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Research Article

Skin Biopsies as a Sensitive Non-Lethal Technique for the Ecotoxicological Studies of Great White Shark (*Carcharodon carcharias*) Sampled in South Africa

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Abstract

Top-predators may be extremely vulnerable to environmental contaminants, such as organochlorines (OCs) and polycyclic aromatic hydrocarbons (PAHs), mostly because of their position in the trophic web. In this study, the use of skin biopsy is proposed as a sensitive non-lethal technique for the toxicological assessment of white shark (*Carcharodon carcharias*) living off the South African coasts. In 2012, 15 specimens of great white shark were sampled in the waters off Dyer Island and Geyser Rock. Then OCs and PAHs were extracted from muscle and biomarkers techniques for the evaluation of the cytochrome P4501A (CYP1A), Vitellogenin (Vtg) and Zona Radiata Proteins (Zrp) in the skin have been developed for the first time. The results showed levels of OCs higher than those found in the literature, ranging in ng/g dry weight (d.w.) from 6.80 to 21.26 for hexachlorobenzene (HCB), from 86.72 to 1416.97 for DDTs and from 379.76 to 11284.31 for polychlorinated biphenyls (PCBs). Furthermore, the values of both pp'DDE/pp'DDT and pp'DDE/DDTs ratios suggest a recent DDT introduction in the environment, probably related to its use against malaria during the period 2000-2005 in KwaZulu-Natal. However, PAHs showed the highest levels, almost double compared to OCs, almost certainly due to the big oil traffic present in South Africa. Regarding biomarkers results, important responses for CYP1A have been highlighted, possibly due to a contamination by planar compounds such as PAHs. Finally, the preliminary results of Vtg and Zrp, biomarkers of estrogenic effects, showed the presence of these proteins in sexually immature females and males.

Keywords

White shark; Skin biopsy; OCs; PAHs; Biomarkers

Introduction

The white shark (*Carcharodon carcharias*) is one of the most important cosmopolitan and epipelagic top predators of the marine environment that is able to maintain the marine biodiversity trough the predation effects. From 1996 it has been indicated as vulnerable by the IUCN Red List of Threatened Species, and South African

population counts less than 1000 animals [1]. South Africa is one of the global centres of abundance of white sharks, especially the Natural Reserve of Dyer Island, where there is a huge population of fur seals (*Arctocephalus pusillus pusillus*), almost 50000 specimens, natural preys of white sharks [2,3]. The threats for white sharks in this area are the same present in the rest of the world [4], but the contamination problem is quite unexplored [5]. Moreover, South Africa is a developing country showing many agricultural and industrial activities, especially regarding the oil traffic and it's derivate: almost the 28% of the oil exported in the Middle East passes near Good Hope Cape [6]. Sharks, as all marine top predators, are particularly susceptible to accumulate high levels of toxic compounds coming from these activities [7-9]. The main aim of this study is to propose a non-lethal technique to assess the toxicological status of South-African white sharks, represented by skin biopsy. This non-lethal and non-harmful technique permits a large number of scientific investigations [10]. In this research the skin biopsy, including integument and muscle, is used for the contaminant analysis and biomarker responses: some organochlorine contaminants (OCs) and polycyclic aromatic hydrocarbons (PAHs) were analysed in the muscle, while the integument was used for the assessment of Cytochrome P4501A (CYP1A), biomarker of exposition to lipophilic contaminants such as OCs and PAHs, and for the development of Vitellogenin (Vtg) and Zona Radiata Proteins (Zrp) analysis, two biomarkers that evaluate the estrogenic effects. In fact, the major part of the analysed OCs in this study is known as endocrine disrupting chemicals (EDCs): environmental estrogens, environmental androgens, anti-estrogens and anti-androgens [11-13]. Environmental estrogens are the most common and most widely studied EDCs and are present with very high levels in marine mammals, particularly in pinnipeds and odontocetes [14,15], preys of the white shark. Consequently this important topic for the toxicological status of the white shark cannot be ignored.

Materials and Methods

Sample collection

Biopsy samples were taken from 15 free-ranging white sharks (Table 1) in the area of the Dyer Island Natural Reserve (South Africa) (Figure 1).

Each specimen was brought near the boat chumming the water with a preparation made of tuna offal in order to recreate the conditions of scavenging (presence of a rotten carcass in the water). No animal was fed during the sampling. The sex of white shark specimens was assessed during cage diving sessions, even though sometimes this was not possible due to the poor visibility of the water (animal without sex assessment are indicated as not determined). All males with a total length body longer than 3.5 meters and all females longer than 4.5 meters were considered sexually mature [4,16]. Biopsies were taken through the use of a pole with a modified tip, able to penetrate the thick and coriaceous skin of the white shark, avoiding any contact with sensitive organs of the animals as eyes or gills. The samples were immediately stored in liquid nitrogen.
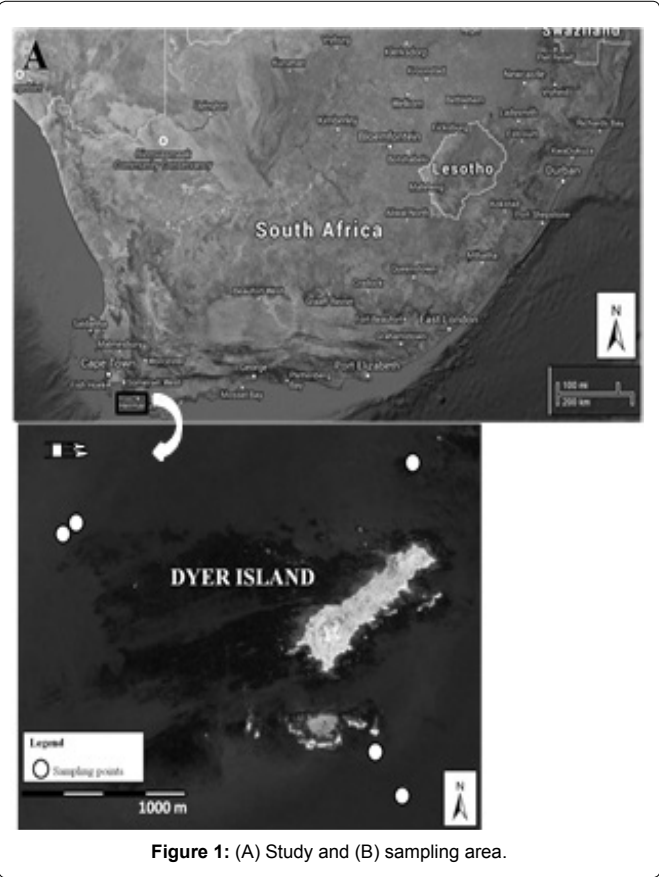
OC analysis

Analysis for HCB, DDTs and PCBs were performed according to method of U.S. Environmental Protection Agency (EPA) 8081/8082

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Table 1: Sex, length and sexual maturity of 15 free-ranging white sharks sampled in Dyer Island Natural Reserve (M= male; F= female; nd= not determined).

Sample ID	Sampling Date	Sex	Length (m)	Sexual Maturity
WSSA1	april 2012	M	4	Yes
WSSA3	april 2012	F	3.5	No
WSSA4	april 2012	F	2.8	No
WSSA5	april 2012	F	2	No
WSSA6	may 2012	M	4	Yes
WSSA7	april 2012	M	1.8	No
WSSA8	may 2012	M	4.2	Yes
WSSA9	april 2012	F	3.7	No
WSSA10	may 2012	nd	2.5	No
WSSA11	may 2012	M	3.8	Yes
WSSA12	may 2012	nd	2	No
WSSA13	may 2012	M	3	No
WSSA14	may 2012	nd	2.5	No
WSSA15	may 2012	nd	3.2	No
WSSA16	may 2012	nd	3	No

Figure 1: (A) Study and (B) sampling area.

with modifications [17]. The sample of subcutaneous muscle (about 0.5 g) was lyophilized in an Edwards freeze drier for 2 days and extracted with n-hexane for gas chromatography (Merck) in a Soxhlet apparatus for analysis of organ chlorines compounds. Whatman cellulose thimble (i.d. 25 mm, e.d. 27 mm, length 100 mm) to be used for extraction of the sample was preheated for about 30 min to 110°C and pre extracted for 9h in a Soxhlet apparatus with n-hexane, in order to remove any organochlorine contamination. The sample was extracted with n-hexane in the thimble in the Soxhlet apparatus for 9 h. The sample was then purified with sulphuric acid to obtain first lipid

sedimentation. The extract then underwent liquid chromatography on a column containing Florisil that had been dried for 1 h in an oven at 110°C. This further purified the apolar phase of lipids that could not be saponified, such as steroids like cholesterol. The sample was spiked with surrogate compound (2,4,6-trichlorobiphenyls - IUPAC number 30) prior to extraction. This compound was quantified and its recovery calculated. Decachlorobiphenyl (DCBP - IUPAC number 209) was used as an internal standard, added to each sample extract prior to analysis, and included in the calibration standard, a mixture of specific compounds (Arochlor 1260, HCB and pp'- and op'-DDT, DDD and DDE). The analytical method used was High Resolution Capillary Gas Chromatography with a Agilent 6890N and a 63Ni ECD and an SBP-5 bonded phase capillary column (30 m long, 0.2 mm i.d.). The carrier gas was N_2 with a head pressure of 15.5 psi (splitting ratio 50/1). The scavenger gas was argon/methane (95/5) at 40 mL/min. Oven temperature was 100°C for the first 10 min, after which it was increased to 280°C at 5°C/min. Injector and detector temperatures were 200°C and 280°C respectively. The extracted organic material (EOM %) from freeze-dried samples was calculated in all samples. Capillary gas-chromatography revealed op'- and pp'- isomers of DDT and its derivatives DDD and DDE, and 30 PCB congeners. Total PCBs were quantified as the sum of all congeners (IUPAC no. 95, 101, 99,151, 144, 135, 149, 118, 146, 153, 141, 138, 178, 187, 183, 128, 174, 177, 156, 171, 202, 172, 180, 199, 170, 196, 201, 195, 194, 206). These congeners constituted 80% of the total peak area of PCBs in the muscle sample. Total DDTs were calculated as the sum of op'DDT, pp'DDT, op'DDD, pp'DDD, op'DDE and pp'DDE. EDCs were calculated as the sum of all OCs with known endocrine disruptors' capacity (DDTs: op'DDT, pp'DDT, op'DDD, pp'DDD, op'DDE, pp'DDE; PCBs IUPAC no. 95, 99, 101, 118, 153). The results are expressed in ng/g dry weight (d.w.). The detection limit was 0.1ng/kg for all the OCs analysed. The extracted organic material (EOM%: mean level=8.30%; Standard Deviation (SD=3.29) and the water content (H_2O%: mean level=84.6%; SD=1.69) have been evaluated in all samples to normalise the results in ng/g lipid weight (l.w.) and wet weight (w.w.) respectively.

PAH analysis

PAHs were extracted from lyophilized muscle (about 0.5 g) according to [18] with some modifications [19]. Samples were extracted with a mixture of KOH/methanol (1/4) in a Soxhlet apparatus for 4 h. Extraction with 200 mL of cyclohexane was performed to obtain the PAH fraction, which was purified in a chromatographic column packed with Florisil. The organic fraction was concentrated to 1 mL in acetonitrile and analyzed in a reversed-phase column (Supelcosil LC-18, 25 cm × 4.6 mm i.d., 0.5 μm particle size) and an acetonitrile/water gradient was used. Initial gradient was 60% acetonitrile and increased to 100% over 20 min, then remaining stable for 10 min. Flow-rate was 1 mL/min. The external standard consisted of 16 PAHs from Supelco (EPA 610 PAH mixture). Results were expressed as the sum of fourteen PAHs (naphtalene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a) anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene). Carcinogenic PAHs were the sum of six PAHs with this propriety (benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k) fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene). Assay reproducibility was determined by five repeated analyses (variation coefficient ranged 1-3%); recoveries of standard ranged 80-98% and no PAHs were detected in blanks. The detection limit was 0.1ng/g for all PAHs. For PAHs also, the results are expressed in ng/g d.w.

Western blot of CYP1A, VTG and ZRP

Sample preparation, standard homogenization procedures and Western Blot (WB) analysis were performed as described in Fossi et al. [20]. The primary polyclonal antibodies used were: rabbit anti-fish specific for CYP1A (Bio sense, Norway) diluted 1:500 in 1% gelatin in TTBS, rabbit anti-salmon specific for Vtg (Bio sense, Norway) diluted 1:1000 and rabbit anti-salmon specific for Zrp (Bio sense, Norway) diluted 1:500. Secondary antibody used was goat anti-rabbit, conjugated with horseradish peroxidase (HRP, Bio rad) diluted 1:3000. Each WB was scanned and captured as TIF files in order to be processed by quantity one (Bio Rad) software. In each WB analysis an internal standard was inserted to normalize the data obtained.

Data analysis

Data were processed with Shapiro-Wilks test to evaluate the distribution using STATISTA 7.0 software. The Shapiro–Wilk test utilizes the null hypothesis principle: the null-hypothesis is that the population is normally distributed (p>0.05). All the investigated groups analyzed with Shapiro-Wilks test were non–normal distributed, but the small sample size did not allow estimating the statistical significance of the evaluated differences among data groups using non-parametric tests. The descriptive statistics (mean, standard deviation, minimum and maximum) are only used to present the data.

Results

OC levels

HCB, ΣDDTs, ΣPCBs, as well as pp'DDE/ΣDDTs, pp'DDE/pp'DDT and ΣDDTs/ΣPCBs ratios, in muscle of the 15 free-ranging white shark specimens were summarized in Table 2, where levels were also separated by gender: males (n=6), females (n=4) and sex not determined (n=5). All contaminant levels were expressed in ng/g d.w. since variations in lipid and water content among organisms that could affect the compound concentrations was not relevant (see OC analysis in Material and Methods).

Among OCs, HCB was the compound with the lowest levels (0.48% of ΣOCs) with similar values among individuals. ΣPCBs was

the class of contaminants with the highest mean (83.08% of ΣOCs), followed by ΣDDTs (16.44% of ΣOCs). Male white sharks had the highest levels for HCB, ΣDDTs and ΣPCBs than females and not determined; females had higher levels than not determined, except for ΣDDTs.

In terms of congener composition, the PCBs found in the muscles of all white shark specimens were reported in Figure 2. This composition was comparable to what was reported for Arochlor 1260 (reference standard for PCBs) and reflected the prevalence of hexa-CBs (50%) and hepta-CBs (26%). In particular hexa-CB congener 141 (2,2',3,4,5,5'- CB) was the most abundant, accounting for 32% of the total CBs content, while in Arochlor 1260 the same congener is accounted just for 2.5%. In Figure 2 the mean % concentration of PCB congeners was also separated by sex. In males hexa-CBs (64.4%), the most abundant, were followed by hepta-CBs (18.1%), penta-CBs (8.1%), octa-CBs (7.1%) and nona-CBs (2.2%); in females the same pattern were present but in different percentages: hexa-CBs (40.6%)>hepta-CBs (24.7%)>penta-CBs (22.2%)>octa-CBs (9.1%)>nona-CBs (3.4%).
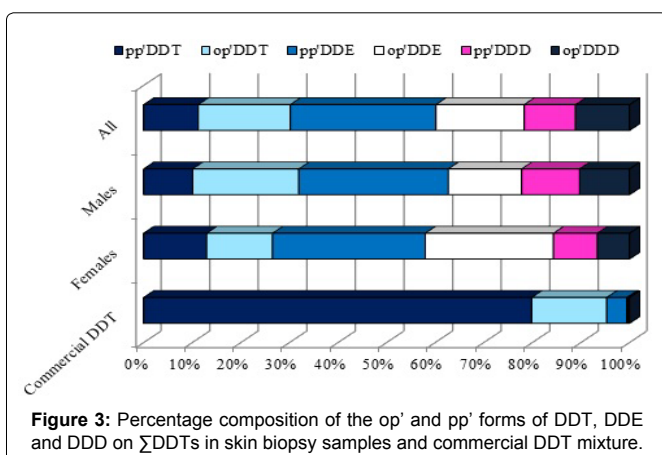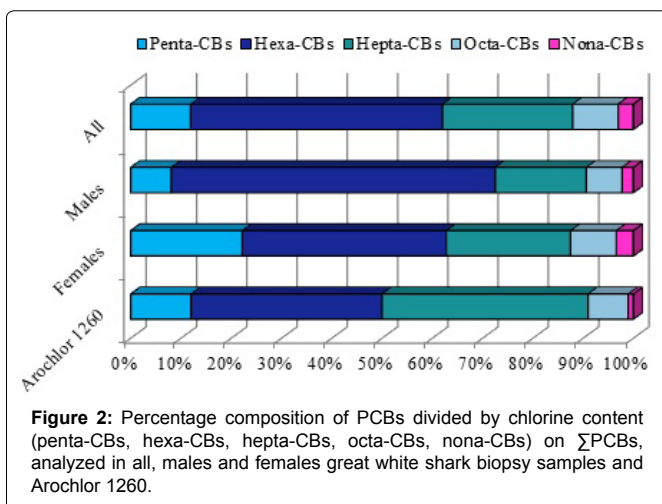
The percentage of pp'DDT and its metabolites evaluated in muscle samples of white sharks and in commercial pesticide were reported in Figure 3. In all groups of samples, the percentage composition was very different respect to the commercial mixture: pp'DDE accounted for about 30% of the total DDT concentration in sharks, while it was present only for 4% in the DDT pesticide; pp'DDT, that is the major and active component of contaminant (77.1% in commercial mixture), accounted from 10 to 13% in the animals. The percentage of op'DDE in all animals (18.2 %) was interesting, particularly in females where it accounted for 26.4%.

Another interesting result is given by the value of the pp'DDE/pp'DDT ratio (Table 2): in the commercial mixture this ratio is 0.05; if this ratio has high values, it means that the major part of the active compound has been degraded and thus there are no recent inputs of the pesticide in the investigated environment [21]. In the muscle of white sharks, the pp'DDE/pp'DDT ratio was 3.05 in males, 2.42 in females and 2.36 in not determined specimens, indicating a very recent DDT input in the environment. Also pp'DDE/ΣDDTs is

Table 2: Mean concentrations (with number of analyzed specimens (n), Standard Deviation (SD) and minimum – maximum range) of organochlorine Contaminants (HCB, ΣDDTs and ΣPCBs), ΣEDCs and PAHs (all measured in ng/g dry weight) detected in the muscle of *Carcharodon carcharias*, collected at Dyer Island (South Africa). 15 samples were analyzed.

	Total	Males	Females	Not determined
HCB (ng/g d.w.)	n=15 13.56 (4.61) 6.80–21.26	n=6 15.43 (3.80) 12.74–21.26	n=4 14.87 (7.00) 6.80–19.23	n=5 10.26 (2.04) 8.61–12.85
ΣDDTs (ng/g d.w.)	n=15 475.73 (353.17) 86.72–1416.97	n=6 546.20 (461.77) 234.89–1416.97	n=4 403.31 (265.91) 86.72–709.41	n=5 449.11 (321.57) 290.70–1023.93
ΣPCBs (ng/g d.w.)	n=15 2554.45 (2854.51) 379.76–11284.31	n=6 3479.65 (4142.84) 615.15–11284.31	n=4 2009.79 (1495.33) 379.76–4005.71	n=5 1879.93 (1799.60) 817.43–5082.88
pp'DDE/ΣDDTs	0.31	0.32	0.33	0.28
pp'DDE/pp'DDT	2.66	3.05	2.42	2.36
ΣDDTs/ΣPCBs	0.19	0.16	0.20	0.24
ΣEDCs	1044.99 (58.63) 38.67–191.44	1022.62 (784.07) 402.29–2360.71	981.75 (608.89) 155.73–1587.80	806.31 (468.58) 572.14–1644.11
ΣPAHs (ng/g d.w.)	n=7 5013.47 (1888.54) 2769.20–7278.40	n=2 6329.30 (1124.25) 5534.33–7124.27	n=3 5399.82 (2097.24) 3137.04–7278.40	n=2 3118.13 (493.47) 2769.20–3467.07
ΣCarPAHs (ng/g d.w.)	n=7 465.75 (174.89) 136.93–623.02	n=2 506.39 (164.94) 389.76–623.02	n=3 431.79 (255.36) 136.93–580.22	n=2 15.11 (2.02) 13.68–16.54

Figure 2: Percentage composition of PCBs divided by chlorine content (penta-CBs, hexa-CBs, hepta-CBs, octa-CBs, nona-CBs) on ∑PCBs, analyzed in all, males and females great white shark biopsy samples and Arochlor 1260.

Figure 3: Percentage composition of the op' and pp' forms of DDT, DDE and DDD on ∑DDTs in skin biopsy samples and commercial DDT mixture.

an indicator of new DDT inputs in the environment, but also of a metabolic "weathering" of DDT; a value of 0.6 is considered critic [22] and values higher than this imply that there are no new inputs. The pp'DDE/∑DDTs ratio had always values much lower than 0.6 (males=0.32; females=0.33; not determined=0.28) confirming the hypotheses of recent inputs of DDT in the environment.

The ratio between DDTs and PCBs (∑DDTs/∑PCBs) (Table 2) was used to characterize the magnitude of the contributions from agricultural and industrial sources to white shark contamination [23], because generally it is higher in water masses closer to agricultural areas and lower in waters closer to industrialized areas, independently of congener number determined. For all the investigated shark classes ∑DDTs/∑PCBs values (between 0.16 and 0.24) showed a high PCB preponderance.

In Figure 4 a comparison between sexual mature specimens has been made. Mature animals (n=4), all males, had levels higher than immature (n=11) for HCB, ∑DDTs and ∑PCBs.

Table 2 reported also the values of ∑EDCs for the white shark specimens. Males had much higher levels of ∑EDCs than females and not determined sex specimens, following the pattern: males>females>not determined. The percentage of ∑EDCs on ∑OCs is 39.9% for all specimens (36.8% for males and 44.0% for females). A comment is necessary about the type of endocrine disrupting compounds composing the group of ∑EDCs in relation to statistical

analysis with sex as an explanatory factor: pp'DDT, op'DDT, pp'DDE and op'DDE and PCB congeners 95, 99, 101 and 153 are EDCs with known estrogenic and anti-androgenic capacity, which can affect male reproductive processes [24-29]; pp'DDE and op'DDT are also androgenic and anti-estrogenic and, with PCB congener 118, could affect female reproductive processes, but they are predominantly estrogenic and anti-androgenic. These pollutants were the major sources of hazard due to OCs in male specimens of white shark. The percentage of ∑EDCs (as environmental estrogens plus anti-androgens and environmental androgens plus anti-estrogens) respect to the OCs total burden were reported in Figure 5. The highest percentage was for the group of environmental estrogens plus anti-androgens both in males and females (70.4% and 76.1% respectively).
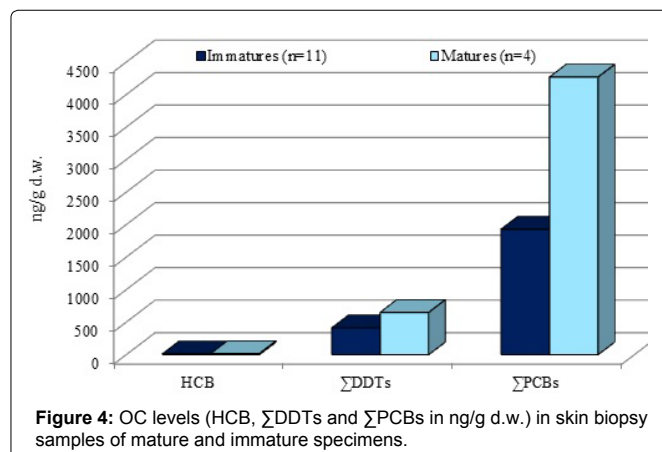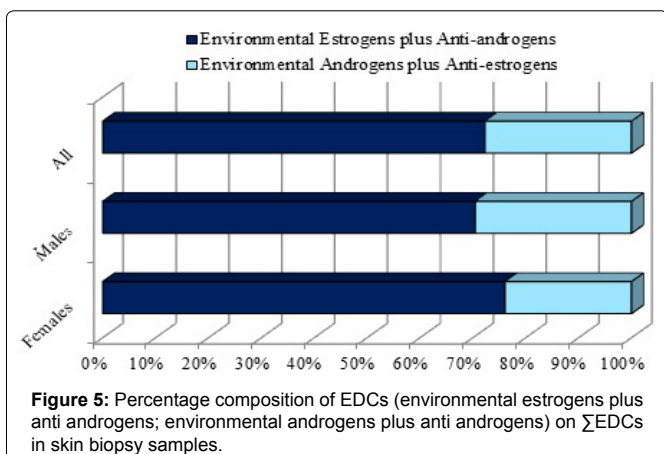
Total and carcinogenic PAH

Due to the low quantity of biological material, PAH levels were analysed only on 7 of the 15 specimens. Males (n=2) showed again the highest levels, followed by females (n=3) and not determined sex specimens (n=2) (Table 2). It is very important to evidence the presence of carcinogenic PAHs (∑CarPAHs), which represented respectively 7.89 ± 0.85% of ∑PAHs in males and 7.45 ± 1.65% in females (Figure 6). Carcinogenic PAHs were also higher in males compared to females. Regarding not carcinogenic PAHs, naphthalene was the compound present with the highest percentage in all groups (all specimens, males and females), followed by acenap hthene>phenanthrene>fluorene>pyrene>fluoranthene>benzo (g,h,i) perylene>anthracene. The fingerprint of carcinogenic PAHs (Figure 7) showed a different pattern between the groups with males that had very high percentage of benzo(a)pyrene (36.7%), while females of benzo(b)fluoranthene (38.3%).

Finally, ∑PAHs and ∑OCs were compared to evaluate the predominant class of contaminants in the skin biopsies (Figure 8). PAHs are present in all the three groups in percentage higher than OCs, particularly in females.

CYP450, VTG and ZRP

Three biomarkers (CYP1A, Vtg and Zrp) were investigated in the skin of three biopsied specimens, all sexually immature respectively female (WSSA4), male (WSSA7) and sex not determined (WSSA10), by Western Blot methodology. The presence of CYP1A (59 kDa molecular weight) was evidenced in all three specimens (Figure 9). The presence of CYP1A was evaluated by the semi-quantitative method and Cytochrome P4501A levels were reported as Adj. Vol.


Figure 4: OC levels (HCB, ∑DDTs and ∑PCBs in ng/g d.w.) in skin biopsy samples of mature and immature specimens.

Figure 5: Percentage composition of EDCs (environmental estrogens plus anti androgens; environmental androgens plus anti androgens) on ∑EDCs in skin biopsy samples.
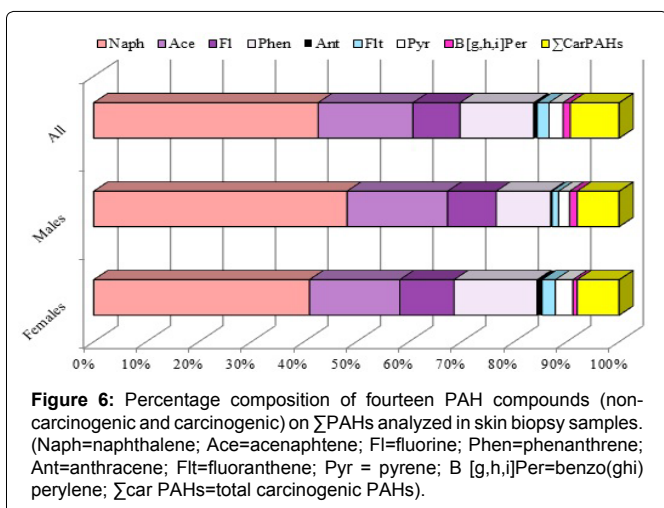
Figure 6: Percentage composition of fourteen PAH compounds (non-carcinogenic and carcinogenic) on ∑PAHs analyzed in skin biopsy samples. (Naph=naphthalene; Ace=acenaphtene; Fl=fluorine; Phen=phenanthrene; Ant=anthracene; Flt=fluoranthene; Pyr = pyrene; B [g,h,i]Per=benzo(ghi)perylene; ∑car PAHs=total carcinogenic PAHs).
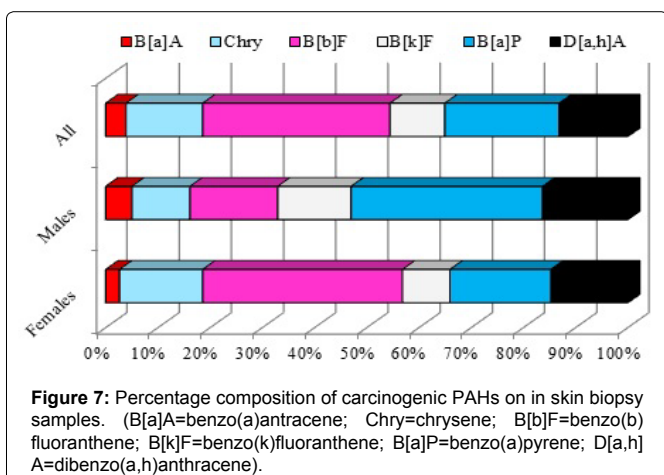
Figure 7: Percentage composition of carcinogenic PAHs on in skin biopsy samples. (B[a]A=benzo(a)anthracene; Chry=chrysene; B[b]F=benzo(b)fluoranthene; B[k]F=benzo(k)fluoranthene; B[a]P=benzo(a)pyrene; D[a,h]A=dibenzo(a,h)anthracene).

INT*mm^2 (Figure 9). The rabbit anti-salmon Vtg primary antibody cross reacted with proteins in a range between 50 and 75 KDa, potentially precursors of the Vitellogenin itself (Figure 9); WSSA4 and WSSA7 showed an evident band in this range. The last biomarker examined was the Zona Radiata Proteins (Figure 9). Three bands ranging between 37 and 50 KDa were found that probably correspond to the three ZRP isoforms α, β and γ. It is very important to underline that the smallest white shark sampled (WSSA7) showed the highest presence of Zrp.

Discussion

The distribution pattern (PCBs>DDTs>HCB) found in all the analysed specimens and showed in Table 2, was very similar to the pattern found in other marine top predators (shark, tuna, swordfish) [30-34]. Furthermore, the highest levels found in male white sharks were in accordance with male specimens of other marine fishes [35,36]. The differences between males and females were not evaluated statistically due to the small sample size; however all females were still immature and therefore have not discharged their load of contaminants through the production of eggs yet [37].

In the commercial mixture (Arochlor 1260), the PCB fingerprint evidenced the prevalence of hexa and hepta-chlorinated congeners (Figure 2); these PCBs are more difficult to metabolise and have a higher bio magnification potential [38]. This fact could explain why there was a prevalence of these chlorinated congeners in their muscle.

The pattern of DDT and its metabolites was very interesting with not only high percentages of pp'DDE but also of op'DDE and, although to a lesser extent, of pp'DDD and op'DDD (Figure 3). The pp'DDE isomer is more biologically active than the op'DDE, but this metabolite is also toxicologically important being a mutagenic, carcinogenic, teratogenic [39] and endocrine disruptor (estrogen and estrogen receptor (ER) agonist) compound. Pp'DDE has also occurred as a compound in commercial-grade DDT but in particular represent the DDT daughter compound. In fact pp'DDE may result from aerobic degradation, abiotic dehydrochlorination or photochemical decomposition [40] and in animals; pp'DDT is mainly metabolized by dehydrochlorination to pp'DDE. In a normal metabolic process, pp'DDT is oxidized to 2,2-bis(4-chlorophenyl) acetic acid (pp'DDA), the major excreted metabolite in animals, but unfortunately pp'DDE, and to a lesser extent other metabolites of DDT, accumulate in animal tissues. There is also a role of CYP in the microsomal reduction of pp'DDT though this mechanism of microsomal reductive dechlorination is not known in detail. pp'DDT, the isomer of pp'DDT, is known to contaminate technical-grade DDT about 20%. Similar enzymatic and non-enzymatic reducing activities are observed toward Op'DDT [41]. So it can be assumed that the op'DDE, resulting also from the degradation of op'DDT present in the commercial pesticide, was probably introduced in recent times, although all these molecules are very persistent. In fact, in the 2000-2005 period 274 tons of DDT were used against *Anopheles* mosquitoes to fight malaria in the KwaZulu-Natal region [42]. This explanation was validated also by pp'DDE/pp'DDT and pp'DDE/DDTs ratios, which indicated a recent and continue DDT input in
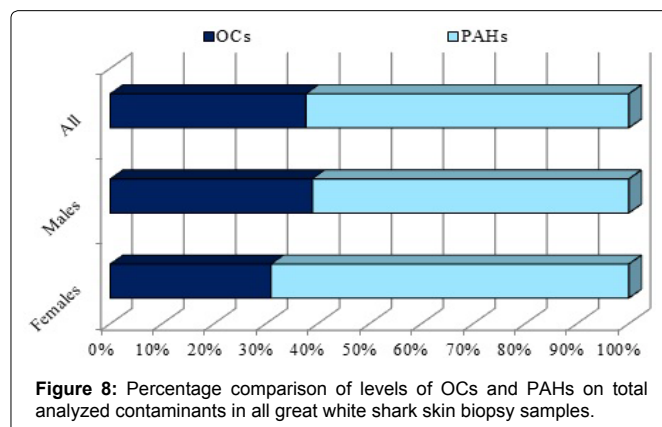
Figure 8: Percentage comparison of levels of OCs and PAHs on total analyzed contaminants in all great white shark skin biopsy samples.
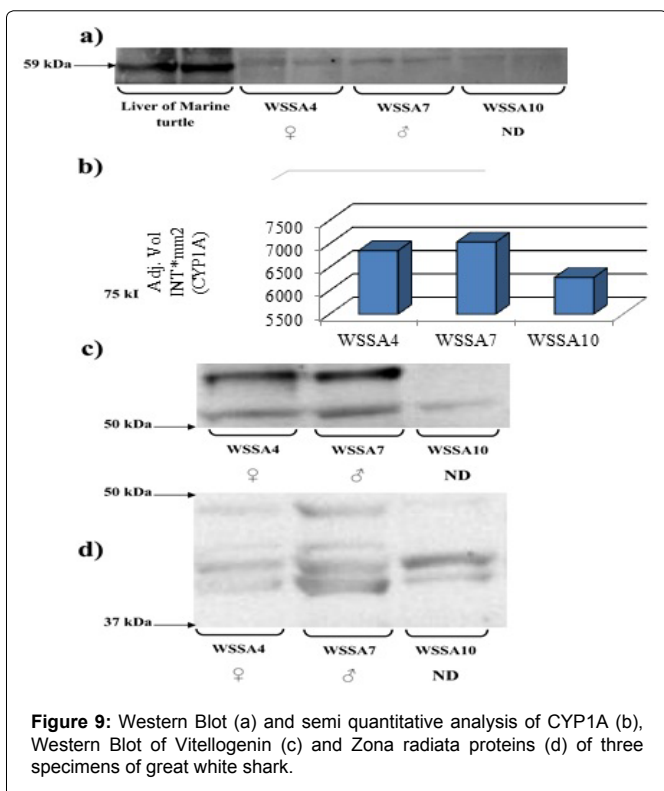
Figure 9: Western Blot (a) and semi quantitative analysis of CYP1A (b), Western Blot of Vitellogenin (c) and Zona radiata proteins (d) of three specimens of great white shark.

the environment (Table 2). The high levels of pp'DDE and op'DDE present in muscles of white sharks reflected not only the stability of these molecules [43], but can also indicate very efficient metabolic processes of this shark population.

Despite the recent DDT input against malaria vector in the South African environment, PCBs were the most present chlorine xenobiotic that is an index of a mainly industrial contamination (Table 2, Figure 4). Conflicting results were found in two other Mediterranean shark species: gulper shark (*Centrophorus granulosus*) and longnose spurdog (*Squalus blainvillei*), which were entangled in gillnets in Adriatic Sea, where PCB levels were lower than DDT levels [44].

Four male sharks, all sexually matures, showed OC levels higher than immature (n=11) (Figure 4). This result was in line with what could be expected with only male mature specimens. In fact females unload part of their contaminant burden to pups with the formation of the eggs, while males accumulate throughout their entire life. This difference in the levels of organochlorines between mature and immature animals could also be explained by diet differences between juveniles and adults [45]. White sharks predate mainly fishes in the early years (when juvenile) until they reach the suitable dimensions to hunt bigger and more energetic preys with major lipids quantity, where lipophilic contaminants like OCs accumulated. The preferential preys of adult white sharks are small-medium size cetaceans or marine mammals like sea lions, which showed high OC levels in the blubber (almost 1000 ng/g lipid bases in South African fur seals (*Arctocephalus pusillus pusillus*).

The UNEP volume entitled Global Assessment of the State-of-the-Science of Endocrine Disruptors [46] proposed a powerful framework to examine the hypothesis that chemicals with endocrine activity are causing adverse effects in wildlife populations; in particular EDC-related sex ratio imbalances have been seen in wild fish and molluscs, and the effects of EDCs on sex ratios in some of these species are also supported by laboratory evidence. Male fish have high incidences of intersex in rivers receiving sewage treatment works effluents that contain oestrogens and anti-androgens [47], and this condition decreases their reproductive success when in competition with normal males [48]. For this reason in this study OC-EDCs known as oestrogens and anti-androgens were separated from androgens and anti-oestrogens in function of the shark gender (Figure 4). Males and females had about the same percentage of these OC-EDCs. In particular male sharks had about 70% of environmental oestrogens and anti-androgens in the muscle. Many laboratories and several field studies clearly show that exposure to oestrogens is also consistent with effects in female fish [49,50]. Masculinizing effects of pollution on female fish, though apparently less prevalent, have also been reported. In fact, the first evidence of ED on fish was provided by Howell et al. [51], an observation that has been confirmed in other countries [52].

Vtg and Zrp biomarkers, important signals of estrogenic alterations used in other top predators species like swordfish [53] were developed and assessed for the first time in this species, and nothing can be found in literature about any kind of utilization in *C. carcharias*. With this preliminary study, there is the evidence of both these proteins not only in a female shark but also in a male shark and in an immature female (Figure 9).

PAHs were evaluated in white sharks because South Africa is on one of the most frequented oil shipping routes, and 28% of the Middle East oil passes from there. This class of organic molecules are known for their genotoxicity, but several lines of evidence indicate correlation between high levels of certain PAHs in the environment and the increasing incidence of carcinogenesis and mutagenesis in exposed organisms [54]. Mostly low molecular weight PAHs were found in the muscle of white shark specimens, in particular naphthalene which is the simplest PAH ($C_{10}H_8$) (Figure 6). As these dissolved low molecular weight PAHs are more quickly adsorbed by biota than those adsorbed on sediment, they are considered more toxic for marine biota [55]. The percentage of carcinogenic PAHs on ΣPAHs in these top predators, both males and females (Figure 7), was very high compared to that found in other top marine predators.

Biota–sediment accumulation factor (BSAF) and bioaccumulation factor (BAF) for fish is very different for organochlorines and PAHs, with these values greater for OCs than those for PAHs [56]. Despite this, the mean percentage of PAHs on total contaminants was higher than the mean percentage of OCs (Figure 8). This result was very surprising since generally the OCs is the priority contaminants in the marine environment. This could be partially explained by the intense oil traffic present in the South African area [57].

CYP1A is a good biomarker of exposure to lipophilic compounds and it was used for the first time in this work to evaluate its presence in white shark biopsies. A higher number of samples will be necessary to confirm these results.

Conclusions

Skin biopsy appeared a good non-lethal technique to evaluate the Eco toxicological status of free-ranging great white shark. The present study was the first to investigate both environmental contaminants (OCs and PAHs) and biomarkers (CYP1A, Vtg protein and Zrp proteins) in white sharks, which showed very high levels of PAHs

compared to other marine animals. The pattern of DDT and its metabolites was very interesting with high percentages of not only pp'DDE but also op' DDE that, probably, result from the recent introduction of DDT to fight malaria in the KwaZulu-Natal region. Also pp'DDE/pp'DDT and pp'DDE/DDTs ratios indicate a recent and continue DDT input in the environment. Anyway, PCBs were the most present chlorine xenobiotic that is index of a mainly industrial contamination. Regarding the biomarker responses, the present results confirmed that induction of Vtg and Zrp could be used as a diagnostic and prognostic tool for the exposure assessment of South African white shark population exposed to OCs with ED capacity. These data suggest the need for continuous monitoring as a tool of conservation and management of this population.

Acknowledgements

References

1. Towner AV, Wcisel MA, Reisinger RR, Edwards D, Jewell OJD (2013) Gauging the Threat: The First Population Estimate for White Sharks in South Africa Using Photo Identification and Automated Software. PLoS ONE 8: e66035.

2. Sperone E, Micarelli P, Andreotti S, Spinetti S, Andreani A, et al. (2010) Social interactions among bait-attracted white sharks at Dyer Island (South Africa). Marine Biology Research 6: 408-414.

3. Sperone E, Micarelli P, Andreotti S, Brandmayr P, Bernabò I, et al. (2012) Surface behaviour of bait-attracted white sharks at Dyer Island (South Africa). Marine Biology Research 8: 982-991.

4. Compagno LJV (2001) Sharks of the World. An annotated and illustrated catalogue of the shark species known to date. FAO Species Catalogue for Fishery Purposes 2: 269.

5. Schlenk D, Sapozhnikova Y, Cliff G (2005) Incidence of organochlorine pesticides in muscle and liver tissues of South African great white sharks Carcharodon carcharias. Marine Pollution Bulletin 50: 208-211.

6. Moldan A, Jackson LF (2005) International Oil Spill Conference. Oil Spill contingency planning in South Africa. Miami Beach Florida USA.

7. Serrano R, Fernández MA, Hernández LM, Hernández M, Pascual P, et al. (1997) Coplanar polychlorinated biphenyl congeners in shark livers from the North-Western African Atlantic Ocean. Bulletin of Environmental Contamination and Toxicology 58: 150-157.

8. Serrano R, Fernández M, Rabanal R, Hernández M, Gonzales MJ (2000) Congener-speci?c determination of polychlorinated biphenyls I shark and grouper livers from the Northwest African Atlantic Ocean. Archives of Environmental Contamination and Toxicology 38: 217-224.

9. Strid A, Jörundsdóttir H, Päpke O, Svavarsson J, Bergman A (2007) Dioxins and PCBs in Greenland shark (Somniosus microcephalus) from the North-East Atlantic. Mar Pollut Bull 54: 1514-1522.

10. Jaime-Rivera M, Caraveo-Patino J, Hoyos Padilla M, Galvan-Magana F (2013) Evaluation of biopsy systems for sampling white shark Carcharodon carcharias (Lamniformes: Lamnidae) muscle for stable isotope analysis. Revista de Biología Marina y Oceanografía 48: 345-351.

11. Colborn T, vom Saal FS, Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101: 378-384.

12. Colborn T, Dumanoski DE, Myers JP (1996) Our Stolen Future: Are We Threatening Our Fertility, Intelligence and Survival? A Scientific Detective Story. In Penguin Books Dutton New York 306.

13. Colborn T, Smolen MJ, Rolland R (1998) Environmental neurotoxic effects: the search for new protocols in functional teratology. Toxicol Ind Health 14: 9-23.

14. Jimenez B, Gonzalez MJ, Hernandez LM, Eljarrat E, Rivera J, et al. (1999) 2,3,7,8-substituted PCDDs and PCDFs in sea lion (Otaria flavescens) skin biopsies from two South-western Atlantic populations. Chemosphere 38: 507-515.

15. Vetter W, Weichbrodt M, Scholz E, Luckas B, Oelschlager H (1999) Levels of organochlorines (DDT, PCBs, toxaphene, chlordane, dieldrin, and HCHs) in blubber of South African fur seals (Arctocephalus pusillus pusillus) from Cape Cross/Namibia. Marine Pollution Bulletin 38: 830-836.

16. Francis MP (1996) Observation on a pregnant white shark with a review of reproductive biology. AP Klimley, DG Ainley. Great white sharks, The Biology of the White Shark, Carcharodon carcharias. Academic Press San Diego CA USA 157-172.

17. Marsili L, Focardi S (1997) Chlorinated hydrocarbon (HCB, DDTs and PCBs) levels in cetaceans stranded along the Italian coasts: an overview. Environmental Monitoring and Assessment 45: 129-180.

18. Griest WH, Caton JE (1983) Extraction of polycyclic aromatic hydrocarbons for qualitative analysis. In A Biørseth. Handbook of polycyclic aromatic hydrocarbons 95-148.

19. Marsili L, Caruso A, Fossi MC, Zanardelli M, Politi E, et al. (2001) Polycyclic aromatic hydrocarbons (PaHs) in subcutaneous biopsies of Mediterranean cetaceans. Chemosphere 44: 147-154.

20. Fossi MC, Casini S, Ancora S, Moscatelli A, Ausili A, et al. (2001) Do endocrine disrupting chemicals threaten Mediterranean swordfish? Preliminary results of vitellogenin and zona radiata proteins in Xiphias gladius. Marine Environmental Research 52: 477-483.

21. Aguilar A (1984) Relationship of DDE/SDDT in Marine Mammals to the Chronology of DDT Input into the Ecosystem. Canadian Journal of Fisheries and Aquatic Sciences 41: 840-844.

22. Tsydenova O, Minh TB, Kajiwara N, Batoev V, Tanabe S (2004) Recent contamination by persistent organochlorines in Baikal seal (Phoca sibirica) from Lake Baikal, Russia. Mar Pollut Bull 48: 749-758.

23. Aguilar A, Borrell A, Pastor T (1999) Biological factors affecting variability of persistent pollutant levels in cetaceans. Journal of Cetacean Research Management (special issue) 1: 83-116.

24. Adami HO, Lipworth L, Titus-Ernstoff L, Hsieh CC, Hanberg A, et al. (1995) Organochlorine compounds and estrogen-related cancers in women. Cancer Causes Control 6: 551-566.

25. Wong PW, Pessah IN (1996) Ortho-substituted polychlorinated biphenyls alter calcium regulation by a ryanodine receptor mediated mechanism. Structural specificity toward skeletal and cardiac type microsomal calcium release channels. Molecular Pharmacology 49: 740-751.

26. Hansen PD, Dizer H, Hock B, Marx A, Sherry J, et al. (1998) Vitellogenin: a biomarker for endocrine disruptors. Trends Analytic Chemistry 17: 448-451.

27. Sohoni P, Sumpter JP (1998) Several environmental oestrogens are also anti-androgens. J Endocrinol 158: 327-339.

28. Hilscherova K, Machala M, Kannan K, Blankenship AL, Giesy JP (2000) Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environmental Science Pollution Research 7: 159-171.

29. Safe SH (2000) Endocrine disruptors and human health--is there a problem? An update. Environ Health Perspect 108: 487-493.

30. Stefanelli P, Ausili A, Di Muccio A, Fossi C, Di Muccio S, et al. (2004) Organochlorine compounds in tissues of swordfish (Xiphias gladius) from Mediterranean Sea and Azores islands. Marine Pollution Bulletin 49: 938-950.

31. Storelli MM, Storelli A, Marcotrigiano GO (2005) Concentrations and hazard assessment of polychlorinated biphenyls and organochlorine pesticides in shark liver from the Mediterranean Sea. Mar Pollut Bull 50: 850-855.

32. Fossi MC, Casini S, Marsili L (2007) Potential toxicological hazard due to endocrine-disrupting chemicals on Mediterranean top predators: state of art, gender differences and methodological tools. Environmental Research 104: 174-182.

33. Storelli MM, Casalino E, Barone G, Marcotrigiano GO (2008) Persistent organic pollutants (PCBs and DDTs) in small size specimens of bluefin tuna (Thunnus thynnus) from the Mediterranean Sea (Ionian Sea). Environ Int 34: 509-513.

34. Moon HB, Choi HG (2009) Human exposure to PCDDs, PCDFs and dioxin-like PCBs associated with seafood consumption in Korea from 2005 to 2007. Environ Int 35: 279-284.

35. Fossi MC, Casini S, Marsili L, Ancora S, Mori G, at al. (2004) Evaluation of eco toxicological effects of endocrine disrupters during a four-year survey of the Mediterranean population of swordfish (Xiphias gladius). Marine Environmental Research 58: 425-429.

36. Moon HB, Kim HS, Choi M, Yu J, Choi HG (2009) Human health risk of polychlorinated biphenyls and organochlorine pesticides resulting from seafood consumption in South Korea, 2005-2007. Food and Chemical Toxicology 47: 1819-1825.

37. Mull CG, Lyons K, Blasius ME, Winkler C, O'Sullivan JB, et al. (2013) Evidence of Maternal Offloading of Organic Contaminants in White Sharks (Carcharodon carcharias). PLoS ONE 8: e62886.

38. Sawhney BL (1986) Chemistry and properties of PCBs in relation to environmental effects. JS Waid. PCBs and the Environment CRC Press Boca Raton 47-64.

39. Marsili L, D'Agostino A, Bucalossi D, Malatesta T, Fossi MC (2004) Theoretical models to evaluate hazard due to organochlorine compounds (OCs) in Mediterranean striped dolphin (Stenella coeruleoalba). Chemosphere 56: 791-801.

40. Thomas JE, Ou LT, All-Agely A (2008) DDE remediation and degradation. Rev Environ Contam Toxicol 194: 55-69.

41. Kitamura S, Shimizu Y, Shiraga Y, Yoshida M, Sugihara K, et al. (2002) Reductive metabolism of p,p'-DDT and o,p'-DDT by rat liver cytochrome P450. Drug Metab Dispos 30: 113-118.

42. Mudzunga JM (2006) Technical Advisor Department of Agriculture South Africa.

43. Borrell A, Aguilar A (1987) Variations in DDE percentage correlated with total DDT burden in the blubber of fin whales. Marine Pollution Bulletin 18: 70-74.

44. Storelli MM, Marcotrigiano GO (2001) Persistent organochlorine residues and toxic evaluation of polychlorinated biphenyls in sharks from the Mediterranean Sea (Italy). Mar Pollut Bull 42: 1323-1329.

45. Hussey NE, McCann HM, Cliff G, Dudley SFJ, Wintner SP, et al. (2012) Chapter 3: Size-based analysis of diet and trophic position of the white shark, Carcharodon carcharias, in South African waters. ML Domeier Global perspectives on the biology and life history of the white shark. Taylor and Francis Group Boca Raton FL USA 27-49.

46. IPCS (2002). Global Assessment of the State-of-the-Science of Endocrine Disruptors. Geneva: World Health Organization.

47. Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, et al. (2006). Predicted exposures to steroid estrogens in UK Rivers correlate with widespread sexual disruption in wild fish populations. Environmental Health Perspectives 114: 32-39.

48. Harris CA, Hamilton PB, Runnalls TJ, Vinciotti V, Henshaw A, et al. (2011) The consequences of feminization in breeding groups of wild fish. Environ Health Perspect 119: 306-311.

49. Jobling S, Coey S, Whitmore JG, Kime DE, Van Look KJ, et al. (2002) Wild intersex roach (Rutilus rutilus) have reduced fertility. Biol Reprod 67: 515-524.

50. Tyler CR, Jobling S (2008) Roach, Sex, and Gender-Bending Chemicals: The Feminization of Wild Fish in English Rivers. BioScience 58: 1051-1059.

51. Howell WM, Black DA, Bortone SA (1980) Abnormal expression of secondary sex characters in a population of mosquitofish Gambusia affinis. Evidence for environmentally-induced masculinization. Copeia 4: 676-681.

52. Larsson Å, Haux C, Sjöbeck ML, Lithner G (1984) Physiological effects of an additional stressor on fish exposed to simulated heavy-metalcontaining effluent from a sulfide ore smeltery. Ecotoxicological Environmental Safety 8: 118-128.

53. Fossi MC, Marsili L, Neri G, Natoli A, Politi E, (2003) The use of nonlethal tool for evaluating toxicological hazard of organoclorine contaminants in Mediterranean cetaceans: new data ten years after the first paper published in MPB. Marine Pollution Bulletin 46: 964-971.

54. NRCC (1983) Polycyclic aromatic hydrocarbons in the aquatic environment. Formation, sources fate and effects on aquatic biota. National Research Council Canada 18981: 1-209.

55. Kwok CK, Liang Y, Leung SY, Wang H, Dong YH et al. (2013) Biota-sediment accumulation factor (BSAF), bioaccumulation factor (BAF), and contaminant levels in prey fish to indicate the extent of PAHs and OCPs contamination in eggs of waterbirds. Environmental Science Pollution Research 20: 8425-8434.

56. Neff JM (1979) Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. London: Applied Science Publishers LTD 262.

57. Houlobek I, Paasivirta J, Maatela P, Lahtiperä M, Holoubkovà I, et al. (1990) Comparison of extraction methods for polycyclic aromatic hydrocarbon determination in sediments. Toxicology Environmental Chemistry 25: 137-154.

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