PRELIMINARY RESULTS OF ORGANOCHLORINES LEVELS AND BIOMARKER RESPONSES IN SKIN BIOPSIES OF THE COMMON BOTTLENOSE DOLPHINS FROM THE ADRIATIC SEA (MEDITERRANEAN SEA)

FOSSI, M.C. (1), HOLCER, D.(2, 3), MALTESE S. (1), FORTUNA C. (2,4), WIEMANN, A. (2,5), CANESE, S. (4), RENAUD DE STEPHANIS (6), MACKELWORTH P (2), MARSILI, L. (1).

(1) Department of Environmental Sciences, University of Siena, Via Mattioli 4, 53100 Siena, Italy,
(2) Blue World Institute of Marine Research and Conservation, Kaštel 24, 51551 Veli Lošinj, Croatia
(3) Croatian Natural History Museum, Demetrova 1, 10000 Zagreb, Croatia
(4) Istituto Superiore per la Protezione e la Ricerca Ambientale, Via Casalotti 300, Roma, Italy
(5) Evolutionary Biology/Special Biology, Institute for Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24-25, Haus 26, 14476 Golm, Germany
(6) CIRCE, Calle Cabeza de Manzaneda 3, 11390 Pelayo-Algeciras, Cádiz, Spain

ABSTRACT
The main objective of this study was to apply a sensitive non-lethal "multi-trial-diagnostic–tool" in skin biopsy of common bottlenose dolphin (Tursiops truncatus), combining molecular biomarkers (western blot of CYP1A1, CYP2B) with analysis of OCs, in subcutaneous blubber, to evaluate the toxicological status of this odontocete species in the Adriatic Sea (Mediterranean Sea). The results of these analyses were compared with those obtained on samples collected in two other areas: the Strait of Gibraltar and the Sicily Channel. The multi-trial biomarker tool applied to skin biopsies underlined differences in OCs and molecular biomarker responses between genders (male greater than female) and among these three areas, revealing a lower toxicological stress in the bottlenose dolphins of the central Adriatic Sea.

KEYWORDS
MONITORING, COMMON BOTTLENOSE DOLPHIN (TURSIOPS TRUNCATUS), ADRIATIC SEA, MEDITERRANEAN SEA, POLLUTANTS, BIOMARKERS.

INTRODUCTION

The Adriatic Sea is the northernmost part of the Mediterranean Sea. Its length is about 780 km and average width is 240 km. The bathymetric characteristics divide the Adriatic into three parts: the shallow northern Adriatic (avg. depth of 40 m), the middle Adriatic (max. depth 266 m) and the southern Adriatic (max. depth 1233 m). The eastern middle Adriatic region is occasionally exposed to strong Mediterranean water inflow (Buljan, 1957; Buljan and Zore-Armanda, 1976). Based on its origin, the water mass is defined as Levantine intermediate water (LIW). The outflow of Adriatic water to the Mediterranean occurs along the western coast and is supported by the Etesian wind (Zore-Armanda, 1969).

The Adriatic Sea is influenced by numerous human activities. Intensive and fast growing maritime traffic (both merchant and tourist ships), oil and gas exploration and drilling (over 1,000 platforms, either operating or dismissed, were build over 40 years) and heavy tourism in summer. Moreover, the introduction of invasive marine species into this ecosystem by ships and via other vectors, has been identified as one of the greatest threats. In addition, its coastal area experiences a heavy pressure due to fast growing urbanisation and different land based aspects of the above mentioned economic activities. At the same time the anthropogenic impact on the Adriatic sea is not evenly distributed along its coasts. In general, the eastern Adriatic side is considered relatively pristine (Degobbis *et al.* 1986, Caricchia *et al.* 1993, Limić and Valković 1996, Tankere and Statham 1996 Kljaković-Gašpić *et al.* 2002) compared to the western; the same applies to deep central and southern Adriatic compared to the shallow northern Adriatic. Such uneven distribution of the impact is due to the number of factors like general water circulation (counter clockwise) (Poulain 1999, 2001); huge difference in the volume of water between north and south, the position of the Po river delta (north-western "corner") that conveys pollutants from one of the most industrialised and inhabited area of southern Europe (*Pianura Padana*); and a relatively low density of coastal urban agglomerates on the eastern side compared to the Italian side.

Vis island is the farthest inhabited island in the Adriatic, located in the open part of the middle Adriatic about 55 km off the mainland. It is strongly influenced by incoming Mediterranean water masses (Zore-Armanda 1963) known as Levantine Intermediate Water (LIW) (Artegiani *et al*. 1993) and seems not affected by land-derived materials (Marasović *et al* 2006). Total number of inhabitants is about 3,700 (census 2001). On the island there are no industrial facilities. Similar to other Adriatic islands, there are no surface water flows. The situation is similar for all of the neighbouring islands (Hvar, Korčula, Lastovo), making the local influence on pollution of the central Adriatic sea area minimal. The strongest impact on pollution of this area is through untreated sewage that is released in the sea and the main anthropogenic impact on this area are related with tourism and fisheries.

The common bottlenose dolphin (*Tursiops truncatus*) is the only resident species of cetaceans in the Adriatic Sea, where it inhabits both coastal areas and open waters. Although it has been studied on a number of locations including Croatia (for example, the Cres-Lošinj archipelago, the Kornati archipelago, north-western coast of Istrian peninsula, Vis and Lastovo islands), Slovenia and Italy (for example, Venice bay, Bay of Trieste and along the coast of Emilia Romagna) there is no information on the overall population status. Culling was one of the main threats in past times, but it does not represent a threat today. However, bottlenose dolphins inhabiting the Adriatic Sea are believed to be facing negative influences of habitat degradation and fragmentation, general overfishing, high environmental pollution, incidental mortality in fishing gear and most recently, a newly emerging threats, noise pollution and disturbance.

As shown in many research sub-lethal exposure to OCs may directly reduce fitness of an individual and, consequently, viability of whole populations (Safe, 1993; Smith and Hall, 1994; O'Shea and Tanabe, 2003; Reijnders, 2003; Keller et al., 1999/2000; Gardner and Oberdörster, 2006). Hence OCs present a potential threat to wildlife. This is of particular concern for long-living and low-fecundity species, such as cetaceans.

The main objective of this project was to apply a suite of sensitive non-lethal biomarkers in skin biopsy of bottlenose dolphin to evaluate the toxicological status of this odontocetes in the Adriatic Sea (Mediterranean Sea). We developed a "multi-trial-biomarker-tool", combining molecular biomarkers (western blot of CYP1A1, CYP2B) with analysis of OCs (DDTs, PCBs) aiming to the ecotoxicological assessment of this odontocete population.

MATERIALS AND METHODS

Sampling - Integument biopsies (epidermis, dermis and blubber) were obtained from 14 common bottlenose dolphins (males N=5, females N=8, unknown N=1) inhabiting the waters around Vis island (Croatia), in the central Adriatic Sea. The skin biopsying was performed using a 54 kg draw crossbow with biopsy arrows collecting tissue sample about 1.5 cm long and 0.6 cm in diameter from distances between 5 and 15 m from the animal. The biopsy tips and arrows were designed and manufactured by F. Larsen from the Danish Institute for Fisheries Research. Samples were immediately placed in liquid nitrogen or stored in cell medium (Qiagen, RNA Later) for the "slice" experiments. Adriatic samples were compared with 3 samples of common bottlenose dolphins from Gibraltar (Spain) collected in 2005 and 4 samples of common bottlenose dolphins collected in Lampedusa (Italy) in 2006.

Contaminants analysis

Organochlorine Compounds (OCs): The analytical method used for quantitative and qualitative analysis of HCB, DDTs and PCBs was High Resolution Capillary Gas chromatograph equipped with an electron capture detector (63Ni ECD)(AGILENT 6890/N), according to the U.S. Environmental Protection Agency (EPA) 8081/8082, modified by us (Marsili and Focardi, 1996). The gas chromatograph had a SPB-5 bonded phase in a 30 m long fused silica capillary column. The "EDC-OCs" consisted in analysed organochlorines known as endocrine disruptors such as: HCB, PCB95, PCB99, PCB101, PCB118, PCB153, pp'DDT, op'DDT, pp'DDE, op'DDE, pp'DDD, op'DDD.

Biomarkers analysis

CYP1A1 and CYP2B western blot* - *CYP1A and ***CYP2B*** have been detected in cetacean skin and induction of these isoforms was found after exposure to lipophylic contaminants such as OCs, PAHs and BFR both *in vitro* and in field studies (Fossi *et al.*, 2006; Fossi *et al.*, 2008). For WB analysis, S9 fractions of tissue homogenates (biopsy and slice biopsies, in duplicate for each sample) were separated by SDS-PAGE (10% polyacrylamide gels – Criterion XT Precast Gel - BioRad) and blotted onto nitrocellulose sheets for 1 hour at the constant voltage of 200 V. The membranes were saturated by incubating them with a blocking solution (3% gelatin dissolved in Tris Buffered Saline containing 0.05% Tween-20, TTBS) for 1 hour at room temperature. Primary polyclonal rabbit antibodies from Oxford Biochemical Research were used (Oxford MI, USA). Goat anti-rabbit CYP1A1 and anti CYP2B4, diluted 1:5000 and 1:1000, respectively, in TTBS-1% gelatin, were incubated overnight at room temperature with cetacean proteins. Incubation with anti-rabbit HRP-labelled secondary

antibody (1:3000 final dilution) was performed for 1.5 hours at room temperature and protein detection was done according to the BioRad Immun-Star HRP Chemiluminescent Kit booklet, using standardized times (Fossi *et al.,* 2008). Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (BioRad, 1-D Analysis Software) using the methods proposed by Fossi *et al.* (2008) (Fig. 1).
Gender was determined genetically according to Berube and Palsboll (1996).

RESULTS AND DISCUSSIONS

Two main preliminary considerations can be done to this preliminary investigation: a) Gender differences; b) Population differences.

Gender differences

- Higher levels of PCBs, DDTs and OCs-EDCs were found in male of Adriatic bottlenose dolphins, compared to females from the same area (Fig. 2 a,b,c). The difference in bioaccumulation levels between males and females is likely related to the high capacity of females to excrete lipophylic contaminants during lactation.

- Exploring molecular biomarker responses, the high induction of CYP1A1 (Fig. 3a) and CYP2B (Fig. 3b) in male Adriatic bottlenose dolphins, compared to that of females, seems to be related to the presence of higher levels of planar OCs, such as coplanar PCBs, and of OC globular insecticides such as DTTs and its metabolite pp'DDE in the blubber. This data confirmed the high sensitivity of these two different Cytochrome as diagnostic tools of planar and globular xenobiotics.

Population differences

- The three populations of *T. truncatus* investigated in this study showed differences in organochlorine, OCs-EDCs (organochlorines with Endocrine Disruption potential), and biomarker responses (Fig. 4-5). Not all statistically significant.

- Higher levels of PCBs, DDTs and OCs-EDCs were found in males from the Strait of Gibraltar, compared to those of the Adriatic Sea and the Sicily Channel (Fig.4 a,b,c). This seems to confirm a high toxicological stress to which the bottlenose dolphins inhabiting the Strait of Gibraltar are exposed to.

- When exploring molecular biomarker responses, the induction of CYP1A1 and CYP2B in Gibraltar bottlenose dolphin, compared to those from the other two locations, seem to indicate the presence of higher levels of planar OCs, such as coplanar PCBs, and of OC globular insecticides such as DTTs and its metabolite pp'DDE in the blubber. This data show the high sensitivity of this diagnostic methodology to define the "ecotoxicological status" of cetacean populations inhabiting different areas.

Limitations of this study

The sample size is generally small, particularly that of comparing areas (Lampedusa island and Gibraltar Strait).

CONCLUSIONS

The multi-trial-diagnostic-tool applied to skin biopsies, underlined differences in OCs, OCs-EDCs levels and molecular biomarker responses between the three populations, revealing a low toxicological stress in the central eastern Adriatic *T. truncatus*. These results suggest that the state of the environment in the eastern central Adriatic Sea is under lower anthropogenic influence.
Nevertheless, the small sample prevents further conclusions on the impact of OCs pollution on this population and point to the need to increase the sample size. Furthermore, the sample does not cover different age groups, which is important to evaluate the actual burden throughout this bottlenose dolphin's population.
Monitoring of the burden of OCs, OCs-EDCs levels and molecular biomarker responses in this population coupled with population ecology study could both reveal the long term effects of pollution on this population and build up an indirect dataset on the status of the local environment.
Also, the results underline the need to carry out more complete study of the influence of the toxicological stress on the common bottlenose population(s) inhabiting northern and western parts of the Adriatic Sea basin.

As animals with delayed maturity, longevity and low fecundity, cetaceans are extremely vulnerable to anthropogenic threats. Therefore, the hidden impact the OCs accumulated in the tissues could have on this population of a species of conservation concern should be further monitored.

ACKNOWLEDGEMENTS
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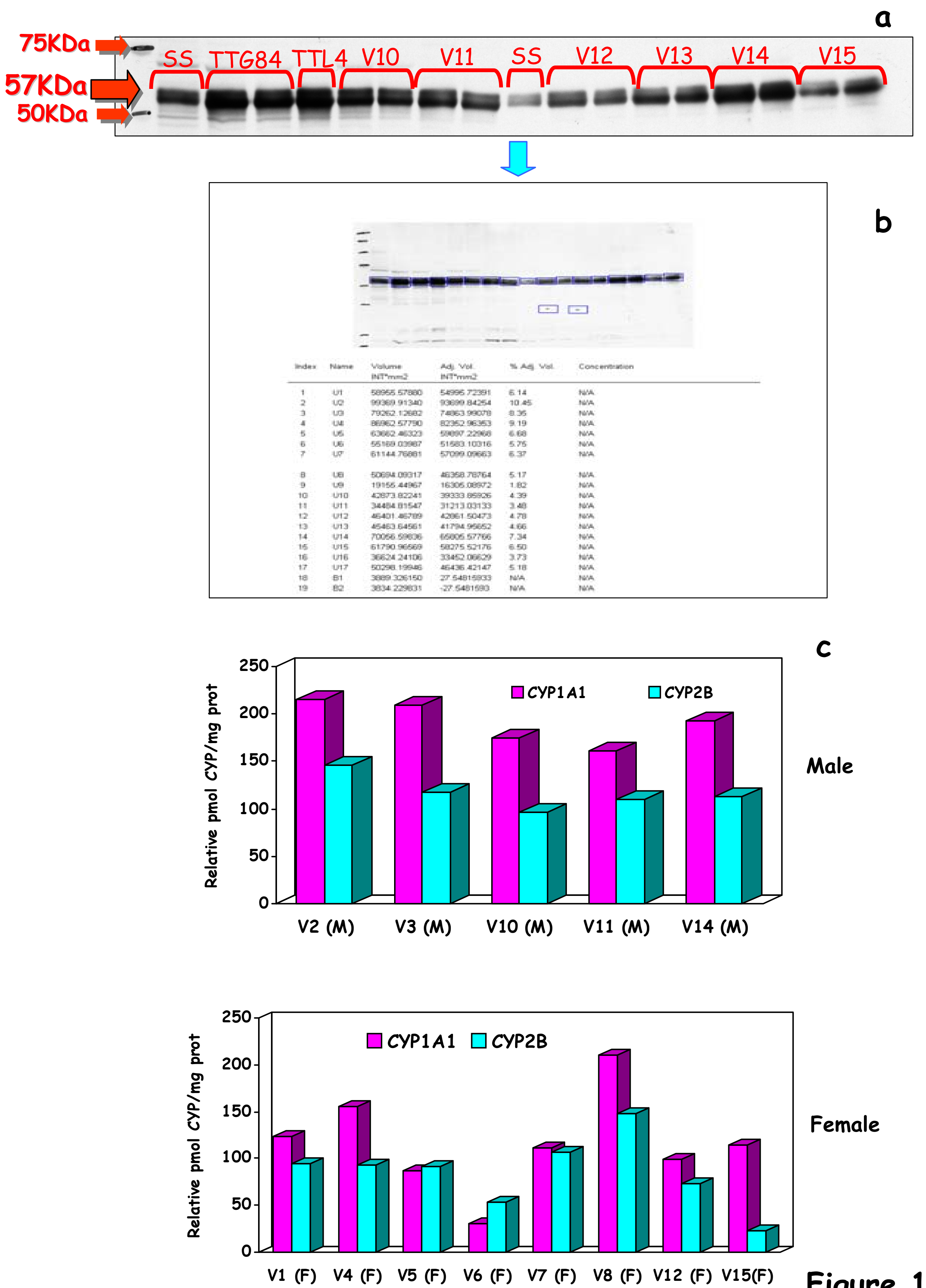
CAPTIONS

Figure 1. Western blot (WB) analysis of CYP1A1 in specimens of Adriatic common bottlenose dolphin (*Tursiops truncatus*) (biopsy samples). Different steps of the application of the diagnostic methodology (WB, Quantity one analyses, and Relative pmol CYP1A1) are reported. The results are express as Relative pmol CYP/mg prot.

Figure 2. ***Contaminant levels: genders differences*** - DDTs(a), PCBs(b) and OCs-EDCs (c), levels in skin biopsy of male and female specimens of common bottlenose dolphin (*Tursiops truncatus*) collected in Adriatic Sea.

Figure 3. ***Biomarker responses: genders differences*** - Western blot of CYP1A1 (a), CYP2B(b), in skin biopsy of male and female specimens of common bottlenose dolphin (*Tursiops truncatus*) collected in Adriatic Sea (* = $p < 0.1$; ** = $p < 0.05$).

Figure 4. ***Contaminant levels: populations differences*** - DDTs(a), PCBs(b) and OCs-EDCs (c), levels in skin biopsy of male and female specimens of common bottlenose dolphin (*Tursiops truncatus*) collected in the three study areas. (* = $p < 0.1$; ** = $p < 0.05$).



Index	Name	Volume INT*mm2	Adj. Vol. INT*mm2	% Adj. Vol.	Concentration
1	U1	58955.57880	54995.72391	6.14	N/A
2	U2	99369.91340	93699.84254	10.45	N/A
3	U3	79262.12682	74863.99078	8.35	N/A
4	U4	86962.57790	82352.96353	9.19	N/A
5	U5	63662.46323	59897.22968	6.68	N/A
6	U6	55169.03987	51583.10316	5.75	N/A
7	U7	61144.76881	57099.09663	6.37	N/A
8	U8	50694.09317	46358.78764	5.17	N/A
9	U9	19156.44967	16306.08972	1.82	N/A
10	U10	42873.82241	39333.86926	4.39	N/A
11	U11	34484.81547	31213.03133	3.48	N/A
12	U12	46401.46789	42861.50473	4.78	N/A
13	U13	45463.64561	41794.95652	4.66	N/A
14	U14	70056.59836	65806.57766	7.34	N/A
15	U15	61790.96569	58275.52176	6.50	N/A
16	U16	36624.24106	33452.06629	3.73	N/A
17	U17	50298.19946	46436.42147	5.18	N/A
18	B1	3889.326150	27.54815933	N/A	N/A
19	B2	3834.229831	-27.5481593	N/A	N/A

Figure 1

Contaminant levels

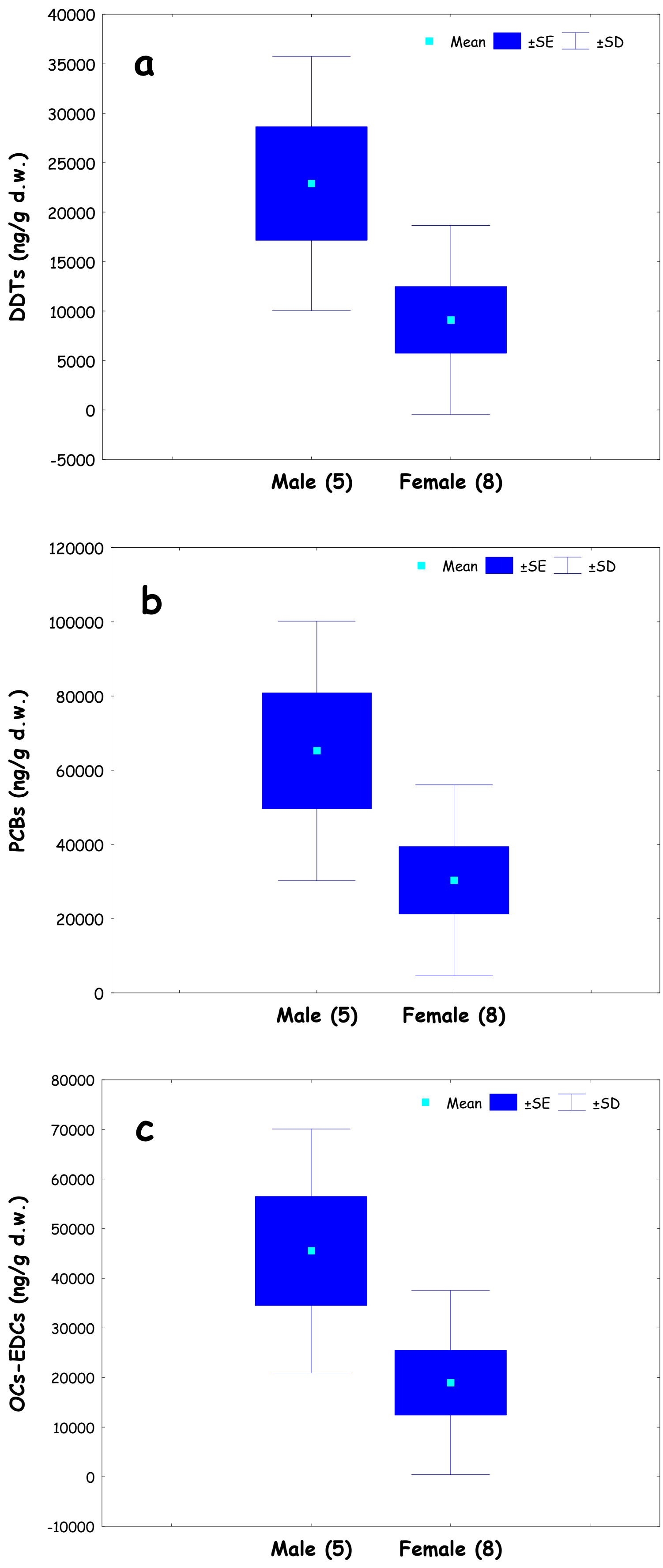


Figure 2

Biomarker responses

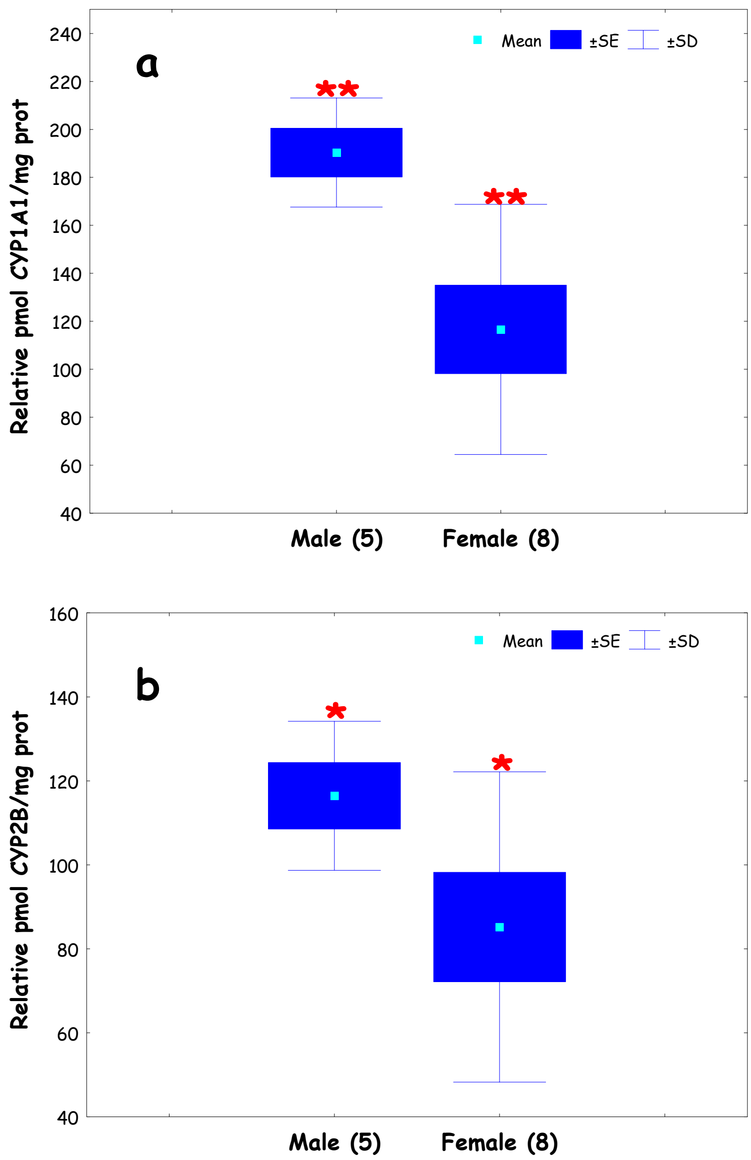


Figure 3

Contaminant levels

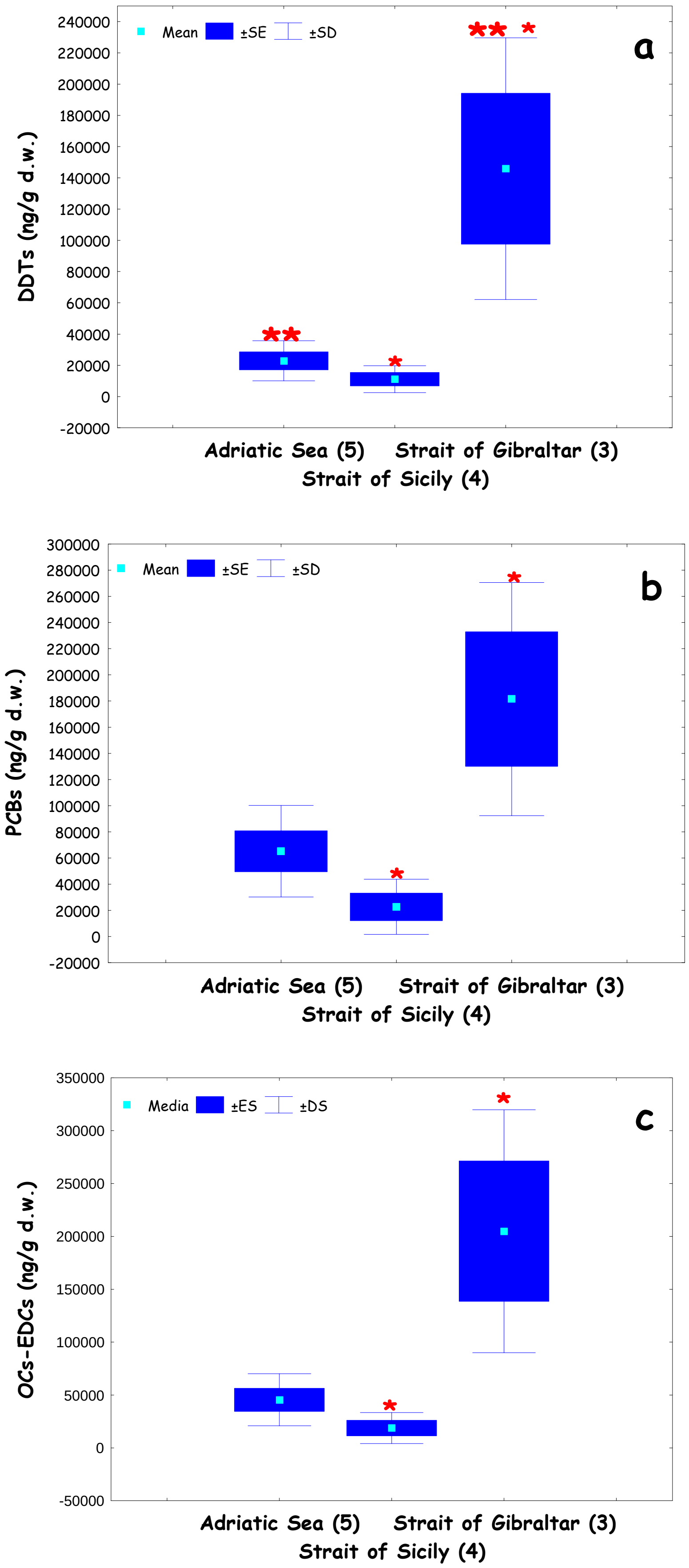


Figure 4