NEW-TOOLS TO ASSESS THE TOXICOLOGICAL HAZARD OF ENDOCRINE DISRUPTOR ORGANOCLORINE CONTAMINANTS IN MEDITERRANEAN CETACEANS

M. Cristina Fossi[1], Letizia Marsili[1], Silvia Casini[1]

[1]Dep. of Environmental Sciences, University of Siena, Italy

Introduction

The Mediterranean top predators, and particularly cetacean odontocetes, accumulate high concentrations of organochlorine contaminants (OCs), incurring high toxicological risk. Some organochlorine compounds, now with worldwide distribution, are known as endocrine disrupting chemicals (EDCs).

Four types of organochlorine endocrine disruptors [1,2,3] are commonly found in Mediterranean cetaceans [4,5,6]: 1) environmental estrogens, 2) environmental androgens, 3) anti-estrogens and 4) anti-androgens. Endocrine disruptors act by mimicking sex steroid hormones, both estrogens and androgens, by binding to hormone receptors or influencing cell pathways (environmental estrogens and androgens), or by blocking and altering hormone receptor binding (anti-estrogens, anti-androgens). Environmental estrogens are the most common and most widely studied EDCs [7]. The relative estrogenic power of these chemicals, identified by *in vitro* and *in vivo* screening methods [8] is rather weak (10^{-3} or less) compared with the reference power of 17-estradiol or DES. However, the high levels of organochlorine compounds detected in marine mammals, particularly in pinnipeds and odontocetes, and consequently, the high levels of organochlorines with ED capacity, cannot be ignored.

Some general considerations on the potential hazard to these Mediterranean species can be drawn from comparison of the levels of EDCs commonly detected in Mediterranean cetaceans and that of other cetacean species with known reproductive impairment [9]. Several examples suggest that exposure to OC insecticides and PCBs has affected endocrine function and reproduction in marine mammals. For example, transformation of epididymal and testicular tissue has been observed in north Pacific minke whales (*Balaenoptera acutorostrata*) [10] . Tumours and reproductive problems are documented in beluga whales of the St. Lawrence estuary, now among the most contaminated animals on earth [11,12]. De Guise et al. [13] reported a true hermaphrodite beluga whale. Here it is worth noting that levels of PCBs found in Mediterranean free ranging odontocetes sampled in the period 1992-1999 (striped dolphin, bottlenose dolphin and common dolphin, mean value = 54587 ng/g l.w.; 44924 ng/g l.w.; 25032 ng/g l.w. respectively) [6] are similar to those detected in the population of beluga whales of the St. Lawrence estuary in which a hermaphrodite specimen was

detected (mean value = 78900 ng/g l.w.) [14]; levels of PCBs detected in Mediterranean free ranging fin whales in the same period (mean value = 7331 ng/g l.w.) [6] are approximately 10 times higher than those found in the population of bowhead whales (*Balaena mysticetus*) in which were detected pseudohermaphroditism and other reproductive dysfunctions (mean value = 610 ng/g l.w.) [15,16]. These observations suggest the potential risks associated with EDC exposure in Mediterranean cetaceans.
Here the hypothesis that some Mediterranean cetaceans (*Stenella coeruleoalba, Delphinus delphis, Tursiops truncatus* and *Balaenoptera physalus*) are "potentially at risk" due to organochlorines with endocrine disrupting capacity is investigated using new non-lethal tools. As "diagnostic" tool we use benzo(a)pyrene monooxygenase (CYP1A1) activity in skin biopsies (non-lethal biomarker) as a potential indicator of exposure to organochlorines, with special reference to the compounds with endocrine disrupting capacity. As "prognostic" tool we propose the immunofluorescence technique in fibroblast cell cultures, for a qualitative and quantitative evaluation of the target proteins as CYP450 1A1-1A2, CYP450 2B4 and ER receptor.

Materials and methods

Sampling. Subcutaneous tissues (skin and blubber) were obtained from *Stenella coeruleoalba, Tursiops truncatus, Delphinus delphis and Balaenoptera physalus* from the western Ligurian Sea, between Corsica and the French-Italian coast, and Ionian Sea using biopsy darts launched with a crossbow. The biopsy dart, a regular aluminium crossbow bolt with a modified stainless steel collecting tip and floater, was fired into the whale with a Barnett Wildcat II crossbow with a 150-pound test bow [6]. To avoid the possibility of infection, the bolt tip was sterilised with alcohol before shooting. Biopsy specimens were taken in the dorsal area near a dorsal fin and on the upper part of the caudal peduncle. All material was immediately placed in liquid nitrogen.

Sex identification. Sex determination in cetaceans was carried out by genetic investigations according to Berube & Palsboll [17].

Biomarkers. The small size of the biopsy samples (between 0.200 g and 0.002 g) did not permit isolation of the microsomal fractions. Benzo(a)pyrene monooxygenase (CYP1A1) activity (BPMO activity) was detected in whole tissue. Since the connective tissue was very tough, the epidermis was homogenized in 1.15% KCl buffer at pH 7.5 by thermal shock and separated by freezing in liquid N_2 and pulverizing in a Potter apparatus with ultrasound. BPMO activity was assessed using the incubation mixture proposed by Fossi *et al.* [18] incubating each sample (plus the blanks) in a shaking bath for 2 h at 37°C. The activity was expressed in arbitrary units of fluorescence (A.U.F./h/g tissue).

Organochlorines. The samples of subcutaneous blubber (about 0.3 g) were freeze-dried and extracted with n-hexane in a Soxhlet apparatus for analysis of chlorinated hydrocarbons [19]. Sample purification was carried out by adding concentrated sulphuric acid to the extracts; after elimination of "black" residues, the extracts were reconcentrated and purified by Florisil column chromatography. The analytical method used was High Resolution Capillary Gas Chromatography with a Perkin-Elmer Series 8700 GC and a 63Ni ECD. Capillary gas-chromatography revealed the presence of op'- and pp'- isomers of DDT and its derivatives DDD and DDE, and about 30 PCB congeners.

Fibroblasts cell culture. The development of a non-invasive sampling method for obtaining viable tissue samples to cell cultures from skin biopsies of free-ranging cetaceans was described in a study published by Marsili et al (2000). The skin sample was stored in sterile medium MEM Eagle Earle's salts w/L-glutamine and sodium bicarbonate (Mascia Brunelli, Milan, Italy)+10% gamma irradiated fetal calf serum (Mascia Brunelli)+1% MEM not essential aminoacids (NEAA) solution 100× (Mascia Brunelli)+1% Penicillin/Streptomycin 100× (Mascia Brunelli)+0.1% Amphotericin B 100× (Mascia Brunelli) at ambient temperature, and was processed within 24 h of collection. In the laboratory, each sample was washed with Earle's balanced salt solution (EBSS; Mascia Brunelli) containing antibiotic (Penicillin/Streptomycin 100× [Mascia Brunelli]) and antimycotic (Amphotericin B 100× [Mascia Brunelli]) solutions. All specimens were handled using sterile techniques. First, the collected tissue was cut into small pieces with curved surgical scissors, placed in 30-mm Petri dishes and incubated with Trypsin-EDTA solution 1× (Mascia Brunelli) for 15 min at 37°C. The biopsy fragments were washed again and then placed in Falcon 25 flasks, moistened with medium. After 24 h at 37°C in an incubator with 5% CO_2, the cultures were covered with 1 ml of medium. Half of the culture medium was replaced every 48 h with fresh medium.

Results and Discussion

In this project skin biopsy was used, as "diagnostic" tool to explore OCs bioaccumulation processes and CYP1A1 activity (BPMO) in four free-ranging species of Mediterranean cetaceans. As a "prediction" model fibroblast cultures were also used as an alternative *in vitro* method of evaluating interspecies susceptibility and gender differences (CYP450 1A1-1A2, CYP450 2B4 and *Zona Pellucida* proteins responses) to a mixture of OCs with endocrine disrupting capacity.

BPMO (CYP1A1) activity and Organochlorines in skin biopsies

We evaluated CYP1A1 (BPMO) activity in skin biopsies of marine mammals *(Stenella coeruleoalba, Tursiops truncatus, Delphinus delphis* and *Balaenoptera physalus)* as a potential indicator of exposure to EDCs, such as organochlorines (OCs) (Fig.1).

Organochlorine concentrations (HCB, DDTs and PCBs) and BPMO activities, in the skin biopsies of odontocetes and mysticetes sampled in the Mediterranean sea, are reported as descriptive statistics (means and standard deviations) in Figures 1 A, B, C and D. Confirming literature data and results obtained in our lab before 1994 [18, 19, 20] , indicated that marked differences in levels of all contaminants exist between fin whales and odontocete species (Fig. 1A, B and C). The same was found for BPMO activity (Fig. 1D) but differences between fin whale and odontocete species were smaller [18]. This difference was remarkable only for striped dolphins. The main explanation for these results is their different position in the food chain with odontocetes as terminal consumers and fin whales as macroplanktophages.

There was a linear correlation between OCs known as endocrine disruptors and BPMO activity (Pearson test) in striped dolphins and common dolphins. Gender differences in BPMO induction was also investigated. In striped dolphins a linear correlation was found between op'DDT/BPMO and PCB153/BPMO [6]. In the common dolphin there were identified five linear correlations with the BPMO activity: DDTs, pp'DDE, op'DDT, PCBs and PCB153. The main result in this species was no induction of BPMO in females with increasing levels of contaminants. A similar result was obtained in fin whales sampled in the Ligurian Sea from 1992 to 1995 [21]. A statistically significant correlation was found between BPMO activity and organochlorine levels (DDTs/BPMO p=0.0319; PCBs/BPMO p=0.0220; DDTs+PCBs/BPMO p=0.0155); in male skin biopsy specimens but not in

females or males and females considered together. This difference in the inductive capacity of skin BPMO between males and females of this species is interesting but more research is required in order to explain it.
These results suggest that BPMO induction may be an early sign of exposure to EDCs such as OCs and a potential alert for transgenerational effects. It is therefore a powerful "prognostic" indicator [9].

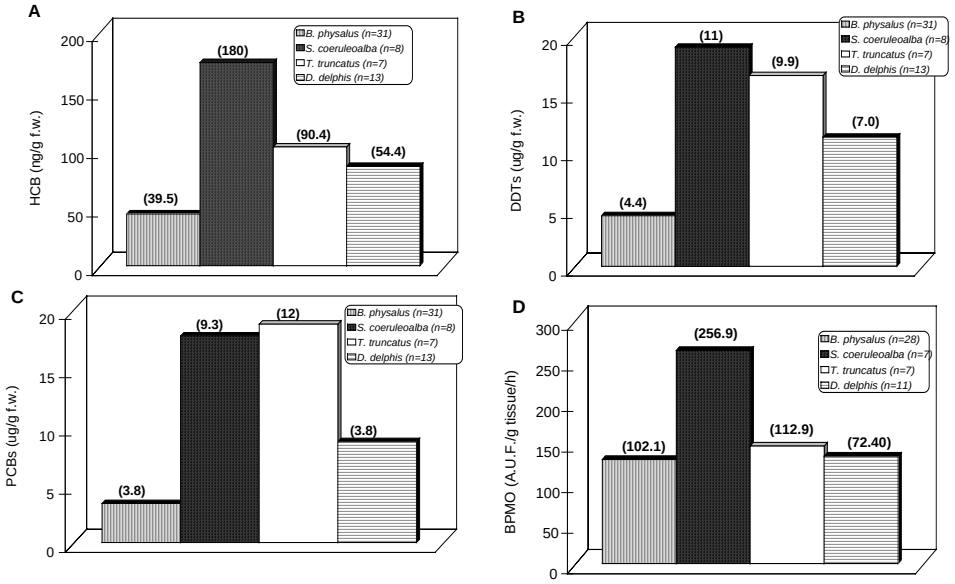
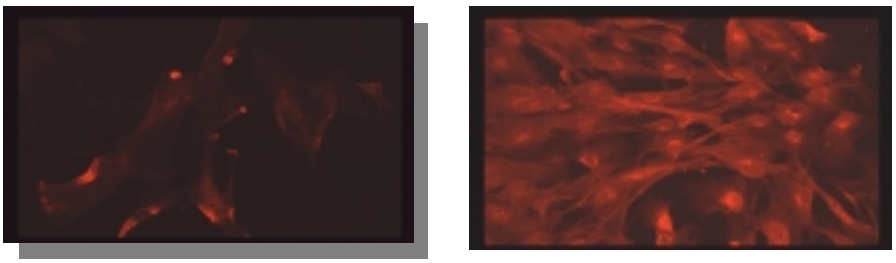


FIGURE 1- A, B, C, D. HCB, DDT and PCB concentration (ng/g and µg/g f.w.) and BPMO activity (AUF/g tissue/h) in skin biopsies from Mediterranean cetaceans. Arithmetic mean and S.D. in brackets; n= number of samples. (Fossi et al. 2003, modified).

New Methodological tools In the study of Marine Mammals: fibroblast cell cultures
Non-destructive biomarker approach is extremely useful for the study of interspecies susceptibility and gender susceptibility to contaminants in Mediterranean cetaceans. The justification for this research comes from the observation that Mediterranean species, such as the *Delphinus delphis* (common until this century), have almost completely disappeared from the Mediterranean sea. To explore the role of detoxification enzymes (and the related biochemical susceptibility) and the ER receptor role, we are conducting a project using fibroblast cell cultures of different Mediterranean species to explore interspecies susceptibility to EDCs contaminants.
Samples of different species of Mediterranean cetaceans have already been collected in several parts of the Mediterranean. Skin biopsy samples are stored in a cell medium and taken to the lab within 24-36 h. Successful cell cultures were obtained from *S. coeruleoalba*, *T. truncatus* and *D. delphis*. The first fibroblasts were observed after 7–21 days. Cultures reached 90% confluence in 15–20 days, then were trypsinized, washed and placed in Falcon 50 and 125 flaskes, after two and three

trypsinizations, respectively. The samples grew for over 4 months, however, there were signs of senescence and increased resistance to trypsin treatment.
We proposed the immunofluorescence technique in fibroblast cell cultures, for a qualitative and quantitative evaluation of the target proteins as CYP450 1A1-1A2, CYP450 2B4 and estrogen receptor (ER)[22] . In this pilot project, fibroblast cell cultures (third generation) of bottlenose dolphin (*T. truncatus*) and striped dolphin *(S. coeruleoalba)* sampled with a biopsy dart, were subjected, for 24 h, to this experimental design, using CYP450 inducers with EDCs potency: a mixture of Arochlor 1260, pp'DDT e pp'DDE solubilized in DMSO (0,05%) added at three different doses: 1µg/ml, 5µg/ml and 25µg/ml, plus a DMSO (0,05%) control. After a first reaction with the primary antibodies for CYP450 1A1-1A2 and 2B4 and with the primary antibody for human estrogen receptor (hER), the cells were treated with the respective secondary antibodies marked with a fluorochrome. The main results of this pilot experiments were: 1) the detection of presence of the cytochromes 1A1-1A2 and 2B4 (Figure 2) and of the estrogen receptor in bottlenose dolphin and striped dolphin fibroblast cells, revealed from the crossreaction of the antibody used and from the presence of fluorescence in the fibroblasts; 2) the increase of fluorescence (cytochromes 2B) in relation to the treatment doses of contaminants; 3) the differences in increase of fluorescence (cytochromes 2B) between the two species, in relation to the treatment doses of contaminants, with high induction responses in *S. coeruleoalba* than *T. truncatus*.



A **B**

Figure 2- Immunofluorescence technique: fibroblast cell cultures of bottlenose dolphin (*T. truncatus*) (A) and striped dolphin *(S. coeruleoalba)* (B) treated for 24h with a mixture (25µg/ml) of Arochlor 1260, pp'DDT e pp'DDE solubilized in DMSO. Cyp450 2B4 primary Rabbit Anti-goat 2B4 P450 antibody provided by Oxford Biomedical Research

The information obtained in this preliminary experiment will represent the basis for further application and validation of this methodology in the study of the susceptibility of marine mammals to endocrine disruptors.

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