Potential toxicological hazard due to endocrine-disrupting chemicals on Mediterranean top predators: State of art, gender differences and methodological tools

Article *in* Environmental Research · June 2007

DOI: 10.1016/j.envres.2006.06.014 · Source: PubMed

CITATIONS
59

READS
144

3 authors, including:

Maria Cristina Fossi
Università degli Studi di Siena
281 PUBLICATIONS **6,163** CITATIONS
SEE PROFILE

Letizia Marsili
Università degli Studi di Siena
197 PUBLICATIONS **3,571** CITATIONS
SEE PROFILE

3B2v8.06a/w (Dec 5 2003).51c + model | YENRS : 4928 | Prod.Type:FTP pp.1–9(col.fig.:1–4) | ED:RajeshGupta PAGN:Kaushik SCAN:

ARTICLE IN PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Environmental Research

ELSEVIER

Environmental Research ▮ (▮▮▮▮) ▮▮▮–▮▮▮

www.elsevier.com/locate/envres

Potential toxicological hazard due to endocrine-disrupting chemicals on mediterranean top predators: State of art, gender differences and methodological tools ☆, ☆☆

M.C. Fossi*, S. Casini, L. Marsili

Department of Environmental Sciences, University of Siena, Via PA Mattioli 4, 53100 Siena, Italy

Received 15 November 2005; received in revised form 9 June 2006; accepted 9 June 2006

Abstract

Man-made endocrine-disrupting chemicals (EDCs) range across all continents and oceans. Some geographic areas are potentially more threatened than others: one of these is the Mediterranean Sea. Levels of some xenobiotics are much higher here than in other seas and oceans. In this paper we review the final results of a project supported by the Italian Ministry of the Environment, in which the hypothesis that Mediterranean top predator species (such as large pelagic fish and marine mammals) are potentially at risk due to EDCs was investigated. We illustrate the need to develop and apply sensitive methodological tools, such as biomarkers (Vitellogenin, Zona Radiata proteins and CYP1A activities) for evaluation of toxicological risk in large pelagic fish top predators (Swordfish, (*Xiphias gladius*), Bluefin Tuna (*Thunnus thynnus thynnus*)) and nondestructive biomarkers (CYP1A activities and fibroblast cell culture in skin biopsy), for the hazard assessment of threatened marine mammals species (Striped Dolphin, (*Stenella coeruleoalba*), Bottlenose Dolphin (*Tursiops truncatus*), Common Dolphin (*Delphinus delphis*) and Fin Whale (*Balaenoptera physalus*))exposed to EDCs. Differential gender susceptibility to EDCs is also explored both in large pelagic fish and in cetaceans. In cetaceans, male specimens showed higher cytochrome P450 induction (BPMO in skyn biopsies, CYP2B in fibroblasts cell cultures) by xenobiotics with respect to females.

Keywords: Endocrine-disrupting chemicals; Mediterranean Sea; Top predators; Biomarkers; Fibroblast cell cultures

1. Introduction

Endocrine disrupting chemicals (EDCs) are a structurally diverse group of compounds that may adversely affect the health of humans, wildlife and fisheries, or their progenies, by interaction with the endocrine system (Colborn et al., 1993, 1996, 1998; Gillesby and Zacharewski, 1998). They include chemicals used heavily in the past, in industry and agriculture, such as polychlorinated biphenyls and organochlorine pesticides, and currently used, such as plasticisers and surfactants. Many of the known EDCs are estrogenic, affecting particularly reproductive functions. Because of the lipophilic and persistent nature of most xenobiotic estrogens and their metabolites, many bioaccumulate and biomagnify (Colborn, 1998; Arukwe et al., 1997).

Man-made EDCs range across all continents and oceans. Some geographic areas, such as the Mediterranean Sea, are potentially more threatened than others. This basin has limited exchange of water with the Atlantic Ocean, and is surrounded by some of the most heavily populated and industrialised countries in the world. Levels of some xenobiotics are therefore much higher here than in other seas and oceans (Aguilar et al., 2002). Mediterranean marine fauna could therefore be a target of EDCs. In this peculiar environment, top predators (such as large pelagic fish and cetaceans) accumulate large quantities of organochlorine contaminants (OCs) and toxic metals (Corsolini et

☆This project was supported by grants from the Italian Ministry for the Environment and ICRAM (Central Institute for Scientific and Technological Research Applied to the Sea), Rome.

☆☆During this research any studies involving humans or experimental animals were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

*Corresponding author. Fax: +39 0577 232913.
E-mail address: fossi@unisi.it (M.C. Fossi).

0013-9351/$ - see front matter © 2006 Published by Elsevier Inc.
doi:10.1016/j.envres.2006.06.014

al., 1995; Marsili, 2000). The levels of OCs in a top predator of the Mediterranean, the striped dolphin (*Stenella coeruleoalba*), are 1–2 orders of magnitude higher than in Atlantic and Pacific dolphins of the same species (Marsili, 2000; O'Shea and Aguilar, 2001; Aguilar and Borrell, 2005). In stranded striped dolphin geometric mean levels of DDTs and PCBs from six Italian study areas were 101 ppm dry weight (d.w.) and 152 ppm d.w., respectively (Marsili, 2000). In other Mediterranean areas, levels of DDTs and PCBs in stranded striped dolphins were 71–456 ppm lipid basis (l.b.) and 67–846 ppm l.b., respectively (Alzieu and Duguy, 1979; Aguilar and Borrell, 1994). Levels of DDTs and PCBs in the Atlantic stranded specimens of the same species were 30–36 and 39–59 ppm l.b., respectively, (Taruski et al., 1975; Borrell, 1993a). In the same species stranded on Pacific coasts, DDTs levels were 21–43 and PCBs 6–29 ppm l.b. (Tanabe et al., 1983; Loganathan et al., 1990). Excluding the dolphins dying in Catalonia as result of *Morbillivirus* (Aguilar and Borrell, 1994), concentrations of these compounds in blubber of striped dolphins living in the Mediterranean are much higher than in specimens of the same species living in oceans. This suggests that Mediterranean top predator species are potentially "at risk" due to EDCs contamination.

In this paper we review the final results of a project, supported from 1999 to 2002 by the Italian Ministry of the Environment, in which the potential estrogenic effects of polyhalogenated aromatic hydrocarbons on Mediterranean top predator was investigated.

We focus on gender differences in biochemical responses, contaminants bioaccumulation and susceptibility to OCs both in large pelagic fish and in cetaceans.

Sensitive biomarkers were used, such as Vitellogenin (Vtg), Zona Radiata proteins (Zrp) and CYP1A activities, for evaluation of toxicological risk in *Xiphias gladius* and *Thunnus thynnus thynnus* (*Large Pelagic Fish Project*), and non-lethal techniques, such as non-destructive biomarkers (CYP1A (BPMO) activities and fibroblast cell culture in skin biopsy), for the hazard assessment of threatened marine mammals species exposed to EDCs. Species examined included cetaceans (*Stenella coeruleoalba, Tursiops truncatus, Delphinus delphis* and *Balaenoptera physalus*) (*Marine Mammals Project*).

2. Materials and methods

2.1. Large pelagic fish

2.1.1. Sampling

Swordfish (*X. gladius*), 98 males and 94 females, were caught in the summers of 1999–2002 in the Straits of Messina (Sicily, Italy) during the spawning period (June–August). They were caught by harpoon from the traditional Sicilian "Passerella" fishing boats which have a long bridge for the harpooners and a tall tower for sighting swordfish. The fish were of different class ages, the range of weights was between 30 and 150 kg. Twenty Atlantic specimens were also captured in the summer of 1999 in Azores coastal waters, the range of weights was between 25 and 125 kg.

The swordfish is a marine predator with worldwide distribution, preying primarily on cephalopods and secondarily on teleosts. Information on swordfish growth is limited and somewhat contradictory. There is good evidence that males and females have different growth patterns, females attaining the larger size. Swordfish live to at least 9 years of age (Stillwell and Kohler, 1995).

Bluefin tuna (*Thunnus thynnus thynnus*) ($n = 20$) were caught in summer 2000 in the Straits of Messina (Sicily, Italy) during the spawning period.

Bluefin tuna is a large top predator present throughout the Mediterranean and Black sea and also widely distributed in the Atlantic Ocean. It is fast swimming and has transoceanic migration in schools. Outside the spawning season, it is a voracious predator of all kinds of fish, crustaceans and molluscs (Fischer, 1973). Maximum age is at least 15 years (Arena et al., 1980).

2.1.2. Sex identification

Sex identification of swordfish and tuna fish specimens was carried out firstly in the field immediately after capture by direct observation of gonads. Successively histological analysis were performed in gonadal tissue sections stored in Bouin fixative.

2.1.3. Biomarkers

Vitellogenin (Vtg) and *Z. radiata* proteins (Zrp) were detected in plasma samples of swordfish and bluefin tuna using western blot and ELISA analysis. For western blot analysis, plasma polypeptides from different fish (20 μg/lane) were separated by SDS-PAGE (7.5% polyacrylamide gels) and blotted onto nitrocellulose sheets (0.45 μm BioRad) for 1 h at a constant voltage of 100 V. The membranes were saturated by incubating with blocking solution (3% gelatine dissolved in Tris Buffered Saline containing 0.05% Tween-20, TTBS) for 45 min at room temperature. Primary polyclonal rabbit antibodies were purchased from Biosense Laboratories (Bergen, Norway). PO-2 anti-sea bream Vtg and O-173 anti-salmon Zrp antibodies, diluted 1:500 and 1:1000, respectively, in TTBS-1% gelatin, were allowed to incubate with fish proteins for 15 h at room temperature. Incubation with the anti-rabbit HRP labelled secondary antibody (1:3000 final dilution) was performed for 1 h at room temperature and detection was carried out as outlined in the Amersham ECL kit booklet. Immunochemical analysis of Vtg and Zrp in plasma samples was performed by indirect ELISA (Goksoyr, 1991). A 96-well microplate was used for the test, each sample was tested in triplicate, adding 10 μg of protein to each well. Dilution of the primary antibodies was 1:1000 for anti-Vtg (PO-2) and 1:3000 for anti-Zrp (O-173). Results were expressed as Absorbance at 492 nm wavelength.

CYP1A activities were evaluated in the microsomal fraction of fish liver by assaying benzo(a)pyrene hydroxylase (BPMO) and ethoxyresorufin-O-deethylase (EROD) activities. All assays were carried out at 30 °C. BPMO activity was measured by the method of Kurelec et al. (1977), activity was expressed in A.F.U./mg prot/h. EROD activity was measured by the method of Lubet et al. (1985), activity was expressed in pmol/mg prot/min. Samples for biomarker analysies were assayed sequentially in the different years and then re-assayed simultaneously in 2002 giving reproducible results.

2.1.4. Organochlorines

Individual tissues of each specimen (liver, muscle and gonads) were freeze-dried and extracted with *n*-hexane in a Soxhlet apparatus for analysis of organochlorines, using the method proposed by Marsili (2000). Water content was found to be 76% in liver, 63% in muscle, and 81% in gonads.

The analytical method used was High-Resolution Capillary Gas Chromatography with a Perkin-Elmer Series 8700 GC and a 63Ni ECD. A mixture of specific isomers was used to calibrate the system, evaluate recovery and confirm the results which were expressed in μg/g d.w. Capillary gas-chromatography revealed op′- and pp′-isomers of DDT and its derivatives DDD and DDE, and about 30 PCB congeners. Samples for organochlorines analysies were assayed sequentially in the different years and the reproducibility of methods over the 4 years was examined.

All data were processed by parametric statistical analysis (ANOVA) using Statistica software. The multiple range test was used to obtain significant differences between means (level of significance, 0.05). The correlations between parameters were examined using the non-parametric test Tau b Kendall. The significant level was $P<0.05$.

2.2. Marine mammals

2.2.1. 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 Ionic Sea using a pole or a biopsy dart 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 body with a Barnett Wildcat II crossbow with a 150-pound test bow (Fossi et al., 2003). 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.

2.2.2. Sex identification

Sex determination in cetaceans was carried out by genetic investigations according to Berube and Palsboll (1996).

2.2.3. Biomarkers

The small size of the biopsy samples (between 0.200 and 0.020 g) did not permit isolation of the microsomal fractions. BPMO (CYP1A1) activity was detected in whole tissue. Since the connective tissue was very tough, the epidermis was homogenised in 1.15% KCl buffer at pH 7.5 by thermal shock and separated by freezing in liquid N_2 and pulverising in a Potter apparatus with ultrasound. BPMO activity was assessed using the incubation mixture proposed by Fossi et al. (1992) 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).

2.2.4. 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 (Marsili, 2000). 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.

Data was processed using Statistica 5.0 (Microsoft), and Pearson product–moment coefficient was used to estimate the linear relationship between two variables (Fossi et al., 2003).

2.2.5. 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.

3. Results and discussion

3.1. Large pelagic fish

The first warning about adverse toxicological risk to large Mediterranean pelagic fish due to EDCs was sounded for swordfish (*Xiphias gladius*) (Fossi et al., 2001a) and blue fin tuna (*Thunnus thynnus thynnus*) (Fossi et al., 2002). These authors used vitellogenin (Vtg) and zona radiata proteins (Zrp) as diagnostic and prognostic biomarkers. Induction of vitellogenin and *zona radiata* protein transcription and translation are well-known major responses to estrogens in fish (Arukwe et al., 1996).

Dramatic induction of these typically female proteins was detected by ELISA and western blot in adult males of the two species (Figs. 1–3).

Four years surveys on the Mediterranean population of *Xiphias gladius* confirmed the finding of dramatic induction in adult male swordfish (Fossi et al., 2004). Several Mediterranean swordfish males specimens showed values of Zrp (33%) (Fig. 4A) and Vtg (11%)(Fig. 4B) which were higher than males average values and/or in the same range as those of reproductive females, which suggests that this species is exposed to xenoestrogen in the Mediterranean Sea.

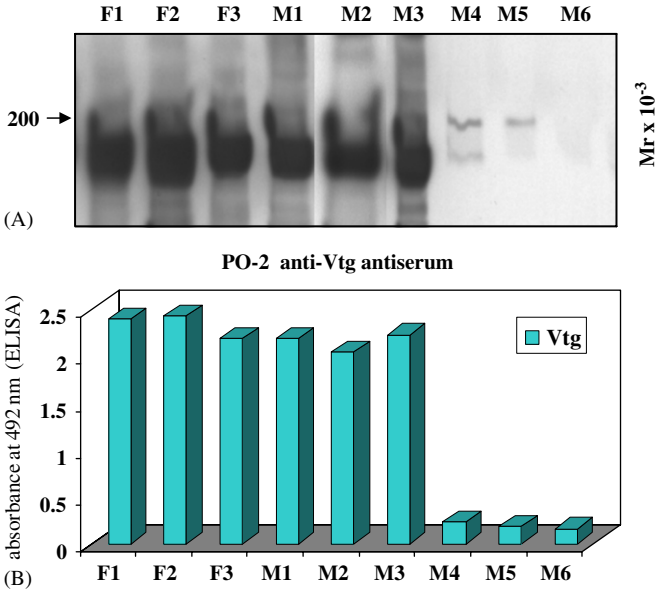

Fig. 1. Vitellogenin (Vtg) of swordfish (*Xiphias gladius*) captured in the Mediterranean sea (Straits of Messina, Sicily, Italy) in July 1999, during the spawning period. The specimens were 3 females (F1–F3) and 6 males (M1–M6) with different Lower Jaw Fork Length and approximate age. Western blot analysis of Vtg (Fig. 1A). Immunochemical analysis (ELISA) of Vtg (Fig. 1B) (Fossi et al., 2001a).

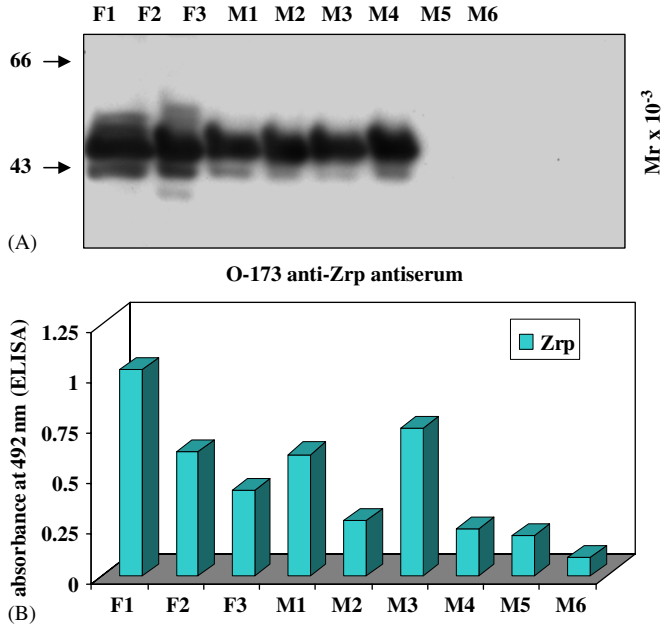

Fig. 2. *Zona radiata* proteins (Zrp) of swordfish (*Xiphias gladius*) captured in the Mediterranean sea (Straits of Messina, Sicily, Italy) in July 1999, during the spawning period. The specimens were 3 females (F1–F3) and 6 males (M1–M6) with different Lower Jaw Fork Length and approximate age. Western blot analysis Zrp (Fig. 2A). Immunochemical analysis (ELISA) Zrp (Fig. 2B) (Fossi et al., 2001a).

Vtg and Zrp were dramatically induced in adult male Mediterranean swordfish in comparison with 20 Atlantic specimens examined. The values of Vtg detected by the ELISA technique in swordfish captured in summer 1999 in Azores coastal waters (Portugal) were several times lower than in the Mediterranean samples (Fossi et al., 2001b).

A role of some organochlorine compounds (PCBs in liver ranged 128–22,847 ppb d.w.) in this induction phenomenon is suggested by the statistically significant correlations between Zrp levels in plasma and PCB concentrations in muscle (Tau b Kendall = 0.312; $P<0.032$) and Vtg levels in plasma and PCB concentrations in liver (Tau b Kendall = 0.618, $P<0.034$) of male specimens. PCBs levels in gonads were positively correlated with Vtg (Tau b Kendall = 0.472, $P<0.002$), and DDTs in gonads were positively correlated with Zrp in female specimens (Tau b Kendall = 0.406, $P<0.042$). Organochlorine levels (PCBs in liver) were also correlated with total length of male specimens (Tau b Kendall = 0.377, $P<0.021$) (Fossi et al., 2004).

The mean values of BPMO activity (A.F.U./mg protein/min), measured in the different years, ranged in male specimens between 0.32 and 1.77 and in female between 0.22 and 0.95. The mean values of EROD activity (pmol/mg protein/min) measured in the different years ranged in male specimens between 10.55 and 36.75 and in female between 4.40 and 18.88. No correlation was found between EROD or BPMO activities and contaminant levels. An interesting periodic variation in several parameters was

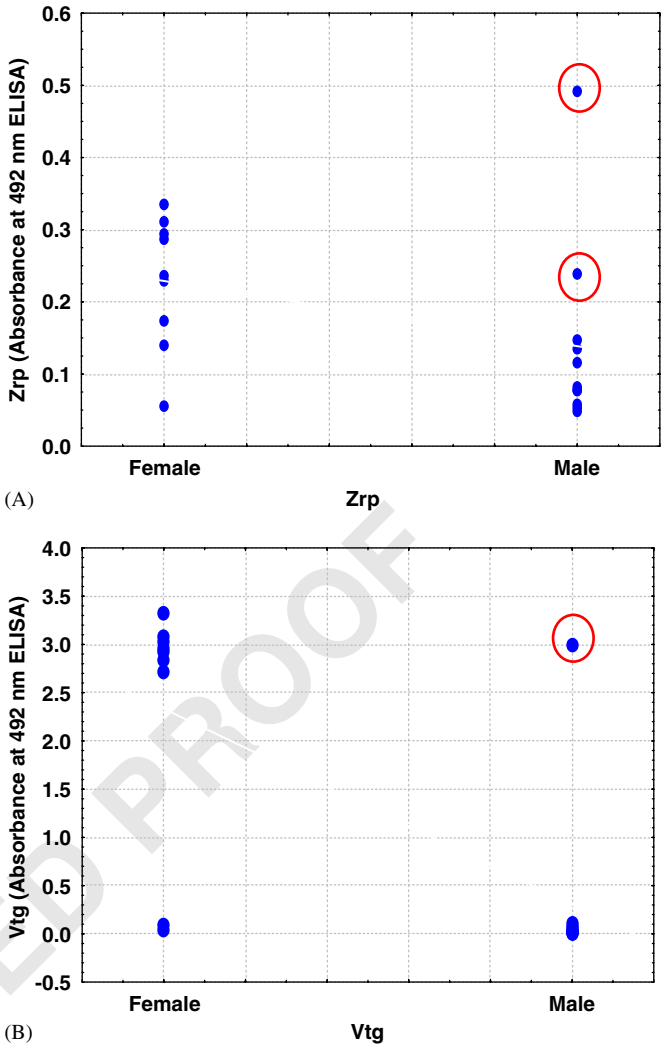

Fig. 3. *Zona radiata* proteins (Zrp) and vitellogenin (Vtg) of male and female bluefin tuna (*Thunnus thynnus thynnus)* captured in the Mediterranean Sea (Straits of Messina, Sicily, Italy) in summer 2000, during the spawning period. Circles indicate male samples, the values of which were nevertheless in the same range as those of reproductive females (Fossi et al., 2002, modified).

detected between years (Fossi et al., 2004). The male swordfish specimens show higher CYP1A1 activities than female, suggesting a possible gender differences in the susceptibility to xenobiotic contaminants.

The present results confirm that induction of Vtg and Zrp can be used as a diagnostic and prognostic tool for exposure assessment of Mediterranean swordfish and tuna stocks exposed to OCs with ED capacity. These data, and those published by De Metrio et al. (2003) and Desantis et al. (2005), demonstrating also a high percentage of intersex in Mediterranean swordfish, sound a warning about potential reproductive alterations in large pelagic fish and suggest the need for continued monitoring to avoid reductions in their populations.

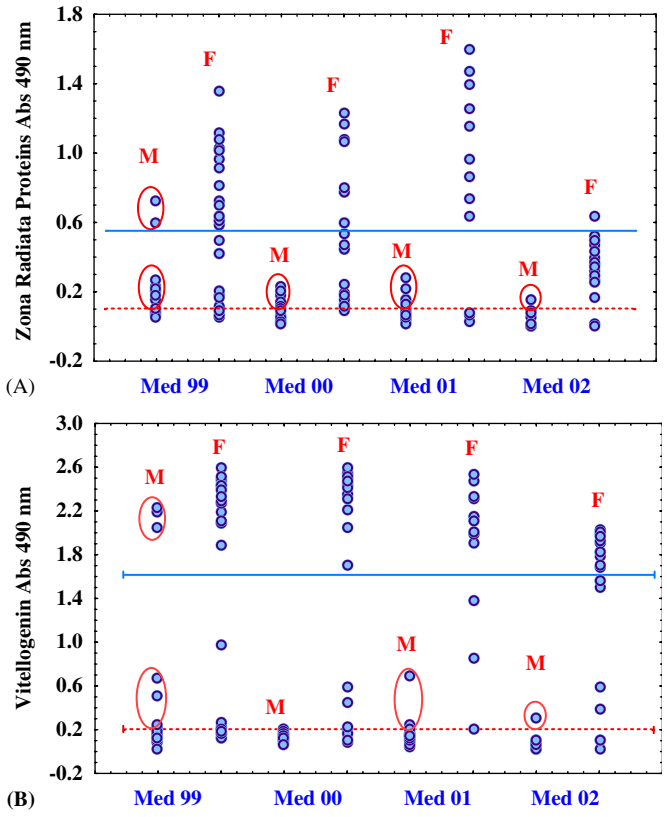

Fig. 4. (A) *Zona radiata* proteins (Zrp) and (B) vitellogenin (Vtg) of male and female swordfish (*Xiphias gladius*) captured in the Mediterranean Sea (Straits of Messina, Sicily, Italy) in summer 1999, 2000, 2001, 2002, during the spawning period. Circles indicate male specimens showing values higher than males average values (line) and/or in the same range as those of reproductive females. (Fossi et al., 2004).

3.2. Marine mammals

One of the main aims of this project was to explore the potential use of skin biopsy as "diagnostic" and "prognostic" tools for the ecotoxicological hazard due to EDCs in Mediterranean cetaceans (Fossi et al., 2000). Skin biopsy as "diagnostic tool" was used to explore OCs bioaccumulation processes and CYP1A1 activity (BPMO) in four free-ranging species of Mediterranean cetaceans (Fig. 5). As a "prognostic" model fibroblast cultures were also used as an alternative in vitro method of evaluating interspecies susceptibility and gender differences (CYP1A1 responses, ER receptor) to contaminants such as PCBs, DDTs and PAHs. We evaluated CYP1A1 (BPMO) activity in skin biopsies (non-destructive biomarker) of cetaceans (*Stenella coeruleoalba, Tursiops truncatus, Delphinus delphis* and *Balaenoptera physalus*) as a potential indicator of exposure to EDCs, such as organochlorines (OCs) (Fig. 5).

Four types of organochlorine endocrine disruptors (Adami et al., 1995; Kelce et al., 1995; Vonier et al., 1996; Wong and Pessah, 1996; Hansen Larry, 1998; Sohoni and Sumpter, 1998; Hilscherova et al., 2000) are commonly found in Mediterranean cetaceans (Aguilar and Borrell, 1994; Marsili, 2000, Fossi et al., 2003): (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 (Colborn et al., 1993, 1996, 1998). The relative estrogenic power of these chemicals, identified by in vitro and in vivo screening methods (Safe, 1995; Environmental Agency, 1998) is rather weak (10^{-3} or less) compared with the reference power of 17-estradiol or DES (Miyamoto and Klein, 1998). 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.

Organochlorine concentrations (HCB, DDTs and PCBs) and BPMO (CYP 1A1) activities, in the skin biopsies of odontocetes and mysticetes sampled in the Mediterranean sea, are reported as descriptive statistics (means and standard deviations) in Fig. 5A, B, C and D. Confirming literature data and results obtained in our lab before 1994 (Fossi et al., 1992; Borrell, 1993a, b; Marsili, 2000), indicated that marked differences in levels of all contaminants exist between fin whales and odontocete species (Fig. 5A, B and C). The same was found for BPMO activity (Fig. 5D) but differences between fin whale and odontocete species were smaller (Fossi et al., 1992). This difference was remarkable only for striped dolphins. The main explanation for these results is 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. In the common dolphin there were identified five linear correlations with the BPMO activity: DDTs, pp'DDE, op'DDT, PCBs and PCB153. Fig. 6A–E shows these correlations: total correlation (males and females continuous line), male correlation (broken line) and female correlation (dotted line). The main result in this species was non 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 (Marsili et al., 1998). 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. The higher cytocrome P450 (CYP2B) induction capability in male than in females was confirmed also in striped dolphin fibroblasts cell cultures (see point 3.3). This difference in the inductive capacity of skin BPMO between

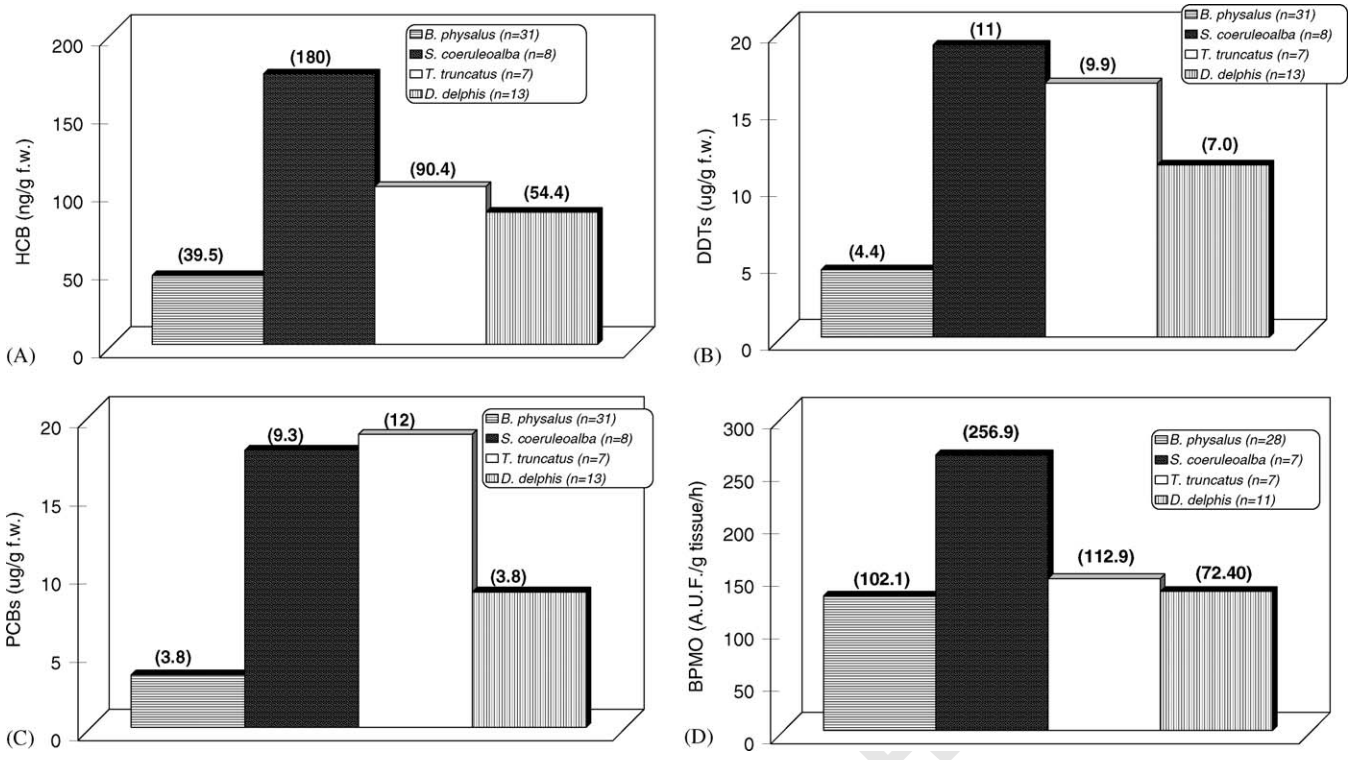

Fig. 5. A, B, C, D. HCB, DDT and PCB concentration (ng/g and μg/g f.w.) and BPMO activity (AUF/g tissue/h) in skin biopsies from Mediterranean cetaceans. Arithmetic mean and SD in brackets; n = number of samples (Fossi et al., 2003, modified).

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, related to exposure of future generations via the placenta and milk. It is therefore a powerful "prognostic" indicator of the health of cetaceans populations (Fossi and Marsili, 2003).

3.3. New methodological tools in the study of marine mammals

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), has drammaticaly reduced in 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 species to explore interspecies susceptibility towards Mediterranean contaminants. Samples of different species of Mediterranean cetaceans have already been collected in several part 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*, *D. delphis* and *Balaenoptera physalus*. The first fibroblasts were observed after 7–21 days. Cultures reached 90% confluence in 15–20 days, then were trypsinised, washed and placed in Falcon 50 and 125 flaskes, after two and three trypsinisations, respectively. The samples grew for over 4 months, however, there were signs of senescence and increased resistance to trypsin treatment. Fibroblasts of the different species are cultured, and when the number of cells is sufficient for testing interspecies differences in susceptibility to the main Mediterranean EDCs contaminants (OCs-EDCs) are investigated (Marsili et al., 2000, 2003).

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) (Marsili et al., 2003). In a pilot project, fibroblast cell cultures (third generation) of bottlenose dolphin (*T. truncatus*) were subjected, for 24 h, to different experimental design, using potent CYP450 inducers: (1) individual dose (5 μg/ml) of HCB, Arochlor 1260, Benzo(a)pyrene and pp'DDE, solubylised in DMSO (0.05%), plus a DMSO (0.05%) control; (2) a mixture of Arochlor 1260, pp'DDT e pp'DDE solubylised in DMSO (0.05%) added at three different doses: 1, 5 and 25 μg/ml, plus a DMSO (0.05%) control. In a second project fibroblasts cell cultures of striped dolphin (*S. coeruleoalba*) and fin whale (*B. physalus*) were subjected for 48 h to the mixture of OCs. After, a first reaction with the primary antibodies for CYP450 1A1–1A2 and 2B4 and for human estrogen

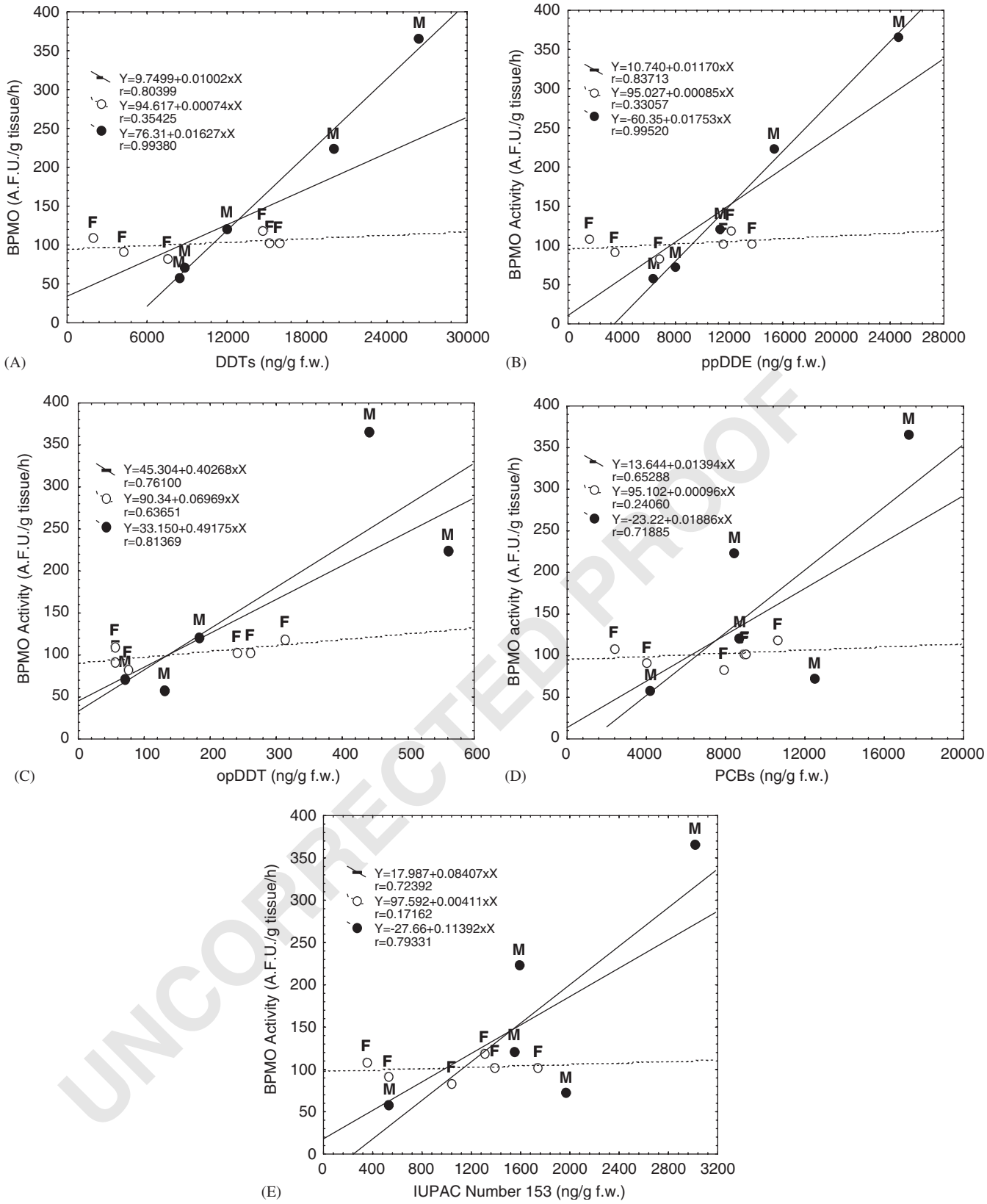

Fig. 6. (A–E) Linear correlations (Pearson test) in the common dolphin between BPMO activity and Total DDT, pp'DDE, op'DDT, Total PCB and PCB153. Total correlation (males and females, continuous line), male correlation (broken line) and female correlation (dotted line) (Fossi et al., 2003).

receptor (hER) were applied, then were treated with the respective secondary antibodies marked with a fluorochrome. The main results of these experiments were: (1) the detection of presence of the cytochromes 1A1–1A2 and 2B4 and of the estrogen receptor in bottlenose dolphin, striped dolphin and fin whale 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) results of immunofluorescence were confirmed by Western Blot investigations (Fossi et al., 2006), (4) higher induction responses were found in odontocetes in comparison to misticetes, (5) gender-related patterns of induction, with higher response capability in males than females, was found in striped dolphin, confirming results obtained for other species for BPMO activity (Fossi et al., 2006). The information obtained in these preliminary experiments will represent the base for further application and validation of this methodology in the study of susceptibility of marine mammals to endocrine disruptors.

4. Conclusions

In conclusion we suggest that diagnostic and prognostic tools can be used for fish stocks hazard assessment and gender susceptibility detection (Vtg, Zrp CYP1A1 activities) and could be helpful for the protection and conservation of endangered species of marine mammals (BPMO (CYP1A1) activity, fibroblast cell cultures) in the Mediterranean Sea. The main outcome of this research represents a first compressive warning signal of the potential reproductive alterations in top predators and suggests the need for continuous monitoring to avoid reductions in population and biodiversity in the Mediterranean Sea.

5. Uncited reference

Arukwe et al., 1998.

Acknowledgments

We thank Dr. Stefania Ancora, Dr. Gabriele Mori, Dr. Teresa Romeo, Dr. Antonella Ausili, for technical support in the sampling activities and in the analytical activities in the *Large Pelagic Fish Project* and all the researchers of the Tethys Research Institute, and Dr. Daniela Bucalossi and Dr. Ada Natoli for technical support in the sampling activities in the *Marine Mammals Project*.

References

Adami, H.O., Lipworth, L., Titus-Ernstoff, L., Chung-cheng, H., Hamberg, A., Anhlborg, U., Baron, J., Trichopoulos, D., 1995. Organochlorine compounds and estrogen-related cancers in women. Cancer Cause Control 6, 551–566.

Aguilar, A., Borrell, A., 1994. Abnormally high polychlorinated biphenyl levels in striped dolphins (*Stenella coeruleoalba)* affected by the 1990–1992 Mediterranean epizootic. Sci. Total Environ. 154 (2–3), 237–247.

Aguilar, A., Borrell, A., 2005. DDT and PCB reduction in the western Mediterranean from 1987 to 2002, as shown by levels in striped dolphins (*Stenella coeruleoalba*). Mar. Environ. Res. 59, 391–404.

Aguilar, A., Borrell, A., Reijnders, P.J.H., 2002. Geographical and temporal variation in levels of organochlorine contaminants in marine mammals. Mar. Environ. Res. 53, 425–452.

Alzieu, C., Duguy, R., 1979. Teneurs en composés organochlorés chez les Cétacés et Pinnipèdes frequentant les côtes françaises. Oceanol. Acta 2 (1), 107–120.

Arena, P., Cefali, A., Munao, F., 1980. Analysis of the age, weight, length and growth of *Thunnus thynnus* (L.) captured in Sicilian seas. Mem Biol. Mar. Ocean. 10 (5), 119–134.

Arukwe, A., Knudsen, F.R., Goksøyr, A., 1997. Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Environ. Health Perspect 105 (4), 418–422.

Arukwe, A., Celius, T., Walther, B.T., Goksoyr, A., 1998. Plasma level of vitellogenin and eggshell zona radiata proteins in 4-nonylphenol and o,p'-DDT treated juvenile Atlantic salmon (*Salmo salar*). Mar. Environ. Res. 46 (1–5), 133–136.

Berube, M., Palsboll, P., 1996. Identification of sex in cetaceans by multiplexing with three ZFX and ZFY specific primers. Mol Ecol 5 (2), 283–287.

Borrell, A., 1993a. PCB and DDTs in blubber of cetaceans from the North-Eastern North Atlantic. Mar. Poll. Bull. 26 (3), 146–151.

Borrell, A., 1993b. Dinamica dels contaminants organoclorats en la balena d'aleta, el cap d'olla d'aleta llarga i el dofi llistat d'aigues atlantiques i mediterranies. Tesi di Doctoral, Departament de Biologia Animal, Facutat de Biologia, Universitat de Barcelona, pp. 1–398.

Colborn, T., 1998. Building scientific consensus on endocrine disruptors. Environm. Toxicol. Chem. 17 (1), 1–2.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect 101, 378–384.

Colborn, T., Dumanoski, D., Myers, J.P., 1996. Our Stolen Future. Dutton, Penguin Books USA, New York pp 306.

Colborn, T., Smolen, M.J., Rolland, R., 1998. Environmental neurotoxic effects: the search for new protocols in functional teratology. Toxicol. Ind. Health 14 (1/2), 9–23.

Corsolini, S., Focardi, S., Kannan, K., Tanabe, S., Borrel, A., Tatsukawa, R., 1995. Congener profile and toxicity assessment of polychlorinated biphenils in dolphins, sharks and tuna collected from Italian coastal water. Mar. Environ. Res. 40 (1), 33–53.

De Metrio, G., Corriero, A., Desantis, S., Zubani, D., Cirillo, F., Deflorio, M., Bridges, C.R., Eicker, J., De la Serna, J.M., Megalofonou, P., Kime, D.E., 2003. Evidence of high percentage of intersex in the Mediterranean swordfish (*Xiphias gladius*). Mar. Poll. Bull. 46 (3), 358–361.

Desantis, S., Corriero, A., Cirillo, F., Deflorio, M., Brill, R., Griffiths, M., Lopata, A.L., de la Serna, J.M., Bridges, C.R., Kime, D.E., De Metrio, G., 2005. Immunohistochemical localization of CYP1A, vitellogenin and radiata proteins in the liver of swordfish (*Xiphias gladius* L.) taken from the Mediterranean Sea, South Atlantic, South Western Indian and Central North Pacific Oceans. Aquat. Toxicol. 71, 1–12.

Environmental Agency, 1998. Endocrine-Disrupting Substances in Wildlife: A Review of the Scientific Evidence and Strategic Response. Publishing Organisation Environmental Agency, Bristol.

Fischer, W., 1973. FAO Species Identification Sheets for Fishery Porpoises. Mediterranean and Black Sea (Fishing area 37). 1. FAO, Rome.

Fossi, M.C., Marsili, L., Leonzio, C., Notabartolo di Sciara, G., Zanardelli, M., Focardi, S., 1992. The use of non-detructive biomarker in Mediterranean cetaceans: preliminary data on MFO activity in skin biopsy. Mar. Poll. Bull. 24 (9), 459–461.

Fossi, M.C., Marsili, L., Neri, G., Casini, S., Bearzi, G., Politi, E., Zanardelli, M., Panigada, S., 2000. Skin biopsy of Mediterranean cetaceans for the investigation of interspecies susceptibility to xenobiotic contaminants. Mar. Environ. Res. 50 (1–5), 643–647.

Fossi, M.C., Casini, S., Ancora, S., Moscatelli, A., Ausili, A., Notarbartolo di Sciara, G., 2001a. Do endocrine disrupting chemicals threaten Mediterranean swordfish? Preliminary results of vitellogenin and zona radiata proteins in *Xiphias gladius*. Mar. Environ. Res. 52 (5), 477–483.

Fossi, M.C., Casini, S., Marsili, L.A., Ausili, A., Notarbartolo Di Sciara, G., 2001b. Are the mediterranean top predators exposed to the toxicological risk due to endocrine disruptors? Ann. NY Acad. Sci. 948, 67–73.

Fossi, M.C., Casini, S., Marsili, L., Neri, G., Mori, G., Ancora, S., Moscatelli, A., Ausili, A., Notarbartolo-di-Sciara, G., 2002. Biomarkers for endocrine disruptors in three species of Mediterranean large pelagic fish. Mar. Environ. Res. 54 (3–5), 667–671.

Fossi, M.C., Marsili, L., Neri, G., Natoli, A., Politi, E., Panigada, S., 2003. The use of non-lethal tool fore evaluating toxicological hazard of organochlorine contaminants in Mediterranean cetaceans: new data 10 years after the first paper published in MPB. Mar. Poll. Bull. 46, 972–982.

Fossi, M.C., Casini, S., Marsili, L., Ancora, S., Mori, G., Neri, G., Romeo, T., Ausili, A., 2004. Evaluation of ecotoxicological effects of endocrine disrupters during a 4-year survey of the mediterranean population of swordfish (*Xiphias gladius*). Mar. Environ. Res. 58, 425–429.

Fossi, M.C., Marsili, L., 2003. Effects of endocrine Disruptors in Aquatic Mammals. Pure Appl. Chem. 75 (11–12), 2235–2247.

Fossi, M.C., Marsili, L., Casini, S., Bucalossi, D., 2006. Development of new-tools to investigate toxicological hazard due to endocrine disruptor organochlorines and emerging contaminants in Mediterranean cetaceans. Mar. Environ. Res. in press, available on line.

Gillesby, B., Zacharewski, T., 1998. Exoestrogens: mechanisms of action and strategies for identification and assessment. Environ. Toxicol. Chem. 17 (1), 3–14.

Goksoyr, A., 1991. A semi-quantitative cytochrome P450IA1 ELISA: a simple method for studying the monooxygenase induction response in environmental monitoring and ecotoxicological testing of fish. Sci. Total. Environ. 101, 255–262.

Hansen Larry, G., 1998. Stepping backward to improve assessment of PCB congener toxicities. Environ. Health Perspect 106 (1), 171–189.

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A.L., Giesy, J.P., 2000. Cell bioassays for detection of Aryl Hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environ. Sci. Pollut. Res. 7 (3), 159–171.

Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., 1995. Persistent DDT metabolite pp'DDE is a potent androgen receptor antagonist. Nature 375, 581–586.

Kurelec, B., Britvic, S., Rijavec, M., Muller, W.E.G., Zahn, R.K., 1977. Benzo(a)pyrene monooxygenase induction in marine fish—molecular response to oil pollution. Mar. Biol. 44, 211–216.

Loganathan, B.G., Tanabe, S., Tanaka, H., Watanabe, S., Miyazaky, N., Amano, M., Tatsukawa, R., 1990. Comparison of organochlorine residue levels in the striped dolphin from the western north Pacific, 1978–79 and 1986. Mar. Poll. Bull. 21, 435–439.

Lubet, R.A., Nims, R.W., Mayer, R.T., Cameron, J.W., Schechtman, L.M., 1985. Measurement of cytochrome P450 dependent dealkylation of alkoxyphenoxazones in hepatic S9 s and hepatocyte homogenates: effects of dicumarol. Mutat. Res. 142, 127–131.

Marsili, L., 2000. Lipophilic contaminants in marine mammals: review of the results of ten years work at the Department of Environmental Biology, Siena University (Italy). "The Control of Marine Pollution: Current Status and Future Trends". IJEP 13, 416–452.

Marsili, L., Fossi, M.C., Notarbartolo di Sciara, G., Zanardelli, M., Nani, B., Panigada, S., Focardi, S., 1998. Relationship between organochlorine contaminants and mixed function oxidase activity in skin biopsy specimens of mediterranean fin whales (*Balaenoptera physalus*). Chemosphere 37 (8), 1501–1510.

Marsili, L., Fossi, M.C., Neri, G., Casini, S., Gardi, C., Palmeri, S., Tarquini, E., Panigada, S., 2000. Skin biopsies for cell cultures from Mediterranean free-ranging cetaceans. Mar. Environ. Res. 50 (1–5), 649–652.

Marsili, L., Fossi, M.C., Casini, S., Bucalossi, D., Ciampolini, F., Moscatelli, A., Lauriano, G., 2003. Evaluation of presence of CYTP450 and ER receptor in fibroblast cell cultures of bottlenose dolphin (*Tursiops truncatus*) using immunofluorescence technique. Europ. Soc. Comp. Physiol. Biochem. 22nd Conference, Alessandria 15–17 December 2003, p. 157.

Miyamoto, J., Klein, W., 1998. Environmental exposure, species differences and risk assessment. Pure Appl. Chem. 70, 1829–1845.

O'Shea, T., Aguilar, A., 2001. Cetaceans and Sirenians. In: Shore, R.F., Rattner, B.A. (Eds.), Ecotoxicology of Wild Mammals. Wiley, Chichester, UK, pp. 427–496.

Safe, S.H., 1995. Environmental and dietary estrogens and human health: is there a problem? Environ. Health Perspect. 103 (4), 346–351.

Sohoni, P., Sumpter, J.P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158, 327–339.

Stillwell, C., Kohler, N., 1995. Food and feeding ecology of the swordfish *Xiphias gladius* in the western North Atlantic Ocean with estimates of daily ration. Mar. Ecol. Prog. Ser. 22, 239–247.

Tanabe, S., Mori, T., Tatsukawa, R., Miyazaki, N., 1983. Global pollution of marine mammals by PCBs, DDTs and HCHs (BHCs). Chemosphere 12, 1269–1275.

Taruski, A.G., Olney, C.E., Winn, H.E., 1975. Chlorinated hydrocarbons in cetaceans. J. Fish. Res. Board. Canada 32, 2205–2209.

Vonier, P.M., Crain, D.A., McLachlan, J.A., Guillette Jr., L.J., Steven, F.A., 1996. Interaction of environmental chemicals with the estrogen and progesterone receptor from the oviduct of the american Alligator. Environ. Health Perspect. 104, 1318–1322.

Wong, P.W., Pessah, I.N., 1996. *ortho*-Subsituted PCBs alter calcium regulation by a ryanodine receptor-mediated mechanism: structural specificity toward skeletal- and cardiac-type microsomal calcium release channels. Mol. Pharmacol. 49, 740–751.