REVIEW

The use of non-destructive biomarkers in the study of marine mammals

M. Cristina Fossi and Letizia Marsili

Marine mammals have been subject to heavy anthropogenic pressure by direct killing and chemical pollution all over the world. Most studies of contamination and biomarker responses in marine mammals have been conducted using animals killed by hunting (out of a total of 12 cetacean species studied, 45% of the specimens were obtained by sacrificing the animal; out of a total of eight pinniped species studied, 40% of the specimens were obtained by killing). The development of a series of non-destructive techniques to evaluate biomarker responses and residue levels is recommended for the hazard assessment and conservation of endangered species of marine mammals. Here we review the current status of the non-destructive biomarker approach in marine mammals, describing the biological materials available for non-destructive tests in stranded (brain, liver, blood, skin, subcutaneous blubber, muscle and fur) and free-ranging animals (blood, skin biopsy, fur and faeces) and the respective biomarker techniques (mixed function oxidase activity and DNA damage in skin biopsy samples; porphyrins in faeces and fur; esterases, porphyrins, clinical biochemical parameter, vitamin A and micronuclei in blood samples). Residue analysis can be carried out in the various biological materials. We also report the results of applying this methodological approach to cetaceans (minke whale - *Balaenoptera acutorostrata*, fin whale –– *Balaenoptera physalus*, beluga whale –– *Delphinapterus leucas*, short-finned pilot whale –– *Globicephala macrorhynchus*, harbour porpoise –– *Phocoena phocoena*, Risso's dolphin –– *Risso's Grampus griseus*, Dall's porpoise –– *Phocoenoides dalli dalli*, melon-headed whale –– *Peponocephala electra*, bottlenose dolphin –– *Tursiops truncatus*, striped dolphin –– *Stenella coeruleoalba*, spinner dolphin –– *Stenella longirostris*, killer whale –– *Orcinus orca)* and pinnipeds (northern fur seal –– *Callorhinus ursinus*, hooded seal –– *Cystophora cristata*, grey seal –– *Halichoerus grypus*, harbour seal –– *Phoca vitulina*, ringed seal –– *Phoca hispida*, harp seal –– *Phoca groenlandica*, ribbon seal –– *Phoca fasciata*, largha seal –– *Phoca largha*, southern sea lion –– *Otaria flavescens)* in field studies for prognostic and diagnostic purposes.

Keywords: *biomarkers, marine mammals, endangered species, ecotoxicological risk, contaminants.*

M. Cristina Fossi *and* **Letizia Marsili** *(authors for correspondence) are in the Department of Environmental Biology. Siena University, Via delle Cerchia 3, 53100 Siena, Italy.*

Abbreviations: *ACTH, adrenocorticotropic hormone; ALAD, aminolevulinic acid dehydratase; BaP, benzo(a)pyrene; BPMO, benzo(a)pyrene monooxygenase; BROD, benzyloxyresorufin-O-deethylase; CBs, carbamates; ConA, concanavalin A; CYP, cytochrome P-450; 3,4-didehydroretinol, vitamin A_2; ELISA, enzyme linked immunosorbent assay; EROD, 7-ethoxyresorufin-O-deethylase; FT4, free thyroxin; γ-GT, γ-glutamil transferases; HgSe, mercury selenide; IgG, immunoglobulin G; MC, 3-methylcholanthrene; MFO, mixed function oxidase; OCs, organochlorines; OPs, organophosphates; PAHs, polycyclic aromatic hydrocarbons; PB, phenobarbital; PCBs, polychlorinated biphenyls; PDV, phocid distemper virus; PHAHs, polyhalogenated aromatic hydrocarbons; PROD, pentoxyresorufin-O-deethylase; TCB, ^{14}C-labelled 3,3´,4,4´-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo(p)dioxin; TT3, triiodothyronin; TT4, total thyroxin.*

Introduction

In the last 100 years, many species of marine mammals (right whales –– *Eubalaena* spp., blue whale –– *Balaenoptera musculus,* humpback whale –– *Megaptera novaeangliae,* beluga whale –– *Delphinapterus leucas*) have undergone a drastic decrease in numbers on a world scale (Martin *et al.* 1990), certainly due to human activities such as hunting and fishing, and potentially due to environmental contamination and destruction of habitat. Chemical pollution is the main source of ecotoxicological risk to these animals. As top predators of the marine food chain many marine mammals (such as odontocetes and pinnipeds) tend to bioaccumulate high concentrations of anthropogenic contaminants, such as organochlorine contaminants (OCs), heavy metals and polycyclic aromatic hydrocarbons (PAHs) (Neff *et al.* 1976, Geraci and St Aubin 1980, Tanabe *et al.* 1983, Boon *et al.* 1992, Leonzio *et al.* 1992, Marcovecchio *et al.* 1994, Marsili and Focardi 1997). The ecotoxicological risk of some species is also related to their 'biochemical vulnerability' to lipophilic xenobiotics. Tanabe and Tatsukawa (1992) reported that '... these animals have a low capacity for degradation of organochlorines due to a specific mode of their cytochrome P450 enzyme system'. Particularly '... recent studies using cetacean liver microsomes demonstrated that these animals have much lower activities of aldrin epoxidase (phenobarbital (PB)-inducible P-450 enzymes) than the rat, whereas activities of 7-ethoxyresorufin O-deethylase (3-methylcholanthrene (MC)-inducible P-450 enzymes) are comparable to those of the rat, supporting the above observations from PCB isomer and congener compositions (Watanabe *et al.* 1989)'. World-wide alarm about this state of affairs has prompted the scientific community to study the ecotoxicological risk to which these animals are exposed. Questions such as whether xenobiotics in the marine environment are a risk factor for these vertebrates have been raised with increasing frequency.

A comprehensive answer to such questions can be obtained by the use of biomarkers. Biomarkers are defined as a change in a biological system that can be related to an exposure to, or effect of, an environmental chemical

1354–750X/97 $12·00

al. 1993). However most researchers who have used this approach so far in ecological studies, have used destructive methods (using animals hunted) which involved decreasing the populations of species already at risk. An on-line bibliographic search from 1982 to 1996 showed that a large percentage of the biomarker studies in marine mammals have been conducted in a destructive way, using animals obtained by hunting or killed for the purpose of analysis (Tables 1 and 2). Out of a total of 12 cetacean species studied, 45% of the specimens were obtained by sacrificing the animal (Goksøyr *et al.* 1986, 1988, 1992, Tanabe *et al.* 1988, Watanabe *et al.* 1989, Ray *et al.* 1991, White *et al.* 1994) and 55% in a non-destructive way, analysing stranded and free-ranging animals (Table 1). In some papers the number of specimens analysed or the method of obtaining the biological material were not specified. The situation for pinnipeds is equally serious: of the eight species studied, ~40% of the specimens were obtained by killing the animal (Engelhardt 1982, Addison and Brodie 1984, Addison *et al.* 1986, Tanabe *et al.* 1988, Harder *et al.* 1992, Lund 1994) (Table 2).

Conservationist and ethical motives have prompted the development and the application of non-destructive methods of investigation (Lambertsen 1987, Fossi *et al.* 1992, 1996a, 1997, Aguilar and Borrell 1994, Fossi 1994, Fossi and Leonzio 1994, Fossi and Marsili 1996, Marsili and Focardi 1996). The biological materials (blood, faeces, fur and skin biopsy specimens) used in this new approach are obtained with minimal stress to individual animals and to the population (Lambertsen *et al.* 1994). The need for non-destructive markers (e.g. routine blood chemistry and haematology) in clinical and veterinary medicine/toxicology and experimental toxicology, is at the present time very well understood.

The aim of this paper is to review the state of art of the non-destructive biomarker approach in marine mammals and to lay the conceptual and methodological foundations for this technological proposal. Moreover we provide indications, accompanied by field validation, of the main biological materials that can be obtained in different species of cetaceans and pinnipeds and the respective biochemical techniques (biomarkers) that can be used in the different materials in order to evaluate the toxicological risk to which a species or population is exposed.

Non-destructive methodology in marine mammals

The biomarker approach and residue analysis can be used in marine mammals in two kinds of samples: those from stranded specimens and free-ranging specimens.

Stranded specimens

In theory, all stranded cetaceans and pinnipeds, in a good state of conservation, can be used for biomarker analysis. Brain, liver, blood, skin, subcutaneous blubber, muscle and fur are suitable materials. A wide range of biomarkers can be investigated in these samples. Most can only be evaluated if the specimen is sampled within 60–120 min of death. Table 3 shows the principal biomarkers and the respective times and methods of conservation possible for the biological materials provided by stranded animals. Various environmental pollutants such as OCs, heavy metals and PAHs can also be analysed in these samples and the lag phase between death and sampling is less critical for these chemical analyses.

Free-ranging specimens

From an ecotoxicological perspective it is preferable to use healthy live free-living animals with samples being collected with minimum disturbance. The main biological materials obtainable from free-ranging pinnipeds and the respective biomarkers are shown in Figure 1. Blood, skin biopsy, fur and faeces can be obtained, under anaesthesia, with minimum stress to individuals or populations (Lambertsen *et al.* 1994). In skin biopsy samples mixed function oxidase (MFO) activity and DNA damage can be detected. In faeces and fur porphyrins can be analysed. Esterases, porphyrins, clinical biochemical parameters (γ-glutamyl transferases (γ-GT), triglycerides, cholesterol, etc.), vitamin A and micronuclei can be analysed in blood samples. Residue analysis can be carried out in the various biological materials.

The most useful samples for non-destructive studies in cetaceans are skin biopsy specimens, obtained by dart, and faeces (Figure 2). MFO activity, DNA damage and residue analysis can be evaluated in the former and porphyrins and residue analysis in the latter.

Biomarker techniques

Several biomarker techniques can be applied in the study of marine mammals in a non-destructive way. Table 4 shows the biological significance of the main possible biomarkers in relation to the class of contaminants responsible for the biological response and their destructive and non-destructive use. Particular attention must be given to the techniques that are unique for marine mammals such as MFO and DNA damage in skin and porphyrins in faeces and fur. Moreover most common biomarkers, such as esterases, clinical biochemistry parameters, etc. can also be applied in these animals.

State of the art of non-destructive biomarkers in marine mammals

Studies in which these methods were used to evaluate toxicological risk due to man-made contaminants in populations of marine mammals have been few. Most of these studies are summarized below. The data of stranded, captive and free-ranging mammals will be discussed separately.

Stranded specimens

Several papers are focused on the study on MFO activity in marine mammals. In short-finned pilot whales (*Globicephala macrorhyncus*), striped dolphins (*Stenella coeruleoalba*) and killer whales (*Orcinus orca*) (Watanabe *et al.* 1989) and in minke whales (*Balaenoptera acutorostrata*) (Goksøyr *et al.* 1985) killed by hunting, 7-ethoxyresorufin-O-deethylase (EROD) microsomal activity was fou

Authors	Species	Number of specimens	Sampling	Biomarker	Tissues	Non-destructive	Destructive
Betti and Nigro 1996	Bottlenose dolphin (*Tursiops truncatus*)	1	Reared at the Adratic Sea World	Comet Assay	Blood	+	
Carvan *et al.* 1995	Bottlenose dolphin (*Tursiops truncatus*)	1	Spontaneously aborted	BaP–DNA adduct levels; DNA excision repair	Epithelial cell line from kidney	+	
Fossi *et al.* 1992	Striped dolphin (*Stenella coerueoalba*)	7	Free-ranging	BPMO activity	Biopsy	+	
	Fin whale (*Balaenoptera physalus*)	9					
Goksøyr *et al.* 1986	Minke whale (*Balaenoptera acutorostrata*)	7	Caught in 1983	Microsomal cytochrome P-450 system	Liver		+
Goksøyr *et al.* 1988	Minke whale (*Balaenoptera acutorostrata*)	10	Caught in 1985	Cytochrome P-450 transferase activities	Liver, kidney		+
Koopman *et al.* 1995	Harbour porpoise (*Phocoena phocoena*)	31	Released from herring weirs	Blood chemistry values	Blood	+	
Lahvis *et al.* 1995	Bottlenose dolphin (*Tursiops truncatus*)	5	Free-ranging	Cellular immune function	Blood	+	
Marsili *et al.* 1996	Striped dolphin (*Stenella coerueoalba*)	18	Free-ranging	BPMO activity	Biopsy	+	
	Fin whale (*Balaenoptera physalus*)	14					
Marsili 1995	Striped dolphin (*Stenella coerueoalba*)	1	Dying at a mammal rehabilitation centre	MFO activity	Liver	+	
Martineau *et al.* 1994	Beluga whale (*Delphinapterus leucas*)	9	Stranded in the period 1983–1990	DNA adducts	Brain, liver	+	
Murk *et al.* 1994	Harbour porpoise (*Phocoena phocoena*)	1	Dying at a mammal rehabilitation centre	EROD activity	Liver	+	
Nigro and Leonzio 1996	Bottlenose dolphin (*Tursiops truncatus*)	4	Stranded	HgSe granules	Liver	+	
	Risso's dolphin (*Risso's Grampus griseus*)	4					
Ray *et al.* 1991	Beluga whale (*Delphinapterus leucas*)	18	14 hunted and 4 stranded	DNA adducts	Liver	+	+
Shugart 1990	Beluga whale (*Delphinapterus leucas*)	5	Stranded	DNA adducts	Brain, liver	+	
Tanabe and Tatsukawa 1992	Short-finned pilot whale (*Globicephala macrorhynchus*)	?	?	EROD	Liver		+
Tanabe *et al.* 1988	Dall's porpoise (*Phocoenoides dalli dalli*)	4	Hunted from 1982 to 1984	PB and MC-type enzyme activities as metabolic Index	Fat		+
	Melon-headed whale (*Peponocephala electra*)	3					
	Striped dolphin (*Stenella coerueoalba*)	2					
	Spinner dolphin (*Stenella longirostris*)	6					
Thomson and Geraci 1985	Bottlenose dolphin (*Tursiops truncatus*)	10 2 given ACTH died	Captured using a net	Levels of cortisol, aldosterone and eosinophils	Blood	+	+
Watanabe *et al.* 1989	Short-finned pilot whale (*Globicephala macrorhynchus*)	33	Caught in 1986	Microsomal cytochrome P-450 system	Liver		+
	Striped dolphin (*Stenella coerueoalba*)	5					
	Killer whale (*Orcinus orca*)	3					
White *et al.* 1994	Beluga whale (*Delphinapterus leucas*)	13	Hunted in 1989	Microsomal cytochrome P-450 system	Liver		+

Table 1. Biomarker studies in cetaceans using destructive and non-destructive sampling methods.

Authors	Species	Number of specimens	Sampling	Biomarker	Tissues	Non-destructive	Destructive
Addison and Brodie 1984	Grey seal (*Halichoerus grypus*)	12	Killed in 1984	EROD	Liver		+
Addison *et al.* 1986	Harbour seal (*Phoca vitulina*)	18	Killed in 1985	MFO activity	Liver, lung, kidney, pancreas		+
Brouwer *et al.* 1989	Harbour seal (*Phoca vitulina*)	24	Housed at a centre for seals	Vitamin A, Thyroid hormone concentrations	Plasma	+	
De Swart *et al.* 1994 De Swart *et al.* 1995a Ross *et al.* 1995a Ross *et al.* 1995b De Swart *et al.* 1995b De Swart *et al.* 1995c De Swart *et al.* 1996	Harbour seal (*Phoca vitulina*)	22	Housed at a centre for seal rehabilitation and research	Natural killer-cell activity Mitogen-induced proliferative T-cell responses Levels of circulating polymorphonuclear granulocytes Clinical chemistry parameters	Blood	+	
Engelhardt 1982	Ringed seal (*Phoca hispida*)	8	Captured by net and hunted	MFO activity Cortisol levels	Plasma, liver, kidney		+
Fossi *et al.* 1996b	Southern sea lion (*Otaria flavescens*)	1	Stranded	MFO activity	Liver	+	
Fossi *et al.* 1996b Fossi unpublished data	Southern sea lion (*Otaria flavescens*)	15	Free-ranging	BPMO activity Porphyrin concentrations Triglyceride levels Esterase activities	Blood, biopsy, fur, excreta	+	
Goksøyr *et al.* 1992	Harp seal (*Phoco groenlandica*) Hooded seal (*Cystophora cristata*)	21 26	Hunted in 1989 and 1990	Cytochrome P-450	Liver		+
Harder *et al.* 1992	Harbour seal (*Phoca vitulina*)	12 (6 dead)	Captured, 10 seals inoculated with a cell culture-propogated PDV isolate	PDV-Antigen Humoral immune response	Different biological material	+	+
Jenssen *et al.* 1994	Grey seal (*Halichoerus grypus*)	17	Free-ranging	Thyroid hormones	Blood	+	
Kendall *et al.* 1992	Harbour seal (*Phoca vitulina*) Grey seal (*Halichoerus grypus*)	35 20	Free-ranging	Thymulin levels	Blood	+	
Lund 1994	Grey seal (*Halichoerus grypus*)	8	Hunted in 1989	Adrenal bioactivation Hydroxylase assay	Adrenal, liver		+
Mazzaro *et al.* 1995a Mazzaro *et al.* 1995b	Northern fur seal (*Callorhinus ursinus*)	10	Housed at an aquarium	Vitamin E Vitamin A_2	Blood	+	
Murk *et al.* 1994	Harbour seal (*Phoca vitulina*)	1	Dying at a mammal rehabilitation centre	EROD activity	Liver	+	
Nigro and Leonzio 1996	Southern sea lion (*Otaria flavescens*)	5	Stranded	HgSe granules	Liver	+	
lafson and Thompson 1974	Grey seal (*Halichoerus grypus*) Northern fur seal (*Callorhinus ursinus*)	1 1	? ?	Metallothionein	Liver	?	?
oss *et al.* 1993	Harbour seal (*Phoca vitulina*)	24	Free-ranging	ELISA Lymphocyte function	Blood	+	
anabe *et al.* 1988	Ribbon seal (*Phoca fasciata*) Largha seal (*Phoca largha*) Harbour seal (*Phoca vitulina*)	5 4 4	Hunted from 1982 to 1984	PB and MC-type enzyme activities as Metabolic Index	Fat		+
ohyama *et al.* 1986	Harbour seal (*Phoca vitulina*)	15	Killed in 1984	Metallothionein	Liver, kidney		+

Table 2. Biomarker studies in pinnipeds using destructive and non-destructive sampling methods.

Biological materials	Biomarkers	Conservation
Liver	MFO activity	0–2 h at –80°C
	DNA damage	0–2 h at –80°C
	Porphyrins	0–2 h at –20°C
	Stress proteins	0–2 h at –80°C
Brain	Acetylcholinesterase	0–2 h at –80°C
	Stress proteins	0–2 h at –80°C
Blood	Blood chemistry	0–2 h at –80°C
	Esterases	0–2 h at –80°C
	Porphyrins	0–2 h at –20°C
Skin	MFO activity	0–2 h at –80°C
Fur	Porphyrins	0–2 h at –20°C

Table 3. Biological materials available for sampling in stranded specimens of marine mammals and the main biomarkers applicable for each tissue.

of magnitude as in the rat, and aldrin epoxidase activity (cytochrome P-450 (CYP)2B) one order of magnitude lower than in rodents. This may explain the high retention of polychlorinated biphenyl (PCB) isomers and congeners with adjacent non-chlorinated *meta* and *para* carbons in biphenyl rings by some species of cetaceans; these PCBs are in fact metabolized by PB-inducible cytochrome P-450 isozymes (Tanabe 1988). Similar results were obtained by Marsili (1995). Comparison of EROD and aldrin epoxidase activities in striped dolphin and rat revealed a metabolic imbalance between high CYP1A–EROD activity and low CYP2A–Aldrin epoxidase activity (Figure 3) (Marsili 1995). The ratio of aldrin to EROD was 0.4 in the dolphin and 8.5 in the rat, in line with the suggestion of Tanabe and Tatsukawa (1992) that animals with a lower ability to metabolize the contaminants (CYP2A) are most vulnerable to their toxic effects. A similar trend of MFO activities was found in a dead southern sea lion (*Otaria flavescens*) from the colony of Mar del Plata in Argentina (Fossi *et al.* 1997). Comparison of its EROD, benzyloxyresorufin-*O*-deethylase (BROD), and pentoxyresorufin-*O*-deethylase (PROD) activities with those of the rat and rabbit showed a metabolic imbalance between high CYP1A enzymes and low CYP2A activity. The BROD/EROD ratios were 0.07 in the sea lion, 2.05 in rat and 6.71 in rabbit. In the same specimen the relationship between the destructive biomarker (benzo(*a*)pyrene monooxygenase (BPMO) in the liver) and the non-destructive biomarker (BPMO in skin biopsy samples) was also investigated. The MFO activity detected in the non-destructive sample (skin biopsy) was only one fifth that in the liver and was found to be suitable for non-destructive studies. The same two isoforms (between 66 and 45 kDa) of cytochrome P-450 were detected by SDS PAGE electrophoresis in the microsomal fraction of liver and skin (C. Savelli, unpublished data).

Hepatic microsomes of two marine mammals environmentally exposed to PCBs: a harbour porpoise (*Phocoena phocoena*) and a harbour seal (*Phoca vitulina*), stranded live on the island of Texel (Netherlands) and dying at a mammal rehabilitation centre respectively, were incubated with ^{14}C-labelled 3,3′,4,4′-tetrachlorobiphenyl (TCB) (Murk *et al.* 1994). These marine mammals metabolized TCB at a rate that correlated with their EROD activity. Moreover, in contradiction to Tanabe *et al.* (1988) who suggested that the

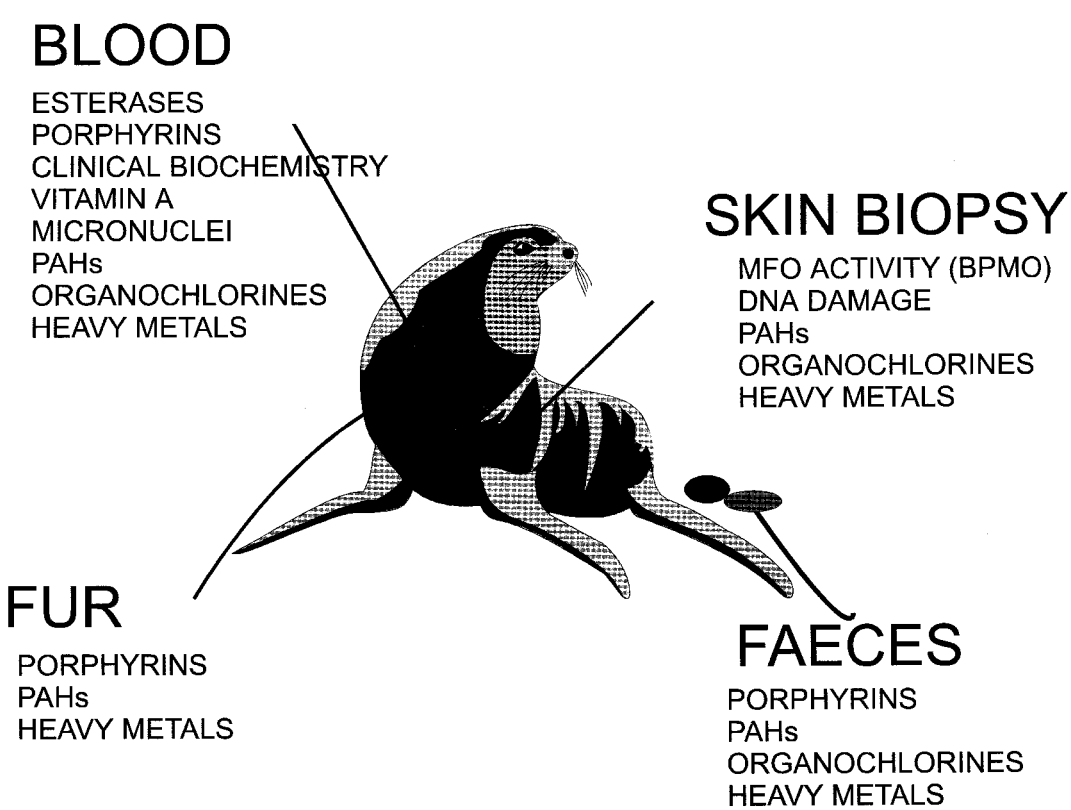

Figure 1. Biological materials obtainable by non-destructive methods in free-ranging pinnipeds, and the respective biomarkers and residu

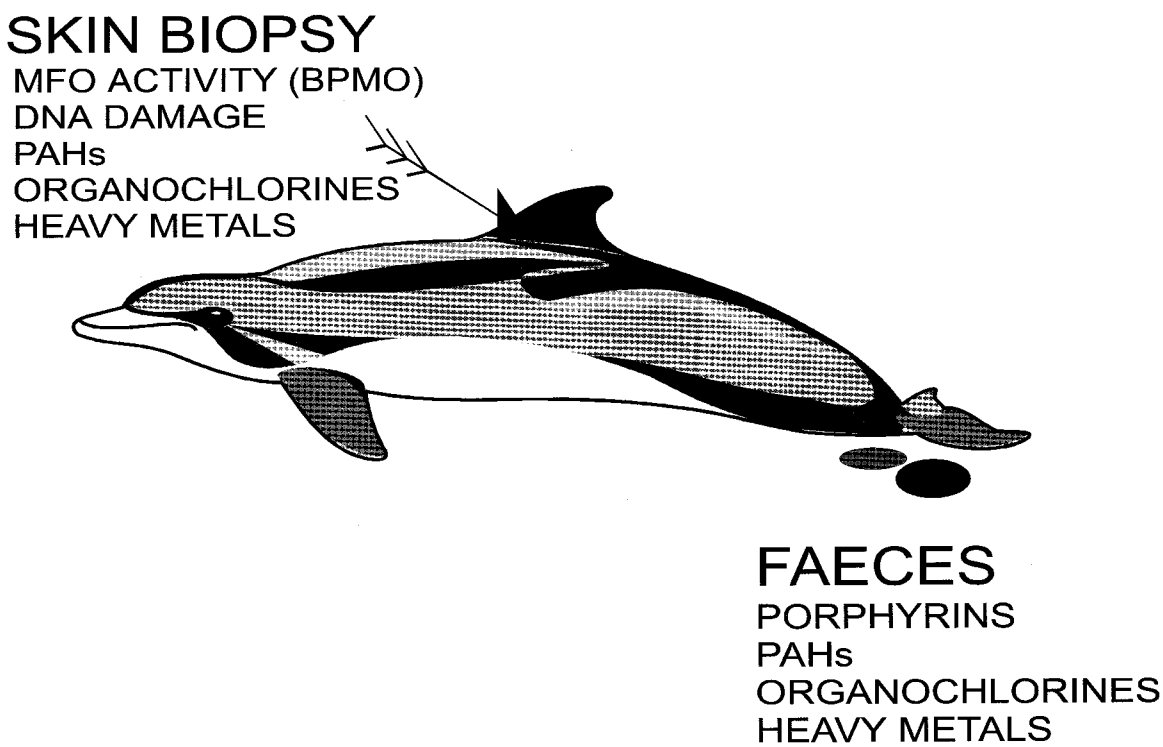

Figure 2. Biological materials obtainable by non-destructive methods in free-ranging cetaceans, and the respective biomarkers and residue analysis.

capacity of small cetaceans to metabolise PCBs is much lower than that of birds and terrestrial mammals, these findings *in vitro* suggest that the metabolic capacity of the environmentally exposed porpoise and seal is similar to that of birds such as the eider duck (*Somateria mollissima*) and about half that of the experimentally induced rat.

Several studies exploring alterations on DNA molecules have been performed on stranded marine mammals. Exposure of an organism to a genotoxic chemical may result in the formation of an attached intermediate covalently bonded to the organism's DNA (DNA adduct). Detection of adducts is therefore a way of documenting exposure. This approach was used with DNA of brain and liver tissues from stranded beluga whales (*Delphinapterus leucas*) of the St Lawrence (highly contaminated area) (Quebec, Canada) and the Mackenzie estuaries (control area) (Canadian Arctic) to determine exposure to benzo(*a*)pyrene (B*a*P) (Shugart 1990). The adducts that form between the DNA and the ultimate carcinogenic form of BaP were detectable in the whales from the St Lawrence estuary but not those from the Mackenzie estuary. Martineau *et al.* (1994) have detected DNA adducts resulting from specific exposure to BaP in eight of the nine St Lawrence belugas, stranded in the period 1983–1990. Adducts were found in six liver tissue samples and seven out of eight brain tissue samples. A similar study was conducted by Ray *et al.* (1991). Aromatic DNA adduct levels were determined in liver tissues of beluga whales from two sites in the Canadian Arctic (East Hudson Bay and Mackenzie Delta) and from the St Lawrence estuary (N.W. Atlantic Ocean). Four liver samples of the St Lawrence belugas were obtained from beached or floating whales dying of natural causes. The other 14 livers were obtained from whales hunted by local Inuit in a subsistence harvest. In contrast to the results of Shugart (1990), detectable levels of aromatic DNA adducts were found in all whales. The presence of adducts in whales from the Canadian Arctic, a relatively clean area, suggests the existence of non pollution-related mechanisms of genetic damage.

The use of cell cultures obtained from stranded organisms represents a powerful tool for non-destructive toxicological studies in these species. To determine the effects of exposure to TCDD (2,3,7,8-tetrachlorodibenzo(*p*)dioxin) and B*a*P in dolphins, an epithelial cell line has been developed from kidney tissues of a spontaneously aborted female bottlenose dolphin (*Tursiops truncatus*) (Carvan *et al.* 1995). Cells pre-treated with TCDD and then exposed to B*a*P exhibited increased B*a*P-DNA adduct levels and increased DNA excision repair. These results indicate that the dolphin cells metabolized B*a*P *in vitro* in relation to cytochrome P450-associated activities and that B*a*P metabolites covalently bound to cellular DNA and initiated excision repair.

The presence of defensive mechanisms toward heavy metals contamination were explored in several species of stranded marine mammals. Selenium and mercury have often been found in a 1:1 molar ratio in the livers of these marine organisms. On the basis of this observation, the interaction with selenium has been supposed to account for the protective effect of this metalloid against mercury toxicity. Martoja and Viale (1977) first reported the presence of mineral granules composed of mercury selenide (HgSe) in the livers of Cuvier dolphins (*Ziphius cavirostris*). Similar granules were later described in the striped dolphin (*Stenella coeruleoalba*) and macrophages were postulated to play a role in mercury selenide biomineralization (Nigro 1994). HgSe was recently reported in the respiratory system of

Biomarkers	Biological effect	Contaminants	Destructive sampling	Non-destructive sampling
DNA alterations				
Chain breakage	Breakage of double helix	PAHs, PHAHs	Different tissues	Blood, skin
Adducts	Adduct formation	PAHs, PHAHs	Different tissues	Blood, skin
Sister chromatid exchanges	Chromosome anomalies	PAHs, PHAHs	Different tissues	Blood
Protein responses				
Esterases	Enzyme inhibition	OPs, CBs	Brain	Blood
MFO	Enzyme induction	PAHs, PHAHs	Liver, kidney	Skin, Mucosa
Stress proteins	Protein induction	Heavy metals, PAHs, PHAHs	Different tissues	Blood
Metallothioneins	Protein induction	Heavy metals	Different tissues	–
ALAD	Enzyme inhibition	Heavy metals (Pb)	–	Blood
Haemoglobin adducts	Protein adduct	PAHs, PHAHs	–	Blood
Blood biochemistry	Modification of various enzymes	Heavy metals, PAHs, PHAHs, OPs	–	Blood
Metabolic products				
Porphyrins	Disruption of haem metabolism	Heavy metals, PHAHs	Liver, kidney	Blood, faeces, fur
Immune system alterations				
Retinol	Change in retinol levels	PHAHs	Liver	Blood
Thyroid function	Change in thyroid hormones	PHAHs	Thyroid	Blood
Immunotoxicology	Various	Heavy metals, PAHs, PHAHs, OPs	Lymphatic cells	Blood

Table 4. Biomarkers applicable in marine mammals.

Key: ALAD=aminolevulinic acid dehydratase; CBs=carbamates; MFO=mixed function oxidase; OPs=organophosphorates, PAHs=polycyclic aromatic hydrocarbons; PHAHs=polyhalogenated aromatic hydrocarbons.

(*Tursiops truncatus*) and short-finned pilot whales (*Globichephala macrorhynchus*), associated with soot particles (Rawson *et al.* 1995). Nigro and Leonzio (1996) found mineral granules consisting of clustered crystalline particles of Hg and Se in stranded cetaceans (bottlenose dolphin – *Tursiops truncatus* and Risso's dolphin – *Risso's Grampus griseus*) and pinnipeds (southern sea lion – *Otaria flavescens*), but not in tuna (*Thunnus thynnus*) and swordfish (*Xiphias gladius*). They were mainly located in the cytoplasm of macrophages. These results suggest that the biosynthesis of mineral granules containing selenium and mercury is common in top marine predators and that excretion of organic mercury might influence the amount of mercury and selenium stored as mineral granules in a particular species.

Animals in captivity

Most of the studies in live animals have been with aquarium mammals, born wild or in captivity. Some of these studies are reported below because they help to validate certain non-destructive biomarkers in marine mammals, however they are not studies in free-ranging populations and the animals are not in natural conditions, being in captivity.

A large number of these studies are mainly based on the collection of blood samples. The activity associated with capturing, restraining and removing bottlenose dolphins (*Tursiops truncatus*) from water stimulates a stress response which is reflected by circulating levels of cortisol, aldosterone and eosinophils (Thomson and Geraci 1985). To capture a dolphin it was corralled into a small area of the sea-pen using a net. The animal could then be restrained manually around the thorax and placed on a stretcher. Blood for haematology was collected from the flukes or dorsal fin by syringe or Vacutainer assembly. Serum cortisol increased from resting levels of about 30 to 110 nmol l^{-1} within 1 h, aldosterone rose from less than 280 pmol l^{-1} to up to 1880 pmol l^{-1} within 3 h, and circulating eosinophils were depressed to less than 40% of their initial numbers within 7 h of removing the animals from the water (calm-capture). This basic response was not enhanced when the capture procedure was prolonged for 3 h (chase-capture) or when the dolphins were given adrenocorticotropic hormone (ACTH) and was similar to that observed in free-ranging dolphins which had been held in a net for up to 5 h. Two dolphins given ACTH died. Dolphins may have a unique sensitivity to stimulation by this hormone. This type of sensitivity may account for often fatal consequences associated with capturing and confining certain species, such as harbour porpoise (*Phocoena phocoena*) (Dudok van Heel 1962), Dall's porpoise (*Phocoenoides dalli*) (Ridgway 1966) and common dolphin (*Delphinus delphis*) (Walker 1975). The rise in cortisol is temporally and consistently associated with an immediate and steady decline in eosinophils. This suggests that eosinophil number is a consistent and practical indicator of stress in dolphins.

Reduction of plasma retinol and thyroid hormone has been used as a sensitive biological effect of PCBs and related environmental pollutants. Brouwer *et al.* (1989) investigated the effect of PCB-contaminated fish on plasma retinol (vitamin A) and thyroid hormone concentrati

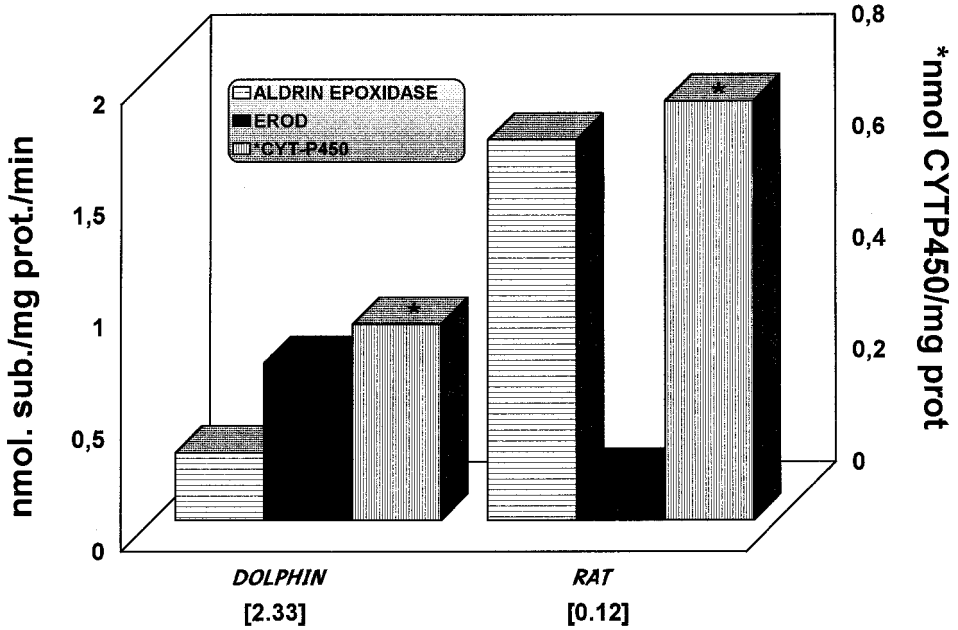

Figure 3. Comparison between microsomal hepatic 7-ethoxyresorufin *O*-deethylase (EROD), Aldrin epoxidase activity and cytochrome-P-450 levels in feral striped dolphin (*Stenella coeruleoalba*) and laboratory outbred rat (*Rattus norvegicus*), Wistar breed. EROD/Aldrin epoxidase ratio in brackets (Marsili 1995).

(*Phoca vitulina*). Seals feeding on fish with high PCB levels showed a drastic reduction in plasma retinol concentrations with respect to seals fed fish with low PCB levels. Significant reductions in plasma total and free thyroxin (TT4 and FT4) and triiodothyronin (TT3) were also observed in the seals with the high PCB diet. The reduced plasma levels of retinol and thyroid hormones were accompanied by significantly reduced reproductive success in these seals (Reijnders 1986).

De Swart *et al.* (1994) reported the results of a prospective study under semifield conditions, in which two groups of harbour seals (*Phoca vitulina*) were fed herring from marine regions with different contamination levels: the highly polluted Baltic Sea and the relatively unpolluted Atlantic Ocean. The seals were housed at a centre for seal rehabilitation and research, in two similar basins. Over a period of 93 weeks, parameters related to immune function were monitored and compared in the two groups. Natural killer-cell activity and mitogen-induced proliferative T-cell responses in the seals fed with Baltic herring were significantly lower. Higher levels of circulating polymorphonuclear granulocytes were also observed which suggest more frequent bacterial infections. This paper was the first demonstration of impaired immunological function in mammals associated with chronic exposure to environmental contaminants accumulated through the marine food chain. Further details on impairment of immune responses in seals fed contaminated Baltic herring may be found in papers by De Swart *et al.* (1995a) and Ross *et al.* (1995a, b). In a 2-week fasting experiment performed at the end of the feeding study, mobilization of organochlorines from the blubber did not lead to a large increase in contaminant levels in the blood, and enhancement of the existing immunosuppression was not observed. These results demonstrate that chronic exposure to environmental contaminants accumulated through the food chain affects immune function in harbour seals, and short fasting periods, which are normal for seals, do not seem to pose additional risk (De Swart *et al.* 1995b). A full set of routine diagnostic parameters was evaluated in the same seals to ascertain that the effects measured by specific immunological assay were due to the chemicals under investigation and not to nutritional status, impaired protein synthesis, or stress (De Swart *et al.* 1995c). The clinical chemistry parameters were found to be insensitive to the effects of chronic exposure, but clear alterations in haematology profiles were observed. The most striking finding was an increase in neutrophil counts in the Baltic group. All the experiments are reviewed in De Swart *et al.* (1996).

Experiments have been conducted to determine the effect of vitamin A supplementation on serum vitamin E in captive adult female northern fur seals (*Callorhinus ursinus*) (Mazzaro *et al.* 1995a). Serum vitamin E, a biochemical index of vitamin E nutritional status, decreased significantly in animals receiving high-level vitamin A supplements (53 μmoles per day). Aquariums should therefore use lower levels of vitamin A supplementation. In two of these seals, vitamin A_2 (3,4-didehydroretinol), a natural analogue of retinol, was used to determine the plasma kinetics of vitamin A (Mazzaro *et al.* 1995b). The objective was to determine whether vitamin A supplementation alters the plasma kinetics of vitamin A_2. If plasma kinetic values are not altered, then vitamin A supplements may not be needed. The northern fur seals in this study consumed between 7.0 and 8.5 μmol of vitamin A per day in their diet. Based on the plasma kinetics observed, Mazzaro's group calculated the PLUS for vitamin A to be 1.3–6.4 μmol per day. Based on the vitamin A content of whole fish, the diet provided to most pinnipeds in captivity will meet their vitamin A requirements. Provi

amounts could lead to oversupplementation and even vitamin A toxicity.

Betti and Nigro (1996) used single cell microgel electrophoresis (Comet assay) to evaluate the genetic effects of methyl-mercury in dolphin (*Tursiops truncatus*) lymphocytes in the dose range 1–8 μg ml^{-1}. The doses of methyl-Hg used in this study were calculated to be the range of concentrations occurring naturally in the blood of wild dolphins. Samples of blood were obtained from a 15-year-old male bottlenose dolphin reared at the Adriatic Sea World (Riccione, Italy). *In vitro* exposure to methyl-Hg induced single-strand DNA breaks and cytotoxicity in a dose-dependent manner. Dolphin lymphocytes were found to have greater resistance to the genotoxic effects of methyl-Hg than human and rats cells.

Free-ranging specimens

As previously mentioned, the most important studies concern healthy free-ranging animals, which can be sampled without killing or disturbance. The non-destructive approach for free-ranging cetaceans and pinnipeds has been developed and validated in the following manner. Fossi *et al.* (1992) proposed non-destructive techniques based on skin biopsy to evaluate residue levels (OCs and heavy metals) and biochemical responses to xenobiotic compounds (BPMO) in two species of Mediterranean cetaceans: striped dolphin (*Stenella coeruleoalba*) and fin whale (*Balaenoptera physalus*). These animals occupy different positions in the marine food chain and are therefore exposed to different levels of risk in relation to organochlorine bioaccumulation. The striped dolphin has recently had a high mortality along the Mediterranean coasts (Aguilar and Raga 1993). The main results of this study concerned BPMO activity in cetacean skin biopsy samples. BPMO activity in the striped dolphin was four times higher than in the fin whale. The two species differed dramatically in levels of DDTs (12 times) and PCBs (9 times) in subcutaneous blubber. The difference in organochlorine bioaccumulation and consequently in BPMO induction between the two species was related by the authors to their different positions in the marine food chain, the dolphin having a fish diet and the whale feeding on macroplankton. Plotting organochlorines against BPMO activity, two species-specific families of points emerged showing a trend of increasing enzyme activity with increasing levels of contamination. These results were confirmed in skin biopsy samples of the same species in 1992–1993 (Marsili *et al.* 1996). Again, two clusters of points characterized by low levels of organochlorines and low levels of BPMO in the whale and high levels of contaminants and high levels of biomarker responses in the dolphin, were obtained.

The non-destructive biomarker approach was recently used to assess the health status of endangered populations of pinnipeds, the southern sea lions (*Otaria flavescens*) in South America. A colony living in a heavily polluted area (Mar del Plata harbour, Argentina) and a colony living in a control area located in a pristine environment (Punta Bermeja, Patagonia, Argentina) were the subject of this preliminary study (Fossi *et al.* 1997). Blood, skin biopsy specimens, fur and excreta were obtained in a non-invasive way by anaesthetizing the sea lions with a mixture of ketamine and xylazine. These biological materials were analysed for a wide range of biomarkers and residues. BPMO activity in skin biopsy samples from the Mar del Plata colony was four times higher than in the one specimen from the control area. The two groups of samples also differed dramatically in levels of DDTs and PCBs. Moreover the differences in BPMO activity in skin biopsy samples from the two colonies were probably due to different PAH levels. The five most carcinogenic PAHs in the skin biopsy samples (Fossi *et al.* 1996b) were several times higher in the Mar del Plata colony. The same trend was confirmed in two fur samples with low PAH levels in the Patagonia sample and high levels in the Mar del Plata sample (Marsili *et al.* 1997). Porphyrin profiles from fur samples have yet to be reported (S. Casini, unpublished data). Porphyrin concentrations in excreta of the Mar del Plata colony, especially protoporphyrin and total porphyrins, were approximately double those in the control colony. This porphyrin disorder is probably related to heavy metal contamination in the harbour. In fact Fossi *et al.* (1997) found high levels of lead, mercury and cadmium in fur samples of these sea lions compared with the control colony. Triglyceride levels in serum samples of the Mar del Plata colony were approximately one-third those of the control colony. Esterase activities in blood samples were slightly lower in the polluted colony.

Comparative blood chemistry can be an important diagnostic and prognostic tool for evaluating the health of marine mammals. Blood chemistry values have been measured in 31 porpoises (*Phocoena phocoena*) released from herring weirs in the Bay of Fundy (Canada) (Koopman *et al.* 1995). Glucose, potassium, creatine kinase, aspartate aminotransferase, haemoglobin, thyroxin, bilirubin and alkaline phosphatase had generally higher activities than those reported for captive and stranded odontocetes.

Jenssen *et al.* (1994) examined the possibility of using blood as a matrix for determining exposure of grey seal pups (*Halichoerus grypus*) to organochlorine compounds from Froan (Norway) and to elucidate correlations between pollutant load and biological variables (age and body mass). Although the two pups with the highest ∑PCB concentrations also had the lowest plasma concentrations of thyroxin, there was no significant correlation between ∑PCB concentrations in blood cells and the corresponding plasma concentrations of any of the thyroid hormones. They concluded that blood-sampling is a good non-destructive method for monitoring OC compounds in seals, and that the use of thyroid hormones as biomarkers should be examined further.

Lahvis *et al.* (1995) reported an inverse correlation between blood levels of polyhalogenated aromatic hydrocarbons (PHAHs) and cellular immune function in five free-ranging bottlenose dolphins (*Tursiops truncatus*).

Immune function in harbour seal (*Phoca vitulina*) mothers and their pups during lactation was studied on Sable Island (Nova Scotia, Canada) (Ross *et al.* 1993). Methods included total white blood cell and differential counts, a Protein A enzyme linked immunosorbent assay (ELISA) for total immunoglobulin G (IgG) quantificat

function testing *in vitro* using the T-cell mitogen concanavalin A (ConA). Lymphocyte function and total IgG levels were reduced in the mothers at the end of lactation, suggesting a reduction in immune function, possibly as a result of the stress of fasting, or hormonal changes associated with lactation and oestrus. By contrast, lymphocyte function and total IgG levels in pups were low at birth and higher at the end of lactation. Pups at birth and females late in lactation may therefore be more susceptible to infection by viral and bacterial agents.

Blood samples of live common seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) from the coasts of Scotland and Northern Ireland obtained during, and immediately after, an epizootic caused by phocid distemper virus (PDV) were analysed for thymulin content (Kendall *et al.* 1992). Thymulin levels were compared with neutralization titres and concentrations of OCs derived from blood and blubber samples obtained by non-invasive methods from the same animals. Thymulin levels were negatively correlated with the logarithm of virus neutralization titre in grey seals. In common seals they varied significantly between age classes. There was no direct relationship between thymulin and contaminant levels in either species. However, when an estimate of time since exposure was included in the regression analysis for common seals, there was a highly significant relationship between thymulin and the two chlorinated biphenyl congeners, the 22′44′55′-hexachlorobiphenyl and the 22′344′55′-heptachlorobiphenyl (IUPAC numbers 153 and 180) with the highest concentrations in blubber. This paper shows that thymulin levels in wild animals provide some indication of individual immune status.

Conclusions

The conclusions of this review on non-destructive biomarkers in marine mammals can be summarized as follows.

Several tissues and biological materials such as skin, fur, blood and faeces, are confirmed to be useful for non-destructive biomarker studies in stranded and free-ranging marine mammals.

A large number of biochemical, molecular and genotoxic biomarkers were found to be detectable and suitable for use in non-destructive samples. Biomarkers such as BPMO in skin, DNA adducts in stranded animals, vitamin A and thyroxin levels in blood and porphyrins in excreta, seem to be particularly useful for demonstrating toxicologically significant contamination of marine mammals.

In some cases a link between cause (environmental contamination) and effect (biomarker responses at different levels of biological organisation) can be established (Brouwer *et al.* 1989, Shugart 1990, De Swart *et al.* 1994, Fossi *et al.* 1997).

The present development and validation of the non-destructive approach in stranded, captive and free-ranging specimens makes it scientifically and ethically unjustified to use marine mammals obtained by hunting for scientific purposes. The use of hunted animals amounts to tacit consent to hunting.

References

Addison, R.F. and Brodie, P.F. (1984) Characterization of ethoxyresorufin *O*-deethylase in grey seal *Halichoerus grypus*. *Comparative Biochemistry and Physiology*, **79C**(2), 261–263.

Addison, R.F., Brodie, P.F., Edwards, A. and Sadler, M.C. (1986) Mixed function oxidase activity in the harbour seal (*Phoca vitulina*) from Sable Is., N.S. *Comparative Biochemistry and Physiology*, **85C**(1), 121–124.

Aguilar, A. and Borrell, A. (1994) Assessment of organochlorine pollutants in cetaceans by means of skin and hypodermic biopsies. In *Nondestructive Biomarkers in vertebrates*, **11**, M.C. Fossi and C. Leonzio, eds (Lewis Publishers, Florida), pp. 245–267.

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

Betti, C. and Nigro, M. (1996) The comet assay for the evaluation of the genetic hazard of pollutants in cetaceans: preliminary results on the genotoxic effects of methyl mercury on the bottle-nosed dolphin (*Tursiops truncatus*) lymphocytes *in vitro*. *Marine Pollution Bulletin*, **32**(7), 545–548.

Boon, J.P., Van Arnhem, E., Jansen, S., Kannan, N., Petrick, G., Schultz, D., Duinker, J.C., Reijnders, P.J.H. and Goksøyr, A. (1992) The toxicokinetics of PCBs in marine mammals with special reference to possible interactions of individual congeners with the cytochrome P-450-dependent monooxigenase system: an overview. In *Persistent Pollutants in Marine Ecosystems*, **6**, C. H. Walker and D. R. Livingstone, eds (Pergamon Press Ltd, Oxford) pp. 119–159.

Brouwer, A., Reijnders, P.J.H. and Koeman, J.H. (1989) Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). *Aquatic Toxicology*, **15**, 99–106.

Carvan III, M.J., Flood, L.P., Campbell, B.D. and Busbee, D.L. (1995) Effects of benzo(*a*)pyrene and tetrachlorodibenzo(*p*)dioxin on fetal dolphin kidney cells: inhibition of proliferation and initiation of DNA damage. *Chemosphere*, **30**(1), 187–198.

De Swart, R.L., Ross, P.S., Vedder, L.J., Timmerman , H.H., Heisterkamp, S., Van Loveren, H., Vos, G.J., Reijnders, P.J.H. and Osterhaus, A.D.M.E. (1994) Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. *Ambio*, **23**, 155–159.

De Swart, R.L., Ross, P.S., Timmerman, H.H., Vos, H.W., Reijnders, P.J.H., Vos, G.J. and Osterhaus, A.D.M.E. (1995a) Impaired cellular immune response in harbour seals (*Phoca vitulina*) feeding on environmentally contaminated herring. *Clinical and Experimental Immunology*, **101**, 480–486.

De Swart, R.L., Ross, P.S., Timmerman, H.H., Hjman, W.C., De Ruiter, E., Liem, A.K.D., Brouwer, A., Van Loveren, H., Reijnders, P.J.H., Vos, G.J. and Osterhaus, A.D.M.E. (1995b) Short-term fasting does not aggravate immunosuppression in harbour seals (*Phoca vitulina*) with high body burdens of organochlorines. *Chemosphere*, **31**, 4289–4306.

De Swart, R.L., Ross, P.S., Vedder, L.J., Boink, F.B.T.J., Reijnders, P.J.H., Mulder, P.G.H. and Osterhaus, A.D.M.E. (1995c) Haematology and clinical chemistry values of harbour seals (*Phoca vitulina*) fed environmentally contaminated herring remain within normal ranges. *Canadian Journal of Zoology*, **73**, 2035–2043.

De Swart, R.L., Ross, P.S., Vos, G.J. and Osterhaus, A.D.M.E. (1996) Impaired immunity in harbour seals (*Phoca vitulina*) exposed to bioaccumulated environmental contaminants: review of a long-term feeding study. *Environmental Health Perspectives*, **104**(4), 823–828.

Dudok van Heel, W.H. (1962) Catching *Phocoena phocoena* for scientific purposes. *Bulletin Institute Oceanographic, Monaco*, Special No. IA, 23–27.

Engelhardt, F.R. (1982) Hydrocarbon metabolism and cortisol balance in oil-exposed ringed seals, *Phoca hispida*. *Comparative Biochemistry and Physiology*, **72C**(1), 133–136.

Fossi, M.C., Marsili, L., Leonzio, C., Notarbartolo di Sciara, G., Zanardelli, M. and Focardi, S. (1992) The use of non-destructive biomarker in Mediterranean Cetaceans: preliminary data on MFO activity in skin biopsy. *Marine Pollution Bulletin*, **24**(9), 459–461.

Fossi, M.C. (1994) Nondestructive biomarkers in ecotoxicology. *Environmental Health Perspectives*, **102**(12), 49–54.

Fossi, M.C. and Leonzio, C. (eds) (1994) *Nondestructive Biomarkers in Vertebrates* (Lewis Publishers, Florida).

Fossi, M.C. and Marsili, L. (1996) L'utilizzo dei biomarkers non distruttivi nello studio dei Mammiferi marini. *Societa' Italiana di Ecologia*, **17**, 387–390.

Fossi, M.C., Marsili, L., Castello, H. and Junin,

biomarkers in the hazard assessment of endangered species of marine mammals. *Sixth SETAC-Europe Annual Meeting*, 19–22 May 1996, Taormina, Italy.

Fossi, M.C., Castello, H., Junin, M., Lorenzani, J.A., Lorenzani, J., Marsili, L., Leonzio, C., Casini, S., Savelli, C., Lari, L. and Monaci, F. (1996b) Use of a nondestructive biomarker approach for hazard assessment in sea lion (*Otaria flavescens*) populations in the Southwest Atlantic. *Sixth SETAC-Europe Annual Meeting*, 19–22 May 1996, Taormina, Italy.

Fossi, M.C., Marsili, L., Junin, M., Castello, H., Lorenzani, J.A., Casini, S., Savelli, C. and Leonzio, C. (1997) Use of nondestructive biomarkers and residue analysis to assess the health status of endangered species of pinnipeds in the southwest Atlantic. *Marine Pollution Bulletin* (in press).

Geraci, J.R. and St Aubin, D.J. (1980) Offshore petroleum resource development and marine mammals: a review and research recommendations. *Marine Fishery Review*, **42**, 1–12.

Goksøyr, A., Tarlebø, J., Solbakken, J.E. and Klungsøyr, J. (1985) Characteristics of the hepatic microsomal cytochrome P-450- system of the Minke whale (*Balaenoptera acutorostrata*). *Marine Environmental Research*, **17**, 113–116.

Goksøyr, A., Solbakken, J.E. Tarlebø, J. and Klungsøyr, J. (1986) Initial characterization of the hepatic microsomal cytochrome P-450-system of the piked whale (Minke*) Balaenoptera acutorostrata. Marine Environmental Research*, **19**, 185–203.

Goksøyr, A., Andersson, T., Förlin, L., Stenersen, J., Snowberger, E.A., Woodin, B.R. and Stegeman, J.J. (1988) Xenobiotic and steroid metabolism in adult and foetal piked (Minke) whales, *Balaenoptera acutorostrata. Marine Environmental Research*, **24,** 9–13.

Goksøyr, A., Beyer, J., Larsen, H.E., Andersson, T. and Förlin, L. (1992) Cytochrome P-450 in seals: monooxygenase activities, immunochemical cross-reaction and response to phenobarbital treatment. *Marine Environmental Research*, **34**, 113–116.

Harder, T.C., Willhaus, T., Leibold, W. and Liess, B. (1992) Investigations on course and outcome of phocine distemper virus infection in harbour seals (*Phoca vitulina*) exposed to polychlorinated biphenyls. *Journal of Veterinary Medicine B*, **39**, 19–31.

Jenssen, M.B., Skaare, U.J., Ekker, M., Vongraven, D. and Silverstone, M. (1994) Blood sampling as a non-destructive method for monitoring levels and effects of organochlorines (PCB and DDT) in seals. *Chemosphere,* **28**(1), 3–10.

Kendall, M.D., Safieh, B., Harwood, J. and Pomeroy, P.P. (1992) Plasma thymulin concentrations, the thymus and organochlorine contaminant levels in seals infected with phocine distemper virus. *Science of the Total Environment*, **115**,133–144.

Koopman, H.N., Westgate, A.J., Read, A.J. and Gaskin, D.E. (1995) Blood chemistry of wild harbor porpoises *Phocoena phocoena* (L.). *Marine Mammal Science,* **11**(2),123–135.

Lahvis, G.P., Wells, R.S., Kuehl, D.W., Stewart, J.L., Rhinehart, H.L. and Via, C.S. (1995) Decreased lymphocyte responses in free-ranging bottlenose dolphins (*Tursiops truncatus*) are associated with increased concentrations of PCBs and DDT in peripheral blood. *Environmental Health Perspectives*, **103**(4), 67–72.

Lambertsen, R.H. (1987) A biopsy system for large whales and its use for cytogenetics. *Journal of Mammals*, **68**(2), 443.

Lambertsen, R.H., Baker, C.S., Weinrich, M. and Modi, W.S. (1994) An improved whale biopsy system designed for multidisciplinary research. In *Nondestructive Biomarkers in Vertebrates*, **10**, M.C. Fossi and C. Leonzio, eds (Lewis Publisher, Florida), pp. 219–244.

Leonzio, C., Focardi, S. and Fossi, M.C. (1992) Heavy metals and selenium in stranded dolphins of the northern Tyrrhenian (NW Mediterranean*). The Science of the Total Environment*, **119**, 77–84.

Lund, B.O. (1994) In vitro adrenal bioactivation and effects on steroid metabolism of DDT, PCBs and their metabolites in the gray seal (*Halichoerus gripus*). *Environment Toxicology and Chemistry*, **13**(6), 911–917.

Marcovecchio , J.E., Gerpe, M.S., Bastida, R.B., Rodriguez, D.H. and Moron, S.G. (1994) Environmental contamination and marine mammals in coastal waters from Argentina: an overview. *Science of the Total Environment*, **154**, 141–151.

Marsili, L. (1995) Indagini ecotossicologiche e morfometriche su alcune specie di Cetacei del Mediterraneo. PhD Thesis, Dipartimento di Biologia Ambientale, Universita' di Siena, Italy.

Marsili, L. and Focardi, S. (1996) Organochlorine levels in subcutaneous blubber biopsies of fin whales (*Balaenoptera physalus*) and striped dolphins (*Stenella coeruleoalba*) from the Mediterranean Sea. *Environmental Pollution*, **91**(1), 1–9.

Marsili, L. and Focardi, S. (1997) Chlorinated hydrocarbon (HCB, DDTs and PCBs) levels in cetaceans stranded along the Italian coasts: an overview. *Environmental Monitoring and Assessment* (in press).

Marsili, L., Fossi, M.C., Notarbartolo di Sciara, G., Zanardelli, M. and Focardi, S. (1996) Organochlorine levels and mixed function oxidase activity in skin biopsy specimens from Mediterranean cetaceans. *Fresenius Environmental Bulletin* **5**(9–10), 723–728.

Marsili, L., Fossi, M.C., Casini, S., Savelli, C., Jimenez, B., Junin, M. and Castello, H. (1997) Fingerprint of polycyclic aromatic hydrocarbons in two populations of southern sea lions (*Otaria flavescens*). *Chemosphere*, **34**(4), 759–770.

Martin, A.R., Donovan, G.P., Hammond, P.S., Hohn, A.A., Jefferson, T.A., Leatherwood, S., Lockyer, C.H., Mead, J.G., Reeves, R.R., Ross, G.J.B. and Webber, M.A. (1990) *Whales and Dolphins* (Salamander Books Ltd, London, UK).

Martineau, D., De Giuse, S., Fournier, M., Shugart, L., Girard, C., Lagacé, A. and Béland, P. (1994) Pathology and toxicology of beluga whales from the St. Lawrence estuary, Quebec, Canada. Past, present and future. *The Science of the Total Environment* **154**, 201–215.

Martoja, R. and Viale, D. (1977) Accumulation de granules de séléniure mercurique dans le foie d'Odontocètes (Mammifères, Cétacés): un mécanisme possible de détoxication du méthilmercure par le sélénium. *Comptes Rendus Hebdomadaires des Séances de l'Academie des Sciences de Paris. Serie D: Sciences Naturelles*, **285**, 109–112.

Mazzaro, L.M., Dunn, J.L., Furr, H.C. and Clark, R.M. (1995a) Study of vitamin A supplementation in captive northern fur seals (*Callorhinus ursinus*) and its effect on serum vitamin E. *Marine Mammal Science*, **11**(4), 545–553.

Mazzaro, L.M., Dunn, J.L., Furr, H.C. and Clark, R.M. (1995b) Vitamin A plasma kinetics in northern fur seals (*Callorhinus ursinus*), using vitamin A_2. *Canadian Journal of Zoology*, **73**, 10–14.

Murk, A., Morse, D., Boon, J. and 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. *European Journal of Pharmacology, Environmental Toxicology and Pharmacology Section*, **270**, 253–261.

Neff, J.M., Cox, B.A., Dixit, D. and Anderson, J.W. (1976) Accumulation and release of petroleum-derived aromatic hydrocarbons by four species of marine animals. *Marine Biology*, **38**, 279–289.

Nigro, M. (1994) Mercury and selenium localization in macrophages of the striped dolphin, *Stenella coeruleoalba*. *Journal of the Marine Biological Association of the United Kingdom*, **74**, 975–978.

Nigro, M. and Leonzio, C. (1996) Intracellular storage of mercury and selenium in different marine vertebrates. *Marine Ecology Progress Series*, **135**, 137–143.

Olafson, R.W. and Thompson, J.A.J. (1974) Isolation of heavy metal binding proteins from marine vertebrates. *Marine Biology*, **28**, 83–86.

Rawson, A.J., Bradley, J.P., Teetsov, A., Rice, S.B., Haller, E.M. and Patton, G.W. (1995) A role for airborne particulate in high mercury levels of some cetaceans. *Ecotoxicology and Environmental Safety*, **30**, 309–314.

Ray, S., Dunn, B.P., Payne, J.F., Fancey, L., Helbig, R. and Béland, P. (1991) Aromatic DNA–carcinogenic adducts in beluga whales from the Canadian Arctic and Gulf of St Lawrence. *Marine Pollution Bulletin*, **22**(8), 392–396.

Reijnders, P.J.H. (1986) Reproductive failure in common seals feeding on fish from polluted coastal waters. *Nature*, **324**, 456–457.

Ridgway, S.H. (1966) Dall porpoise, *Phocoenoides dalli* (True): observations in captivity and at sea. *Norsk. Hvalfangst-Tidende*, **5**, 97–110.

Ross, P.S., Pohajdak, B., Bowen, W.D. and Addison, R.F. (1993) Immune function in free-ranging harbor seal (*Phoca vitulina*) mothers and their pups during lactation. *Journal of Wildlife Diseases*, **29**(1), 21–29.

Ross, P.S., De Swart, R.L., Reijnders, P.J.H., Van Loveren, H., Vos, G.J. and Osterhaus, A.D.M.E. (1995a) Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbor seals feed herring from the Baltic Sea. *Environmental Health Perspectives*, **103**, 162–167.

Ross, P.S., De Swart, R.L., Timmerman , H.H., Vedder, L.J., Van Loveren, H., Vos

G.J., Reijnders, P.J.H. and Osterhaus, A.D.M.E. (1995b) Suppression of natural killer cell activity in harbour seals (*Phoca vitulina*) fed Baltic Sea herring. *Aquatic Toxicology*, **34**, 71–84.

Shugart, L.R. (1990) Detection and quantification of benzo(*a*)pyrene–DNA adducts in brain and liver tissues of beluga whales (*Delphinapterus leucas*) from the St. Lawrence and Mackenzie estuaries. In *Proceedings of the International Forum for the Future of the Beluga*. (Presses de l'Université du Quebec, Quebec) pp. 219–223.

Shugart, L.R., Peakall, D.B., Walker, C.H. and Everaarts, J.M. (1993) Overview. In *Biomarkers*, D.B. Peakall and L.R. Shugart, eds (Springer-Verlag, Berlin, Heidelberg), NATO ASI Series, Cell Biology, **H68**.

Tanabe, S. (1988) PCB problems in the future: foresight from current knowledge. *Environmental Pollution*, **50**, 5–28.

Tanabe, S. and Tatsukawa, R. (1992) Chemical modernization and vulnerability of cetaceans: increasing toxic threat of organochlorine contaminants. In *Persistent Pollutants in Marine Ecosystems*, **7**, C. H. Walker and D. R. Livingstone, eds (Pergamon Press Ltd, Oxford) pp. 161–177.

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

Tanabe, S., Watanabe, S., Kan, H. and Tatsukawa, R. (1988) Capacity and mode of PCB metabolism in small cetaceans. *Marine Mammal Science*, **4**(2), 103–124.

Thomson, C.A. and Geraci, J.R. (1985) Cortisol, aldosterone, and leucocytes in the stress response of bottlenose dolphins, *Tursiops truncatus*. *Canadian Journal of Fisheries and Aquatic Sciences*, **43**, 1010–1016.

Tohyama, C., Himeno, S., Watanabe, C., Suzuki, T. and Morita, M. (1986) The relationship of the increased level of metallothionein with heavy metal levels in the tissue of the harbor seal (*Phoca vitulina*). *Ecotoxicology and Environmental Safety*, **12**, 85–94.

Walker, W.A. (1975) Review of the live-capture fishery for smaller cetaceans taken in the southern California waters for public display, 1966–73. *Journal of the Fisheries Research Board of Canada*, **32**, 1197–1211.

Watanabe, S., Shimada, T., Nakamura, S., Nishiyama, N., Yamashita, N., Tanabe, S. and Tatsukawa, R. (1989) Specific profile of liver microsomal cytochrome P-450 in dolphins and whales. *Marine Environmental Research*, **27**, 51–65.

White, R.D., Hahn, M.E., Lockhart, W.L. and Stegeman, J.J. (1994) Catalytic and immunochemical characterization of hepatic microsomal cytochromes P 450 in beluga whale (*Delphinapterus leucas*). *Toxicology and Applied Pharmacology*, **126**, 45–57.

Received 29 November 1996, revised form accepted 6 March 1997