SKIN BIOPSY SLICES OF CETACEANS AS A MULTI-RESPONSE "*IN VITRO*" METHOD TO DETECT TOXICOLOGICAL EFFECTS OF LIPOPHILIC CONTAMINANTS

Fossi, M. C.(1), Casini, S.(1), Maltese, S.(1) Panti, C.(2), Spinsanti, G.(2) and Marsili, L.(1)

(1) *Department of Environmental Sciences, University of Siena, Via Mattioli 4, 53100 Siena, Italy*
(2) *Department of Evolutionary Biology, University of Siena, Via A. Moro 2, 53100 Siena , Italy*

ABSTRACT

The need for powerful new tools to detect the effects of POPs, emerging contaminants and PAHs on Mediterranean cetaceans led us to develop and apply a suite of sensitive biomarkers for slice integument biopsies of stranded and free-ranging animals, as a multi-response in vitro method to detect toxicological effects of contaminant mixtures. In this study, we applied an in vitro assay using skin biopsy and liver slices, combining molecular biomarkers (Western blot of CYP1A1, CYP2B) and gene expression levels (qRT-PCR of CYP1A1, HSP70, ERα and E2F-1) in response to chemical exposure (OCs, PBDEs, PAHs) for stranded Mediterranean Stenella coeruleoalba. The main goal of this experiment was to identify among the various assays the biomarker and/or series of biomarkers that best allows us to diagnose the presence of a specific class of pollutants (OCs, PBDEs, PAHs) or a mixture of them for future investigations in field studies.

KEYWORDS

Mediterranean cetaceans; *Stenella coeruleoalba*; slice skin biopsy; biomarkers; lipophilic contaminant treatments.

INTRODUCTION

Pressures on dolphins and whales in Mediterranean waters are higher than they have ever been in the last millennium and have different origins. In the last 20 years there has been growing concern about hazards to Mediterranean cetaceans occasioned by multiple stress factors, due to bioaccumulation and effects of anthropogenic contaminants combined with infectious diseases, climate change, food depletion (overfishing), trapping, noise, ship collisions, whale watching activities and genetic erosion (Reeves and Notarbartolo di Sciara, 2006; Fossi *et al*., 2007; Van Bressem *et al*., 2009). The simultaneous combination of some or all of these pressures for different cetacean species may produce dramatic effects on population stability and consequently on Mediterranean biodiversity. Exposure to contaminants combined with other human impacts can affect survival, recruitment, reproductive success, mutation rates and migration, and may play a significant role in the partitioning of genetic variation between more and less stress-exposed populations (Whitehead *et al*., 2003)

Concerning ecotoxicological stressors, while levels of organochlorine compounds (OCs) are decreasing, emerging contaminants such as polybrominated diphenyl ethers (PBDEs) seem to be increasing in the environment, including the Mediterranean Sea. Several international institutions (IWC, ACCOBAMS *et al*.) have encouraged research and development of sensitive non-lethal tools (biomarkers) for biopsies from free-ranging animals, to define the "toxicological health status" of endangered cetacean species.

In this context, the main aim of our research team over the last decade has been to develop a non-lethal "multi-trial biomarker tool" in a two-phase experimental project. This was first achieved for two in vitro systems (first phase): fibroblast cell culture (Marsili *et al*., 2000; Fossi *et al*., 2006) and skin biopsy slices (Fossi *et al*., 2010). We have since applied this to free-ranging cetacean skin biopsies (second phase) to diagnose "multiple-stress syndromes" related to multiple human toxicological impacts (Spinsanti *et al*., 2006, Fossi *et al*., 2008, Spinsanti *et al*., 2008, Fossi *et al*., 2010). The need for new markers of environmental contaminants in cetaceans, and alternative "in vitro" models to explore differing susceptibilities to several classes of legacy contaminants as well as new contaminants of concern (emerging contaminants), led us to use tissue slices (skin biopsy and liver of stranded animals) from various cetacean species as a new non-lethal investigative tool (Godard *et al*., 2004; Fossi *et al*., 2006).

In this survey we have focused our attention on striped dolphins, which are the most numerous dolphins in the Mediterranean Sea; they are odontocetes at the top of the marine food chain and are therefore potentially subject to biomagnification of persistent contaminants. In the Red List of the IUCN, striped dolphins are classified as Low Risk for extinction (IUCN, 2010). This species was subjected in 1991 to a massive stranding related to Morbillivirus infection (Van Bressem *et al*., 1993; Domingo *et al*., 1995). The 1990–

1992 dolphin Morbillivirus epidemic started along the central coast of Spain in 1990 and ended in Turkey and the Greek Islands in 1992, affecting predominantly Stenella coeruleoalba, which is currently the most abundant odontocete in the Mediterranean Sea (Aguilar and Raga, 1993; Aguilar, 2000; Gomez de Segura *et al*., 2007; Fortuna *et al*., 2007). Between 2007-2008 new cases of Morbillivirus infection were detected in Mediterranean specimens (Raga *et al*., 2008; Fernandez *et al*., 2008). In early July 2007 dead or moribund Stenella coeruleoalba and Globicephala melas animals were found in the Gulf of Valencia (Raga *et al*., 2008). Polychlorinated biphenyl (PCB) loads in Stenella coeruleoalba that had died during the 1990–1992 epidemic were significantly higher than in surviving individuals, and given their well-known immunosuppressive effects in mammals it was suggested that PCBs may have compromised immune responses and increased the severity of the outbreak (Aguilar and Borrell 1994; Marsili *et al*., 2004). Though the role of environmental contaminants in the 2007 epidemic remains inconclusive, recent pollutant data obtained by analyses of biopsies from apparently healthy striped dolphins from 1987 to 2002 suggested that PCB and dichlorodiphenyl trichloroethane (DDT) concentrations have gradually decreased (Aguilar and Borrell, 2005). No data have been reported for these specimens regarding emerging contaminants.

Here we present results from the first phase of the project (in vitro experiments), in which molecular biomarkers (Western blot of CYP1A1 and CYP2B) and gene expression biomarkers (Real Time quantitative Reverse Transcription PCR (qRT-PCR) of CYP1A1, Heat shock protein 70 (HSP70), Estrogen receptor alpha (ERα) and Transcription factor E2F-1) were used in slices of skin biopsy and liver from a stranded Mediterranean target species Stenella coeruleoalba (pelagic odontocete) that had been experimentally exposed to OCs, PBDEs and polycyclic aromatic hydrocarbons (PAHs). The first aim of this experiment was to explore the sensitivity of the multi-response biomarkers in response to treatments with various mixtures of contaminants. In vitro tests using slices are also proposed in this project as an innovative tool to study inter- and intra-species sensitivities to various classes of environmental contaminants present in the Mediterranean environment. This approach was previously proposed by Drahushuk *et al*. (1996; 1998) in human liver slices exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), by Godard *et al*. (2004) in skin biopsy slices of sperm whale (Physeter macrocephalus) exposed for 24 hr to β-naphthoflavone (BnF) and by Harrigan *et al*. (2004; 2006) in rat liver and lung tissue slices exposed to benzo(a)pyrene (BaP).

One of the major aims of ecotoxicological investigations with Mediterranean cetaceans is to develop diagnostic systems for detecting exposure to specific classes of contaminants in the Mediterranean basin. In this context the second objective of this experimental design is to identify, among the various categories of selected biomarkers, the biomarker and/or series of biomarkers that best allows us to diagnose the presence of a specific class of pollutants, or a mixture of them, for future applications in field ecotoxicological monitoring.

To this end several biomarker responses were explored in combination for the first time, in an "in vitro" assay (skin biopsy and liver slices) in response to chemical exposure, such as CYP1A1 and CYP2B detected by Western blot and qRT-PCR techniques and HSP70, ERα and E2F-1 detected by the quantitative real-time PCR (qRT-PCR) technique. Each proposed biomarker represents a different diagnostic signal.

Cytochrome P450, which is composed of hundreds of isoforms, is the most important metabolic/detoxifying enzyme system in mammals. It is substrate-inducible and substrate-specific. CYP1A1 induction is widely used as a biomarker for exposure in animal species (Stegeman *et al*., 1992). Among the few studies of cytochrome P450s in cetaceans, several have examined the metabolism of foreign chemicals in hepatic microsomes or cell culture (Boon *et al*., 1998; Goksøyr *et al*., 1986; Murk *et al*., 1994; White *et al*., 1994; White *et al*., 2000) and a CYP1A1 gene has been identified in several species (Teramitsu *et al*., 2000). Correlations between non-ortho and mono-ortho PCB burdens in blubber and hepatic CYP1A1 content and activity have been observed in beluga whales (White *et al*., 1994). Such correlations generally support the use of CYP1A1 induction as a biomarker of exposure to AHR agonists in cetaceans. The use of skin biopsies for measuring CYP1A1 activity in marine mammals has been advocated as a valid non-destructive method since the early 1990s (Fossi *et al*., 1992; Fossi *et al*., 2003). CYP1A and CYP2B have been detected in cetacean skin and induction of these isoforms was found after exposure to lipophilic contaminants such as OCs, PAHs and brominated flame retardant (BFR) both in vitro and in field studies (Godard *et al*., 2004; Fossi *et al*., 2006; Fossi *et al*., 2008; Montie *et al*., 2008; Hooker *et al*., 2008; Fossi *et al*., 2010). Induction of CYP isoforms can thus be considered as a powerful biomarker of exposure. Furthermore, basal expression and upregulation of extrahepatic CYP enzymes can significantly affect local disposition of xenobiotics or endogenous compounds in peripheral tissues and thus modify their pharmacological and toxicological effects or affect absorption of xenobiotics into systemic circulation.

The E2F transcription factor is a member of the E2F family (E2F1-6), which is important in regulating the cell cycle and has a dual role: controlling some genes that regulate the progression of DNA synthesis or being involved in apoptotic processes (La Thangue, 2003; Attwool *et al*., 2004). E2F-1 overexpression upregulates several genes involved in the activation of apoptosis and seems to interact with and be modulated by the aryl hydrocarbon receptor. DNA damage in general seems to be responsible for induction of the apoptotic pathway by E2F-1 (Stevens and La Thangue, 2004). Stress signals (such as UV exposure or

hypoxia) can induce expression of E2F-1 (O'Connor and Lu, 2000), supporting the use of this gene as a putative biomarker for response to ecotoxicological stress.
The heat shock protein 70 (HSP70) is a stress-related protein belonging to a multigene family. HSPs are stress-related proteins induced by a variety of agents and conditions that either directly damage proteins or indirectly act by causing production of abnormal proteins in cells (Nollen and Morimoto, 2002). HSPs are induced as a first response, their main role being to protect cells exposed to stress. Among all HSP families, HSP70 is often used as an early biomarker for environmental stress assessment in a wide variety of organisms. Nevertheless, most studies that use HSP70 as a biomarker are carried out in invertebrate species such as terrestrial arthropods (e.g., Chilopoda and Diptera; Pyza *et al*., 1997) or marine invertebrates and vertebrates (Cruz-Rodrìguez and Chu, 2002; Porte *et al*., 2001). Studies on vertebrates in the wild are very limited (mainly studies on fishes; Deane and Woo, 2004; Boone and Vijayan, 2002) and information about marine mammals is lacking so far.
Estrogen receptors (ERs) are members of the nuclear receptor superfamily and are ligand-inducible transcription factors. Two isoforms of ERs are known, ERα and ERβ, which have differing tissue distributions and physiological roles (Mueller and Korach, 2001) and are encoded by different genes located on different chromosomes. Ligand-induced signaling is due to binding of estrogen (or a structurally similar compound such as OCs or PBDEs) and a specific transcriptional response is subsequently activated. The affinity of chemicals with estrogenic or antiestrogenic activity is due to the ability of these compounds to interact with ERs (Mueller, 2004). Because of the central role of ERs in cell differentiation and proliferation, abnormalities in ER signaling pathways can interfere with sexual development and the endocrine system, both in wildlife and humans.
The exposure to exogenous compounds (such as endocrine disrupting chemicals (EDCs)) with high affinity for ER may therefore cause impairment of endocrine functions. To date, most studies on ERs and their interactions with xenobiotic compounds have been carried out in vitro to understand better the toxic effects they can have on living organisms (Janošek *et al*., 2006; Tiemann, 2008). Compounds like PCBs and PBDEs that have dioxin-like property can bind estrogen receptors and interfere with signaling pathways, having an agonist potency measured in vitro more preferential for ERα than ERβ. The binding of xeno-estrogens or xeno-antiestrogens to ER, indeed, can enhance responses of endogenous estrogens or agonistically bind the receptor to inhibit the physiological action of those estrogens (Carpenter *et al*., 2002).

METHODS

Sampling - Sub-samples of skin and liver from a striped dolphin (Stenella coeruleoalba) stranded on Tyrrhenian shores were obtained 4-8 hours after death. All material was immediately placed in liquid nitrogen or stored in cell medium for slice experiments.

Slice experimental design - Immediately after collection, we manually cut slices (about 2 mm thick spanning the epidermis and dermis) from samples of skin and liver of the stranded specimens, as previously done by Godard *et al*. (2004). The slices (skin and liver) were subjected to four different experimental protocols (Mix OCs, Mix FR, Mix PAHs and Super Mix). We incubated slices (treated slice) for 24 hr in cell culture media (Fossi *et al*., 2006) with different mixtures of contaminants as reported below. The first was a mixture of Arochlor 1260, pp'DDT and para,para'- dichlorodiphenildichloroethylene pp'DDE in Dimethyl sulfoxide (DMSO) (0.05%) at three doses: 0.01 µg/ml, 0.1 µg/ml and 1 µg/ml, plus a DMSO (0.05%) control. The second was a mixture containing 27 PBDEs, from mono- to deca-brominated, 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. The third was a mixture of PAHs containing benzo(a)pyrene (1 mM) and beta-naphthoflavone (20 mM) in acetone (0.1%) at three doses: Low Dose (0.5 µM BaP + 10 µM BnF); Medium Dose (2.5 µM BaP + 50 µM BnF); and High Dose (12.5 µM BaP + 250 µM BnF). The fourth was a mixture combining the other mixtures, in a mix of carriers (DMSO 0.005%, nonane 0.01% and acetone 0.1%) at two doses: Low Dose (OCs 0.01 µg/ml + PBDEs 0.01 µg/ml + PAHs 0.5 µM) and High Dose (OCs 1 µg/ml + PBDEs 0.1 µg/ml + PAHs 12.5 µM). Incubation was carried out at room temperatures ranging from 24°C to 28°C. After 24 hours incubation in media, untreated and treated slices were placed in liquid nitrogen.

Slice homogenates - Homogeneous subsamples (30-40 mg) of skin slices (including epidermis, dermis and blubber layers; Angell *et al*., 2004; Wilson *et al*., 2007) and liver slices were homogenized in AhR buffer with protease inhibitors (1:10) in a Tissue Lyser (Qiagen) for 5 plus 3 minutes using 5 mm aluminium balls (+4°C). The homogenate was centrifuged at 750 x g for 10 min and then at 9000 x g for 20 min (+4°C). The supernatant (S9) was analyzed for total protein and then by WB (Fossi *et al*., 2008).

CYP1A1 and CYP2B Western blots - For WB analysis of CYP1A1 and CYP2B, S9 fractions of tissue homogenates (slice biopsies and liver, in duplicate for each sample) were separated by SDS-PAGE (10% polyacrylamide gels – Criterion XT Precast Gel, Bio-Rad) and blotted onto nitrocellulose sheets (0.45 µm

Bio-Rad) for 1 hour at 200 V. The membranes were saturated by incubating with blocking solution (3% gelatin dissolved in Tris-Buffered Saline containing 0.05% Tween-20, TTBS) for 1 hour at room temperature. Primary polyclonal rabbit antibodies were from Oxford Biochemical Research (Oxford MI, USA). Goat anti-rabbit CYP1A1 and anti-CYP2B4, diluted 1:5000 and 1:1000, respectively, in TTBS-1% gelatin were incubated with cetacean proteins overnight at room temperature. Incubation with anti-rabbit HRP-labelled secondary antibody (1:3000 final dilution) was performed for 1 hour and 30 minutes at room temperature and detected according to the Bio-Rad Immun-Star HRP Chemiluminescent Kit booklet, using standardized times (Fossi *et al*., 2008). In order to validate WB analysis of CYP1A1 and CYP2B as a semi-quantitative detection tool in cetacean skin biopsies and slices, a series of calibration curves for CYP450 from liver (ranging from 1.2 to 4.8 pmol CYP450) and skin (including epidermis, dermis and blubber layers, ranging from 0.095 to 2.400 pmol of CYP450) from stranded specimens of *Tursiops truncatus* were analysed in triplicate by Fossi and collaborators (2008). A triplicate skin standard (SS) was analyzed within the calibration curve, sub-aliquoted and maintained as an internal standard for subsequent WBs. Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (Bio-Rad, 1-D Analysis Software) using Adj.Vol.Int *mm2 as a quantitative parameter. The data were correlated with increasing pmol concentrations of CYP450 and two Excel Macros were produced (one for CYP1A1 and one for CYP2B) incorporating the normalizing factor of SS for quantification of unknown biopsy samples. A linear correlation was found between relative pmol CYP1A1 and Adj.Vol.Int *mm2 (R = 0.9901) and between relative pmol CYP2B and Adj.Vol.Int *mm2 (R = 0.9914). The two Excel Macros (one for CYP1A1 and one for CYP2B), incorporating the normalizing factor of SS (Skin Standard) will be used for quantification of unknown biopsy samples or biopsy slices (Fossi *et al*., 2008).

CYP1A1- E2F-1 - HSP70 - ERα: Quantitative real-time PCR assay - Subsamples of skin biopsy slices were used for gene expression analyses by qRT-PCR. Each sample was homogenized and RNA was isolated using an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). One microgram of RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). The real-time PCR reaction conditions were set as described in Spinsanti *et al*. (2006) and the reaction was run on an iCycler iQ5 (Bio-Rad) using SYBR Green detection chemistry in 96-well reaction plates. Primers were designed for the specific sequences of Stenella coeruleoalba using Beacon Designer Software (Premier Biosoft International) for both housekeeping genes and genes of interest. Each reaction was carried out in triplicate, and the relative expression of the four genes of interest was normalized to expression of the control genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) and succinate dehydrogenase complex, subunit A (SDHA). The three housekeeping genes were selected according to Spinsanti *et al*. (2008). Raw threshold cycles (Ct) were converted into quantities according to the modified ΔΔCt method (Livak and Schmittgen, 2001).

Sex determination - Cetacean gender was determined genetically according to Berube and Palsboll (1996).

RESULTS AND DISCUSSION

In this study we developed and evaluated several biomarker responses using different methodologies in slices experimentally exposed to increasing concentrations of OCs, PBDEs and PAHs, in order to study the sensitivity of the selected in vitro system to various classes of environmental contaminants present in the Mediterranean environment, and to select a suite of sensitive diagnostic tools to apply to future investigations in field studies.
In order to answer these two experimental questions, the biomarker responses (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70, ERα, AHR and E2F-1 detected by the qRT-PCR technique) are discussed first of all in response to each different chemical exposure, and secondly by exploring the different sensitivities of each biomarker separately in response to the four groups of chemicals mixtures.

OCs treatment for skin and liver slices

CYP1A1 and CYP2B Western blot - In this *in vitro* experiment, we applied the WB technique to detect CYP1A1 and CYP2B induction produced by OC mixtures in liver and skin slice samples (**Fig. 1**). Increasing induction of CYP1A1 was detected in skin slice samples with increasing doses of OC contaminants (**Fig. 1A**). Moderate induction of CYP2B, with respect to vehicle-treated samples, was detected only at the high dose (**Fig. 1B**). No induction of CYP1A1 and CYP2B was detected in liver slice samples with increasing doses of OC contaminants, probably due either to the autolytic state of the samples or to the documented inhibitory potential of PCBs at high concentrations (Gooch *et al*., 1989; Hahn *et al*., 1993).These in vitro data confirm the strong potential of several OCs to induce the CYP1A1 isoform and confirm the potential use of CYP1A1 induction in cetacean skin samples as a “diagnostic” biomarker of exposure to OC mixtures, as previously reported by Fossi *et al*. (2007; 2010) in striped dolphin and fin whale.

CYP1A1- E2F-1 - HSP70 - ERα: Quantitative real-time PCR assay - In this *in vitro* experiment the quantitative real-time PCR assay was applied for the first time to measure expression of several "biomarkers of gene expression" such as CYP1A1- E2F-1 - HSP70 - ERα (**Fig. 1C**) in skin slice samples. Strong induction of CYP1A and ERα was detected at the low dose of OCs, which increased 17- and 15-fold as compared to the control vehicle, respectively. A "bell-shaped" induction phenomenon was shown for both genes at medium and high doses of OC mixtures. A moderate induction was reported for HSP70, with a 4-fold increase at the medium dose with respect to the control vehicle. No variations were reported for E2F-1.

PBDEs treatment for skin and liver slices

CYP1A1 and CYP2B Western blot - In this second *in vitro* experiment we applied the WB technique to detect CYP1A1 and CYP2B induction produced by PBDEs in liver and skin slice samples (**Fig. 2**). Induction of CYP1A1 was detected in both liver and skin slice samples treated with various doses of PBDEs contaminants (**Fig. 2A**). Higher induction responses were detected in liver slice samples treated with a medium dose of PBDEs. Moderate induction of CYP2B was detected for both skin and liver samples in comparison to CYP1A1, and again higher induction responses were detected in slice samples treated with medium doses of PBDEs (**Fig. 2B**). A "bell-shaped" induction phenomenon was shown for both CYPs.

CYP1A1- E2F-1 - HSP70 - ERα: Quantitative real-time PCR assay - Moderate induction phenomena were observed for HSP70 and E2F-1 with respect to the control vehicle (**Fig. 2C**). No variation was reported for CYP1A1 and HSP70 with respect to the control vehicle. These data suggest a potential influence of the vehicle (nonane) on gene expression responses for this *in vitro* experiment.

PAHs treatment for skin and liver slices

CYP1A1 and CYP2B Western blot - In this *in vitro* experiment we applied the WB technique to detect CYP1A1 and CYP2B induction by PAHs in liver and skin slice samples (**Fig. 3**). Increasing induction of both CYP1A1 and CYP2B was detected in skin slice samples with increasing doses of PAH mixtures (**Fig. 3A, B**), with higher induction responses of CYP1A1 as compared to CYP2B. Slight induction of CYP1A1 was detected in liver slice samples treated with high doses of PAHs, with higher induction reported at the low PAHs dose.

CYP1A1- E2F-1 - HSP70: Quantitative real-time PCR assay - In this experiment high induction of the CYP1A gene was detected in skin slice samples with increasing doses of PAH mixtures (**Fig. 3C**), increasing respectively 1.5-, 6- and 9.5-fold at the different doses with respect to the control vehicle. No upregulation was observed for HSP70 and no variation was reported for E2F-1. Harrigan *et al*. (2006) investigated differences in the metabolism and bioactivation of BaP between target (lung) and non-target (liver) tissues, using precision-cut rat liver and lung slices incubated in dynamic organ culture with BaP. The *in vivo* studies with BaP (Harrigan *et al*., 2006) were performed to characterize and validate use of the *in vitro* tissue slice model for assessing tissue-specific induction of CYPs and the formation of chemical-DNA adducts. The expression and induction of CYP1A1 mRNA in rat liver and lung slices incubated with BaP for 24 h was measured by quantitative-competitive RT-PCR. CYP1A1 mRNA levels were greater in lung slices as compared to liver slices at each dose examined. The *in vitro* data from our experiment confirm the high potential of a PAH mixture (BaP and BnF) for inducing CYP1A1 mRNA and protein (Andersson and Forlin, 1992; Goksøyr and Forlin, 1992; Andersson, 1995; Marsili *et al*., 2009) and confirm the potential use of CYP1A1 induction in cetacean skin samples as a "diagnostic" biomarker of exposure to PAH mixtures.

OC, PBDE and PAH mixture treatment for skin and liver slices

CYP1A1 and CYP2B Western blot - In this *in vitro* experiment we applied the WB technique to detect CYP1A1 and CYP2B induction produced by a mixture of OC, PBDE and PAH in liver and skin slice samples (**Fig. 4**). Increasing induction of both CYP1A1 and CYP2B was detected in liver slice samples with increasing doses of contaminant mixtures (**Fig. 4A, B**). A slight induction of CYP1A1 was detected in skin slice samples with increasing doses of contaminants, and no variation was observed for CYP2B in skin slice samples.

CYP1A1- E2F-1 - HSP70 - ERα: Quantitative real-time PCR assay - In this experiment high induction of CYP1A was detected in skin slice samples with a medium dose of contaminant mixture (**Fig. 4C**), showing a "bell-shaped" dose-response curve. Slight upregulation of AHR and HSP70 was detected in skin slice samples with increasing doses of contaminants. No induction phenomena were observed for E2F-1. The high potential of a mixture of multiple contaminants (ecotoxicologically relevant in Mediterranean areas) to induce CYP1A1 mRNA and protein was documented by these *in vitro* data, confirming the potential use of CYP1A1 induction in cetacean skin samples as a "diagnostic" biomarker of exposure to lipophilic contaminants.

Different biomarker sensitivities in response to the four mixtures of chemicals - A major aim of this study was to identify the biomarkers and/or a series of biomarkers that best allow us to diagnose the presence of a specific class of pollutants or a mixture of them. To this end, for each group of contaminants tested in skin biopsy slices we report the various biomarker responses at the different test doses, expressed as Index Numbers. The results are described separately below.

Biomarker responses to OC Mixture - **Figure 5** shows the values for six tested biomarkers in response to treatment with increasing doses of a mixture of OCs, expressed as Index Numbers (assigning a value of 1 to treatment of the slice with vehicle). Among the six biomarkers tested, the most sensitive response was detected for CYP1A1 gene expression, with 17-fold upregulation relative to the control at the lower dose treatment. The upregulation response of CYP1A1 is lower at intermediate and high doses of treatment. The second biomarker, in terms of sensitivity of response, is expression of estrogen receptor (ER) genes, with upregulation of 14-fold relative to control at the lower dose treatment and showing a bell-shaped pattern similar to that seen for CYP1A1 gene expression. A lower sensitivity of the response has been evaluated for induction of CYP1A1 assessed by WB and gene expression of HSP70, with induction and upregulation 2.3-fold and 2.6-fold, respectively, relative to the control at the higher dose treatment. The induction of CYP2B assessed by WB and the gene expression levels of E2-F1 are apparently not affected by OC exposure. These data confirm the close relationship between contamination by OCs and inductive effects on both CYP1A and ERs. This phenomenon was previously highlighted by White *et al*. (1994) and Fossi *et al*. (2006; 2008) for CYP1A1. Correlations between non-*ortho* and mono-*ortho* PCB burdens in blubber and hepatic CYP1A1 content and activity have been observed in beluga whales (White *et al*., 1994). CYP1A and CYP2B have been detected in cetacean skin, and induction of these isoforms was found after exposure to lipophilic contaminants such as OCs and BFR *in vitro* and in field studies (Godard *et al*., 2004; Fossi *et al*., 2006; Fossi *et al*., 2008 ; Montie *et al*., 2008; Hooker *et al*., 2008; Fossi *et al*., 2010; Wilson *et al*., 2010). On the other hand, an inductive response to ERs has rarely been shown in cetaceans (Fossi *et al*., 2010). These data demonstrate a greater sensitivity of CYP1A1 gene expression (assessed by RT-PCR) with respect to the protein induction response detected by the WB technique after exposure to OCs. In conclusion, the data from this experiment show expression of CYP1A1 and ERs genes as the most sensitive diagnostic tests for exposure to mixtures of OCs.

Biomarker responses to PBDE mixture - **Figure 6** shows the values for six tested biomarkers in response to treatment with increasing doses of a mixture of PBDEs, expressed as an Index Number (assigning a value of 1 to treatment of the slice with vehicle). Among the six biomarkers tested, the most sensitive response was detected for protein induction of CYP1A1, with a 2.9-fold induction relative to the control at the medium dose treatment. The upregulation response of CYP1A1 shows a slight bell-shaped pattern. A lower sensitivity has been observed for all other biomarker responses investigated. These data show a close dependence between contamination by PBDEs and inductive effects on CYP1A. This phenomenon was previously highlighted by Fossi *et al*. (2006) during *in vitro* studies after exposure to PBDEs (Fossi *et al*., 2006; Fossi *et al*., 2008). From a diagnostic point of view this experiment suggests that induction of CYP1A1 protein is the most sensitive diagnostic test to show exposure to mixtures of PBDEs.

Biomarker responses to PAH mixture - The metabolism of carcinogenic compounds such as PAHs depends on the relative expression of bioactivating and detoxifying enzymes. Thus induction of bioactivating CYPs may alter the balance between activation and detoxification. One purpose of this study was to investigate the constitutive and inducible expression of CYP1A1 and CYP2B in tissue slices incubated as organ cultures with PAH mixtures. **Figure 7** shows the values for six tested biomarkers in response to treatment with increasing doses of a mixture of PAHs, expressed as an Index Number (assigning a value of 1 to treatment of the slice with vehicle). Among the six biomarkers tested, the most sensitive response was observed for expression of the CYP1A1 gene, with a 9.2-fold upregulation relative to the control at the higher dose treatment. The second biomarkers in terms of response sensitivity are protein induction of CYP1A1 and CYP2B, with 3.5- and 2.9-fold upregulation, respectively, related to control at the higher dose treatment. The upregulation/induction responses of CYP1A1 and CYP2B show a linear relationship with increasing dose of PAHs treatment. The mRNA levels of E2-F1 and HSP70 are not upregulated by PAH mixture. These data confirm for this *in vitro* assay the close dependence between contamination by PAHs and inductive effects on CYP1A. This phenomenon was previously highlighted by Godard *et al*. (2004) in skin biopsy slices of sperm whale (*Physeter macrocephalus*) exposed for 24 hr to β-naphthoflavone (BnF). The inductive response of CYP2B, however, has rarely been shown in cetaceans (Fossi *et al*., 2010). These data demonstrate, as previously showed for OCs exposure, the greater sensitivity of CYP1A1 gene expression (as assessed by RT-PCR) than protein induction detected by the WB technique, with respect to PAHs exposure. In conclusion, the data from this experiment show that expression of the CYP1A1 gene and protein

induction of CYP1A1 and CYP2B are the most sensitive diagnostic tests to show exposure to mixtures of PAHs.

Biomarker responses to OC, PBDE and PAH mixture - **Figure 8** shows values for five tested biomarkers in response to treatment with increasing doses of OC, PBDE and PAH mixtures, expressed as an Index Number (assigning a value of 1 to treatment of the slice with vehicle). Among the five biomarkers tested in this *in vitro* experiment, the most sensitive response was detected for expression of the CYP1A1 gene, with 4.9-fold upregulation relative to the control at the lower dose treatment. The second biomarker, in term of sensitivity of response, is 4.9-fold upregulation of HSP70 relative to the control at the higher dose treatment. The induction of CYP1A1 and CYP2B proteins as assessed by WB are very low. The mixture does not modulated the mRNA levels E2-F1. The results from this last *in vitro* experiment lead us to identify important considerations for the simultaneous effect of contaminant mixtures. The simultaneous presence of three major classes of soluble contaminants presented to cetaceans in the Mediterranean (OC, PBDE and PAH) produces a subtractive effect *in vitro* on the inductive power of some biomarkers such as CYP1A1-PCR. The inductive phenomena change from 17-fold upregulation for OCs exposure and 9.2-fold upregulation for PAHs exposure to a reduced effect of only 4.9-fold upregulation for a mixture of the three classes of chemicals. This suggests a state of "stress" for enzyme systems subjected to multiple doses of pollutants, as evidenced also *in vivo* for situations of "multiple stresses" revealed in cetaceans in the Mediterranean sea and in particular of the Pelagos Sanctuary (Fossi *et al*., 2010).

CONCLUSIONS

This study had two main objectives: 1) to develop and evaluate several biomarker responses using a wide range of methodologies in slices experimentally exposed to increasing concentrations of contaminants such as OCs, PBDEs and PAHs and a mixture of them; and 2) to identify among the various categories of selected biomarkers the biomarkers and/or a series of biomarkers that best allow us to diagnose the presence of a specific class of pollutants, or a mixture of them, in future investigations in field studies.

The main conclusion that we can draw in response to the first objective of this study is that this multi-response biomarker methodology revealed an high sensitivity and selectivity of responses, and is a valid future approach for the study of inter- and intra-species sensitivities to various classes of environmental contaminants (including emerging contaminants) present in the Mediterranean environment. The last decade has seen major successes in terms of global measurement and regulation of persistent, bioaccumulative and toxic chemicals and persistent organic pollutants (POPs) (Muir and Howard, 2006). The Stockholm Convention, a global agreement on POPs, began to be enforced in 2004. There has been a major expansion of monitoring and risk assessment for new chemical contaminants in the global environment, particularly brominated diphenyl ethers and perfluorinated alkyl acids. However, the list of measured chemicals represents only a small fraction of the approximately 30,000 chemicals widely used in commerce (Muir and Howard, 2006). Currently there are few information on the levels and especially the effects of these emerging contaminants for cetaceans. This *in vitro* method is proposed as a valuable forecasting tool to estimate toxicological effects for individual classes of emerging contaminants or mixtures of them.

In order to achieve the second goal of this paper, the potential induction and/or upregulation of the different biomarkers are reported in relation to each class of contaminants investigated in **Table 1**. The response value for each biomarker was calculated in relation to the highest response (expressed as index number) obtained within all treatments (namely CYP1A1 qRT-PCR, index number value 17) being considered as 10. Different classes of response were categorised as follows: 1-2.5 = +; 2.6-5.0 = ++; 5.1-7.5 = +++; 7.6-10 = ++++. The different patterns of induction and/or upregulation for the six biomarkers investigated may allow identification and diagnosis of the main classes of contaminants responsible for the toxicological effect. The simultaneous presence in a sample of skin slices (or skin biopsy for free-ranging specimens) of a slight induction response for CYP1A1 (+; detected by Western blot) accompanied by very strong upregulation of the CYP1A1 (++++) and ERα genes (++++) as well as a slight upregulation of HSP70 genes (+), indicates that the sample was exposed to high concentrations of OCs. In the same way, the simultaneous presence in a sample of skin slices of a slight induction response for CYP1A1 (+) and CYP2B (+; detected by Western blot) accompanied by slight upregulation of HSP70 (+) and E2F-1 genes (+) indicates that the sample was exposed to high concentrations of PBDEs. The simultaneous presence of a slight induction response for CYP1A1 (+) and CYP2B (+; detected by Western blot) accompanied by strong upregulation of the CYP1A1 gene (+++) indicates that the sample was exposed to high concentrations of PAHs. Finally, the simultaneous presence of moderate upregulation of the CYP1A1 gene (++) accompanied by slight upregulation of the HSP70 gene (+) indicates that the sample was exposed to high concentrations of a mixture of several lipophilic contaminants such as OC, PBDE and PAH. This suggests a state of "stress" for enzyme systems subjected to multiple doses of pollutants, as also evidenced *in vivo* in situations of "multiple stresses" (Fossi *et al*., 2010).

These preliminary data, with the obvious limitations presented by an *in vitro* experiment as compared to the complex interactions that characterize the responses of a whole body exposed to mixtures of contaminants under natural conditions, constitute a conceptual basis for both identifying the biochemical-molecular mechanisms that regulate expression of different biomarkers in skin tissues of cetaceans and applying these methods in the future to skin

biopsies from free-ranging cetaceans. This last objective is in fact the main aim of our research team, addressed to develop a non-lethal "multi-trial biomarker tool" (combining molecular biomarkers and gene expression with analysis of OCs, PAHs and PBDEs) and then to apply to free-ranging cetacean skin biopsies for diagnosis of "multiple toxicological stress" related to multiple human toxicological impacts (Spinsanti *et al*., 2006; Spinsanti *et al*., 2008; Fossi *et al*., 2008; Fossi *et al*., 2010). Final development of this methodology could provide a statistical system for obtaining more complete information about the "toxicological-stress-syndrome" in cetacean populations living in highly anthropised areas such as the Mediterranean sea (Fossi, personal communication). This multidisciplinary approach could open new horizons for research, and the final outcome will be a powerful frontier approach to conservation and regulatory actions for major species in Mediterranean biodiversity.

ACKNOWLEDGEMENTS

This project was partially supported by the Italian Ministry for Environment and Territory (ACCOBAMS project).

REFERENCES

Aguilar, A., 2000. Population biology, conservation threats and status of Mediterranean striped dolphins (*Stenella coeruleoalba*). *J. Cetacean Res. Manage*. 2: 17-26.

Aguilar, A., Raga, J.A., 1993. The striped dolphin epizootic in the Mediterranean Sea. *Ambio*. 22(8): 524-528.

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(4): 391-404.

Andersson, T., 1995. Effects of β-naphthoflavone on the cytochrome P450 enzyme system in the mature male rainbow trout kidney. *Mar. Environ. Res*. 39(1-4): 341.

Andersson, T., Forlin, L., 1992. Regulation of the cytochrome P450 enzyme system in fish. *Aquat. Toxicol.* 24: 1–20.

Angell, C.M., Wilson, J.Y., Moore, M.J., Stegeman, J.J., 2004. Cytochrome P450 1A1 expression in cetacean integument: Implications for detecting contaminant exposure and effects. *Mar. Mamm. Sci.* 20 (3): 554-566.

Attwool, C., Lazzerini Denchi, E., Helin, K., 2004. The E2F family: specific functions and overlapping interests. *EMBO J*. 23: 4709-4716.

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.

Boon, J., Sleiderink, H., Helle, M., Dekker, M., van Shanke, A., Roex, E., Hillebrand, M., Klamer, H., Govers, B., Pastor, D., Morse, D., Wester, P.G., de Boer, J., 1998. The use of microsomal in vitro assay to study phase I biotransformation of chlorobornanes (Toxaphene) in marine mammals and birds. Possible consequences of biotransformation for bioaccumulation and genotoxicity. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol*. 121: 385–403.

Boone, A.N., Vijayan, M.M., 2002. Constitutive heat shock protein 70 (HSC70) expression in rainbow trout hepatocytes: effect of heat and heavy metal exposure. *Comp. Biochem. Physiol*. 132C: 223-233.

Carpenter, D.O., Arcaro, K., Spink, D.C., 2002. Understanding the human health effects of chemical mixture. *Environ. Health Perspect*. 110: 25-42.

Cruz-Rodrìguez, L.A., Chu, F.L.E., 2002. Heat-shock protein (HSP70) response I eastern oyster, *Crassostrea virginica*, exposed to PAHs sorbed to suspended artificial clay particles and to suspended field contaminated sediments. *Aquat. Toxicol*. 60: 157-168.

Deane, E.E., Woo, N.Y.S., 2004. Differential gene expression associated with euryhalinity in sea bream. *American Journal of Physiology*. 287: 1054-1063.

Domingo, M., Vilafranca, M., Visa, J., Prats, N., Trudgett, A., Visser, I., 1995. Evidence for chronic morbillivirus infection in the Mediterranean striped dolphin (*Stenella coeruleoalba*). *Vet. Microbiol.* 44 (2-4): 229-239.

Drahushuk, A.T., McGarrigle, B.P., Tai, H.L., Kitareewan, S., Goldstein, J.A., Olson, J.R., 1996. Validation of precision-cut liver slices in dynamic organ culture as an in vitro model for studying CYP1A1 and CYP1A2 induction. *Toxicol Appl Pharmacol.* 140 (2): 393-403.

Drahushuk, A.T., McGarrigle, B.P., Larsen, K.E., Stegeman, J.J., Olson, J.R., 1998. Detection of CYP1A1 protein in human liver and induction by TCDD in precision-cut liver slices incubated in dynamic organ culture. *Carcinogenesis*. 19(8): 1361-1368.

Fernández, A., Esperón, F., Herraéz, P., de Los Monteros, A.E., Clavel, C., Bernabé, A., Sánchez-Vizcaino, J.M., Verborgh, P., DeStephanis, R., Toledano, F., Bayón, A., 2008. Morbillivirus and pilot whale deaths, Mediterranean Sea. *Emerg. Infect. Dis*. 14(5): 792-794.

Fortuna, C.M., Canese, S., Giusti, M., Revelli, E., Consoli, P., Florio, G., Greco, S., Romeo, T., Andaloro. F., Fossi, M.C., Lauriano, G., 2007. An insight into the status of the striped dolphins, *Stenella coeruleoalba*, of the southern Tyrrhenian Sea. *J. Mar. Biol. Assoc. UK*. 87: 1321-1326.

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

Fossi, M.C., Marsili, L., Neri, G., Natoli, A., Politi, E. & Panigada, S., 2003. The use of a non-lethal tool for 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., 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*. 62: 200–204.

Fossi, M.C., Casini, S., Marsili, L. 2007. Potential toxicological hazard due to EDCs on Mediterranean top predators: state of art, gender differences and methodological tools. *Environ. Res*. 104(1): 174-182.

Fossi, M.C., Casini, S., Bucalossi, D., Marsili, L., 2008. First detection of CYP1A1 and CYP2B induction in Mediterranean cetacean skin biopsies and cultures fibroblasts by Western Blot Analysis. *Mar. Environ. Res.* 66(1): 3-6.

Fossi, M.C., Urban, J., Casini, S., Maltese, S., Spinsanti, G., Panti, C., Porcelloni, S., Panigada, S., Lauriano, G., Niño-Torres, C., Rojas-Bracho, L., Jimenez, B., Muñoz-Arnanz, J., Marsili, L., 2010. A multi-trial diagnostic tool in fin whale (*Balaenoptera physalus*) skin biopsies of the Pelagos Sanctuary (Mediterranean Sea) and the Gulf of California (Mexico). *Mar. Environ. Res*. *In press.*

Godard, C.A.J., Smolowitz, R.M., Wilson, J.Y., Payne, R.S., Stegeman, J.J., 2004. Induction of cetacean cytochrome P4501A1 by beta-naphthoflavone exposure of skin biopsy slices. *Toxicol. Sci*. 80: 268-275.

Goksøyr, A., Solbakken, J. E., Tarlebø, J., Klungsøyr, J., 1986. Initial characterization of the hepatic microsomal cytochrome P-450 system of the piked whale (minke) *Balaenoptera acutorostrata*. *Mar. Environ. Res.* 19: 185–203.

Goksøyr, A., Forlin, L., 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquat. Toxicol*. 22: 287–312.

Gómez de Segura, A., Hammond, P.S., Cañadas, A. and Raga, J.A., 2007. Comparing cetacean abundance estimates derived from spatial modelling and line transect sampling. *Mar. Ecol.* 329: 289-299.

Gooch, J.W., Elskus, A.A., Kloepper-Sams, P.J., Hahn, M.E., Stegeman, J.J., 1989. Effects of ortho- and non-ortho-substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotomus chrysops*). *Toxicol. Appl. Pharmacol*. 98(3): 422-433.

Hahn, M.E., Lamb, T.M., Schultz, M.E., Smolowitz, R.M., Stegeman, J.J., 1993. Cytochrome P4501A induction and inhibition by 3,39,4,49-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). *Aquat. Toxicol.* 26:185–208.

Harrigan, J.A., Vezina, C.M., McGarrigle, B.P., Ersing, N., Box, H.C., Maccubbin, A,E., Olson, J.R., 2004. DNA adduct formation in precision-cut rat liver and lung slices exposed to benzo[a]pyrene. *Toxicol. Sci.* 77(2): 307-314.

Harrigan, J.A., McGarrigle, B.P., Sutter, T.R., Olson, J.R., 2006. Tissue specific induction of cytochrome P450 (CYP) 1A1 and 1B1 in rat liver and lung following in vitro (tissue slice) and in vivo exposure to benzo(a)pyrene. *Toxicol. in Vitro*. 20(4): 426-438.

Hooker, S.K., Metcalfe, T.L., Metcalfe, C.D., Angell, C.M., Wilson, J.Y., Moore, M.J., Whitehead, H., 2008. Changes in persistent contaminant concentration and CYP1A1 protein expression in biopsy samples from northern bottlenose whales, *Hyperoodon ampullatus*, following the onset of nearby oil and gas development. *Environ. Poll.* 152(1): 205-216.

International Union for Conservation of Nature and Natural Resources (IUCN), 2010. The IUCN Red List of Threatened Species. www.iucnredlist.org/

Janošek, J., Hilscherová, K., Bláha, L., Holoubek, I., 2006. Environmental xenobiotics and nuclear receptors-Interactions, effects and in vitro assessment. *Toxicol. in Vitro*. 20: 18-37.

La Thangue, N.B., 2003. The yin and yang of E2F-1: balancing life and death. *Nat. Cell Biol*. 5: 587-589.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data usingreal-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* 25: 402-408.

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., D'Agostino, A., Bucalossi, D., Malatesta, T., Fossi, M.C., 2004. Theoretical models to evaluate hazard due to organochlorine compounds (OCs) in Mediterranean striped dolphin (*Stenella coeruleoalba*). *Chemosphere*. 56(8): 791-801.

Marsili, L., Casini, S., Carletti, L., Maltese, S., Porcelloni, S., Nino-Torres, C.A., Rojas-Bracho, L., Urban, J., Fossi, M.C., 2009. 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. *International Whaling Comm.,* SC/61/E13 1-7.

Montie, E.W., Fair, P.A., Bossart, G.D., Mitchum, G.B., Houde, M., Muir, D.C., Letcher, R.J., McFee, W.E., Starczak, V.R., Stegeman, J.J., Hahn, M.E., 2008. Cytochrome P4501A1 expression, polychlorinated biphenyls and hydroxylated metabolites, and adipocyte size of bottlenose dolphins from the Southeast United States. *Aquat. Toxicol.* 86(3): 397-412.

Mueller, S.O., 2004. Xenoestrogens mechanisms of action and detection methods. *Anal. Bioanal.Chem.* 378: 582-587.

Mueller, S.O., Korach, K.S., 2001. Estrogen receptor and endocrine diseases: lesson from estrogen receptor knockout mice. *Curr. Opin. Pharmacol*. 1: 613-619.

Muir, D.C., Howard, P.H., 2006. Are there other persistent organic pollutants? A challenge for environmental chemists. *Environ. Sci. Technol*. 40(23): 7157-7166.

Murk, A., Morse, D., Boon, J., Brouwer, A., 1994. In vitro metabolism of 3,3',4,4'-tetrachlorobiphenyl in relation to ethoxyresorufin-O-deethylase activity in liver microsomes of some wildlife species and rat. *Eur. J. Pharmacol*. 270: 253–261.

Nollen, E.A.A., Moromoto, R., 2002. Chaperoning signaling pathways: molecular chaperones as stress-sensing “heat shock” proteins. *J. Cell Sci.* 115: 2809-2816.

O’Connor, D.J, Lu, X., 2000. Stress signal induce transcriptionally inactive E2F-1 independently of p53 and Rb. *Oncogene*. 19: 2369-2376.

Porte, C., Biosca, X., Solé, M., Albaigés, J., 2001. The integrated use of chemical analysis, cytochrome P450 and stress proteins in mussels to assess pollution along the Galician coast (NW Spain). *Environ. Pollut.* 112(2): 261-268.

Pyza, E., Mak, P., Kramar, P., laskowski, R., 1997. Heat shock Proteins (HSP70) as biomarkers in ecotoxicological studies. *Ecotoxicol. Environ. Saf.* 38: 244-251.

Raga, J.A., Banyard, A., Domingo, M., Corteyn, M., Van Bressem, M.F., Fernández, M., Aznar, F.J., Barrett, T., 2008. Dolphin morbillivirus epizootic resurgence, Mediterranean Sea. *Emerg. Infect. Dis*. 14(3): 471-473.

Reeves, R., Notarbartolo di Sciara, G. 2006. The status and distribution of cetaceans in the Black Sea and Mediterranean Sea. IUCN Centre for Mediterranean Cooperation, Malaga, Spain. 137 pp.

Spinsanti, G., Panti, C., Lazzeri, E., Marsili, L., Casini, S., Frati, F., Fossi, M.C., 2006. Selection of reference genes for quantitative RT-PCR in striped dolphin (*Stenella coeruleoalba*) skin biopsies. *BMC Mol. Biol*. 7: 32-42.

Spinsanti, G., Panti, C., Bucalossi, D., Marsili, L., Casini, S., Frati, F., Fossi, M.C., 2008. Selection of reliable reference genes for qRT-PCR studies on cetacean fibroblast cultures exposed to OCs, PBDEs, and 17β-estradiol. *Aquat. Toxicol*. 87: 178-186.

Stegeman, J., Brouwer, M., Di Gulio, R., Forlin, L., Fowler, B., Sanders, B., Van Veld, P., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effects. In Biomarkers. Biochemical, Physiological, and Histological Markers of Anthropogenic Stress (R. Huggett, R. Kimerle, P. Mehrle, and H. Bergman, Eds.), Lewis Publishers. SETAC Special Publications Series, Boca Raton, FL., 235–335.

Stevens, C., La Thangue, N.B., 2004. The emerging role of E2F-1 in the DNA damage response and checkpoint control. *DNA Repair*. 3: 401-409.

Teramitsu, I., Yamamoto, Y., Chiba, I., Iwata, H., Tanabe, S., Fujise, Y., Kazusaka, A., Akahori, F., Fujita, S., 2000. Identification of novel cytochrome P450 1A genes from five marine mammal species. *Aquat. Toxicol*. 51: 145–153.

Tiemann, U., 2008. *In vivo* and *in vitro* effects of the organochlorine pesticides DDT, TCPM, methoxychlor, and lindane on the female reproductive tract of mammals: a review. *Rep. Toxicol*. 25: 316-326.

Van Bressem, M.F., Visser, I.K., De Swart, R.L., Orvell, C., Stanzani, L., Androukaki, E., Siakavara, K., Osterhaus, A.D., 1993. Dolphin morbillivirus infection in different parts of the Mediterranean Sea. *Arch. Virol.* 129(1-4): 235-242.

Van Bressem, M.F., Raga, J.A., Di Guardo, G., Jepson, P.D., Duignan, P.J., Siebert, U., Barrett, T., Santos, M.C., Moreno, I.B., Siciliano, S., Aguilar, A., Van Waerebeek, K. 2009. Emerging infectious diseases in cetaceans worldwide and the possible role of environmental stressors. *Dis. Aquat. Organ.*, 86(2):143-57.

White, R., Hahn, M.E., Lockhart, W.L., Stegeman, J.J., 1994. Catalytic and immunochemical characterization of hepatic microsomal cytochromes P450 in beluga whale (*Delphinapterus leucas*). *Toxicol. Appl. Pharmacol.* 126: 45–57.

White, R., Shea, D., Schlezinger, J., Hahn, M., Stegeman, J.J., 2000. In vitro metabolism of polychlorinated biphenyl congeners by beluga whale (*Delphinapterus leucas*) and pilot whale (*Globicephala melas*) and relationship to cytochrome P450 expression. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol*. 126: 267–284.

Whitehead, A., Anderson, S.L., Kuivila, K.M., Roach, J.L., May, B., 2003. Genetic variation among interconnected populations of *Catostomus occidentalis*: implications for distinguishing impacts of contaminants from biogeographical structuring. *Mol. Ecol.* 12(10): 2817-2833.

Wilson, J.Y., Wells, R., Aguilar, A., Borrel, A., Tornero, V., Reijnerds, P., Moore, M., Stegeman, J.J., 2007. Correlates of cytochrome P450 1A1 expression in bottlenose dolphin (*Tursiops truncatus*) integument biopsies. *Toxicol. Sci*. 94(1): 111-119.

Wilson, J.Y., Moore, M., Stegeman, J.J., 2010. Catalytic and immunochemical detection of hepatic and extrahepatic microsomal cytochrome P450 1A1 (CYP1A1) in white-sided dolphin (*Lagenorhynchus acutus*). *Aquat. Toxicol*. 96(3): 216–224.

CAPTIONS

Figure 1. Biomarker responses (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70, ERα and E2F-1 detected by the qRT-PCR technique) in skin and liver slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of OCs (Arochlor 1260, pp'DDT and pp'DDE in DMSO (0.05%) at three doses: 0.01 µg/ml, 0.1 µg/ml and 1 µg/ml, plus a DMSO (0.05%) control). Western blot analysis of CYP1A1 (**A**) and CYP2B (**B)** from skin and liver treated slices. Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (Bio-Rad, 1-D Analysis Software) using Adj.Vol.Int *mm2 as a quantitative parameter. Gene expression analyses by the quantitative real-time PCR assay (**C**) in skin slice samples: CYP1A1, E2F-1, HSP70, Erα and AHR. Gene expression was calculated using GenEx software v. 4.3.8 (MultiD Analyses AB).

Figure 2. Biomarker responses (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70, ERα and E2F-1 detected by the qRT-PCR technique) in skin and liver slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of PBDEs (27 PBDEs, from mono- to deca-brominated, 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). Western blot analysis of CYP1A1 (**A**) and CYP2B (**B)** from skin and liver treated slices. Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (Bio-Rad, 1-D Analysis Software) using Adj.Vol.Int *mm2 as a quantitative parameter. Gene expression analyses by the quantitative real-time PCR assay (**C**) in skin slice samples: CYP1A1, E2F-1, HSP70, Erα and AHR. Gene expression was calculated using GenEx software v. 4.3.8 (MultiD Analyses AB).

Figure 3. Biomarker responses (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70, ERα and E2F-1 detected by the qRT-PCR technique) in skin and liver slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of PAHs (benzo(a)pyrene (1mM) and beta-naphthoflavone (20mM), in acetone (0.1%), at three doses: 0.5 µM BaP + 10 µM BnF; 2.5 µM BaP + 50 µM BnF; 12.5 µM BaP + 250 µM BnF, plus an acetone (0.1%) control). Western blot analysis of CYP1A1 (**A**) and CYP2B (**B)** from skin and liver treated slices. Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (Bio-Rad, 1-D Analysis Software) using Adj.Vol.Int *mm2 as a quantitative parameter. Gene expression analyses by a quantitative real-time PCR assay (**C**) in skin slice samples: CYP1A1, E2F-1, HSP70, ERα, and AHR. Gene expression was calculated using GenEx software v. 4.3.8 (MultiD Analyses AB).

Figure 4. Biomarker responses (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70, ERα and E2F-1 detected by the qRT-PCR technique) in skin and liver slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of the three combined mixtures in a mix of carriers (DMSO 0.005%, nonane 0.01% and acetone 0.1%), at two doses: OCs 0.01 µg/ml + PBDEs 0.01 µg/ml + PAHs 0.5 µM and OCs 1 µg/ml + PBDEs 0.1 µg/ml + PAHs 12.5 µM, plus a control with the mixed carriers. Western blot analysis of CYP1A1 (A) and CYP2B (B) from skin and liver treated slices. Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (Bio-Rad, 1-D Analysis Software) using Adj.Vol.Int *mm2 as a quantitative parameter. Gene expression analyses by a quantitative real-time PCR assay (C) in skin slice samples: CYP1A1, E2F-1, HSP70, ERα and AHR. Gene expression was calculated using GenEx software v. 4.3.8 (MultiD Analyses AB).

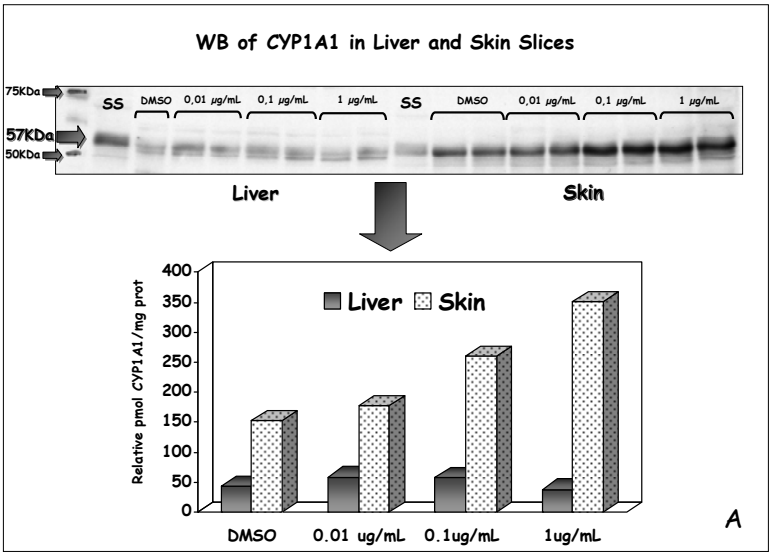
Figure 5. Values for the six tested biomarkers (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70, ERα and E2F-1 detected by the qRT-PCR technique) in skin slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of OCs, expressed as an Index Number (assigning a value of 1 to treatment of the slice with vehicle).

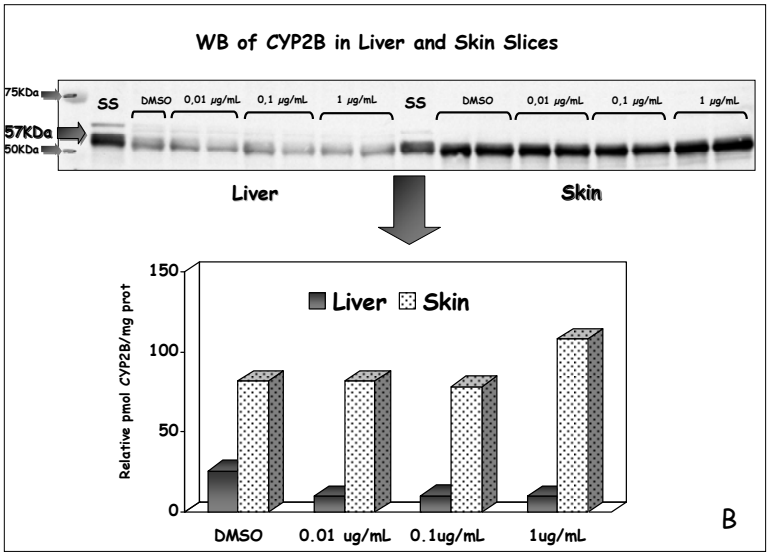
Figure 6. Values for the six tested biomarkers (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70, ERα and E2F-1 detected by the qRT-PCR technique) in skin slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of PBDEs, expressed as an Index Number (assigning a value of 1 to treatment of the slice with vehicle).

Figure 7. Values for the five tested biomarkers (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70 and E2F-1 detected by the qRT-PCR technique) in skin slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of PAHs, expressed as an Index Number (assigning a value of 1 to treatment of the slice with vehicle).

Figure 8. Values for the five tested biomarkers (CYP1A1 and CYP2B detected by Western blot and CYP1A1, HSP70 and E2F-1 detected by the qRT-PCR technique) in skin slices of stranded striped dolphin *(Stenella coeruleoalba*) experimentally exposed to increasing concentrations of an OC, PBDE and PAH mixture, expressed as an Index Number (assigning a value of 1 to treatment of the slice with vehicle).

OC Slice Treatments





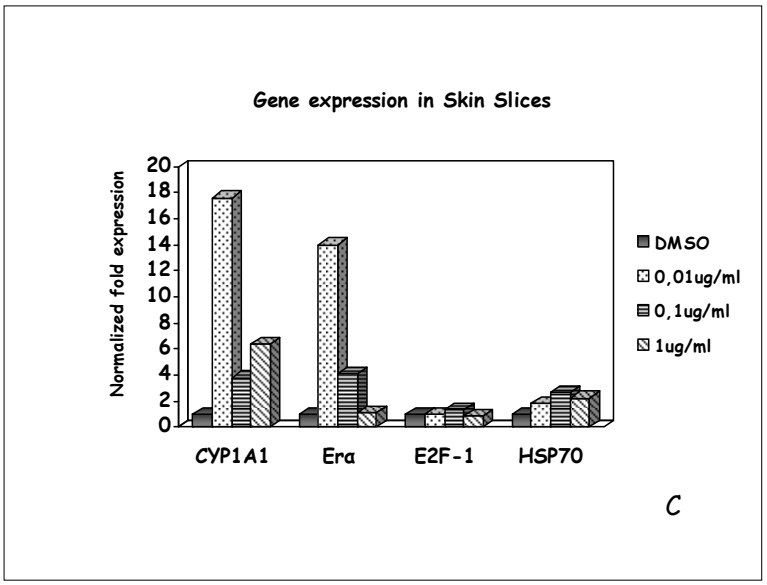
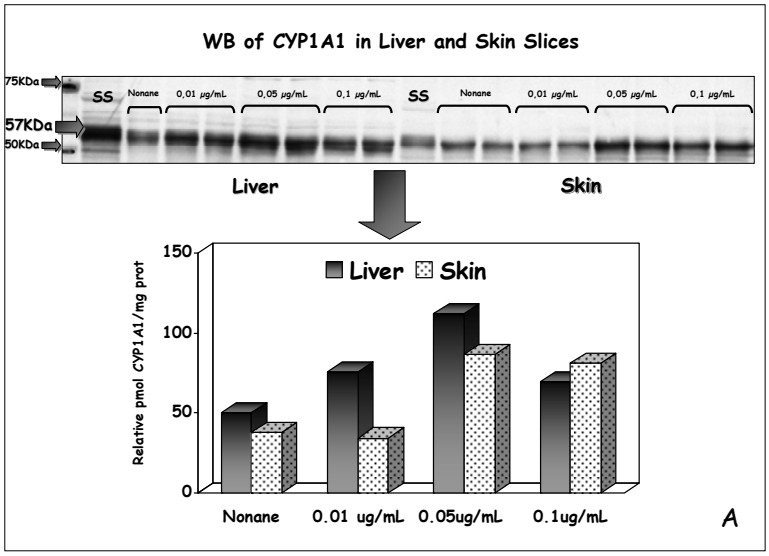
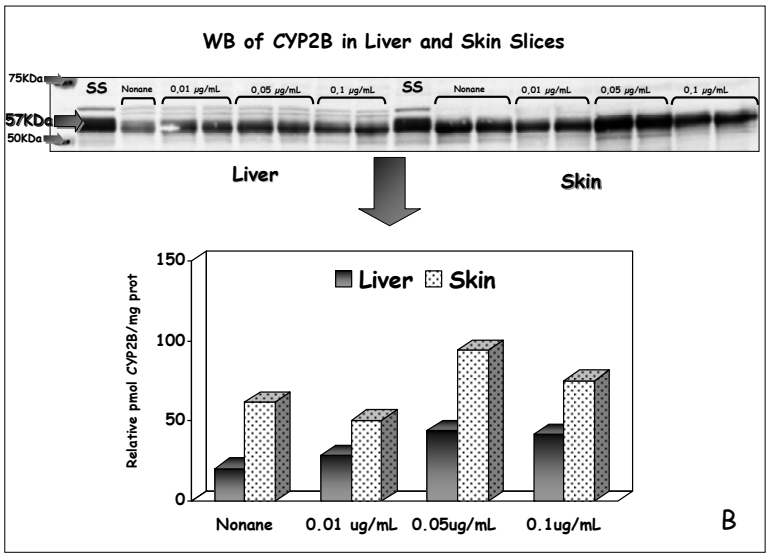


Fig. 1

PBDE Slice Treatments





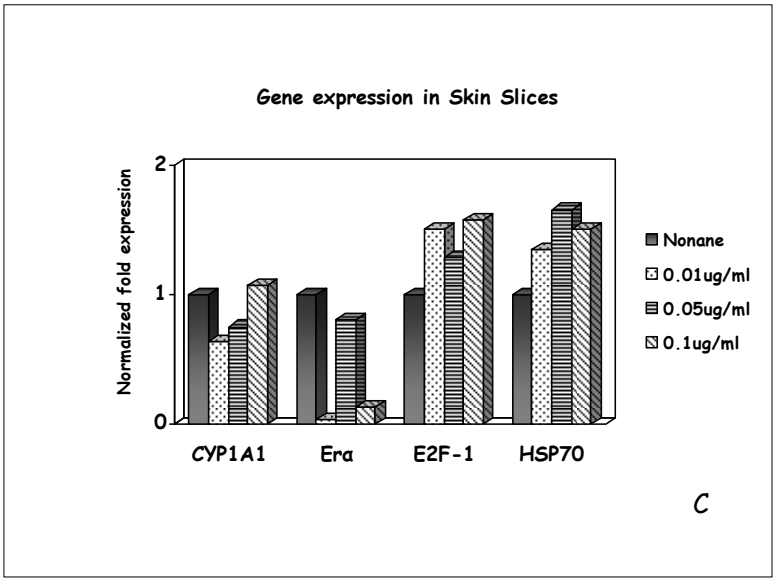
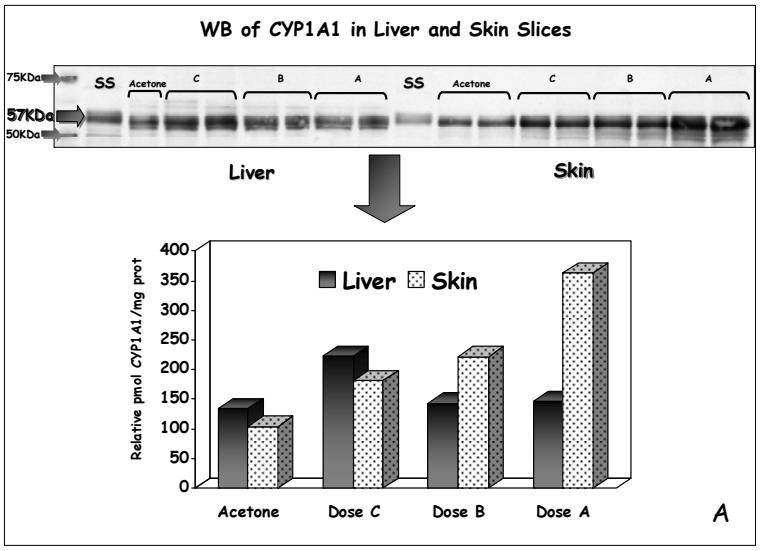
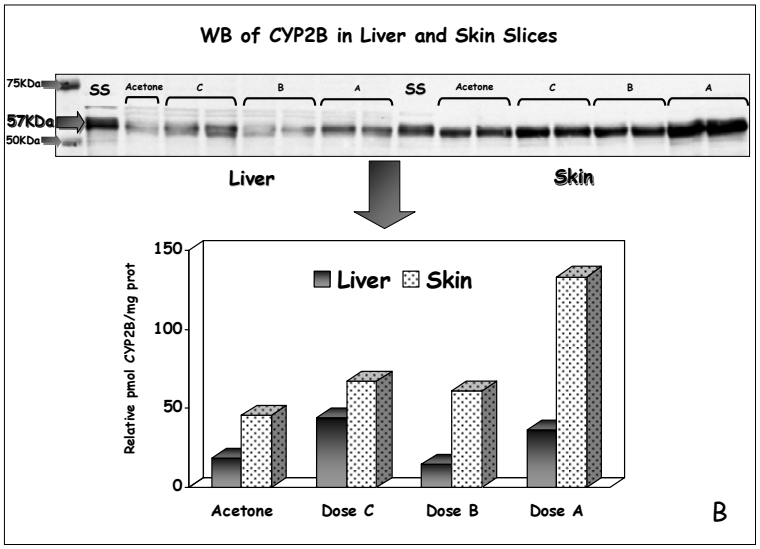


Fig. 2

PAH Slice Treatments





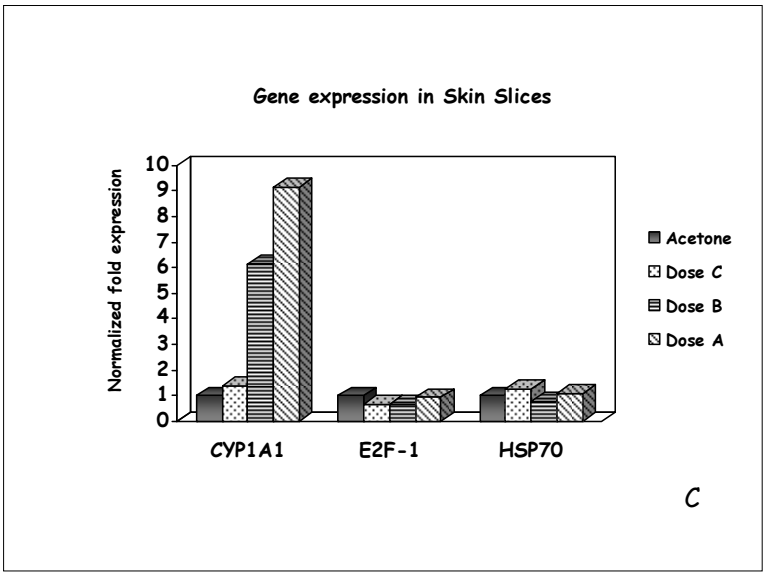
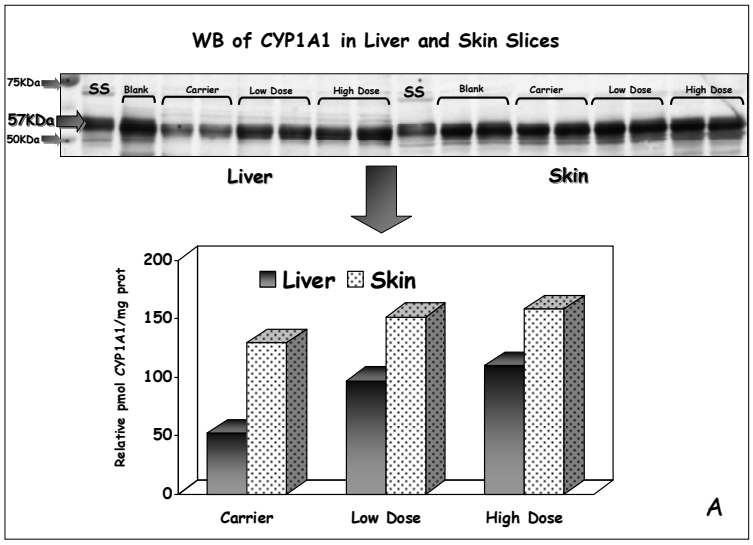
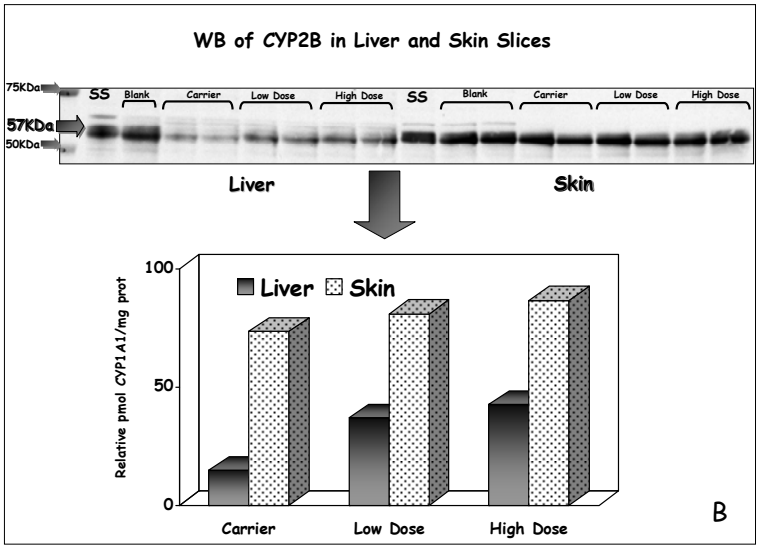


Fig. 3

OC, PBDE and PAH Mixture Slice Treatments





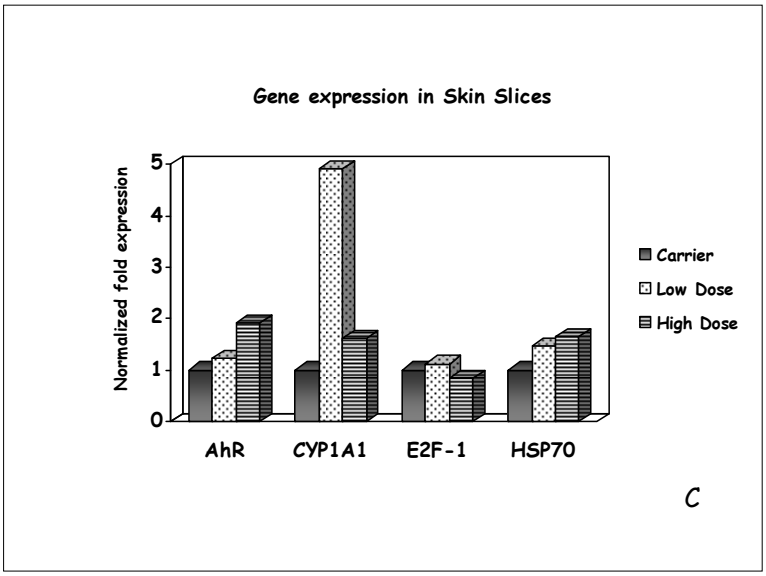


Fig. 4

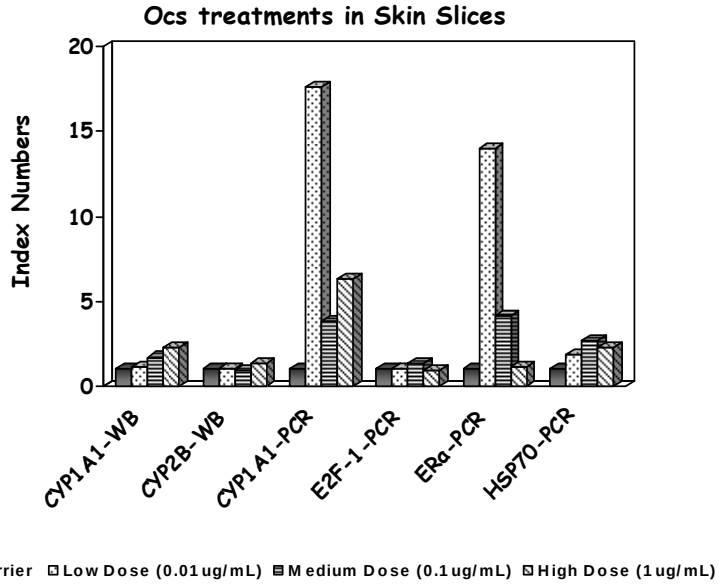


Fig. 5

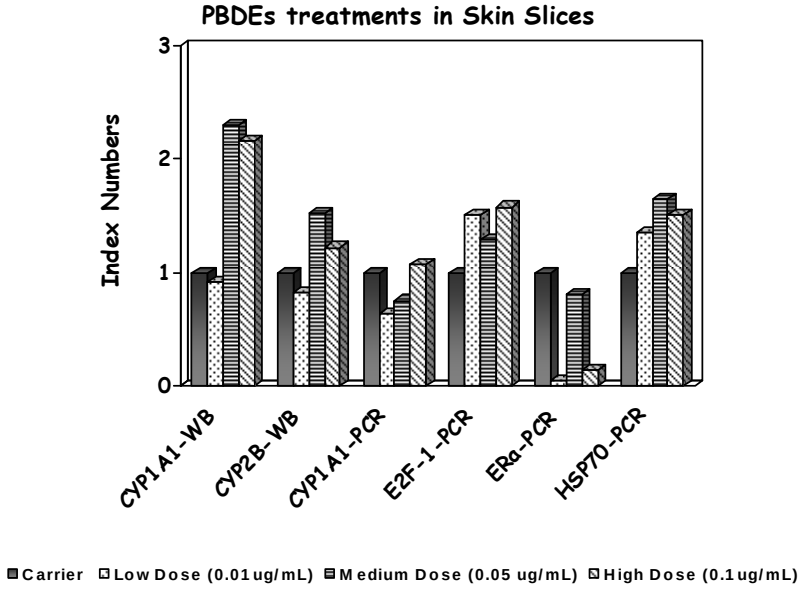


Fig. 6

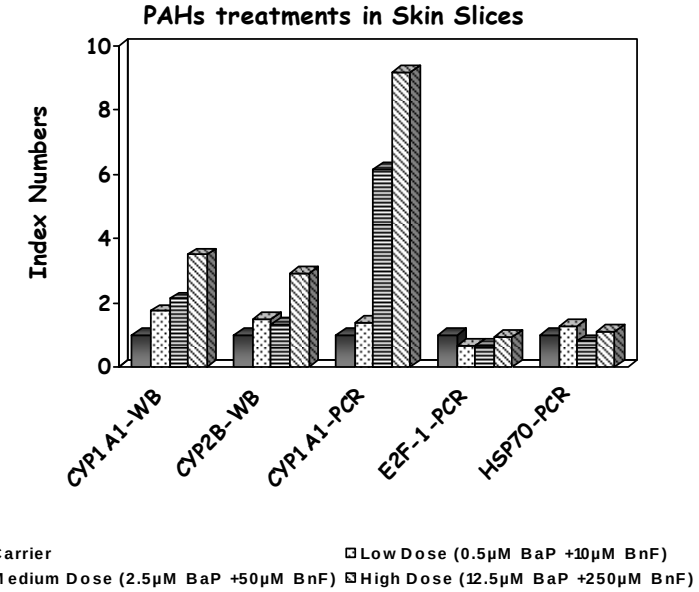


Fig. 7

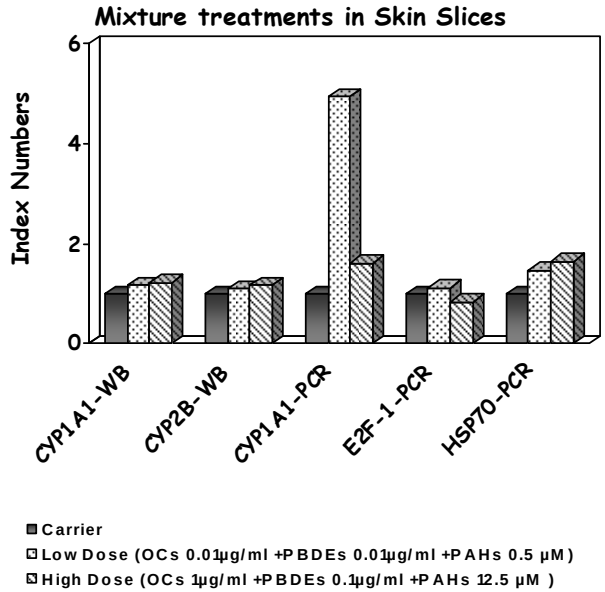


Fig. 8

TABLE

Table 1 – Sensitivity of induction or upregulation responses for the different biomarkers (CYP1A1 and CYP2B detected by Western blot and CYP1A1, ERα, HSP70 and E2F-1 detected by the RT-PCR technique) reported in relation to each class of contaminants investigated in treated skin slices. The response value for each biomarker was calculated in relation to the highest response (expressed as index numbers) obtained within all treatments (i.e., CYP1A1 qRT-PCR, index number value 17) being considered as 10. Different classes of response were categorised as follows: 1-2.5 = +; 2.6-5.0 = ++; 5.1-7.5 = +++; 7.6-10 = ++++.

Biomarkers	**OCs**	**PBDEs**	**PAHs**	**MIX**
CYP1A1 WB	+	+	+	
CYP2B WB		+	+	
CYP1A1 qRT-PCR	++++		+++	++
Erα qRT-PCR	++++			
HSP70 qRT-PCR	+	+		+
E2F-1 qRT-PCR		+		