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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ABSTRACT

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

items/m^3 to 0.14 items/m^3. A Focused PCA analysis was performed to evaluate a possible correlation among the size of the whale sharks, contaminants and CYP1A reponses. Further ecotoxicological investigation on whale shark skin biopsies will be carried out for a worldwide ecotoxicological risk assessment of this endangerd species.

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

1 Introduction

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

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

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

Despite the growing scientific attention on this issue, little scientific investigation has focused on the potential impact of micro- and macroplastics on large filter feeding marine organisms such as baleen whale and planktivorous sharks (Fossi et al., 2014; Besseling et al., 2015; Fossi et al., 2016). In particular, we lack information about inputs, spatial and temporal distributions and interactions with biota in semi-closed basins, such as the Gulf of California.

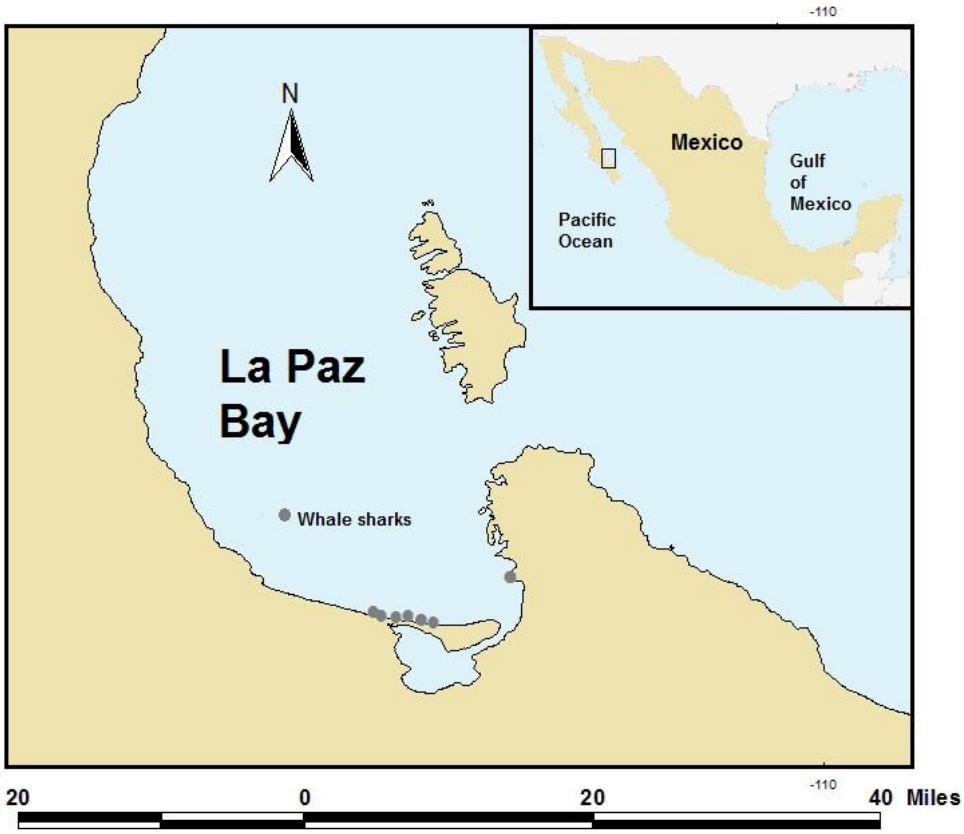
In this paper, we perform the first ecotoxicological investigation on whale sharks sampled in the Gulf of California exploring the potential interaction of this species with plastic debris (macro- and micro-plastics), the levels of PBDEs and OCs and related biomarkers responses (CYP1A) using skin biopsies as target tissue due to the lack of stranded organisms and the protected status of the whale shark. Skin biopsy samples were collected from twelve whale sharks in La Paz Bay and a preliminary investigation on microplastic concentration and polymer composition was also carried out in samples collected in the whale shark ground.

2 Material and Methods

2.1 Study area and collected samples

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

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



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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

131

2.2 POPs determination

2.2.1 Sample treatment

The analysis of HCB (hexachlorobenzene), DDTs (sum of dichloro-diphenyltrichloroethane (DDT) and its main metabolites), PCBs (polychlorinated biphenyls) and PBDEs (polybrominated diphenyl ethers) was carried out in the subcutaneous tissues of freeze-dried skin biopsy samples (n=12). Initially, samples were spiked with isotopic labeled surrogates of HCB, DDTs, PCBs and PBDEs (detailed list to be found at QA/QC) prior to soxhlet extraction for 24 h with a mixture of n-hexane:dicloromethane (9:1, v:v). A subsequent clean-up process was achieved by using open columns packed with neutral and acidic-modified silica gel and the same mixture of solvents as eluting agent. Final extracts were evaporated using a TurboVap® system until ~1 mL, transferred to vials, and dried under a gentle nitrogen steam. Samples were reconstituted in 20 µL of a solution of 13C12-p, p'-DDT, 13C12-PCB-111, 170, 178 and 13C12-BDE-139 in nonane as injection/internal standards for instrumental analysis.

2.2.2 Instrumental analysis

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

2.2.3 QA/QC in POPs determination

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

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

2.3 Cytochrome P450 1A protein determination

Cytochrome P450 1A (CYP1A) protein, used in this study as marker of POPs exposure, was analyzed in the dermal part of the skin biopsies of 8 out of 12 specimens of whale shark using western-blotting (WB) techniques. Four samples were not analyzed due to their small size that could not allow performing the analysis. Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (Bio-Rad, 1-D Analysis Software) using Adjusted Volume (Intensity *mm2) as quantitative parameter. Homogeneous sub-samples of biopsies were homogenized in aryl-hydrocarbon-receptor (AhR) buffer (Wilson et al., 2007) using a Tissue Lyser (Qiagen). The homogenate was centrifuged twice and the supernatant (S9) was analyzed for total proteins and then by WB. For WB analysis, S9 tissue homogenates (in duplicate) were separated by SDS–PAGE (10% polyacrylamide gels) and blotted onto nitrocellulose; the membranes were saturated with blocking solution for 1 h. Primary polyclonal antibodies were used from Biosense Laboratories AS (Norway). There are no specific antibodies for this species, for this reason a Rabbit anti-fish CYP1A peptide Polyclonal antibody (CP-226) from Biosense Laboratories AS (Norway) has been used. This product consists of rabbit polyclonal antibodies (affinity-purified IgG fraction) against peptides 190-204 and 282-296 of rainbow trout (*Oncorhynchus mykiss*) cytochrome P450 1A (CYP1A). Due the detection of CYP1A with a heterologous antibody, the protein detected has been named hereafter as CYP1A-like protein. The antibody was diluted 1:500 in TTBS-1% gelatin and it was incubated with shark proteins overnight. Incubation with goat anti-rabbit IgG HRP-labelled secondary antibody (Bio-Rad) (1:3000) was performed (1.30 h) and detected according to the Bio-Rad Immun-Star-HRP-Chemiluminescent-Kit booklet. Semi-quantitative analysis was performed for each WB with Quantity-One software (Bio-Rad) using Adjusted Volume (Intensity*mm2) as quantitative parameter. The lane-based functions have been used to calculate molecular weights for CYP1A-like peptide with multiple regression models using as a Precision Plus Protein™ Standards (Bio-Rad). Precision Plus Protein All Blue Standards are a mixture of ten blue-stained recombinant proteins (10–250 kDa), including three reference bands (25, 50, and 75 kDa).

2.4 Sampling and characterization of microplastics

2.4.1 *Sampling of microplastics*

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

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

2.4.2 Microplastics analysis

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

2.4.3 Polymer identification: Fourier Transform Infrared Spectroscopy

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

2.5 Statistics on biomarker responses and contaminants

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

3 Results

3.1 POP concentrations

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

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

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

	Average	**Median**	**Range**
HCB	0.192	0.104	0.018 – 0.659
DDTs (6 isomers)	1.31	0.545	0.201 – 6.36
ortho 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

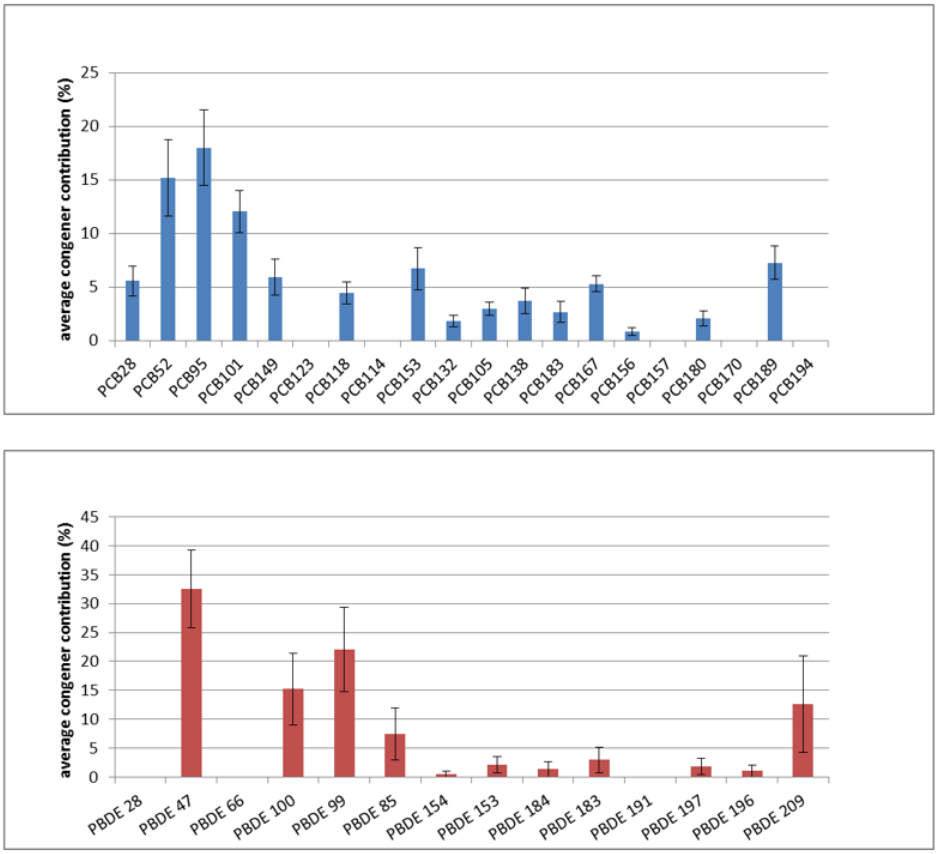

Figure 2. A) Average PCB congener profile skin biopsies of whale sharks (n=12). Error bars represent standard errors (SE). **B**) Average PBDE congener profile in skin biopsies of whale sharks (n=12). Error bars represent standard errors (SE).

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

3.2 Western blot analysis of CYP1A-like protein

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

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

The semi-quantitative analysis of whale shark's CYP1A-like protein was performed with the Quantity One software (Bio-Rad, 1-D Analysis Software) using Adjusted Volume (Intensity *mm2) as quantitative parameter. The Adjusted Volume ranged from 439.85 (intensity*mm2/µg protein) in WS10 to 2273.39 (intensity*mm2/µg protein) in WS9 (Tab.2 and Fig 3 B).

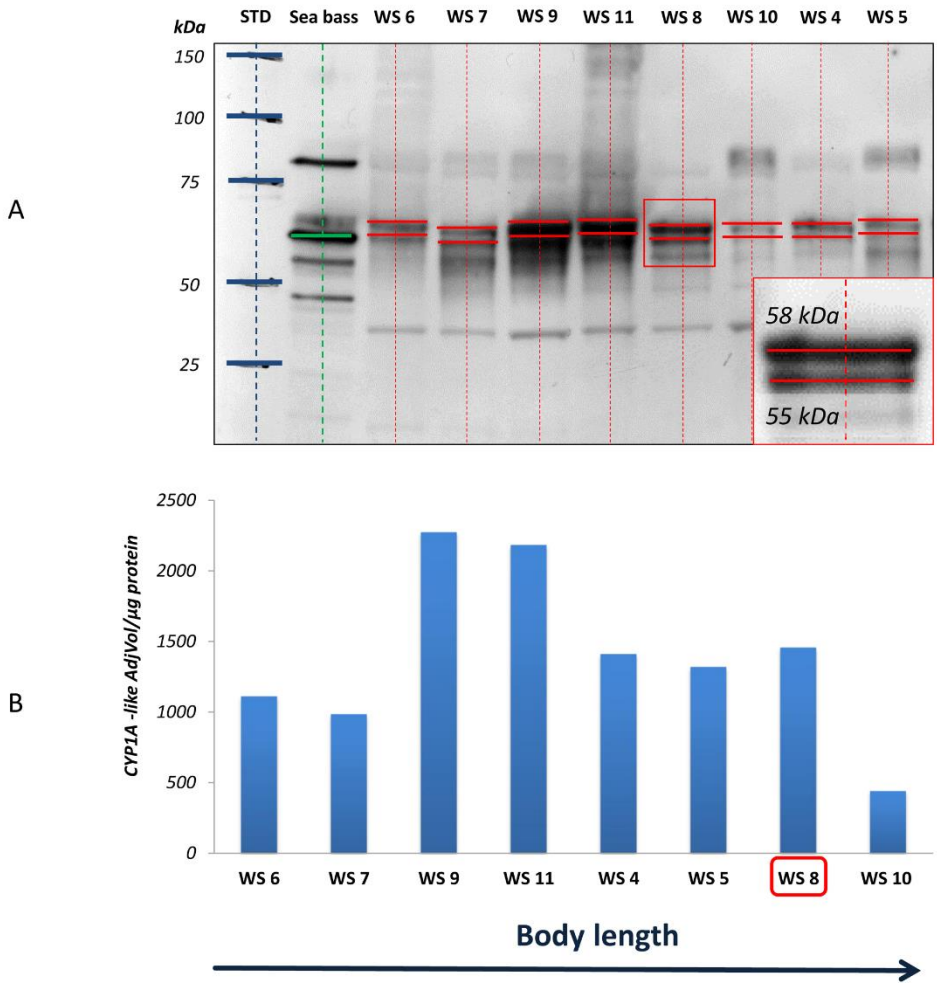

Figure 3. A) Western blot analysis of CYP1A-like protein in skin biopsy of whale sharks (WS) (red) and European seabass liver (green). Precision Plus Protein™ Standards (Bio-Rad) (blue). **B**) Semi-quantitative analysis of WS's CYP1A-like protein performed with Quantity One software (Bio-Rad, 1-D Analysis Software) using Adjusted Volume (Intensity *mm2) in skin biopsies of males and one female (red square).

3.3 **Microplastic abundance and polymer identification**

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

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

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

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

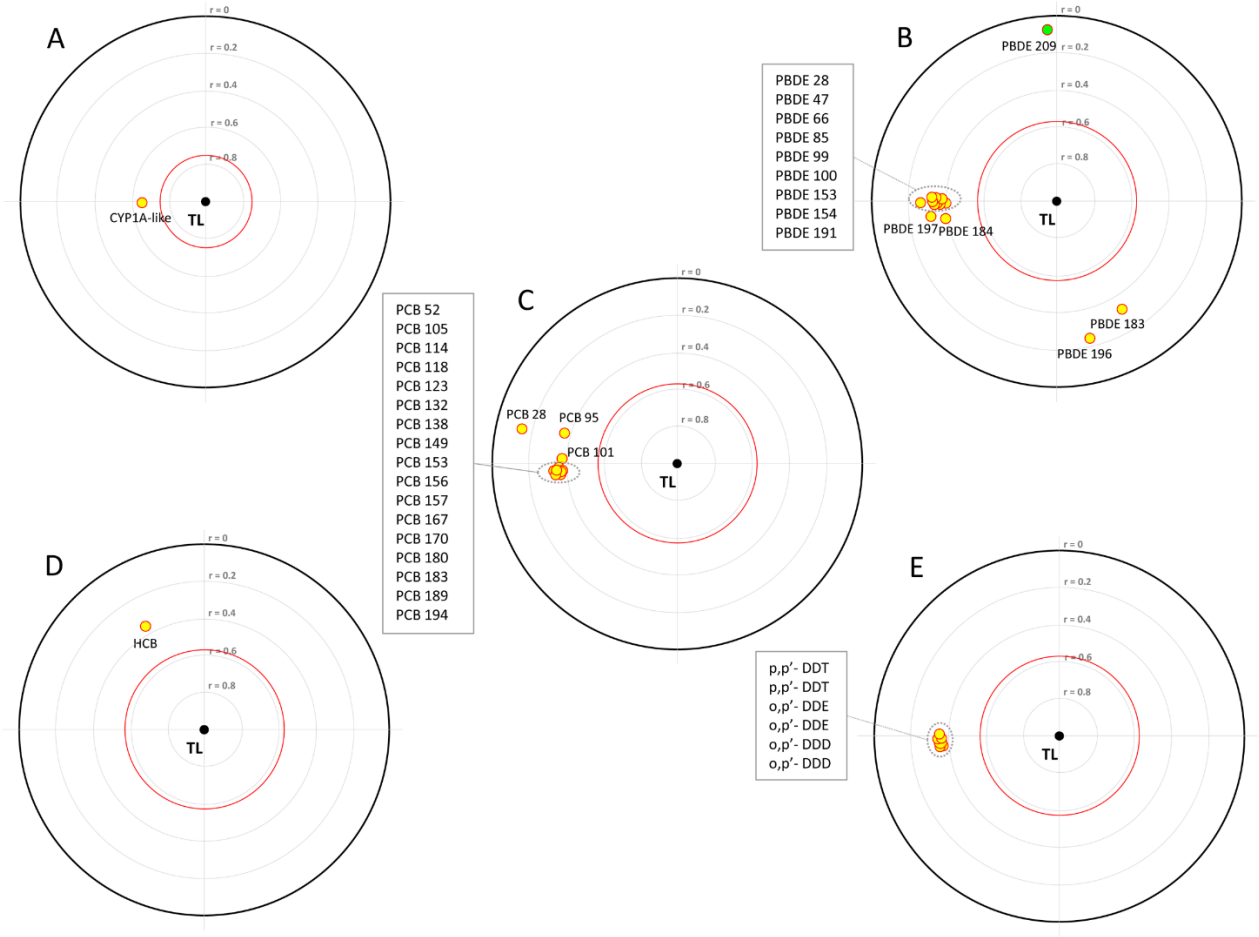

Figure 4. Focused Principal Component Analysis (FPCA). Whale sharks total length (TL) as variable of interest in relation to contaminant and biomarker set variables: **A**) CYP1A-like protein; **B**) PBDEs; **C**) PCBs; **D**) HCB; **E**) DDTs. Red circles indicate the significant level of p = 0.05.

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

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

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

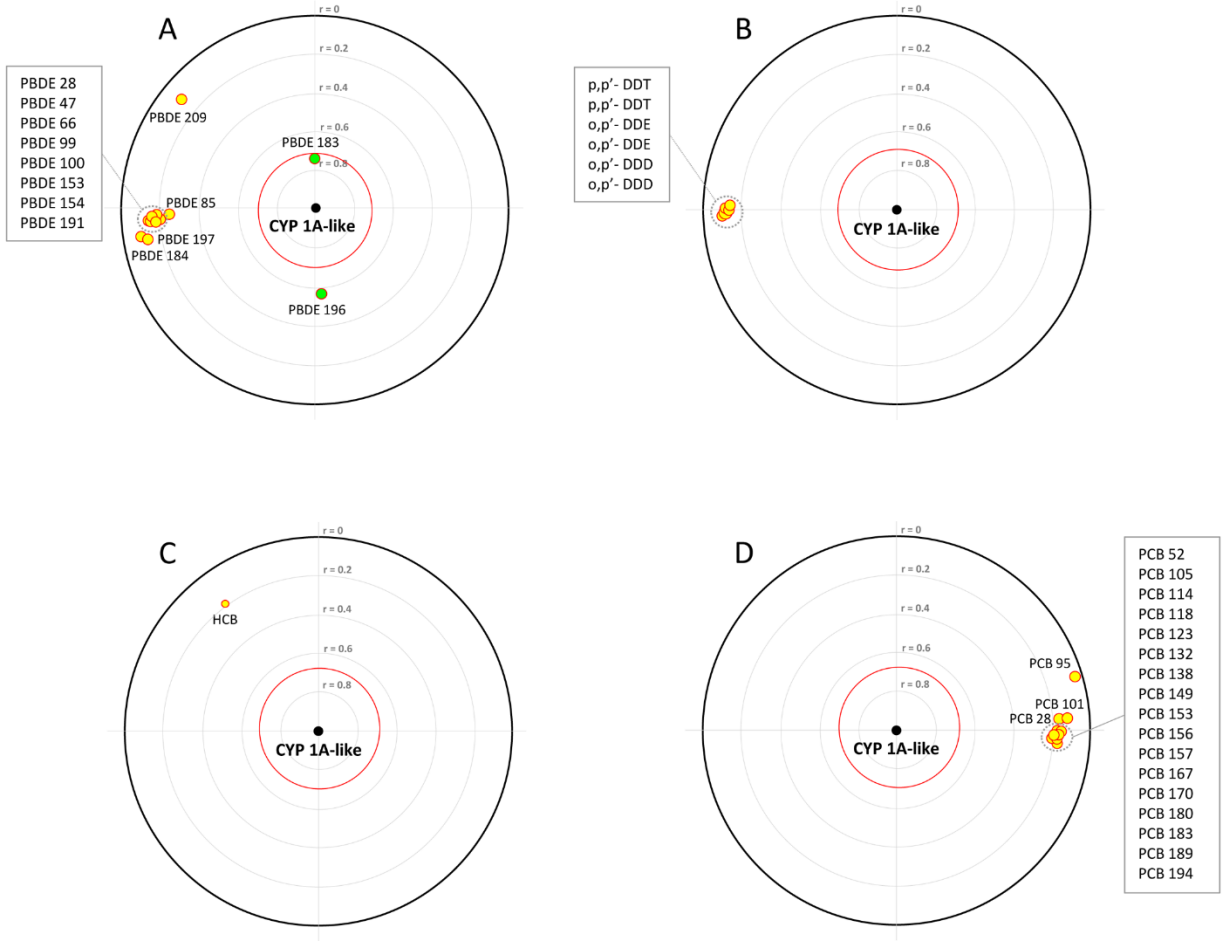

Figure 5. Focused Principal Component Analysis (FPCA). Whale sharks biomarkers response (CYP1A-like protein) as variable of interest in relation to contaminants set variables: **A**) PBDEs; **B**) DDTs; **C**) HCB; **D**) PCBs. Red circles indicate the significant level of $p = 0.05$.

4 Discussion

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

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

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

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

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

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

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

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

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

5 Conclusions

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

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

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

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

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

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642 **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
Present study	**0.29 ± 0.32 ng/g wet w.**	**1.31 ± 1.76 ng/g wet w.**	**11.42 ± 8.60 ng/g wet w.**	**9.73 ± 12.36 ng/g wet w.**	**0.19 ± 0.19 ng/g wet w.**	**Biopsy**	**La Paz Bay**	
Tiger shark (*Galeocerdo cuvier*)	26 ng/g lipid w.	/	480 ng/g lipid w.	/	/	Liver	Southern Coast of Japan	(Haraguchi et al., 2009)
Silvertip Shark (*Carcharhinus albimarginatus*)	12 ng/g lipid w.	/	280 ng/g lipid w.	/	/	Liver	Southern Coast of Japan	(Haraguchi et al., 2009)
Sandbar Shark (*Carcharhinus plumbeus*)	17 ng/g lipid w.	/	280 ng/g lipid w.	/	/	Liver	Southern Coast of Japan	(Haraguchi et al., 2009)
Bull Shark (*Carcharhinus leucas*)	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 (*Somniosus microcephalus*)	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 (*Prionace glauca*)	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 (*Carcharhinus melanopterus*)	11.6 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Spiny dogfish (*Squalus acanthias*)	1.34 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Pelagic thresher shark (*Alopias pelagicus*)	1.47 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Shortfin mako (*Isurus oxyrinchus*)	4.34 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Oceanic whitetip shark (*Carcharhinus longimanus*)	0.55 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)

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Species	ΣPBDEs	ΣDDTs	ΣPCBs	ΣHCHs	HCB	Tissue	Geographic area	References
Present study	**0.29 ± 0.32 ng/g wet w.**	**1.31 ± 1.76 ng/g wet w.**	**11.42 ± 8.60 ng/g wet w.**	**9.73 ± 12.36 ng/g wet w.**	**0.19 ± 0.19 ng/g wet w.**	**Biopsy**	**La Paz Bay**	
Milk shark (*Rhizoprionodon acutus*)	1.21 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Smooth hammerhead (*Sphyrna zygaena*)	2.07 ng/g lipid w.	/	/	/	/	Muscle	Korea	(Lee et al., 2015)
Brazilian sharpnose shark *(Rhizoprionodon lalandii)*	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 (*Scyliorhinus canicula*)	/	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 *(Dalatias licha)*	/	4554 ± 2046 ng/g lipid w.	1827 ± 349 ng/g lipid w.	/	/	Liver	Mediterranean Sea (Italy)	(Storelli et al., 2005)
White shark (*Carcharodon carcharian*)	/	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 (*Carcharhinus obscurus)*	/	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 (*Galeus melastomus*)	/	/	12.76 ± 31.14 ng/g dry w.	/		Muscle	Mediterranean Sea (France)	(Cresson et al., 2016)
Whitespotted Bamboo Shark (*Chiloscyllium plagiosum*)	/	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 (*Centrophorus granulosus*)	/	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 (*Squalus blainville*)	/	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 (*Dasyatis sabina*)	/	/	2415 ng/g lipid w.	/	/	Liver	Southeastern USA	(Weijs et al., 2015)
Bonnetheads shark (*Sphyrna tiburo*)	124 ng/g lipid w.	/	91550 ng/g lipid w.	/	/	Liver	Southeastern USA	(Weijs et al., 2015)
Lemon shark (Negaprion brevirostris)	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 (*Cetorhinus maximus*)	/	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)

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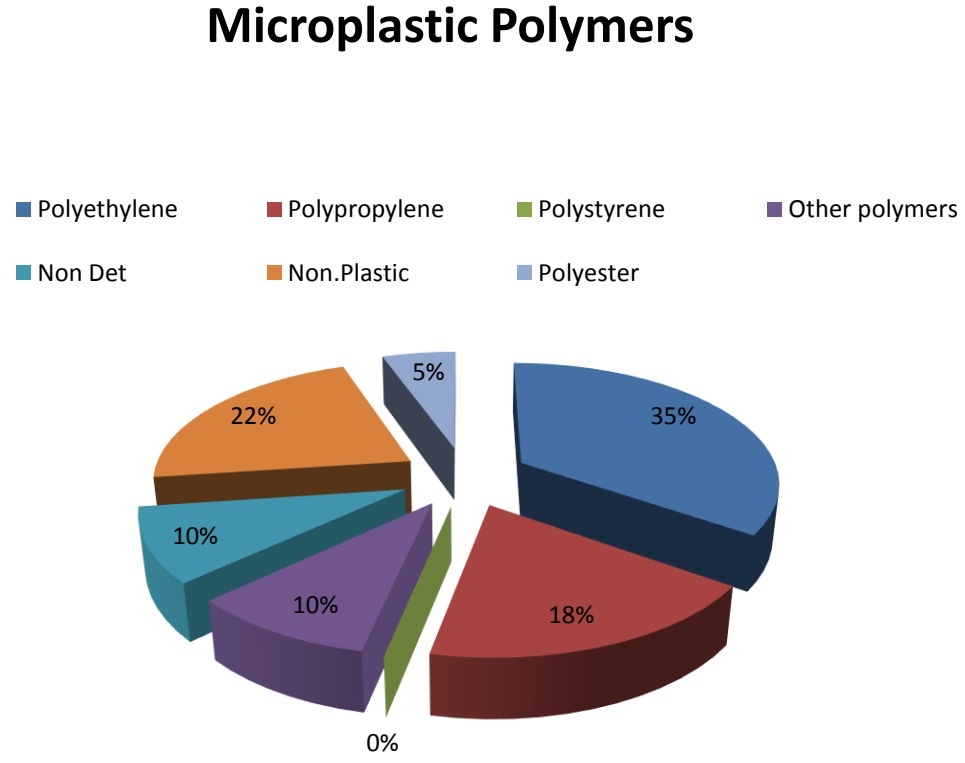

Fig 1 – SI - Polymer composition, identified using Fourier transformed infrared (FT-IR) spectroscopy technique, of microplastic samples collected in inshore waters of La Paz Bay, in the whale sharks feeding ground.

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.