The role of skin biopsy in the detection of exposure to endocrine disrupting chemicals in Mediterranean cetaceans

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ABSTRACT

Use of skin biopsy is proposed as a sensitive non-lethal technique for the hazard assessment of Mediterranean cetaceans exposed to endocrine disrupting chemicals (EDCs). EDCs are a structurally diverse group of compounds that may adversely affect the health of humans and wildlife or their progeny, by interaction with the endocrine system. In the Mediterranean environment top predators accumulate high concentrations of polyhalogenated aromatic hydrocarbons (PHAHs) and toxic metals, incurring high toxicological hazard. In this paper, the hypothesis that Mediterranean cetaceans are potentially at risk due to PHAH-EDCs is investigated using skin biopsy samples. Benzo-α-pyrene monoxigenase (BPMO) activity in skin biopsies was used as a potential indicator of exposure to different organochlorines (OCs) known to have endocrine disrupting properties. The main objective of this paper was to use this non-destructive ecotoxicological tool to define the potential hazard to Mediterranean odontocete and mysicete species, comparing the present data with values detected in other cetaceans from heavily polluted areas, affected by pseudohermaphroditism and other reproductive dysfunction. Subcutaneous tissue consisting of skin and blubber was obtained from striped dolphins (*Stenella coeruleoalba*), bottlenose dolphins (*Tursiops truncatus*), common dolphins (*Delphinus delphis*) and fin whales (*Balaenoptera physalus*) in the Mediterranean basin. Sampling was performed in the western Ligurian Sea, between Corsica and the French-Italian coast, and in the Ionian Sea. High concentrations of DDT metabolites and PCB congeners (known as Endocrine Disruptors) were detected in the different species. Significant differences in BPMO induction and OC levels were found between odontocetes and mysticetes. Differences in organochlorine bioaccumulation and consequently potential risk due to endocrine disruptors were primarily related to different positions in the marine food web. A statistical correlation was found between BPMO activity and organochlorine (op'DDT, a potent estrogen and antiandrogen and pp'DDE, a potent antiandrogen) levels in skin biopsy specimens of the endangered Mediterranean population of common dolphin. Several conclusions on the potential risk to Mediterranean cetaceans can be drawn from comparison of the levels of OC-EDs detected in Mediterranean odontocetes with those in white whales (*Delphinapterus leucas*) of the St Lawrence estuary and bowhead whales (*Balaena mysticetus*) affected by pseudohermaphroditism and other reproductive dysfunction. Finally, these results suggest that BPMO induction may be an early sign of exposure to EDCs such as OCs and a warning of the possibility of transgenerational effects through exposure of future generations via the placenta and milk.

KEYWORDS: BIOPSY SAMPLING; POLLUTANTS; ORGANOCHLORINES; STRIPED DOLPHIN; BOTTLENOSE DOLPHIN; COMMON DOLPHIN; FIN WHALE

INTRODUCTION

Endocrine Disrupting Chemicals (EDCs) have recently attracted much public and scientific attention (Colborn *et al.*, 1993; 1996; 1998). EDCs are a structurally diverse group of compounds that may adversely affect the health of humans and wildlife, or their progeny, by interaction with the endocrine system (Gillesby and Zacharewski, 1998). They include chemicals used heavily in the past in industry and agriculture, such as polychlorinated biphenyls and organochlorine pesticides, and also plasticisers and surfactants. Many known EDCs are estrogenic and affect reproductive function. Most xenobiotic estrogens and their metabolites are lipophilic and persistent which is why they bioaccumulate and biomagnify (Arukke *et al.*, 1997; Colborn *et al.*, 1998; Reijnders *et al.*, 1999).

Man-made EDCs range across all continents and oceans; some geographic areas are potentially more threatened than others: one of these is the Mediterranean Sea. 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 much higher here than in other seas and oceans (Bernard, 1978; Ankley *et al.*, 1998). Mediterranean marine fauna could therefore be a target of EDCs. In this environment, top predators (such as large pelagic fish and marine mammals) tend to accumulate high quantities of polyhalogenated aromatic hydrocarbons (PHAHs) and toxic metals (Leonzio *et al.*, 1992; Corsolini *et al.*, 1995; Marsili and Focardi, 1996; 1997). For example, levels of PHAHs in a top predator of the Mediterranean, the striped dolphin, are 1-2 orders of magnitude higher than in Atlantic and Pacific dolphins of the same species (Marsili, 2000). This suggests that Mediterranean top predator species, and particularly odontocetes, are potentially 'at risk' due to EDC contamination.

The potential effects of EDCs on a Mediterranean fish species of commercial interest, the swordfish (*Xiphias gladius*), were recently investigated by Fossi *et al.* (2001) using vitellogenin (Vtg) and zona radiata proteins (Zrp) as diagnostic and prognostic biomarkers. Significant induction of typically female proteins (Vtg and Zrp) was detected by ELISA and Western Blot in adult males of the species. Vtg and Zrp were found to be significantly more induced in adult male Mediterranean swordfish than 25 Atlantic specimens. These results are the first warning of the potential risk for reproductive function of Mediterranean top predators, and suggest the need for continuous monitoring of this marine environment and other Mediterranean top predator species such as marine mammals.

It is more difficult to explore the ecotoxicological risk to free-ranging Mediterranean cetaceans than to swordfish. When the levels of organochlorines in swordfish (average DDTs in blubber 2.13 μg/g f.w.; average PCBs in blubber 1.97 μg/g f.w.; Ausili, pers. comm.) are compared with those found in free-ranging striped dolphins, levels are found to be

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10 to 20 times higher in the latter (average DDTs in blubber 26.65 μg/g d.w.; average PCBs in blubber 46.67 μg/g d.w.; Marsili, 2000). The use of skin biopsies to assess benzo-α-pyrene monoxigenase (BPMO) activity and organochlorine body loads in cetaceans has been proposed and validated in several papers (Fossi *et al.*, 1992; 1997; 1999; 2000; Fossi and Marsili, 1997; Marsili *et al.*, 1998). The aim of this paper is to define 'potential hazard' to Mediterranean odontocete and mysicete species, using skin biopsies as a non-lethal ecotoxicological tool for the evaluation of BPMO (MFO) activity, as a potential indicator of exposure to different organochlorines (OCs) known to have endocrine disrupting properties. The final assessment involves comparing the data of Mediterranean species with OC-ED levels detected in other cetaceans from heavily polluted areas, affected by pseudohermaphroditism and other reproductive dysfunction.

MATERIALS AND METHODS

Subcutaneous tissue consisting of skin and blubber was obtained from striped dolphins, bottlenose dolphins, common dolphins and fin whales, in the western Ligurian Sea (1992-1999), between Corsica and the French-Italian coast, and in the Ionian Sea. Samples were collected from fin whales using biopsy darts launched with a 150-pound *Barnett Wildcat* II test crossbow. Dolphins were sampled while riding the vessel's bow wave by means of a pole with a biopsy tip. To avoid the possibility of infection, the biopsy tips were sterilised with ethanol before use. Biopsy specimens were taken from the dorsal area surrounding the dorsal fin. The samples were immediately placed in liquid nitrogen. The small size of the biopsy samples (between 0.200g and 0.002g) did not permit isolation of the microsomal fraction. BPMO activity was detected in the 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 with ultrasound in a Potter apparatus. BPMO activity was assessed using the incubation mixture proposed by Fossi *et al.* (1992). Each sample and the blanks were incubated in a shaking bath for 1 hour at 37°C. Activity was expressed in arbitrary units of fluorescence (A.U.F./h/g tissue). Samples of subcutaneous blubber (about 0.3g) were freeze-dried and extracted with n-hexane in a Soxhlet apparatus for analysis of chlorinated hydrocarbons. 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 op'- and pp'- isomers of DDT and its derivatives DDD and DDE, and about 30 PCB congeners.

The data were processed by summary statistics and ANOVA using Statgraphics software (Statistical Graphics Corporation), Statistica and Excel (Microsoft), assigning $p < 0.05$ as significance level.

RESULTS AND DISCUSSION

There are several types of organochlorine endocrine disruptors (Adami *et al.*, 1995; Kelce *et al.*, 1995; Vonier *et al.*, 1996; Wong and Pessah, 1996; Hansen, 1998; Sohoni and Sumpter, 1998; Hilscherova *et al.*, 2000) commonly found in Mediterranean cetaceans (Aguilar and Borrell, 1994; Aguilar, 2000; Marsili, 2000) (Table 1): environmental estrogens, anti-estrogens, environmental androgens and anti-androgens. Endocrine disruptors act by mimicking steroid sex-hormones, both estrogens and androgens, by binding to hormone receptors or influencing cell pathways (environmental estrogens and androgens), they also interfere with steroidogenesis, metabolism, and excretion of hormones, or by blocking and altering hormonal binding to hormone receptors (anti-estrogens, anti-androgens). Environmental estrogens have been more extensively studied than the others and may be the most common EDCs (Colborn *et al.*, 1993; 1996; 1998; Fournier *et al.*, 2000; Colborn, 2002).

Table 1

DDT metabolites and PCB congeners (known as Endocrine Disruptors) most commonly found in Mediterranean cetaceans.

	Activity	Potency[1]	aER binding IC50 (M)[2]	ER binding IC50 (M)[3]	ER binding RBA,%[4]
DDTs	Estrogen				
pp'DDT	Estrogen				
	Antiandrogen				
	ER agonist		>50[a]	>1000	
op'DDT	Estrogen	++			
	Antiestrogen	+			
	Antiandrogen	+++			
	ER agonist		9.1	5	0.1
pp'DDE	Estrogen	+			
	Antiestrogen	+			
	Androgen	+			
	Antiandrogen	++			
	ER agonist		>50[a]	>1000	
	AR agonist				
	AR antagonist				
op'DDE	Estrogen				
	ER agonist		37.25		
pp'DDD	ER agonist			>1000	
op'DDD	ER agonist		2.26		
PCBs					
Arochlor 1260	Estrogen				
	Effect on sexual differentiation				
	Gonadal abnormalities				
95	Estrogen	+			
99	Estrogen	++			
101	Estrogen				<0.001
118	Antiestrogen	++			
153	Estrogen	+++			0.004

[1]The most potent chemical for each activity was assigned a potency of four plus signs (++++), and the potency of all the chemicals was expressed relative to this (Hansen, 1998; Sohoni & Sumpter, 1998).
[2]Inhibitor concentrations necessary for 50% inhibition (IC50) of 3H17-estradiol binding to estrogen receptors (aER) in alligators. The aER binding IC50 value for 17-estradiol was 0.0078M.
[a]Compounds that inhibited 3H17-estradiol but were insoluble at concentrations necessary to achieve 50% inhibition (Vonier *et al.*, 1996).
[3]Inhibitor concentrations necessary for 50% inhibition (IC50) of 3H17-estradiol binding to ERs in rats. The ER binding IC50 value for 17-estradiol was 0.002M (Kelce *et al.*, 1995).
[4]Relative estrogen receptor-binding affinities (RBA). Competitive binding with estradiol in rat uterine ER preparations (Hansen, 1998).
Activity references: Adami *et al.*, 1995; Wong & Pessah, 1996; Hansen, 1998; Sohoni & Sumpter, 1998; Hilscherova *et al.*, 2000).

High concentrations of organochlorines with known EDC properties (Table 1) were detected in the different cetaceans (Table 2). Significant differences (T-student test = $p < 0.05$) in levels were found between odontocetes and mysticetes.

Table 2

Arithmetic mean (ng/g fresh weight) and standard deviations (in brackets) of DDT metabolites and PCB congeners known as EDCs in the four species of Mediterranean cetaceans.

	Fin whale (n=63)	Striped dolphin (n=95)	Bottlenose dolphin (n=7)	Common dolphin (n=13)
pp'DDT	457.2 (301.2)	2676.9 (2058.3)	793.2 (892.8)	336.4 (230.2)
op'DDT	438.9 (348.5)	1306.1 (1085.9)	450.2 (322.1)	400.1 (297.5)
pp'DDE	3679.4 (2560.3)	19972.2 (17557.2)	10138.5 (9973.8)	13453.6 (11437.1)
op'DDE	45.4 (27.6)	288.4 (277.5)	286.2 (227.5)	99.6 (95.8)
pp'DDD	446.2 (318.4)	1884.1 (1472.9)	543.9 (381.3)	467.1 (376.7)
op'DDD	101.5 (64.1)	521.7 (604.6)	71.5 (104.0)	115.5 (205.8)
DDTs	**5168.6 (3844.4)**	**26649.4 (21391.8)**	**12283.5 (10532.3)**	**14872.3 (11801.4)**
PCB 95	154.9 (73.1)	533.8 (343.4)	566.5 (402.6)	344.4 (389.2)
PCB 101	144.8 (70.4)	579.3 (381.2)	562.5 (449.5)	219.9 (179.8)
PCB 99	372.4 (275.8)	1879.2 (1668.5)	820.6 (759.8)	560.2 (313.1)
PCB 118	203.9 (102.8)	994.9 (594.4)	951.0 (718.2)	1788.1 (1710.9)
PCB 153	1100.8 (529.6)	8592.5 (5174.3)	8186.7 (9376.4)	4214.7 (2072.1)
PCBs	**6597.4 (3270.8)**	**49127.6 (29669.2)**	**31446.5 (25129.7)**	**20025.8 (9024.3)**

Highest levels were found in striped dolphins, followed by bottlenose dolphins and common dolphins. Organochlorine values were lower in fin whales than in the dolphin species by a factor of 2-8. Differences in organochlorine bioaccumulation and consequently potential risk due to endocrine disruptors are primarily related to different positions in the marine food web. In this case, the odontocete species may be regarded as terminal consumers that prey mainly on fish and squid; fin whales occupy a lower level, feeding on plankton which contain average PCB levels 5-10 times lower than odontocete prey. This suggests that odontocetes incur greater risk than Mediterranean mysticetes.

Levels of the different OCs with endocrine disrupting properties need to be explored separately (Table 1). The high percentage of op'DDT detected in fin whale samples (Fig. 1a-b) is noteworthy, because this DDT metabolite is a potent oestrogen and anti-androgen and could theoretically affect the low reproductive rate of this mysticete.

This research was prompted by the fact that some species, such as the common dolphin, have almost completely disappeared from the Mediterranean Sea. In considering the potential use of this ecotoxicological tool (skin biopsy) to study inter-species susceptibility to contaminants in Mediterranean cetaceans, a high statistical correlation (Pearson product-moment correlations $p < 0.05$) was found between BPMO activity and organochlorine levels (DDTs) in skin biopsy specimens of the endangered Mediterranean population of common dolphin. op'DDT is a potent Oestrogen and anti-androgen (Table 1) (Fig. 2a) and pp'DDE is a potent anti-androgen (Table 1) (Fig. 2b). This data suggests that EDCs may be one stress factor for common dolphin populations in the Mediterranean Sea.

In an attempt to define the 'potential hazard' of Mediterranean odontocete and mysticete species, a preliminary assessment can be undertaken, comparing the data of the Mediterranean species with those for PCB-EDs detected in other cetaceans from heavily polluted areas, affected by pseudohermaphroditism and other reproductive dysfunction.

Research in other seas and oceans indicates that some cetaceans, particularly odontocetes, have detectable and sometimes extremely high levels of substances known or suspected to be EDCs, such as PCBs, DDTs, chlorinated pesticides, brominated flame retardants and tributyltin (TBT). Biologists have reported a range of effects of these chemicals on cetaceans, including immunosuppression, cancer, skin lesions, secondary infections and diseases, sporadic die-offs, and reduced reproductive success. There are several examples suggesting 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 common minke whales, *Balaenoptera acutorostrata* (Fujise *et al.*, 1998).

Another example is the endangered white whales of the St Lawrence estuary, now among the most contaminated animals on earth, with tumours and reproductive problems

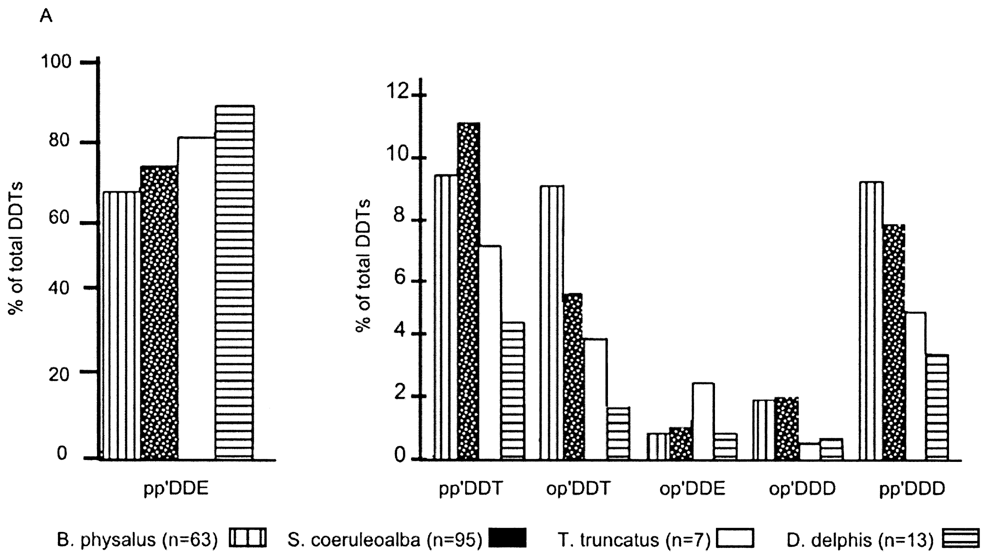

Fig. 1a. Percentage of DDT compounds known to be EDCs (see Table 1) out of total DDTs in skin biopsy specimens from Mediterranean cetaceans.

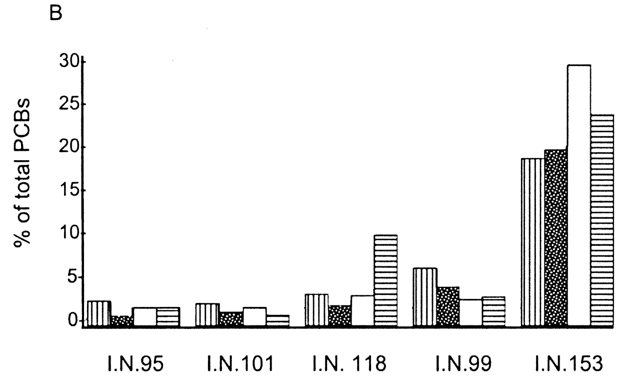

Fig. 1b. Percentage of PCB congeners known to be EDCs (see Table 1) out of total PCBs in skin biopsy specimens from Mediterranean cetaceans.

(Deguise *et al.*, 1995; Martineau *et al.*, 1999; 2002). De Guise *et al.* (1994) reported a true hermaphrodite white whale. This animal had two testicles, two separate ovaries and the complete ducts of each sex; cervix, vagina and vulva were absent. Mature spermatozoa were found in the lumen of seminiferous tubules in the testicles, and many involuted corpora lutea were recognised in the ovaries.

Increasing residue levels of PCBs and DDE in the blubber of Dall's porpoises (*Phocoenoides dalli*) were found to have a negative effect on testosterone levels in blood. Testosterone levels decreased in a statistically significant way with increasing DDE concentrations. These results suggest that current levels of environmental contamination by persistent organochlorines can cause an imbalance in sex hormones and subsequent reproductive abnormalities in wild porpoises. The other hormone measured, aldosterone, which has no sexual function, showed levels which were independent of the effects of PCBs and DDE (Subramanian *et al.*, 1987).

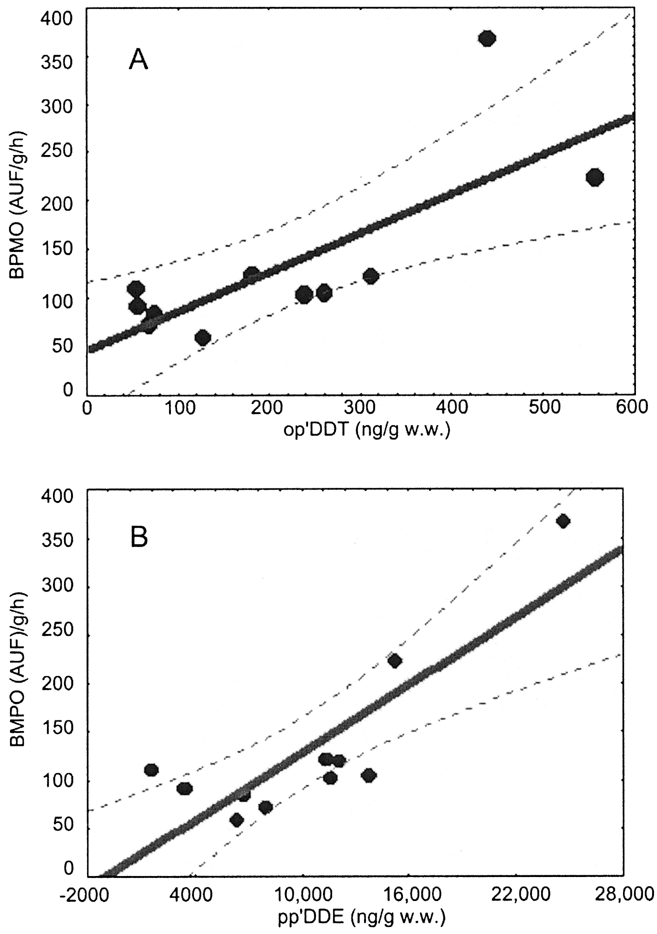

Fig. 2. Statistical correlation ($p < 0.05$) between BPMO activity and organochlorine levels: (A) (op'DDT) and (B) (pp'DDE), in common dolphin skin biopsies.

In the endangered bowhead whale, not all sexual activity leads to conception. Pseudohermaphroditism has been reported in at least two males with testicular feminisation (Tarpley *et al.*, 1995). This is a relatively high incidence, as only 76 bowheads were closely examined between 1980 and 1989 (Philo *et al.*, 1993). Concentrations of most organochlorine contaminants in biological material of 20 bowhead whales from Barrow (Alaska, USA) were low compared to those in tissues of other cetaceans, especially odontocetes, but it is impossible to rule out any link between pseudohermaphroditism and these contaminants.

Despite difficulties in establishing direct cause-effect links (Reijnders *et al.*, 1999) it is worth noting that the levels of PCBs found in Mediterranean odontocetes are similar to those detected in hermaphrodite white whales of the St Lawrence estuary (Fig. 3a); levels of PCBs detected in Mediterranean fin whales are approximately 10 times higher

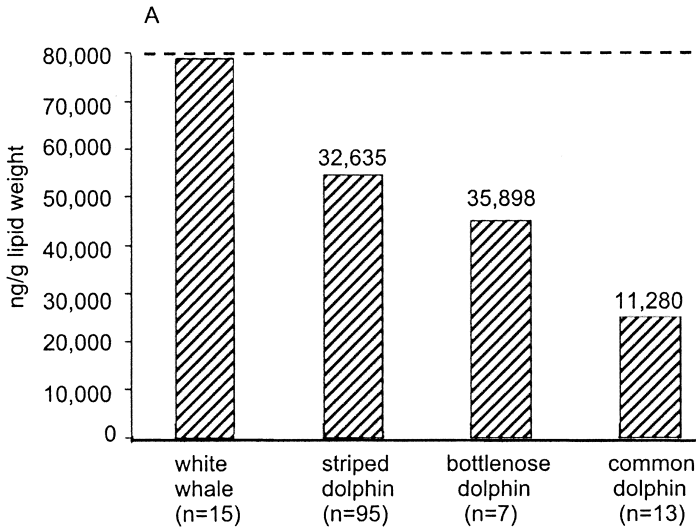

Fig. 3a. Comparison of PCB levels in Mediterannean odontocetes (striped, bottlenose and common dolphins) and odontocete species affected by hermaphroditism. Broken line — levels of PCBs found in populations of white whale manifesting hermaphroditism.

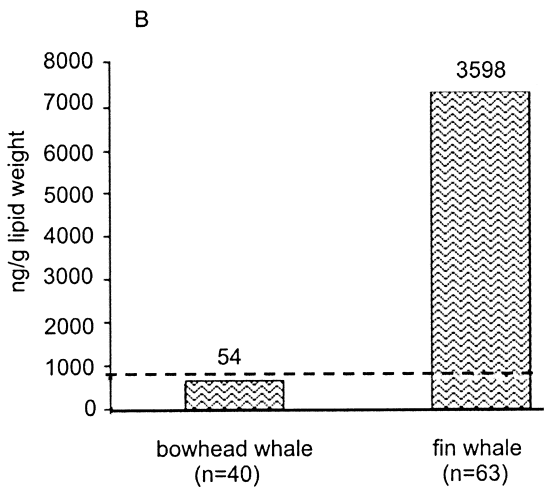

Fig. 3b. Comparison of PCB levels in a Mediterannean fin whale and mysticete species manifesting pseudohermaphroditism. Broken line — levels of PCBs found in populations of bowhead whale affected by pseudohermaphroditism.

than those found in bowhead whales with pseudohermaphroditism and other reproductive dysfunctions (Fig. 3b). This observation illustrates the potential risk to which these species are exposed in the Mediterranean Sea. Future research on stranded animals will help to clarify potential effects of these chemicals on gonad integrity.

Inter-species differences in susceptibility to EDCs will be a major topic to consider in this context. Future studies should explore the role of detoxification enzymes and estrogen receptors (ER) in inter-species susceptibility to EDCs. As an approach to this problem, the authors have been studying cultures of fibroblasts from different cetaceans (striped dolphin, bottlenose dolphin, common dolphin and fin whale; Marsili *et al.*, 2000) sampled in the Mediterranean Sea between 1999 and 2001. Skin biopsy samples were stored in a 'cell medium' and transported to the lab within 24-36 hours where they were processed to obtain fibroblast cultures. Fibroblasts of the different species have already been cultured, and when the number of cells is sufficient for testing, inter-species differences in susceptibility to the main Mediterranean OCs with EDC properties will be investigated. The results will be a first indication of any differences in the susceptibility of the various Mediterranean cetaceans to this dangerous class of chemicals.

ACKNOWLEDGEMENTS

This project was financed by a grant from the Italian Ministry for the Environment and ICRAM. We thank the Tethys Research Institute and Giovanni Neri for scientific and technical support.

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Date received: March 2002.
Date accepted: August 2002.