Chapter 3

"Test Tube Cetaceans": From the Evaluation of Susceptibility to the Study of Genotoxic Effects of Different Environmental Contaminants Using Cetacean Fibroblast Cell Cultures

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Additional information is available at the end of the chapter

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1. Introduction

Cetacean diversity, like all biodiversity worldwide, is seriously threatened; its loss seems to be occurring at a very rapid and increasing rate [1]. In March 2010, the European Commission set a key objective for 2020: halt the loss of biodiversity and the degradation of ecosystem services in the EU [2]. To the objective of improving the effectiveness of conservation strategies it becomes important to know the health status of endangered species and then to develop methods of investigation that are not destructive and the least invasive possible. In the last few years a non destructive sampling method, the skin biopsy, was developed in cetaceans to obtain viable tissue samples from free-ranging animals [3]. With the skin biopsy it is possible to assess the effects of multiple pressures related to bioaccumulation of anthropogenic contaminants, infectious diseases, climate change, food depletion from over-fishing, bycatch, noise, shipping and collision that stress cetacean species. The evaluation of their "health status" is possible using a suite of sensitive tools, such as non-destructive biomarkers, that will enable us to detect the presence and the effects of contaminants, the reproduction alteration, the genotoxicity, the immunosuppression, the feeding ecology and the general stress [4]. Actually, it is very difficult to discern the effects of one threat from those of another when multiple threats are acting simultaneously; for example the incidence of pathology in cetaceans is closely related to the level of pollution in their environments and thus bacterial and viral infections and contaminants should be considered from a holistic point of view [5]. Regarding the effects of anthropogenic

contaminants, it is known that some contaminants, such as organochlorines (OCs), polybrominated diphenyl ethers (PBDEs), bisphenol A (BPA) and phthalates are endocrine disrupting chemicals (EDCs) and immunosuppressors [6, 7, 8, 9, 10]. Others, such as polycyclic aromatic hydrocarbons (PAHs), derived from both natural (e.g., oil spills, forest fires, natural petroleum seeps) and anthropogenic (e.g., combustion of fossil fuels, use of oil for cooking and heating, coal burning) sources, are carcinogenic, teratogenic and mutagenic compounds [11] and some studies have shown that PAHs with four or more rings can induce dioxin-like activity and weak estrogenic responses [12]. Moreover PAHs have attracted scientific interest due to their genotoxicity [13]. But how is it possible to discriminate the effects of a specific toxic in a mixture of many pollutants and assess the susceptibility of a particular cetacean species to just one class of contaminants? The aim of the present study is to use cetacean fibroblast cell cultures, obtained from skin biopsy of free-ranging animals and from skin tissue of stranded animals dead within 12 h [14, 15], as an "in vitro" method, called "***Test Tube Cetaceans***", to investigate the effects of environmental contaminants. In particular we use *Test Tube Cetaceans* to explore the susceptibility to genotoxic effects of different environmental contaminants in these marine mammals. Cell cultures were obtained from several species of cetaceans: fin whale (*Balaenoptera physalus*) and Bryde's whale (*Balaenoptera edeni*) for mysticetes, sperm whale (*Physeter macrocephalus*), killer whale (*Orcinus orca*), Risso's dolphin (*Grampus griseus*), bottlenose dolphin (*Tursiops truncatus*), striped dolphin (*Stenella coeruleoalba*), long-beaked common dolphin (*Delphinus capensis*) and common dolphin (*Delphinus delphis*) for odontocetes. Here we present the results for three different biomarkers of anthropogenic stress in cetacean cell cultures that will enable us to assess: i) exposure to contaminants, ii) immunosuppression and iii) genotoxicity.

i. **Interspecies differences in the mixed function oxidase (MFO) induction as biomarker of exposure to different environmental contaminants:** the evaluation in fibroblast cell cultures with immunofluorescence technique of the presence and the induction of two components (CYP1A1 and CYP2B) of the cytochrome P450 monooxygenase system (MFO), among the most relevant in drug and xenobiotic metabolism, was used to evaluate interspecies sensitivities to various classes of environmental contaminants. In particular CYP1A1 is induced by planar compounds such as planar OCs (coplanar polychlorinated biphenyls (PCBs)) and PAHs [16] and CYP2B by globular compounds such as PBDEs, PCBs and OC insecticides such as dichlorodiphenyltrichloroethane (DDT) and its metabolites [17, 18].
ii. **Qualitative and quantitative major histocompatibility complex (MHC) class I chain related protein A (MICA) expression as toxicological stress marker of the immune system:** the evaluation of the qualitative and quantitative MICA protein expression in fibroblast cell cultures with the immunofluorescence technique was used as toxicological stress marker of the immune system of different species of cetaceans [19]. The genes encoding for MICA and MICB are found within the major histocompatibility complex. Although MIC products have been found in various cells/tissues, the current consensus is that MIC genes are mainly expressed in gastrointestinal epithelium,

endothelial cells and fibroblasts. MIC molecules are considered to be stress-induced antigens that are recognized by cytotoxic T cells and natural killer (NK) cells, which play an important role in the surveillance of transformed infected and damaged cells [20]. Because the cetacean skin is an important tissue of the immune system and contributes to biological structure by acting not only as a protective physical barrier, but also as a target for immune components that mount the initial defense against invading pathogens, noxious stimuli, and resident neoplastic cells, the evaluation of MICA protein expression in cetaceans can be used to evaluate the status of the immune system of different species of cetaceans.

iii. **Detection of DNA damage by Comet assay as genotoxicity biomarker:** the presence of compounds such as PAHs, OCs and heavy metals in the marine environment can damage the DNA of living cells. The loss of DNA integrity can determine genotoxic effects, such as DNA base modifications, strand breaks, depurination and cross-linkages [21]. The comet assay or single cell gel electrophoresis (SCGE) is a sensitive method used as an indicator of genotoxicity and an effective biomarker for detecting DNA damage in living cells of aquatic animals [22]. Compared to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, alkaline elution, and micronucleus assay, the advantages of the Comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 10^{10} Da of DNA), requirement for small number of cells (~10,0000) per sample, flexibility to use proliferating cells, low cost, ease of application, and the short time needed to complete a study [23].

2. Sampling methods

2.1. Free- ranging cetaceans

Samples of skin (epidermis, dermis and blubber) were obtained from free-ranging specimens of long-beaked common dolphin (*Delphinus capensis*; MDC12) and common dolphin (*Delphinus delphis*; DDL1) using an aluminium pole armed with biopsy tips (0.7 cm ø, 3.0 cm length), while skin biopsies from free-ranging specimens of Bryde's whale (*Balaenoptera edeni*; MBE3), killer whale (*Orcinus orca*; MOO12), sperm whale (*Physeter macrocephalus*; PMAS1), bottlenose dolphin (*Tursiops truncatus*; TTA1) and Risso's dolphin (*Grampus griseus*; GGL1) were obtained using a Barnett Wildcat II crossbow with a 150-pound test bow, using a biopsy dart with modified stainless steel collecting tip (0.9 cm ø, 4.0 cm length). Biopsy samples were taken in the dorsal area near the dorsal fin, with CITES authorization (CITES Nat. IT025IS, Int. CITES IT 007) in the Sea of Cortez (MDC12, MBE3 and MOO12) and Mediterranean Sea (DDL1, PMAS1, TTA1 and GGL1). A small fragment of the biopsy was immediately stored in cell medium for the cell cultures.

2.2. Stranded cetaceans

Skin tissue of stranded cetaceans (dead within 2-12 h) were obtained from specimens found dead along the Italian coasts in the period 2005–2009 (CITES Nat. IT025IS, Int. CITES IT 007). Samples were taken under the dorsal fin of stranded specimens of fin whale (RT2 and

RT25), sperm whale (PM6), bottlenose dolphin (TurNic) and striped dolphin (RT1 and RT23), and immediately placed in cell medium.

2.3. Sex identification

Sex determination in cetaceans was carried out by genetic investigations according to Berubè & Palsboll [24].

3. Fibroblast cell cultures

The development of a non-invasive sampling method for obtaining viable tissue samples for cell cultures from skin biopsies of free-ranging and stranded cetaceans was described by Marsili *et al.* [14]. Successful cell cultures were obtained from all the animals. After the biopsy, skin samples were 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 100x (Mascia Brunelli) + 1% Penicillin/Streptomycin 100x (Mascia Brunelli) + 0.1% Amphotericin B 100x (Mascia Brunelli) at room 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 100x [Mascia Brunelli]) and antimycotic (Amphotericin B 100x [Mascia Brunelli]) solutions. All specimens were handled using sterile techniques. Initially, the collected tissue was cut into small pieces with curved surgical scissors, placed in 30-mm Petri dishes and incubated with Trypsin-EDTA solution 1x (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.

4. Indirect immunofluorescence tecnique

Third generation fibroblast cell cultures were exposed to the different mixtures of contaminants reported in the Table 1.

We used immunofluorescence in fibroblast cultures for a qualitative and semi-quantitative analysis of target proteins CYP1A1, CYP2B and MICA. After a first reaction with the primary polyclonal antibodies (goat anti-rabbit cytochrome P450 1A1 and goat anti-rabbit cytochrome P450 2B; Oxford Biochemical Research (Oxford MI, USA); rabbit polyclonal anti-MICA; Abcam), the cells were treated with the respective secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG (H+L) for CYP1A1 and CYP2B; Alexa Fluor 568 rabbit anti-goat IgG (H+L) for MICA; Invitrogen), labelled with red-fluorescent Alexa Fluor dye. Immunofluorescence was quantified with a specially designed Olympus Soft Imaging Systems macro, *DetectIntZ*, which works with the image acquisition, processing and analysis system, *analySIS^B* (Olympus) [15]. The image analysis procedure has the objective of quantifying, with an adimensional index generated for this purpose, the amount of Alexa

Name	Mixture	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5
OC mixture	(Arochlor 1260 + pp'DDT + p'DDE) solubilized in DMSO (0.05%)	0.01 μg/ml	0.1 μg/ml	1 μg/ml	5 μg/ml	25 μg/ml
PAHs	Benzo(a)pyrene (1mM) + beta-naphthoflavone (20mM) solubilized in acetone (0.1%)	0.5μM BaP + 10μM BnF	2.5μM BaP + 50μM BnF	12.5μM BaP + 250μM BnF	/	/
Flame retardants	BDE-MXE solubilized in nonane (0.01 μg/ml)	0.01 μg/ml	0.05 μg/ml	0.1 μg/ml	/	/
BPA	BPA solubilized in ethanol (0.1%)	0.1 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml	/

Table 1. The different mixtures of contaminants and doses to which cell cultures were exposed.

Fluor localized in the membrane of cytoplasmatic area of sample cells. The sample cells are imaged using DAPI and this image is presented to the operator for threshold selection of cytoplasmatic and nuclei Region of Interests (ROIs) across the field. The procedure then utilizes these ROIs to measure fluorescence intensity of Alexa Fluor sample cell and summarizes the results in a worksheet. The system generates index values which are unitless until compared with other units, such as number of cells to obtain mean fluorescence per cell or the area in which it is calculated to obtain mean fluorescence per mm^2. Images are all obtained with a magnification of 20X, a calibration of 0.65 μm/pixel and a resolution of 1360 x 1024 x 8 pixel. Exposure times were maintained fixed while reading the CYP1A1, CYP2B and MICA for each treatment. A series of images of each slide was acquired so that a minimum of 250 cells/slide could be counted. The total fluorescence revealed by the program is divided by number of cells to obtain arbitrary unity of fluorescence (AUF) per cell. Several slides for CYP1A1, CYP2B and MICA were made for each culture: one was a blank (cells treated only with primary and secondary antibodies), one was a secondary blank (cells treated only with secondary antibody), one was a chemical blank (cells treated with contaminant carrier), two were for each treatment dose of contaminants. The blank enabled the natural presence of the target proteins in cultured fibroblasts to be checked. The secondary blank enabled validation of the dose of secondary antibody without cross reaction as the primary antibody was absent.

5. Genotoxicity biomarker: comet assay

Fibroblast cell cultures (third generation) of striped dolphin (RT23) were subjected to this experimental protocol for 4 h. A cell line was exposed to a mixture of benzo(a)pyrene

(1mM) and beta-naphthoflavone (20mM), solubilized in acetone (0.1%), at three doses (Table 1), plus an acetone (0.1%) control. Fibroblast cell cultures were processed for the comet assay after Caliani *et al.* [13], with some modifications. The cells were centrifuged at 1000 g for 10 min, then were embedded in agarose (0.5% low-melting agarose) and layered on conventional slides, predipped in 1% normal melting agarose. The slides were immersed into a freshly made lysis solution (2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, 1% Triton X-100, and 10% DMSO, pH 10) for at least 1 h at 4°C in the dark. The slides were then placed on a horizontal electrophoresis tray previously filled with freshly prepared cold alkaline buffer and left for 20 min to allow DNA unwinding. Electrophoresis was performed at 25 V and 300 mA for 20 min. The DNA migration was evaluated at three different pH conditions (pH 13, pH 12.1, pH 8). Slides were then neutralized in Tris (0.4 M, pH 7.5) for 3x5 min and stained with Sybr safe 1:10.000 in TE (10 mM Tris-HCl pH 7.5 and 500 mM EDTA pH 7.5) buffer. A total of 50 cells per slide were examined under epifluorescence at 40X magnification. The amount of DNA damage was evaluated as the percentage of DNA migrating out of the nucleus using an image analyser (Komet 6.0 Software, Kinetic Imaging Ltd.), connected to a fluorescent microscope (Olympus BX41).

6. Interspecies differences in the Mixed Function Oxidase (MFO) as biomarker of exposure of different environmental contaminants

Fibroblast cell cultures of fin whale (RT2), Bryde's whale (MBE3), sperm whale (PM6), killer whale (MOO12), Risso's dolphin (GGL1), bottlenose dolphin (TurNic), striped dolphin (RT1), long-beaked common dolphin (MDC12) and common dolphin (DDL1) were treated for 48 h with different environmental contaminants and the quantification of the induction of endogenous proteins such as CYP1A1 and CYP2B was used as target of toxicological susceptibility. The presence and the induction of CYP1A1 and CYP2B were evaluated with the indirect immunofluorescence and quantified with the Olympus macro, *DetectIntZ*. CYP1A1 is induced by planar compounds and CYP2B by globular compounds. The treatments were performed with OC mixture; flame retardants; PAHs; and BPA (Table 1). In the total mixture of Arochlor 1260 [25] only the 1.3033% shows a CYP1A1 inductive capacity while the remaining congeners are CYP2B inducers [26, 27, 28, 29, 30]. pp'DDE and pp'DDT are known as CYP2B inducers [31, 32] but an experiment on fibroblast cell culture of sperm whale (PM6) treated only with pp'DDT and pp'DDE has shown a capacity of these compounds to induce also the CYP1A1 (Figure 1). Examining at the bromine substitution patterns in the basic structure of the PBDE molecule, and with the support of the other studies on this topic [33] we can say that in the BDE-MXE mixture, the 18.72% is CYP1A1 inducer and the rest of congeners are CYP2B inducers. Benzo(a)pyrene and beta-naphthoflavone are important planar compounds and CYP1A1 inducers [31]. Finally BPA that may be a human-specific inducer of the CYP3A4 gene [34], but many studies have shown that BPA inhibits several P450-dependent monooxygenases activities (CYP1A2, CYP2A2, CYP2B2, CYP2C11, CYP2D1, CYP2E1 and CYP3A2) [35].

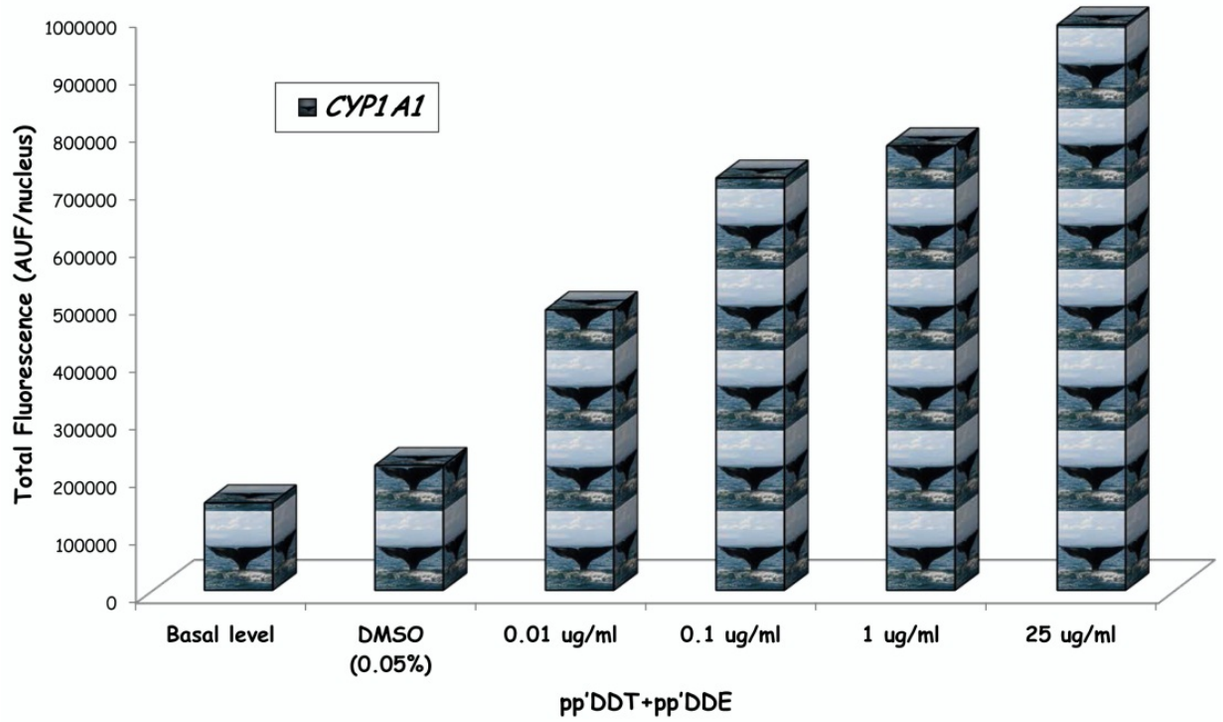

Figure 1. Basal levels of immunofluorescence (AUF/nucleus) of CYP1A1 in fibroblast cells of sperm whale treated with pp'DDT and pp'DDE.

6.1. Basal levels of CYP1A1 and CYP2B in different species

The first result of these experiments in nine cetacean species was the detection of the presence of CYP1A1 and CYP2B in fibroblast cells of all species, revealed by immunofluorescence (Figure 2A-B); a higher basal expression of both proteins was found in Risso's dolphin (GGL1) and bottlenose dolphin (TurNic), while fin whale (RT2) and sperm whale (PM6) showed the lowest levels of these proteins. The Risso's dolphin (GGL1) and the sperm whale (PM6) have a very similar diet, consisting mostly of squid. Nevertheless, they have a very different basal expression of the two cytochromes. As for the other species, very high levels of CYP1A1 were present in the Bryde's whale (MBE3). This mysticete sampled in the Sea of Cortez showed CYP1A1 levels more than 20 times greater than the other mysticete studied, the Mediterranean fin whale (RT2). Regarding the levels of contaminants detected in the blubber of different species, the bottlenose dolphin (TurNic), stranded along the coasts of the Mediterranean Sea, had very high levels of organochlorine contaminants in its blubber (DDTs = 77.4 μg/g lipid weight (l.w.); PCBs = 262.6 μg/g l.w.) that are potent inducers of CYP2B. In fact, especially in this specimen this cytochrome appears to be markedly higher than the levels shown by other species (Figure 2B). But the sperm whale (PM6) was also a stranded specimen found on the Italian coasts (Mediterranean Sea) having high values of these xenobiotics in the blubber. It seems therefore that this basal activity is more species-specific than related to the geographical location, diet, toxicological status, etc. in which the animals were found.

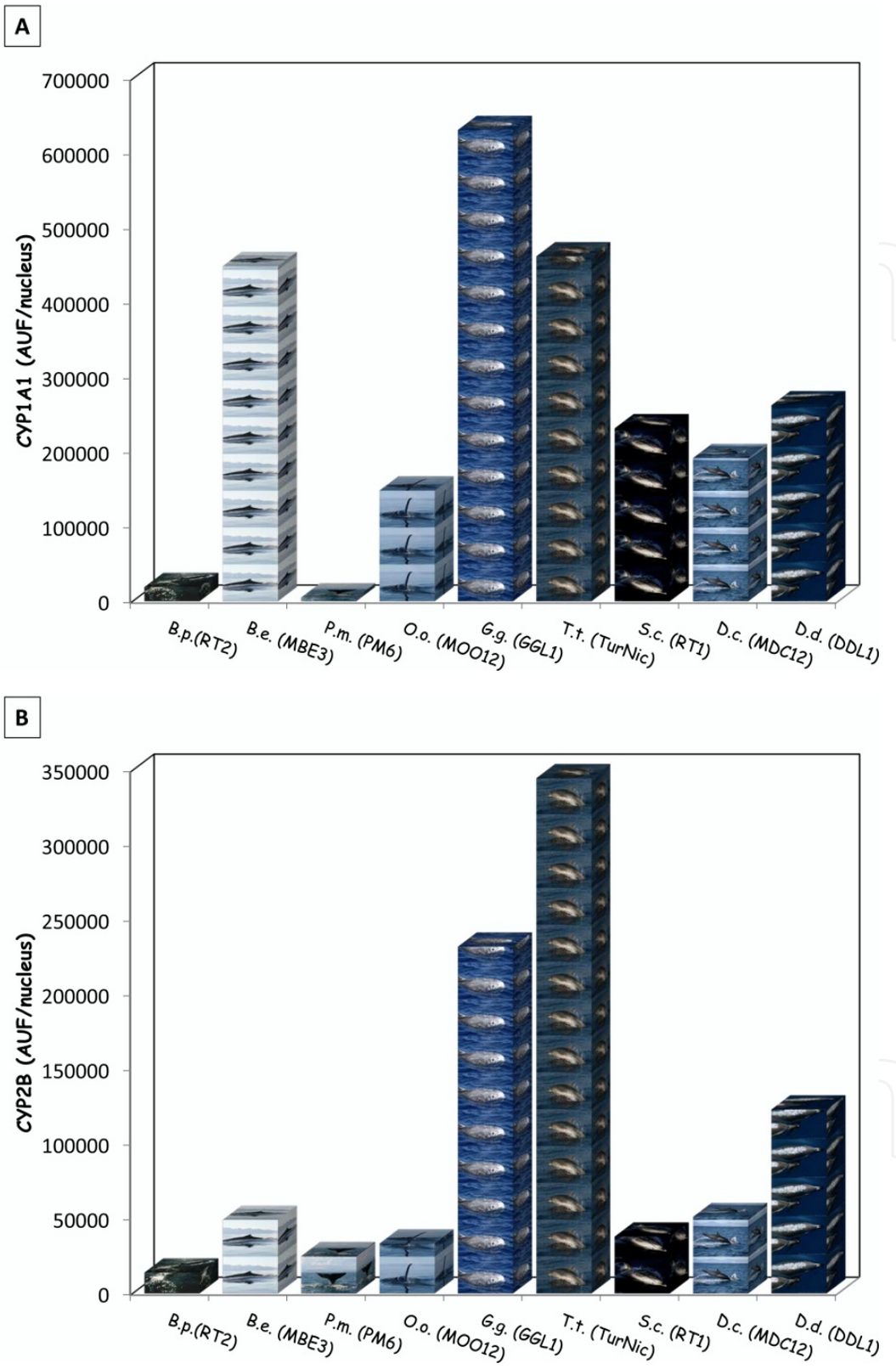

Figure 2. (A-B): Basal levels of immunofluorescence (AUF/nucleus) of CYP1A1 (A) and CYP2B (B) in fibroblast cells of fin whale (B.p.), Bryde's whale (B.e.), sperm whale (P.m.), killer whale (O.o.), Risso's dolphin (G.g.), bottlenose dolphin (T.t.), striped dolphin (S.c.), long-beaked common dolphin (D.c.) and common dolphin (D.d.).

6.2. CYP1A1 and CYP2B in different species after treatment with OC mixture

Results of the mean levels of immunofluorescence of CYP1A1 (A) and CYP2B (B), revealed in cultured fibroblasts of different species treated with OC mixture and expressed as index numbers, are reported in Table 2A-B.

A CYP1A1	*DMSO 0.05%*	*0.01 µg/ml*	*0.1 µg/ml*	*1 µg/ml*	*5 µg/ml*	*25 µg/ml*
RT2 (fin whale)	100	125.1	170.3	148.0	104.3	143.6
MBE3 (Bryde's whale)	100	31.6	106.2	55.8	/	/
PM6 (sperm whale)	100	/	/	15.7	31.9	77.4
MOO12 (killer whale)	100	427.2	288.6	207.8	696.6	49.0
GGL1 (Risso's dolphin)	100	/	/	144.5	224.1	104.7
TurNic (bottlenose dolphin)	100	63.0	96.5	81.4	/	/
RT1 (striped dolphin)	100	56.4	31.0	49.3	94.1	219.6
MDC12 (long-beaked common dolphin)	100	325.9	312.1	836.9	/	/
DDL1 (common dolphin)	100	/	/	111.4	117.3	82.9
B CYP2B	*DMSO 0.05%*	*0.01 µg/ml*	*0.1 µg/ml*	*1 µg/ml*	*5 µg/ml*	*25 µg/ml*
RT2 (fin whale)	100	112.3	110.1	110.4	116.3	146.5
MBE3 (Bryde's whale)	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
PM6 (sperm whale)	100	/	/	134.6	292.1	212.4
MOO12 (killer whale)	100	156.4	300.5	456.0	153.3	104.3
GGL1 (Risso's dolphin)	100	/	/	70.8	45.2	128.1
TurNic (bottlenose dolphin)	100	88.6	136.2	85.7	/	/
RT1 (striped dolphin)	100	399.8	756.2	305.7	141.8	368.0
MDC12 (long-beaked common dolphin)	100	205.2	127.6	189.4	/	/
DDL1 (common dolphin)	100	/	/	112.1	102.0	84.7

Table 2. (A-B): Mean values of immunofluorescence of CYP1A1 (A) and CYP2B (B) revealed in cultured fibroblasts of different species treated with OC mixture. The immunofluorescence is expressed as index numbers respect to solvent control. Different colour of box is related to different increase of these proteins. N.C. = no cells.

The results confirm the capability of this methodology to detect CYP1A1 (Table 2A) and CYP2B (Table 2B) induction with OC mixture in many species of this study; particularly we had induction of CYP1A1 and CYP2B, with respect to chemical blank (DMSO), at all doses in fin whale (RT2) (Figure 3A; D) and long-beaked common dolphin (MDC12); an induction of CYP1A1 was detected at all doses in Risso's dolphin (GGL1) and of CYP2B at all doses in sperm whale (PM6), killer whale (MOO12) and striped dolphin (RT1) (Figure 3F). No induction of CYP1A1 was detected in sperm whale (PM6) and bottlenose dolphin (TurNic), while CYP2B showed OC induction at least at one treatment dose in all species. Different induction responses were given by the different specimens: there was a dose/response induction for CYP1A1 only for long-beaked common dolphin (MDC12) and for CYP2B only for fin whale (RT2) (Figure 3D), while a bell-shaped response was present for CYP1A1 in Risso's dolphin (GGL1) and common dolphin (DDL1), and for CYP2B in sperm whale (PM6), killer whale (MOO12) (Figure 3E), striped dolphin (RT1) (Figure 3F) and common dolphin (DDL1). Discontinuous induction response was showed for CYP1A1 and CYP2B by the other specimens such as CYP1A1 in killer whale (MOO12) (Figure 3B) and striped dolphin (RT1) (Figure 3C). It is interesting to point out that all species, following treatment with the OC mixture, showed a greater response of CYP2B, compared to CYP1A1, confirming that these xenobiotics mostly with globular structure have a major ability to induce this cytochrome.

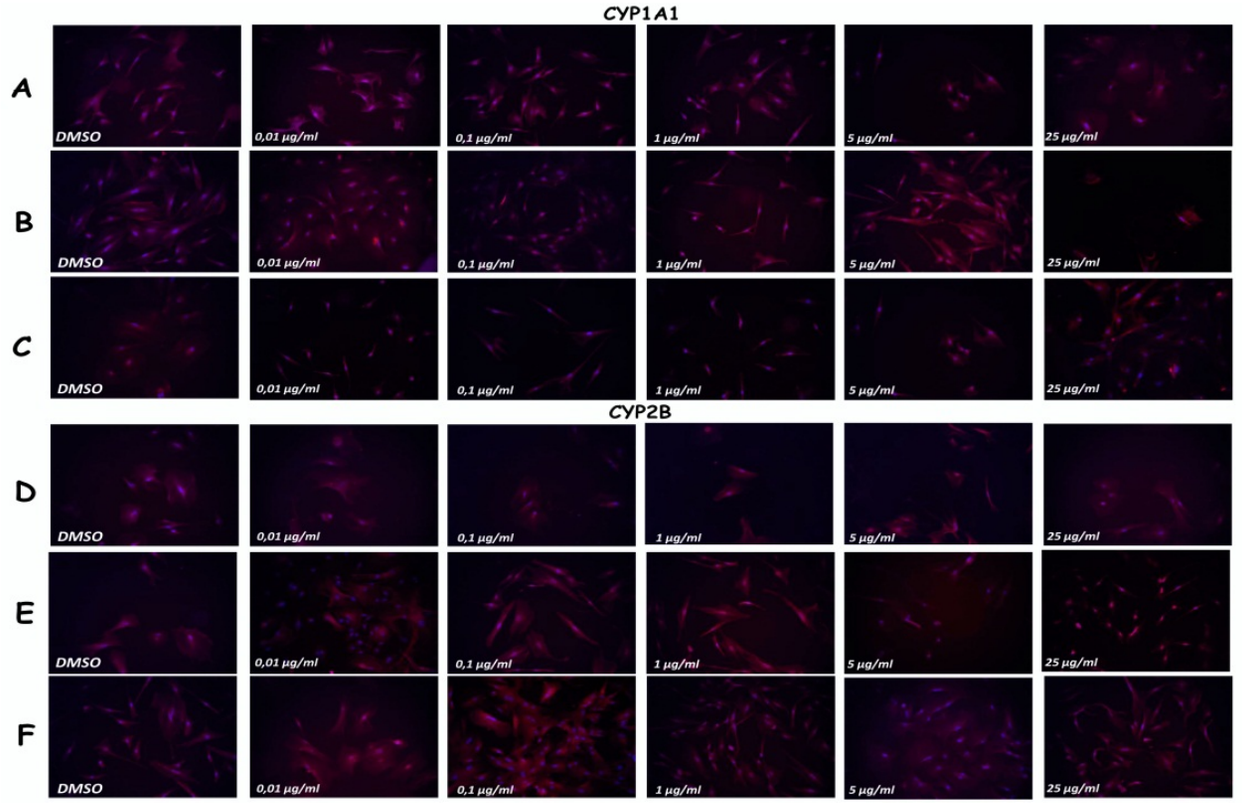

Figure 3. A-F: Immunofluorescence (AUF/nucleus) of CYP1A1 (A-C) and CYP2B (D-F) in fibroblast cells of fin whale (RT2) (A, D), killer whale (MOO12) (B, E) and striped dolphin (RT1) (C, F) treated with OC mixture. DAPI and Alexa Fluor 594 (Intensity 200ms) images of DMSO and the five OC mixture treatments.

6.3. CYP1A1 and CYP2B in different species after treatment with flame retardants

Results of the mean levels of immunofluorescence of CYP1A1 (A) and CYP2B (B), revealed in cultured fibroblasts of different species treated with flame retardants and expressed as index numbers, are reported in Table 3A-B. Marked differences in CYP1A1 (Table 3A) and CYP2B (Table 3B) induction by flame retardants were detected in different species, with higher sensitivity of responses in striped dolphin (RT1) for CYP1A1 (Figure 4A) and killer whale (MOO12) for CYP2B. To be highlighted that we have an inductive response of both cytochromes in the same animals, precisely in sperm whale (PM6), killer whale (MOO12), striped dolphin (RT1) (Figure 4A; C), long-beaked common dolphin (MDC12) (Figure 4B; D) and common dolphin (DDL1). Bottlenose dolphin (TurNic) showed only the CYP1A1 induction.

A CYP1A1	*Nonane 0.01 µg/ml*	*0.01 µg/ml*	*0.05 µg/ml*	*0.1 µg/ml*
RT2 (fin whale)	100	52.1	91.2	51.3
MBE3 (Bryde's whale)	N.C.	N.C.	N.C.	N.C.
PM6 (sperm whale)	100	113.2	105.2	52.0
MOO12 (killer whale)	100	132.2	36.6	131.1
GGL1 (Risso's dolphin)	100	73.3	98.6	82.6
TurNic (bottlenose dolphin)	100	71.1	102.5	131.9
RT1 (striped dolphin)	100	128.4	232.6	273.1
MDC12 (long-beaked common dolphin)	100	205.2	127.6	189.4
DDL1 (common dolphin)	100	135.4	133.9	154.6
B CYP2B	*Nonane 0.01 µg/ml*	*0.01 µg/ml*	*0.05 µg/ml*	*0.1 µg/ml*
RT2 (fin whale)	100	60.2	68.4	54.1
MBE3 (Bryde's whale)	N.C.	N.C.	N.C.	N.C.
PM6 (sperm whale)	100	111.9	70.5	63.1
MOO12 (killer whale)	100	314.7	110.7	52.7
GGL1 (Risso's dolphin)	100	70.2	75.9	48.6
TurNic (bottlenose dolphin)	100	71.5	69.2	94.1
RT1 (striped dolphin)	100	177.4	109.9	128.3
MDC12 (long-beaked common dolphin)	100	149.5	139.3	178.1
DDL1 (common dolphin)	100	118.4	85.8	324.5

Table 3. (A-B): Mean values of immunofluorescence of CYP1A1 (A) and CYP2B (B) revealed in cultured fibroblasts of different species treated with flame retardants. The immunofluorescence is expressed as index numbers respect to solvent control. Different colour of box is related to different increase of these proteins. N.C. = no cells.

A dose dependent induction of CYP1A1 was detected in striped dolphin (RT1) and common dolphin (DDL1) and of CYP2B only in long-beaked common dolphin (MDC12). A bell-shaped response was present for CYP1A1 and CYP2B in sperm whale (PM6), and for CYP2B

in killer whale (MOO12) and striped dolphin (RT1), while discontinuous induction responses were showed for CYP1A1 and CYP2B by the other species. Also these contaminants are mainly with globular structure as OC mixture, but no differences are present in the induction of the two cytochromes.

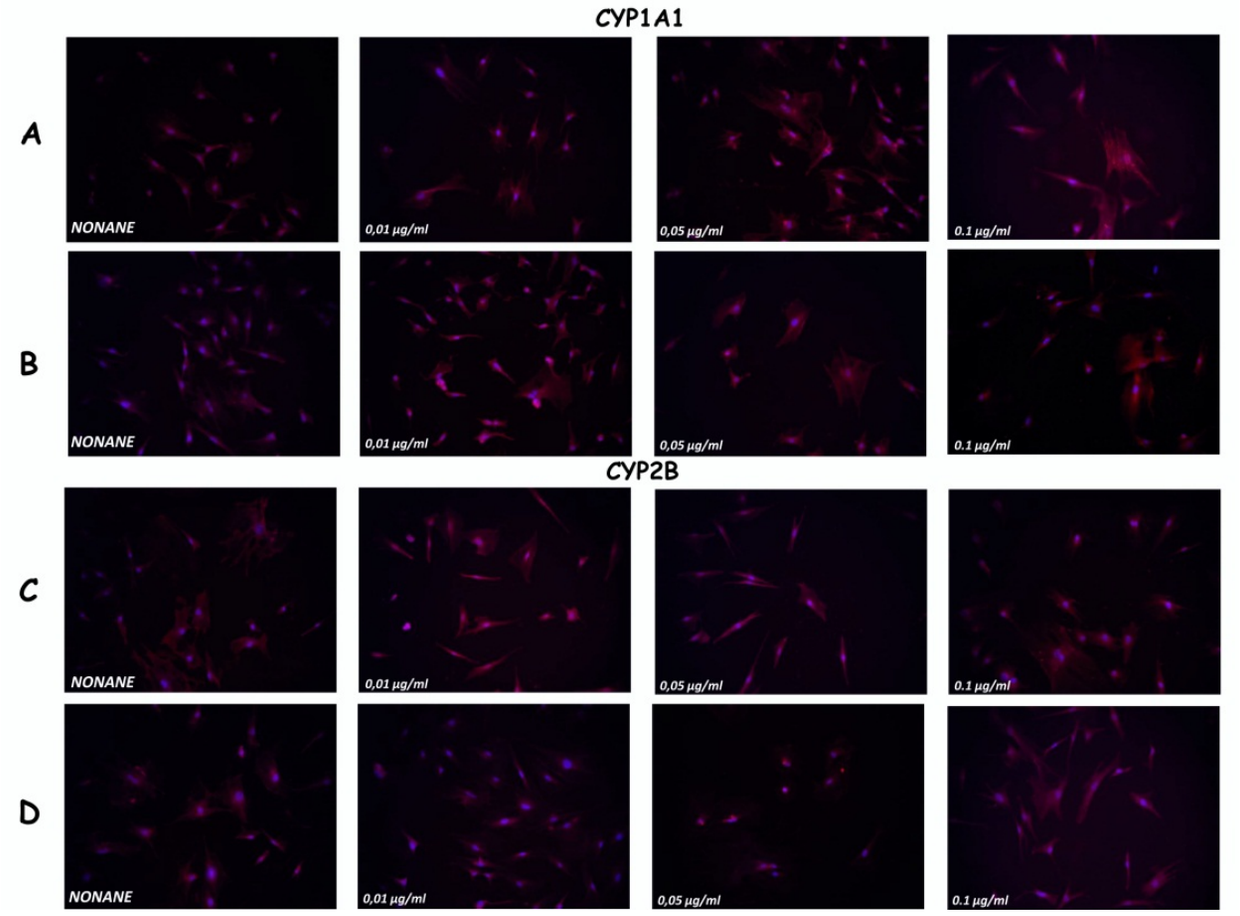

Figure 4. A-D: Immunofluorescence (AUF/nucleus) of CYP1A1 (A-B) and CYP2B (C-D) in fibroblast cells of striped dolphin (RT1) (A, C) and long-beaked common dolphin (MDC12) (B, D) treated with flame retardants. DAPI and Alexa Fluor 594 (Intensity 200ms) images of Nonane and the three flame retardant treatments.

6.4. CYP1A1 and CYP2B in different species after treatment with PAHs

In Table 4A-B we reported the results of the mean levels of immunofluorescence of CYP1A1 (A) and CYP2B (B), revealed in cultured fibroblasts of different species treated with PAHs, expressed as index numbers.

Only the fibroblasts of some species were treated with PAHs due to the fact that there was not a sufficient amount of cells from all of them to perform the various contaminant treatments. These planar contaminants are known to induce CYP1A1. In fact, an increase of CYP1A1 was detected, at least at one dose, in all specimens cultured fibroblasts exposed to PAHs (Table 4A) (Figure 5A-E). In the Risso's dolphin (GGL1) and in the striped dolphin (RT1), the higher dose of PAHs even caused the death of all cells. The fibroblast vitality was

assessed with trypan blue, a quality control test to check the cell preparation. In the event of cell damage, trypan blue penetrates the cell membrane, and dead or damaged cells appear blue [36]. CYP2B also, showed induction in the three species treated with PAHs (Table 4B). The striped dolphin (RT1) showed the same trend for the two cytochromes (Table 4A-B).

A CYP1A1	*Acetone 0.1%*	*Dose C*	*Dose B*	*Dose A*
MBE3 (Bryde's whale)	100	31.6	106.2	55.8
PM6 (sperm whale)	100	111.5	69.6	68.2
GGL1 (Risso's dolphin)	100	107.7	90.2	D.C.
RT1 (striped dolphin)	100	279.8	77.3	D.C.
MDC12 (long-beaked common dolphin)	100	155.6	279.7	123.2
B CYP2B	*DMSO 0.1%*	*0.01 µg/ml*	*0. 1 µg/ml*	*1 µg/ml*
MBE3 (Bryde's whale)	N.C.	N.C.	N.C.	N.C.
PM6 (sperm whale)	100	125.0	64.2	101.6
GGL1 (Risso's dolphin)	N.C.	N.C.	N.C.	N.C.
RT1 (striped dolphin)	100	108.6	25.3	D.C.
MDC12 (long-beaked common dolphin)	100	218.6	65.6	64.3

Table 4. (A-B): Mean values of immunofluorescence of CYP1A1 (A) and CYP2B (B) revealed in cultured fibroblasts of different species treated with PAHs (Dose C = 0.5µM BaP + 10µM BnF, Dose B = 2.5µM BaP + 50µM BnF and Dose A = 12.5µM BaP + 250µM BnF). The immunofluorescence is expressed as index numbers respect to solvent control. Different colour of box is related to different increase of these proteins. N.C. = no cells. D.C. = death cells.

6.5. CYP1A1 and CYP2B in different species after treatment with BPA

In Table 5A-B we reported the results of the mean levels of immunofluorescence of CYP1A1 (A) and CYP2B (B), revealed in cultured fibroblasts of killer whale (MOO12) treated with BPA, expressed as index numbers.

A CYP1A1	*Ethanol 0.1%*	*0.1 µg/ml*	*1 µg/ml*	*10 µg/ml*	*100 µg/ml*
MOO12 (killer whale)	100	800.7	886.3	747.3	D.C.
B CYP2B	*Ethanol 0.1%*	*0.1 µg/ml*	*1 µg/ml*	*10 µg/ml*	*100 µg/ml*
MOO12 (killer whale)	100	48.3	40.4	49.2	D.C.

Table 5. (A-B): Mean values of immunofluorescence of CYP1A1 (A) and CYP2B (B) revealed in cultured fibroblasts of different species treated with BPA. The immunofluorescence is expressed as index numbers respect to solvent control. Different colour of box is related to different increase of these proteins. D.C. = death cells.

Only one species, the killer whale, was treated with this potent estrogenic compound since the study to evaluate the cetacean susceptibility to BPA has just started in our laboratories. However, this preliminary experiment confirms that BPA is a potent CYP2B inhibitor [35]

while it gives information on CYP1A1 inductive capacity (Figure 6 A-B). To be highlighted that the highest used dose (100 µg/ml) is 100% lethal for fibroblast cells.

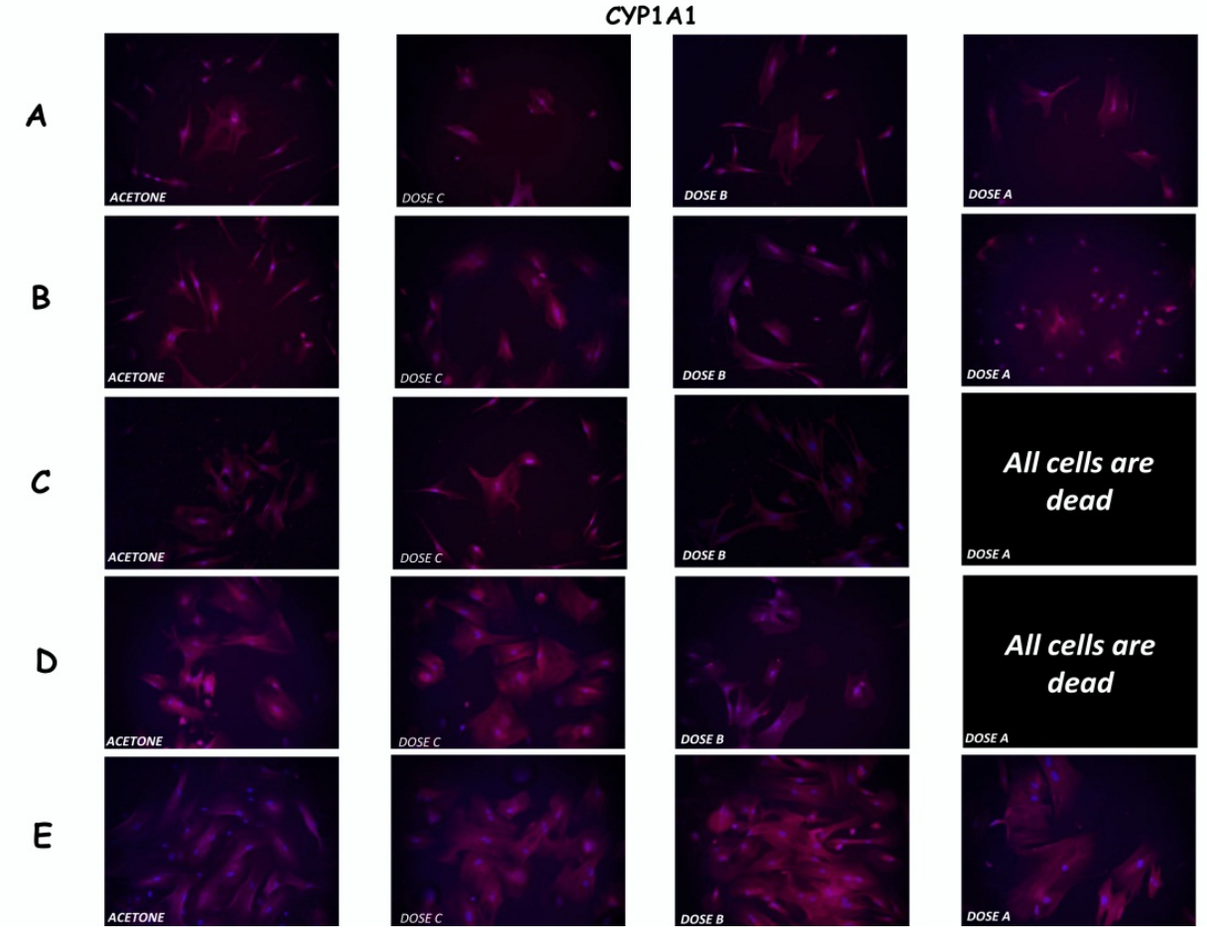

Figure 5. A-E: Immunofluorescence (AUF/nucleus) of CYP1A1 (A-E) in fibroblast cells of Bryde's whale (MBE3) (A), sperm whale (PM6) (B), Risso's dolphin (GGL1) (C), Striped dolphin (RT1) (D) and long-beaked common dolphin (MDC12) (E) treated with PAHs. DAPI and Alexa Fluor 594 (Intensity 200ms) images of Acetone and the three PAH treatments.

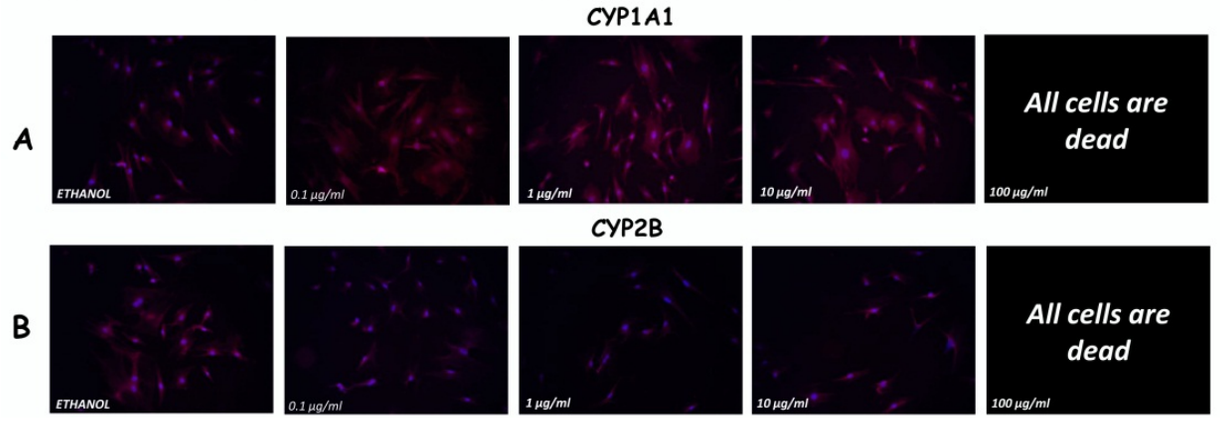

Figure 6. A-B: Immunofluorescence (AUF/nucleus) of CYP1A1 (A-B) and CYP2B (C-D) in fibroblast cells of killer whale (MOO12) (A, B) treated with BPA. DAPI and Alexa Fluor 594 (Intensity 200ms) images of Ethanol and the four BPA treatments.

7. Qualitative and quantitative mica protein expression as toxicological stress marker of the immune system

Cetacean morbilliviruses and papillomaviruses as well as *Brucella* spp. and *Toxoplasma gondii* are thought to reduce population abundance by inducing high mortalities, lowering reproductive success or by synergistically increasing the virulence of other diseases. Severe cases of lobomycosis and lobomycosis-like disease (LLD) may contribute to the death of some dolphins [37]. Environmental contamination seems to play a role in these diseases because many pollutants are known immunosuppressants and can markedly affect the immune status of the cetacean specimens [38, 39, 40, 41, 42]. As already mentioned, the cetacean skin is an important tissue of the immune system and the MICA protein, used in this study as toxicological stress marker of the immune system, is expressed in fibroblasts. In fact, the aim of this study was to evaluate the MICA protein expression in fibroblast cell cultures of cetaceans (skin biopsies of free-ranging animals and skin samples of stranded cetaceans dead within 2-12 h). Here we present the immunofluorescence technique in cultured fibroblasts used for qualitative and quantitative evaluation of MICA expression, induced by treatment with OC mixture, flame retardants, PAHs and BPA, as toxicological stress marker of the immune system of different species of odontocetes (sperm whale (PMAS1), killer whale (OO12), striped dolphin (RT23), long-beaked common dolphin (MDC12)) and mysticetes (fin whale (RT25), Bryde's whale (MBE3)).

7.1. Basal levels of MICA in different species

The basal level of MICA, evaluated with immunofluorescence technique in the fibroblasts of different cetacean species before treatment with different mixtures, is the first important result of this research step as it provides an indication of the immune status of these marine mammals. The results, expressed as immunofluorescence for cell (AUF/nucleus) mean values are presented in the Table 6 and, as histograms, in the Figure 7.

	MICA (UAF/nucleus)
Bryde's whale (MBE3)	50482.0
Long-beaked common dolphin (MDC12)	94751.0
Killer whale (MOO12)	195378.0
Sperm whale (PMAS1)	12647.0
Bottlenose dolphin (TTA1)	31473.0
Striped dolphin (RT23)	18511.0
Fin whale (RT25)	31325.0

Table 6. Basal levels of immunofluorescence (AUF/nucleus) of MICA in fibroblast cells of Bryde's whale, long-beaked common dolphin, killer whale, sperm whale, bottlenose dolphin, striped dolphin and fin whale.

We can highlight that the three specimens belonging to the three species sampled in the Sea of Cortez (Bryde's whale, long-beaked common dolphin and killer whale) showed higher basal activity of MICA with respect to all Mediterranean specimens, regardless of the

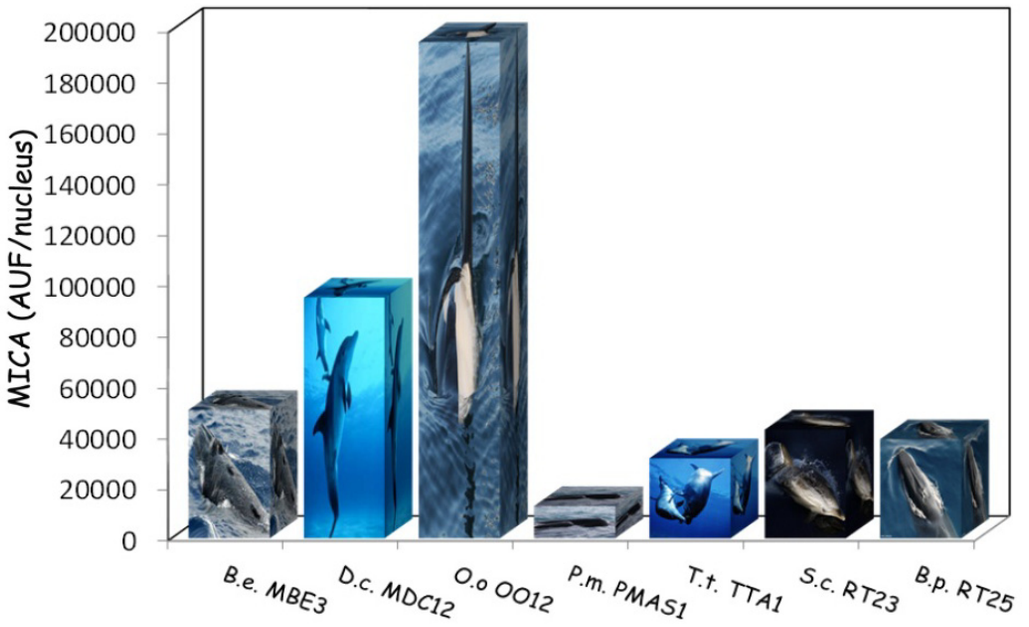

Figure 7. Basal levels of immunofluorescence (AUF/nucleus) of MICA in fibroblast cells of Bryde's whale (B.e), long-beaked common dolphin (D.c.), killer whale (O.o.), sperm whale (P.m.), bottlenose dolphin (T.t.), striped dolphin (S.c.) and fin whale (B.p.).

species and the fact that they were sampled free-ranging or found stranded alive and then died. Moreover, the basal activity of MICA in the three Mexican species seems to be related to their different diet, with an increasing activity with the increase of the trophic level. Bryde's whale is not strictly planktophagus as is the fin whale in the Mediterranean, feeding mainly on blue fish. So this species in the food chain is definitely closer to long-beaked common dolphin and the other toothed whales in this study than to the other mysticete species. The free-ranging specimen of sperm whale sampled in the surrounding water of Asinara Island (Mediterranean Sea) showed a basal activity very similar to that showed by the striped dolphin, but lower than the other species sampled in the Mediterranean Sea (bottlenose dolphin and fin whale).

This sharp distinction between the activity of MICA found in the Sea of Cortez and Mediterranean Sea specimens is probably the most important result to be highlighted: it seems that the environment in which specimens live and, therefore, the anthropogenic stress to which they are subjected are determinant in the response of this protein. In the light of this result we can hazard the conclusion that the lower the anthropic stress of the specimens, the higher the basal activity of MICA. Regarding the Mediterranean species, the two stranded specimens (striped dolphin RT23 and fin whale RT25), were both affected by *morbillivirus*; it would be very interesting to know the basal activity in the same species sampled free-ranging, to understand whether in case of immunosuppression the activity of MICA increases or decreases. To assess whether MICA increases or decreases, because of the presence of an inducer or a repressor of the immune system, we treated fibroblast cell cultures with cyclosporine A (CsA), a drug that belongs to the category of immunosuppressants, and with β-glucan, a polysaccharide known to increase the response

of the immune system. The results of each specimen whose cells were treated with the two compounds are showed in Table 7.

Killer whale							
	n° cells	**Mean**	**Median**	**Minimum**	**Maximum**	**S.D.**	**S.E.**
BA	98	195378	151675	42646	592187	162020	46176
Inducer	151	189172	202628	50932	350323	109320	30320
Repressor 0.8μg/ml	135	106656	86680	46865	248599	61607	19481
Repressor 80μg/ml	79	279350	250709	20631	636284	174779	50454.4
Bottlenose dolphin							
	n° cells	**Mean**	**Median**	**Minimum**	**Maximum**	**S.D.**	**S.E.**
BA	87	31473	30832	26302	39107	4196.4	1713.2
Inducer	72	19230	19319	9871	44949	10153	3210
Repressor 0.8μg/ml	66	21673	19480	19637	55989	13259	4193
Repressor 80μg/ml	71	20647	17264	10492	56499	13536	4280
Striped dolphin							
	n° cells	**Mean**	**Median**	**Minimum**	**Maximum**	**S.D.**	**S.E.**
BA	75	18511	17896	8825	42083	9254	3055
Inducer	76	17606	18690	8556	26951	5977	1890
Repressor 0.8μg/ml	69	20677	124525	41662	329043	13440	4250
Repressor 80μg/ml	**All cell death**						
Fin whale							
	n° cells	**Mean**	**Median**	**Minimum**	**Maximum**	**S.D.**	**S.E.**
BA	114	31325	25172	18459	63324	16085	5086
Inducer	244	17409	17867	9673	23938	4270	1350
Repressor 0.8μg/ml	78	28304	29231	13275	37051	69853	21061
Repressor 80μg/ml	35	19031	18254	7299	56686	14102	4252

Table 7. Descriptive statistics of immunofluorescence per cell (AUF/nucleus) of MICA revealed in cultured fibroblasts of Bryde's whale (MBE3), striped dolphin (RT23), bottlenose dolphin (TTA1), and killer whale (OO12) treated with the inducer (β-glucan) and the repressor (Cyclosporine A). BA represents the blank (cultured fibroblasts treated only with primary and secondary antibodies).

In all cases, with the exception of fibroblasts of killer whale treated with the highest dose of repressor and fibroblasts of striped dolphin treated with the lower dose of repressor (highlighted in dark grey), we can see a decrease of the response of the MICA compared to

the basal activity. After the treatment with these doses, it therefore remains difficult to understand the response of MICA which we must expected from a toxicological stress. Probably the choice of the two compounds, known to have such capabilities in relation to the immune system but not specifically in respect to MICA, should be reassessed.

7.2. MICA in different species after treatment with OC mixture

From Table 8 it appears evident that the carrier of OC mixture (DMSO) confounds the evaluation of the responses of the cells to the treatments, as previously demonstrated in other studies [15, 43].

MICA	*DMSO 0.05%*	*0.01 µg/ml*	*0.1 µg/ml*	*1 µg/ml*	*5 µg/ml*	*25 µg/ml*
MBE3 (Bryde's whale)	100	100	174	62	/	/
MDC12 (long-beaked common dolphin)	100	104	98	29	/	/
MOO12 (killer whale)	100	31	25	26	47	22
PMAS1 (sperm whale)	100	137	94	291	2523	11195
RT23 (striped dolphin)	100	53	N.C.	59	54	N.C.
RT25 (fin whale)	100	119	96	100	120	69

Table 8. Mean values of immunofluorescence of MICA revealed in cultured fibroblasts of different species treated with OC mixture. The immunofluorescence is expressed in index numbers respect to solvent control. Different colour of box is related to different increase of this protein.

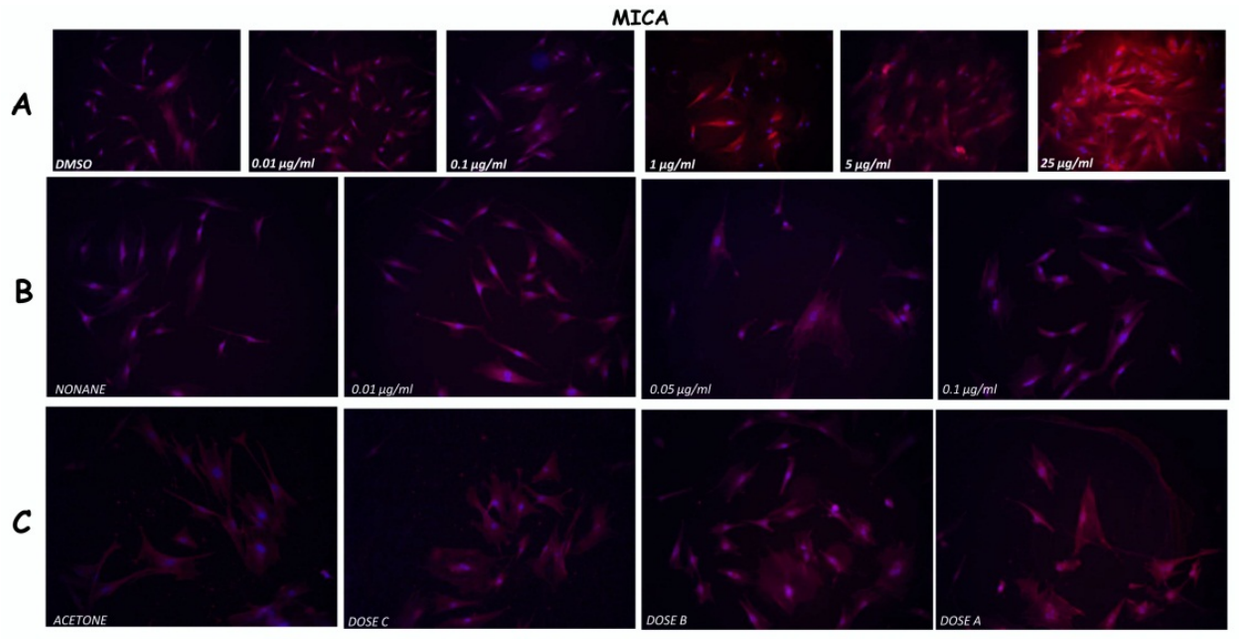

Figure 8. A-C: Immunofluorescence (AUF/nucleus) of MICA in fibroblast cells of sperm whale (PMAS1).DAPI and Alexa Fluor 594 (Intensity 200ms) images of DMSO and the five OC mixture treatments (A); Nonane and three flame retardant treatments (B) and Acetone and three PAH treatments (C).

Only the sperm whale demonstrated a clear inductive effect on MICA due to OCs, compared to DMSO (Figure 8A). In fibroblasts of killer whale (MOO12) and striped dolphin (RT23) no dose caused an increase of the MICA with respect to the carrier (DMSO). A dose dependent induction of MICA was present in sperm whale (PMAS1), a bell-shaped response was present in Bryde's whale (MBE3) and in long-beaked common dolphin (MDC12) while discontinuous induction response was showed by fin whale (RT25).

7.3. MICA in different species after treatment with Flame Retardants

In the Table 9 are reported the results of the mean levels of immunofluorescence of MICA, revealed in cultured fibroblasts of different species treated with the flame retardants, expressed as index numbers.

MICA	*Nonane 0.01 µg/ml*	*0.01 µg/ml*	*0.05 µg/ml*	*0.1 µg/ml*
MBE3 (Bryde's whale)	100	49	35	55
MDC12 (long-beaked common dolphin)	100	69	96	209
MOO12 (killer whale)	100	66	54	96
PMAS1 (sperm whale)	100	102	74	102
RT23 (striped dolphin)	100	103	132	107
RT25 (fin whale)	100	101	134	120

Table 9. Mean values of immunofluorescence of MICA revealed in cultured fibroblasts of different species treated with flame retardants. The immunofluorescence is expressed in index numbers respect to solvent control. Different colour of box is related to different increase of this protein.

In striped dolphin (RT23) and fin whale (RT25) the highest response of MICA was related to flame retardants, with a bell-shaped response in both species. Discontinuous induction response is shown by sperm whale (PMAS1) (Figure 8B) and long-beaked common dolphin (MDC12), while Bryde's whale (MBE3) and killer whale (MOO12) showed no induction response.

7.4. MICA in different species after treatment with PAHs

In Table 10 are reported the results of the mean levels of immunofluorescence of MICA, revealed in cultured fibroblasts of different species treated with the PAHs, expressed as index numbers.

MICA	*Acetone 0.1%*	*Dose C*	*Dose B*	*Dose A*
MDC12 (long-beaked common dolphin)	100	68	93	55
PMAS1 (sperm whale)	100	101	181	189

Table 10. Mean values of immunofluorescence of MICA revealed in cultured fibroblasts of different species treated with PAHs. The immunofluorescence is expressed in index numbers respect to solvent control. Different colour of box is related to different increase of this protein.

Only two species, the long-beaked common dolphin (MDC12) and the sperm whale (PMAS1) were treated with PAHs. However, even with this treatment, there is a significant dose/response type increase in the level of MICA in the sperm whale (PMAS1) (Figure 8C). Long-beaked common dolphin (MDC12) showed no induction response.

7.5. MICA in different species after treatment with BPA

Table 11 shows the results of the mean levels of immunofluorescence of MICA, revealed in cultured fibroblasts of different species treated with the BPA, expressed as index numbers.

MICA	*Ethanol 0.1%*	*0.1 µg/ml*	*1 µg/ml*	*10 µg/ml*	*100 µg/ml*
RT23 (striped dolphin)	100	84	99	85	D.C.
RT25 (fin whale)	100	62	68	153	D.C.

Table 11. Mean values of immunofluorescence of MICA revealed in cultured fibroblasts of different species treated with BPA. The immunofluorescence is expressed in index numbers respect to solvent control. Different colour of box is related to different increase of this protein.

The results showed that the higher dose caused the death of all cells, thus focusing all its toxicity. Only fin whale (RT25) fibroblasts had an inductive phenomenon with respect to solvent control.

8. Presence of DNA damage by comet assay as genotoxicity biomarker

We treated fibroblast cells with three different doses of a mixture of benzo(a)pyrene and beta-naphthoflavone for 4 h. The viability of fibroblast cells, assessed by the trypan blue test, was very high in control (>95%) and slightly decreased following the different treatments. We analysed DNA damage by the Comet assay. The principle of the Comet assay is that smaller DNA molecules migrate faster in an electric field than larger molecules. The treated cells are encapsulated in gel and lysed by alkali, which also denatures the DNA. Subsequent electrophoresis causes migration of the DNA. While the undamaged DNA appears as a "head", fragmented DNA move faster, giving the characteristic appearance of a comet tail. Figure 9 shows four normal cells at increasing degree of damaged DNA.

The cells were processed at different conditions of electrophoresis, to evaluate different types of strand breaks. Varying the pH during lysis and electrophoresis effects the type of strand breaks expressed. When cells are lysed and subjected to electrophoresis under neutral conditions (pH 8) only double strand breaks were detected. Under pH 12.1 conditions the double and single strand breaks were detected while under pH > 13 conditions the double strand breaks, single strand breaks and alkali labile lesions were detected [44].

The results of Comet assay evaluated in cells processed at pH 12.1 are shown in Figure 10. An increase of DNA migration was observed in fibroblasts exposed for 4 h at doses C and B, while a slight decrease was observed at dose A in comparison to the control. The acetone, used as carrier for benzo(a)pyrene and beta-naphthoflavone exposure, showed DNA fragmentation values very similar to the control.

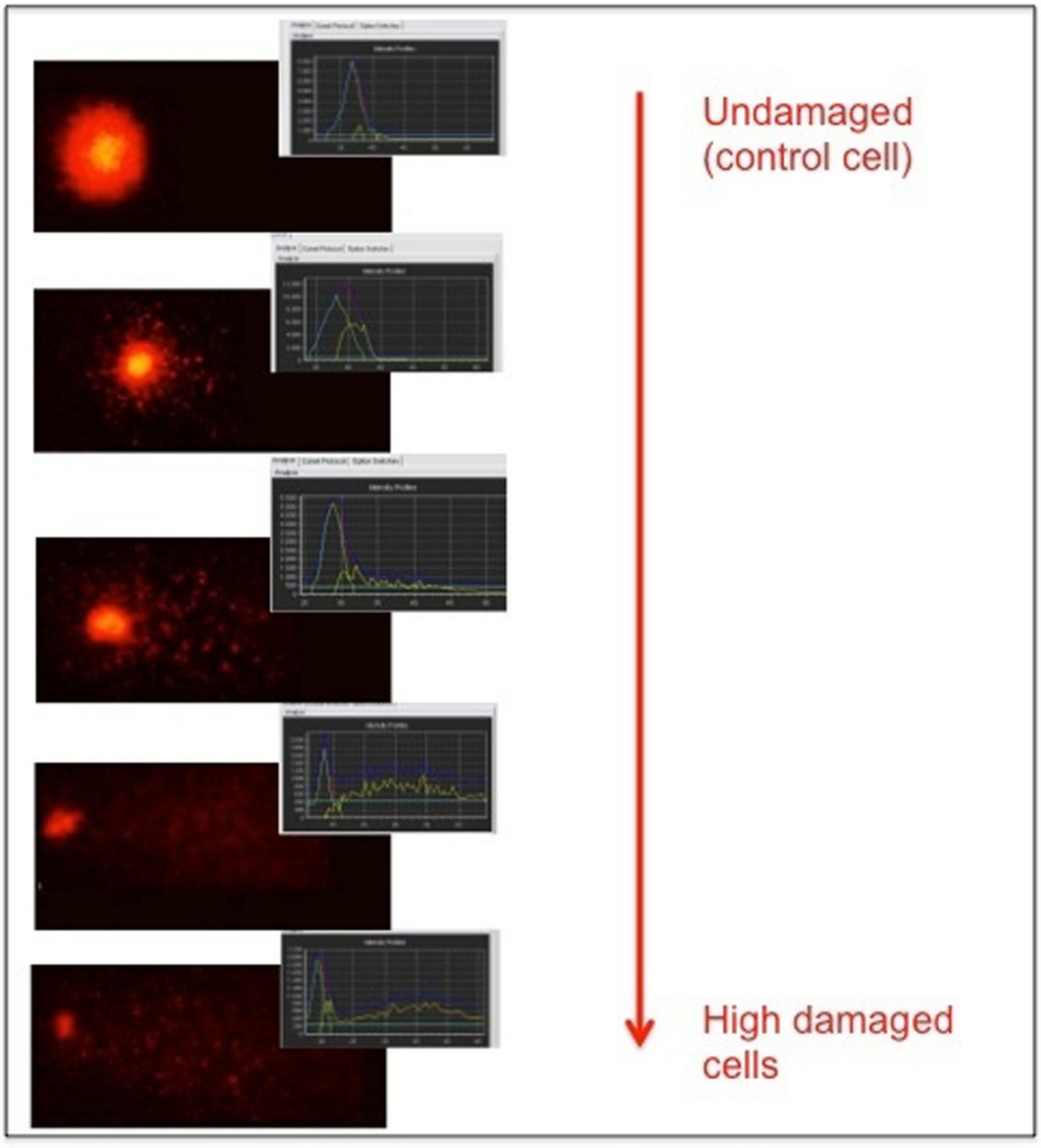

Figure 9. A photomicrograph of fibroblast cells of striped dolphin processed for the DNA comet assay. Four normal cells at increasing degree of damaged DNA: normal DNA, low-level DNA damage, DNA with a long tail, DNA almost completely fragmented.

The highest DNA fragmentation was observed at dose C and decreasing DNA tail values were observed from dose C to dose A. The results of the Comet assay evaluated in striped dolphin fibroblast cells processed at three different pHs showed a similar trend (Table 12), although the trend was more evident for the pH 12.1. Our results, although preliminary, suggest that alkaline Comet assay (pH 12.1) is the optimal version capable of detecting the DNA damage in fibroblast cells for future analysis.

Electrophoresis condition	Tail DNA %		
	Dose C	Dose B	Dose A
pH 8	30.91	19.68	24.8
pH 12.1	30.98	24.88	17.25
pH 13	35.09	27.54	25.71

Table 12. DNA migration evaluated in fibroblast cells of striped dolphin at three different pH conditions.

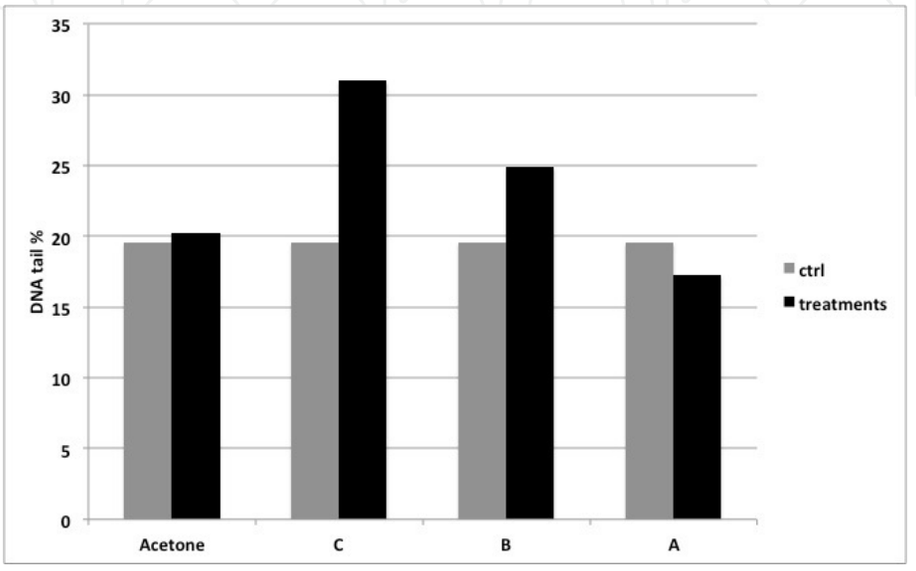

Figure 10. Effects of different doses of the mixture benzo(a)pyrene and beta-naphthoflavone at three doses (C = (0.5μM BaP + 10μM BnF), B = (2.5μM BaP + 50μM BnF) and A = (12.5μM BaP + 250μM BnF)) exposure on the DNA integrity of striped dolphin fibroblast cells after 4 h exposure.

The induction of DNA fragmentation was higher at the lowest dose (Dose C), while decreased at higher doses (Dose B and A), a result in contrast to other studies on cell mammal cultures [45, 46]. These investigations have demonstrated that an increase in the percentage of DNA in the tail region of the comets occurred in a concentration-dependent manner after exposure to different classes of genotoxic compounds, such as PAHs, methyl methanesulfonate (MMS) and H_2O_2. Our earlier Comet assay data on dolphin fibroblast cells exposed to benzo(a)pyrene are in agreement with the bibliography data. Thus, the decrease observed in our present data could be probably due to the action of the beta-naphthoflavone. This hypothesis is consistent with Gravato *et al.* [47] who demonstrated a decrease of DNA damage after exposure of specimens of *Anguilla anguilla* to beta-naphthoflavone. However, further studies are needed to confirm the genotoxic potential of mixture of PAHs for cetacean fibroblasts and investigate the potential genotoxicity of other classes of contaminants.

9. Conclusion

The aim of the present study was to propose cetacean fibroblast cell cultures as an "in vitro" method, called “Test Tube Cetaceans”, to investigate effects of environmental contaminants

in these marine mammals. The data reported in this chapter confirm that the use of Test Tube Cetaceans is a good non destructive surrogate of "in vivo" cetacean test (killing) to evaluate the different hazards of cetaceans to pollution.

Regarding the toxicological susceptibility to some xenobiotic compounds and to PAHs, the main results showed that the basal level of CYP1A1 and CYP2B of different cetacean species is very dissimilar and this seems to be especially species-specific rather than related to the geographic range, diet, toxicological status, etc. in which the specimens were found. All pollutants, at different level depending on the species and of the dose of treatment, showed an inductive capacity of these cytochromes. At times the response was dose dependent, other was bell-shaped response and other was a discontinuous induction response.

The qualitative and quantitative MICA protein expression as toxicological stress marker of the immune system showed that the three species sampled in the Sea of Cortez (Bryde's whale, long-beaked common dolphin and killer whale) showed higher basal level of MICA in respect to all Mediterranean specimens, regardless of the species and the fact that they were sampled free-ranging or found stranded alive and then died. This sharp distinction between the activity of MICA found in the Sea of Cortez and Mediterranean Sea specimens is probably the most important result to be highlighted: it seems that the environment in which specimens live and, therefore, the anthropogenic stress to which they are subjected, are determinant in the response of this protein of the immune system. In the light of this result we can hazard the conclusion that the lower the anthropic stress of the specimens, the higher is the basal level of MICA.

The Comet assay proved to be a very useful tool for assessing the potential genotoxicity of PAHs in cetacean fibroblast cell cultures. Future investigations will be conducted to investigate the genotoxic effects of different classes of contaminants in striped dolphin and other cetacean species. This technique led to the evaluation of possible DNA damage in species never studied before in this field, in order to investigate the different susceptibility to various contaminants, using fibroblast cell cultures.

In conclusion, "Test Tube Cetaceans" can be proposed to the scientific community as the "in vitro" method used to replace the "scientific whaling" to study the toxicological threats of different species of cetaceans, primarily of endangered species such as fin whale and Mediterranean common dolphin, since the research priorities is the conservation for the maintenance of cetacean biodiversity.

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