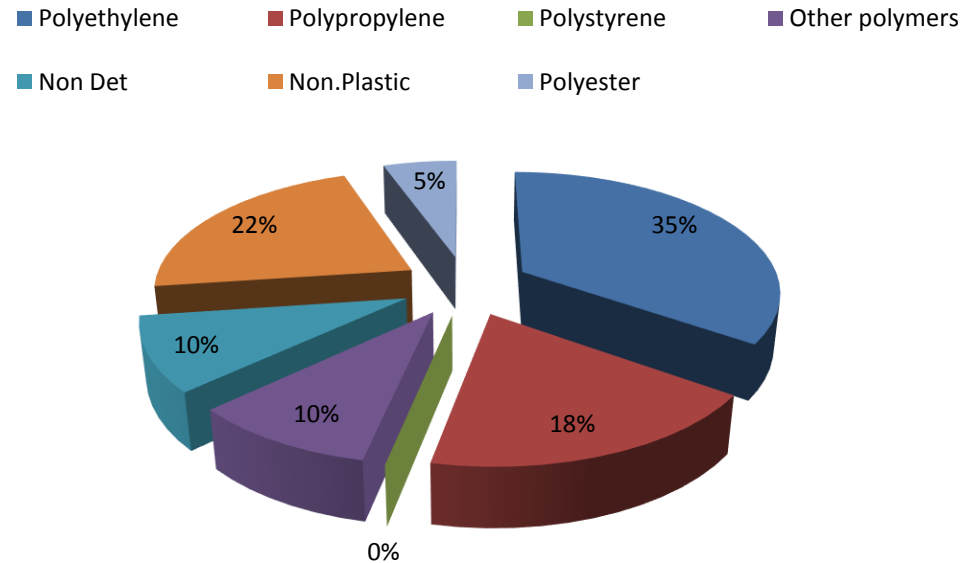


**Table 3.** Bibliographic research on POPs (PCBs, DDTs, PBDEs and HCB) presence and concentrations in other shark species world wide

Species	ΣPBDEs	ΣDDTs	ΣPCBs	ΣHCHs	HCB	Tissue	Geographic area	References
<b>Present study</b>	<b>0.29 ± 0.32 ng/g wet w.</b>	<b>1.31 ± 1.76 ng/g wet w.</b>	<b>11.42 ± 8.60 ng/g wet w.</b>	<b>9.73 ± 12.36 ng/g wet w.</b>	<b>0.19 ± 0.19 ng/g wet w.</b>	<b>Biopsy</b>	<b>La Paz Bay</b>	
Tiger shark ( <i>Galeocerdo cuvier</i> )	26 ng/g lipid w.	/	480 ng/g lipid w.	/	/	Liver	Southern Coast of Japan	(Haraguchi et al., 2009)
Silvertip Shark ( <i>Carcharhinus albimarginatus</i> )	12 ng/g lipid w.	/	280 ng/g lipid w.	/	/	Liver	Southern Coast of Japan	(Haraguchi et al., 2009)
Sandbar Shark ( <i>Carcharhinus plumbeus</i> )	17 ng/g lipid w.	/	280 ng/g lipid w.	/	/	Liver	Southern Coast of Japan	(Haraguchi et al., 2009)
Bull Shark ( <i>Carcharhinus leucas</i> )	850 ng/g lipid w.	/	35000 ng/g lipid w.	/	/	Liver	Southern Coast of Japan	(Haraguchi et al., 2009)
	1467 ng/g lipid w.	27310 ng/g lipid w.	51700 ng/g lipid w.	/	9 ng/g lipid w.	Liver	Southeastern USA	(Weijs et al., 2015)
Greenland shark ( <i>Somniosus microcephalus</i> )	764 ± 962 pg/g wet w.	0.195 ± 0.063 ng/g wet w.	31.1 ± 11.1 ng/g lipid w.	/	0.017 ± 0.023 ng/g wet w.	Red Muscle	Greenland	(Corsolini et al., 2014)
	1365 ± 1845 pg/g wet w.	0.594 ± 0.664 ng/g lipid w.	57.8 ± 41.9 ng/g lipid w.	0.011 ± 0.013 ng/g wet w.	0.026 ± 0.027 ng/g wet w.	White Muscle	Greenland	(Corsolini et al., 2014)
	35 ng/g lipid w.	/	/	/	/	Muscle	NE Greenland	(Strid et al., 2010)
Blue shark ( <i>Prionace glauca</i> )	5.7 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
	0.0543 ± 0.0310 ng/g lipid w.	/	1.22 ± 1.12 ng/g lipid w.	/	/	Muscle	Southwest of Portugal	(Alves et al., 2016)
	/	2392 ± 1439 ng/g lipid w.	2482 ± 1020 ng/g lipid w.	/	/	Liver	Mediterranean Sea (Italy)	(Storelli et al., 2005)
Blacktip reef shark ( <i>Carcharhinus melanopterus</i> )	11.6 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Spiny dogfish ( <i>Squalus acanthias</i> )	1.34 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Pelagic thresher shark ( <i>Alopias pelagicus</i> )	1.47 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Shortfin mako ( <i>Isurus oxyrinchus</i> )	4.34 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Oceanic whitetip shark ( <i>Carcharhinus longimanus</i> )	0.55 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)

Species	ΣPBDEs	ΣDDTs	ΣPCBs	ΣHCHs	HCB	Tissue	Geographic area	References
<b>Present study</b>	<b>0.29 ± 0.32 ng/g wet w.</b>	<b>1.31 ± 1.76 ng/g wet w.</b>	<b>11.42 ± 8.60 ng/g wet w.</b>	<b>9.73 ± 12.36 ng/g wet w.</b>	<b>0.19 ± 0.19 ng/g wet w.</b>	<b>Biopsy</b>	<b>La Paz Bay</b>	
Milk shark ( <i>Rhizoprionodon acutus</i> )	1.21 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Smooth hammerhead ( <i>Sphyrna zygaena</i> )	2.07 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Brazilian sharpnose shark ( <i>Rhizoprionodon lalandii</i> )	10.4 ± 4.78 ng/g lipid w.	111 ± 40 ng/g lipid w.	1019 ± 267 ng/g lipid w.	< 1.96 ng/g lipid w.	< 3.48 ng/g lipid w.	Liver	Southeastern coast of Brazil	(Cascaes et al., 2014)
Small spotted dogfish ( <i>Scyliorhinus canicula</i> )	/	1171 ± 471 ng/g lipid w.	1292 ± 577 ng/g lipid w.	/	/	Liver	Mediterranean Sea (Italy)	(Storelli et al., 2006)
	/	/	17 ± 18.11 ng/g dry w.	/	/	Muscle	Mediterranean Sea (Italy)	(Cresson et al., 2016)
Kitefin Shark ( <i>Dalatias licha</i> )	/	4554 ± 2046 ng/g lipid w.	1827 ± 349 ng/g lipid w.	/	/	Liver	Mediterranean Sea (Italy)	(Storelli et al., 2005)
White shark ( <i>Carcharodon carcharian</i> )	/	20.4 ng/g dry w.	38.5 ng/g dry w.	0.02 ng/g dry w.	0.2 ng/g dry w.	Muscle	South Africa	(Beaudry et al., 2015)
	/	/	3.8077 ug/g lipid w.	/	/	Liver	South-eastern Australia	(Gilbert et al., 2015)
	/	86.76-1416.97 ng/g lipid w.	379.76-11284.31 ng/g lipid w.	/	6.80-21.26 ng/g lipid w.	Biopsy	South Africa	(Marsili et al., 2016)
Dusky shark ( <i>Carcharhinus obscurus</i> )	/	9.5 ng/g dry w.	30.5 ng/g dry w.	0.02 ng/g dry w.	0.1 ng/g dry w.	Muscle	South Africa	(Beaudry et al., 2015)
Blackmouth Catshark ( <i>Galeus melastomus</i> )	/	/	12.76 ± 31.14 ng/g dry w.	/		Muscle	Mediterranean Sea (France)	(Cresson et al., 2016)
Whitespotted Bamboo Shark ( <i>Chiloscyllium plagiosum</i> )	/	2.3957 ng/g wet w.	2.0901 ng/g wet w.	0.022 ng/g wet w.	/	Liver	Southern waters of Hong Kong, China.	(Cornish et al., 2007)
Gulper shark ( <i>Centrophorus granulosus</i> )	/	49.3 ± 12.6 ng/g wet w.	28.3 ± 11.3 ng/g wet w.	/	3.5 ± 2.1 ng/g wet w.	Muscle	Mediterranean Sea (Italy)	(Storelli and Marcotrigiano, 2001)
Longnose Spurdog ( <i>Squalus blainville</i> )	/	10.8 ± 6.6 ng/g wet w.	16.8 ± 9.2 ng/g wet w.	/	2.5 ± 1.1 ng/g wet w.	Muscle	Mediterranean Sea (Italy)	(Storelli and Marcotrigiano, 2001)
Atlantic stingray ( <i>Dasyatis sabina</i> )	/	/	2415 ng/g lipid w.	/	/	Liver	Southeastern USA	(Weijs et al., 2015)
Bonnetheads shark ( <i>Sphyrna tiburo</i> )	124 ng/g lipid w.	/	91550 ng/g lipid w.	/	/	Liver	Southeastern USA	(Weijs et al., 2015)
Lemon shark ( <i>Negaprion brevirostris</i> )	98 ng/g lipid w.	816 ng/g lipid w.	1950 ng/g lipid w.	/	/	Liver	Southeastern USA	(Weijs et al., 2015)
Mediterranean basking shark ( <i>Cetorhinus maximus</i> )	/	1667.84 ng/g lipid w.	1483.06 ng/g lipid w.		20.58 ng/g lipid w.	Muscle	Mediterranean Sea (Italy)	(Fossi et al., 2014b)

## Microplastic Polymers



**Fig 1 – SI** - Polymer composition, identified using Fourier transformed infrared (FT-IR) spectroscopy technique, of microplastic samples collected in inshore waters of La Paz Bay, in the whale sharks feeding ground.

**Fig 2- SI -**  
La Paz Bay (Mexico).  
Human impacts in  
the whale sharks  
feeding ground.





**Fig. 3 SI** - La Paz Bay, the whale sharks feeding ground. Images of whale sharks ingesting macroplastics in the study area.