SUCCESS IN FIBROBLAST CELL CULTURES FROM LONG-BEAKED COMMON DOLPHIN (*Delphinus capensis*) AND BRYDE'S WHALE (*Balaenoptera edeni*) SKIN BIOPSIES OF THE GULF OF CALIFORNIA (MEXICO): POTENTIAL APPLICATION FOR ECOTOXICOLOGICAL STUDIES.

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ABSTRACT

The aim of this study was to develop fibroblast cell cultures from skin biopsies of free-ranging cetaceans from the Gulf of California (Sea of Cortez-Mexico): long-beaked common dolphins (Delphinus capensis) and Bryde's whales (Balaenoptera edeni), as methodological tool in ecotoxicological studies. The biopsy dart, a regular aluminium crossbow bolt with a modified stainless steel collecting tip, was used in different ways depending on the species. The tissue was kept in tissue culture medium at ambient temperature and processed in 72h. The main result was the success for the first time in fibroblast cultures of two long-beaked common dolphin and two Bryde's whale specimens. The growth of first fibroblasts were observed after 10 days both in dolphins and in whales. The difference was that fibroblast cultures reached 90% confluence in 50 ml Falcon flasks in 15 days time for long-beaked common dolphins while fibroblasts from Bryde's whales grew slowly and do not reached confluence in 50 ml flasks. The dolphin fibroblasts were trypsinized, washed and placed in 250 and 550 ml flasks, after two and three trypsinizations, respectively. The cultures thus obtained can be used for many purposes, including genetic, biochemical and toxicological studies. In particular, fibroblasts can be used to test the susceptibility of these cetaceans to different environmental contaminants such as organochlorine compounds (OCs) and polybrominated diphenyl ethers (PBDEs). Fibroblast cell cultures of Mexican cetaceans, treated with different mixtures of OCs, PBDEs and PAHs, will be analysed by immunofluorescence and western blotting techniques for a qualitative and quantitative evaluation of target proteins such as CYP1A1-1A2 and CYP2B4.

KEYWORDS

MONITORING, LONG-BEAKED COMMON DOLPHIN (*Delphinus capensis*), BRYDE'S WHALES *(Balaenoptera edeni,)*, GULF OF CALIFORNIA (SEA OF CORTEZ-MEXICO), POLLUTANTS, BIOMARKERS.

INTRODUCTION

Cetaceans, especially odontocetes, are particularly subject to chemical stress from xenobiotic compounds, because they accumulate large quantities of these contaminants. The toxicological status of cetaceans of Gulf of California (Mexico) is at the moment not very well-known. The development of a series of nondestructive techniques to evaluate biomarker responses and residue levels is strongly recommended for the hazard assessment, protection and conservation of these marine mammals (Fossi & Marsili, 1997). A non-invasive sampling method is represented by skin biopsy (Fossi *et al.*, 1992). Among the various techniques applied to integument biopsies, developed at the University of Siena (Fossi *et al.*, 2006), here we present the immunofluorescence technique in cultured fibroblasts (Marsili *et al.*, 2008) as a new "*in vitro*" tool to explore the susceptibility at the environmental contaminants of two cetaceans from the Gulf of California: long-beaked common dolphins (*Delphinus capensis*) and Bryde's whales (*Balaenoptera edeni*).

Long-beaked common dolphins. The long-beaked common dolphi is widely distributed from tropical to temperate oceans worldwide. This species is often sympatric in the eastern North Pacific and Gulf of California with the shortbeaked common dolphin, *Delphinus delphis*. Of all the studies that have described the basic ecology of common dolphins, little work has been done on *D. capensis*, chiefly because of the failure to recognize this species as being different from *D. delphis* until the 1980s. In Mexico the long-beaked common dolphin is a protected species by low; more information about habitat, feeding ecology, reproduction and treats are needed to carry out adequately management and conservation plans.

Bryde's whale. The Bryde's whales are distributed in tropical and subtropical Waters in the world. Based on genetics studies it has been suggested that possibly in the Gulf of California cohabit two different populations, one resident and

other associated with the Pacific population.The best estimation of Bryde's whales in the Gulf of California was made by Gerrodette and Palacios (2004), ~952 animals made with data from 1993. The feeding ecology of this species is based principally on mesopelagic fishes such as anchovy (*Opisthonema libertate*), sardins (Sardinops sagax), and occasionally euphausiids. The UICN mentioned the Bryde's whale as insufficiently known. In Mexico, this whale are under special protection NOM-059-Semarnat-2001. Further research would be needed to known multiple aspects of this species

MATERIAL AND METHODS

Sampling. Subcutaneous tissues (skin and blubber) were obtained from two specimens of *D. capensis* using an aluminium pole armed with biopsy tips and from two specimens of *B. edeni* with a biopsy dart launched with a crossbow (150-pound). Biopsy samples were taken in the dorsal area near a dorsal fin and on the upper part of the caudal peduncle. A little piece of biopsy was immediately stored in cell medium for the cell cultures.

Sex identification. Cetacean gender was determined genetically according to Gaspari *et al.* (2007).

Fibroblasts cell culture. The development of a non-invasive sampling method for obtaining viable tissue samples for cell cultures from skin biopsies of free-ranging cetaceans was described by Marsili *et al.* (2000). Success cell cultures were obtained both *D. capensis* and *B. edeni.* The first fibroblasts were observed after 10 days but the *B. edeni* cells were less vital and they has never reached the confluence in the 50ml Falcon flask. *D. capensis* cultures reached 90% confluence in 15 days, when they were trypsinized, washed and placed in 250ml and 550ml Falcon flasks, after two and three trypsinizations respectively.

Experimental design. Fibroblast cultures (third generation) from *D. capensis* (n=2) and and *B. edeni* (n=2) were subject to this experimental protocol for 48 h. The different cell lines were subjected to a mixture of organochlorines (OCs) (Arochlor 1260, pp'DDT and pp'DDE) solubilized in DMSO (0.05%), at three doses: 0,1 µg/ml, 0,5 µg/ml and 1 µg/ml, plus a DMSO (0.05%) control; to a commercial mixture containing 27 polybrominated diphenyl ethers (PBDEs), from mono- to deca-brominated, solubilized in nonane (0.01 µg/ml), at three doses: 0.1 µg/ml, 0.05 µg/ml and 0.01 µg/ml, plus a nonane (0.01 µg/ml) control; to a mixture of benzo(a)pyrene (1mM) and beta-naphthoflavone (20mM), solubilized in acethon (0.1%), at three doses: C = (0,5µM BaP + 10µM BnF), B = (2,5µM BaP + 50µM BnF) and A = (12,5µM BaP + 250µM BnF), plus a acethon (0.1%) control.

Immunofluorescence. We used immunofluorescence in fibroblast cultures for a qualitative and semi-quantitative analysis of target proteins CYP450 1A1 and CYP450 2B. Exposure to planar molecules (e.g. PAHs and planar halogenated aromatic hydrocarbons (PHAHs) such as non-*ortho* and mono-*ortho* PCBs) results in increased activity of the CYP1A subfamily. More three-dimensional molecules (e.g. PBDE, DDT, DDE and 2-*ortho*-PCBs) usually cause an induction of the CYP2B subfamily. After a first reaction with the primary polyclonal antibodies: goat *anti rabbit cytochrome P450 1A1* and goat *anti rabbit cytochrome P450 2B* purchased from Oxford Biochemical Research (Oxford MI, USA), the cells were treated with the with the respective secondary antibodies (Alexa Fluor 594 goat *anti rabbit* IgG (H+L)), labelled with red-fluorescent Alexa Fluor 594 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). (Marsili *et al.*, 2008). The image analysis procedure has the objective of quantifying, with an adimensional index generated for this purpose, the amount of Alexa Fluor 594 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 594 sample cell and summarizes the results in a worksheet. Results are also saved with associated images in an internal database, for future reference and re-analysis.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 mm2. 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 and CYP2B for each species and for each treatment. A series of images of each slide was acquired so that a minimum of 250 cells/slide could be counted. Nine slides for CYP1A1 and CYP2B were made for each culture, making a total of 18 slides for each toxicological treatment: of the nine, one was a blank (only primary and secondary antibodies), one was a secondary blank (only with secondary antibody), one was a chemical blank (treated with contaminant vehicle), two were for the first dose, two for the second dose and two for the third dose of contaminants. The blank enabled the natural presence of cytochromes 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.

Statistical analysis - Data was processed using Statistica 5.0 (Microsoft).

RESULTS AND DISCUSSIONS

The main results of these pilot experiments were the success for the first time in fibroblast cultures of long-beaked common dolphin and Bryde's whale specimens and the detection of presence of the cytochromes 1A1 and 2B in *D. capensis* (fig. 2A-B) and *B. edeni* (fig. 3A-B) fibroblast cells, revealed by immunofluorescence.
In addition, preliminary detection in *D. capensis* of differences in the induction phenomena between different mixture of contaminant (Table 1) was detected, such as:

- OC induction of Cyp 1A1 and 2B in female at all doses with strong induction for Cyp 1A1 at dose 3;
- OC induction of Cyp 1A1 in male at all doses and dose dependent induction of Cyp 2B (fig. 4);
- induction of Cyp 2B in male and female exposed to PBDEs at all doses;
- the male exposed to PAHs showed the highest Cyp 1A1 induction at dose 1, while Cyp 2B showed a bell-shaped trend;
- the female exposed to PAHs showed induction of Cyp 1A1 at all doses and induction of Cyp 2B only at dose 1.

On the opposite, no induction in male and female Cyp 1A1, after treatment with PBDEs, was detected.

CONCLUSION

In conclusion the information obtained in this pilot experiment will be the basis for further applications and validation of *in vitro* methodologies (immunofluorescence) to study susceptibility of cetaceans from the Gulf of California to different mixtures of old and new environmental contaminants.

ACKNOWLEDGEMENTS

This project was supported by the Italian and Mexican Ministry for Foreign Affair.

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CAPTIONS

Table 1. Mean values of immunofluorescence of CYP1A1 and CYP2B revealed in cultured fibroblasts of one male and one female specimens of *Delphinus capensis* treated with the OC, PBDE and PAH mixture. The immunofluorescence is expressed in index numbers respect to solvent control. Explication about treatment doses in Materials and Methods.

Figure 1. Map of sampling area.

Figure 2. Immunofluorescence (AUF) of CYP1A1 and CYP2B in cultured fibroblasts of *Delphinus capensis*. DAPI (Intensity = 6ms) and Alexa Fluor 594 (Intensity = 200ms) images.

Figure 3. Immunofluorescence (AUF) of CYP1A1 and CYP2B in cultured fibroblasts of *Balaenoptera edeni*. DAPI (Intensity = 6ms) and Alexa Fluor 594 (Intensity = 200ms) images.

Figure 4. Immunofluorescence (AUF) of CYP2B in cultured fibroblasts of *Delphinus capensis* treated with the OC mixture. DAPI (Intensity = 6ms) and Alexa Fluor 594 (Intensity = 200ms) images of DMSO and the three treatments.

CYP 1A1 – Organochlorine mixture				
	Solvent control	**Dose 1**	**Dose 2**	**Dose 3**
Delphinus capensis ♂	100	313.8	118.5	344.6
Delphinus capensis ♀	100	325.9	312.1	836.9
CYP 2B – Organochlorine mixture				
	Solvent control	**Dose 1**	**Dose 2**	**Dose 3**
Delphinus capensis ♂	100	49.80	130.9	356.6
Delphinus capensis ♀	100	205.2	127.6	189.4
CYP 1A1 – PBDEs				
	Solvent control	**Dose 1**	**Dose 2**	**Dose 3**
Delphinus capensis ♂	100	146.7	69.20	45.70
Delphinus capensis ♀	100	55.40	20.10	63.70
CYP 2B – PBDEs				
	Solvent control	**Dose 1**	**Dose 2**	**Dose 3**
Delphinus capensis ♂	100	230.3	140.5	185.4
Delphinus capensis ♀	100	149.5	139.3	178.1
CYP 1A1 – PAHs				
	Solvent control	**Dose 1**	**Dose 2**	**Dose 3**
Delphinus capensis ♂	100	161.7	98.1	106.9
Delphinus capensis ♀	100	155.6	279.7	123.2
CYP 2B – PAHs				
	Solvent control	**Dose 1**	**Dose 2**	**Dose 3**
Delphinus capensis ♂	100	80.90	536.5	72.60
Delphinus capensis ♀	100	218.6	65.60	64.30

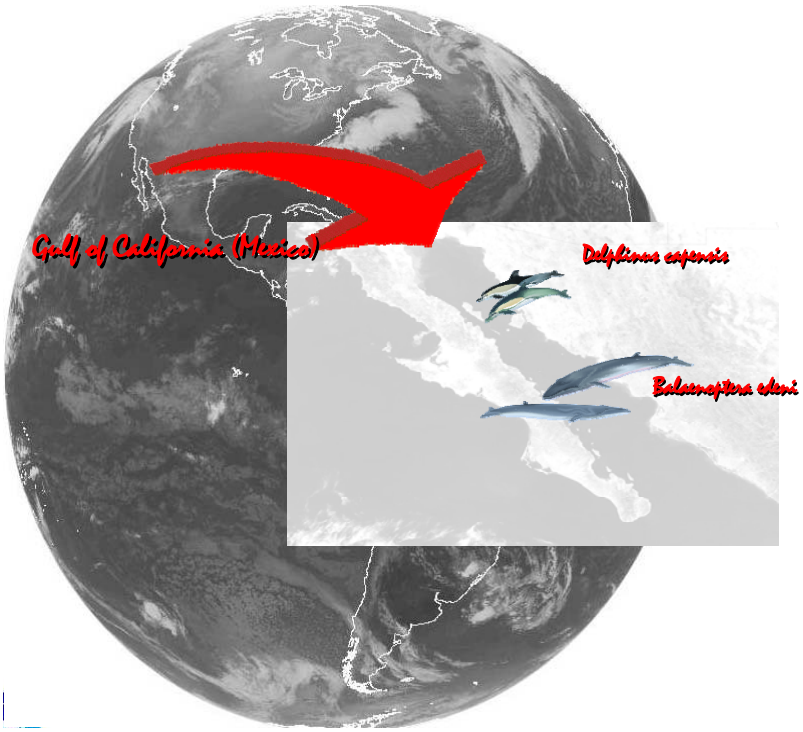
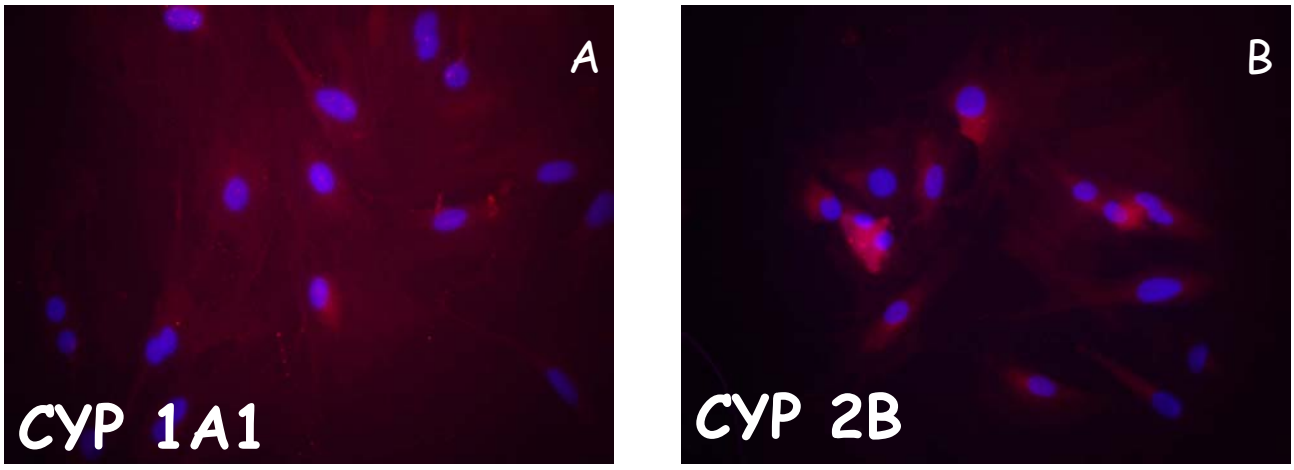


Figure 1



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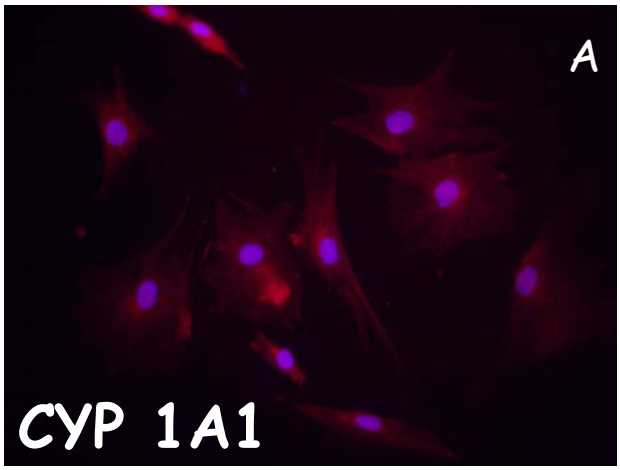


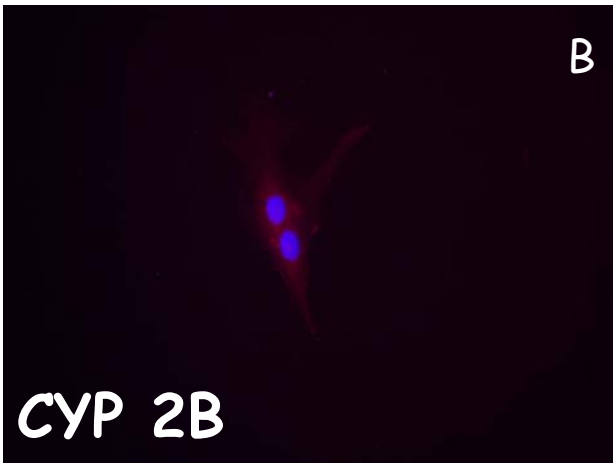
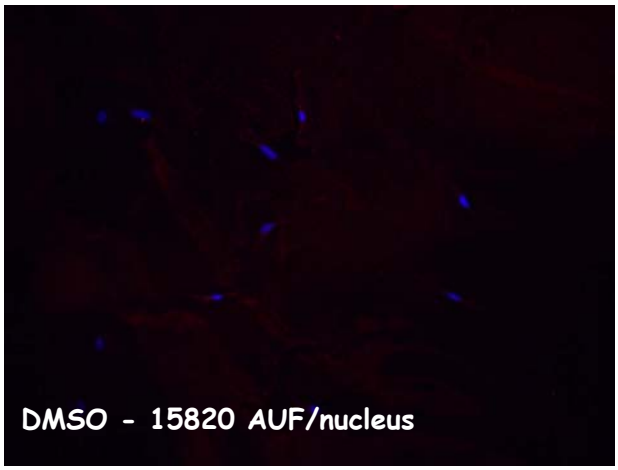
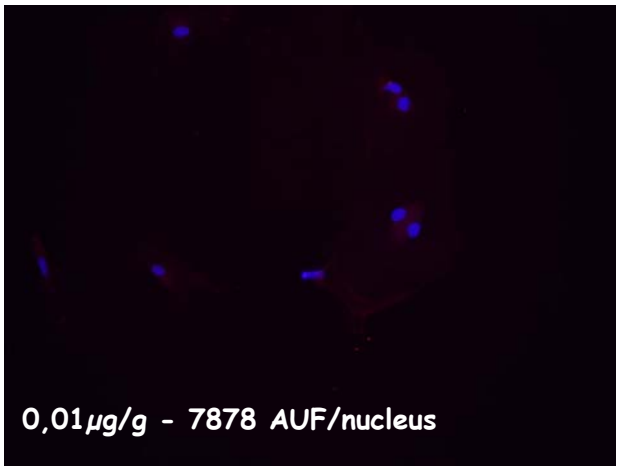
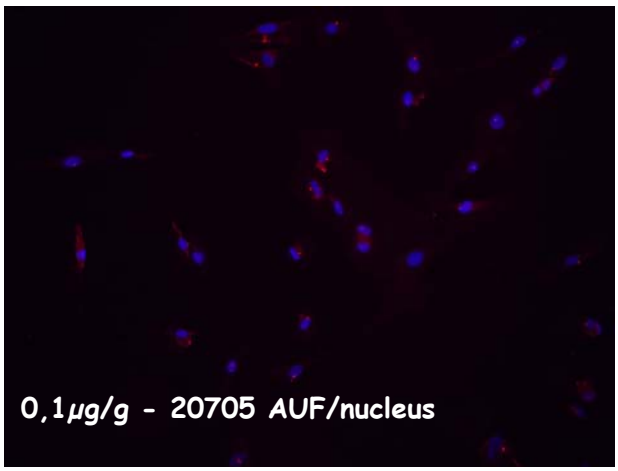


Figure 3







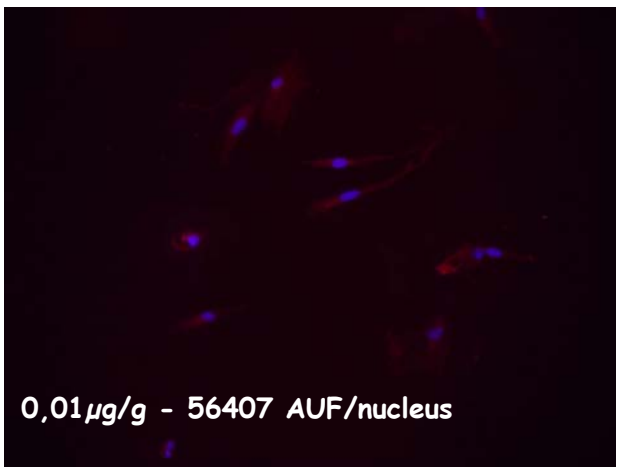


Figure 4