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Sea Mammal Research Unit and Centre for Biomolecular Sciences
University of St Andrews

**Molecular characterisation of cytochrome
P450 isoenzymes in marine mammals:
their suitability to reflect environmental
exposure to PCBs**

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October 2001

Submitted for the Degree of Doctor of Philosophy



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Abstract

It has been demonstrated that polychlorinated biphenyls (PCBs) have adverse health effects on marine mammals and are inducers of cytochrome P450 isoenzymes (CYP). This research study aimed to investigate marine mammal CYPs as biomarkers of PCB exposure.

Inter-specific and intra-specific differences in total blubber levels of 27 PCB congeners ($\Sigma 27\text{PCB}$) were observed for adult and pup hooded and harp seals. Furthermore a selective transfer of PCBs was apparent from mother to pup. Inter-specific differences were demonstrated for CYP1A-mediated EROD and PROD activities, and apoprotein levels of CYP1A and -2B. However, within each species neither the CYP-monoxygenase (MO) activities nor apoprotein levels correlated with total mono-*ortho* PCBs or sum of 2,3,7,8 TCDD toxic equivalents.

An investigation of the CYP-MO system of harbour porpoise and grey seal was performed, and the PCB levels analysed. EROD and PROD activities were mediated by CYP1A as determined by inhibition studies, and CYP1A and CYP2B were detected by immunoblotting in both species. The $\Sigma 24\text{PCB}$ blubber concentrations reflected their respective EROD, but not PROD activities.

The loss of EROD activities was demonstrated by freezing liver samples for >24 hours. Preparing post-nuclear supernatants prevented the reduction in EROD activities. The preparation of fresh liver prior to storage is a viable fieldwork option.

Harp and grey seal CYP1A1 and -1A2 cDNA were cloned and sequenced and comparative analysis with other mammalian CYP1As was performed. The deduced amino acid sequence data enabled the design and characterisation of species-specific anti-peptide antibodies. A distinct band(s) at ~52kDa was detected by both antibodies in harp, hooded and grey seals and harbour porpoise.

A non-destructive biomarker assay was preliminarily investigated using skin samples from seals. However, interference of the EROD activities by another NADPH-dependent enzyme was observed. A candidate enzyme is NADPH-quinone oxidoreductase.

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Abbreviations

3-MC	3-methylcolanthrene	DRRE	Dexamethasone-repsponsive regulatory element
7ER	7-ethoxyresorufin	DTT	Dithiothreitol
7PR	7-pentoxyresorufin	ECOD	Ethoxycoumarin O-deethylase
A	Adenosine (or adenine)	EDTA	Diaminethanetetra-acetic acid
a.u.	Arbitrary unit	ELISA	Enzyme linked immunosorbent assay
ACTH	Adrenocorticotropic hormone	EROD	Ethoxyresorufin o-deethylase
AEBSF	4-(2-aminoethyl)benzene sulphonyl fluoride	Fe²⁺	Reduced iron ion
AHH	Aryl hydrocarbon hydroxylase	Fe³⁺	Oxidised iron ion
AhR	Aryl hydrocarbon receptor	G	Guanine
α-NF	α-naphthoflavone	HCB	Hexachlorobenzene
Arnt	Aryl hydrocarbon receptor nuclear translocator	HCH	Hexachlorocyclohexane
B(a)P	Benzo(a)pyrene	HCl	Hydrochloric acid
B(a)PMO	Benzo(a)pyrene monooxygenase	HRP	Horse radish peroxidase
β-NF	β-naphthoflavone	hsp90	Heat shock protein 90
bp	Base pairs	IgG	Immunoglobulin G
BSA	Bovine serum albumin	IPTG	β-D-isopropyl-thiogalactopyranoside
C	Cytosine	K₂HPO₄	Di-potassium hydrogen phosphate
CAR	Constitutive androstane receptor	KCl	Potassium chloride
CB	Chlorobiphenyl	KH₂PO₄	Potassium dihydrogen phosphate
cDNA	Complementary DNA	KLH	Keyhole limpet haemocyanin
CO	Carbon monoxide	MgCl₂	Magnesium chloride
CYP	Cytochrome P450	MO	Monooxygenase
Da	Dalton	MRNA	Messenger RNA
δ-ALA	δ-aminolevulinic acid	MSFs	Methylsulphones
DDT	Dichlorodiphenyl trichloroethane	NaCl	Sodium chloride
df	Degrees of freedom	NADH	Nicotinamide adenine dinucleotide reduced form
DMP	Dimethylpimelimidate	NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
DMSO	Dimethylsulfoxide	NaOH	Sodium hydroxide
DNA	Deoxyribonucleic acid	OCs	Organochlorines
DNase	Deoxyribonuclease	PAGE	Polyacrylamide gel electrophoresis
DNTP	Deoxynucleotide triphosphate	PAHs	Polyaromatic hydrocarbons
DRE	Dioxin responsive element	PB	Phenobarbital
		PBDE	Polybrominated diphenyl ether

PBS	Phosphate buffered saline	RT-PCR	Reverse transcriptase polymerase chain reaction
PCA	Principal component analysis		
PCBs	Polychlorinated biphenyls	RXR	Retinoid x receptor
PCDD	Polychlorinated dibenzo dioxin	SAR	Structure activity relationship
PCDF	Polychlorinated dibenzo furan	SDS	Sodium dodecyl sulphate
PCR	Polymerase chain reaction	SE	Standard error
PDV	Phocine distemper virus	T	Thymidine (or thymine)
PES	Phenazine ethosulphate	T6H	Testosterone 6B-hydroxylase
PHAH	Polyhalogenated hydrocarbon	TAE	Tris-acetate-EDTA
PMSF	Phenylmethylsulphonyl fluoride	Taq	<i>Thermus aquaticus</i>
PPARα	Peroxisome proliferator activated receptor	TCDD	Tetrachlorodibenzo-p-dioxin
PPRE	Peroxisome proliferator chemicals response element	TEF	Toxic equivalent factor
PROD	Pentoxoresorufin O-deethylase	TEQ	2,3,7,8-TCDD toxic equivalent
PVDF	Polyvinylidene difluoride	Tm	Melting temperature
PXR	Pregnane X receptor	Tris	Tris-hydroxymethylethylene diamine tetra-acetic acid
QR	Quinone-oxidoreductase	u	Unit
RNA	Ribonucleic acid	X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

Symbols for Amino Acids

A	Ala	Alanine	M	Met	Methionine
B	Asx	Asparagine or Aspartic acid	N	Asn	Asparagine
C	Cys	Cysteine	P	Pro	Proline
D	Asp	Aspartic acid	Q	Gln	Glutamine
E	Glu	Glutamic acid	R	Arg	Arginine
F	Phe	Phenylalanine	S	Ser	Serine
G	Gly	Glycine	T	Thr	Threonine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine	W	Trp	Tryptophan
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine	Z	Glx	Glutamine or glutamic acid

I

General Introduction

Molecular characterisation of cytochrome P450 isoenzymes in marine mammals: their suitability to reflect environmental exposure to PCBs

1.1. Introduction

In recent years the physiological and the putative ecological effects of environmental contaminants in many animals, including marine mammals, has provoked a great deal of concern. Observed physiological effects in marine mammals include reproductive dysfunction (De Guise *et al.*, 1994; DeLong *et al.*, 1973; Reijnders, 1986; Subramanian *et al.*, 1987; Tanabe *et al.*, 1982) immunosuppression (LeBlanc, 1997; Ross, 1996), tumours (De Guise *et al.*, 1994) and effects on thyroid hormone metabolism and retinoid levels (Brouwer *et al.*, 1989; Jenssen, 1995). However, much of the evidence for these effects in marine mammals is associative, and more definitive links between the cause and effect need to be elucidated. Determining molecular and cellular processes that underlie the pathological changes in the whole animal would be one approach.

Marine environmental contaminants include polyaromatic hydrocarbons (PAHs, such as benzo(a)pyrene) and dioxins, e.g. polychlorinated dibenzo dioxins (PCDDs) and -furans (PCDFs). These compounds are formed as combustion by-products of organic matter from industrial and municipal incineration, heating, traffic and fire (Bumb *et al.*, 1980; Gribble, 1992). In addition to these naturally occurring contaminants are the synthesised, anthropogenic polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and pesticides such as dichlorodiphenyl trichloroethane (DDT) (and its metabolite *p,p'*-DDE), dieldrin, chlordane, toxaphene, hexachlorocyclohexanes (HCHs) and hexachlorobenzene (HCB) (e.g. Lindstrom *et al.*, 1999; Oehme *et al.*, 1995). 'Environmental contaminants', in this thesis, refers to the organic, particularly halogenated compounds and does not include inorganic contaminants such as metals.

Furthermore the organic environmental contaminants can be divided into accumulated, or non-accumulated compounds. Both groups can induce cytochrome P450 (CYP) isoenzymes.

PCBs have a multitude of applications because of their good insulation properties, such as in transformers and capacitors and are used as plasticisers in food bags and polystyrene cups (Solomans, 1992). PBDEs are used as flame retardants in a variety of consumer products including electronic boards in computers, radios and television sets (Lindstrom *et al.*, 1999). Some pesticides such as DDT were banned in the western world in the early 1970s, however continue to be sprayed over thousands of hectares in third world countries. Other pesticides are still applied in vector control operations and for other agricultural purposes (Mowbray, 1986).

These environmental contaminants have entered the environment by leeching from land – fill sites, as well as released accidentally during chemical and oil spillage's, during oil field development, in outflow waters from industrial and municipal wastes, and directly into the environment (in the case of pesticides) (Anon., 2000; Norstrom and Muir, 1994). Pathways of transport of environmental contaminants from industrial areas to remote 'pristine' areas such as the Arctic, have occurred though long range atmospheric transport, transfer in marine organisms and biota, as well as by the oceans currents, resulting in the ubiquitous distribution of these pollutants (Macdonald *et al.*, 2000).

Distribution estimates indicated that 50,000-60,000 tons of PCBs were produced per year between 1930 – 1970, and 20% of the worlds production is present in upper layers of the ocean (Tateya *et al.*, 1988). Furthermore in 1992, the world annual production of PBDEs was 40,000 tons (WHO, 1994).

Hydrophobic contaminants accumulate in the lipid tissues of marine organisms (Boon *et al.*, 1992; Muir *et al.*, 1999) thus top predators of marine trophic webs including marine mammals, which have thick layers of subcutaneous fat, show high bioaccumulated concentrations of organochlorine contaminants (Fossi *et al.*, 1997b). However, the contaminant mixtures to which marine mammals are exposed to differ greatly from the original industrial mixtures. This is because the organisms at various trophic levels differentially accumulate and metabolise the discrete chemical types (Ross, 2000).

Some environmental contaminants are almost completely metabolised, including the PAHs, 2,3,7,8 TCDD and 2,3,7,8-PCDD/Fs, the coplanar PCBs that are approximate isostereomers of 2,3,7,8-TCDD, as well as some of the lower chlorinated non-planar PCBs, and therefore do not bioaccumulate. Investigating the relative levels of pollutants in tissues and organs is not an accurate indication of the exposure of marine mammals to these groups of environmental contaminants (Fossi *et al.*, 1992).

Determining the exposure of populations to the range of contaminants found in the marine environment without having to measure the individual chemical compounds and to find out something about the response of exposed individuals requires a more integrated approach: One method has developed the drug metabolising system in the tissues of animals as a biomarker of environmental contamination. Xenobiotics, including environmental contaminants, induce specific enzymes involved in their metabolism and since this is indicative of exposure to many inducers, can be used in this integrated way. Biomarkers are defined as molecular, biochemical and cellular changes caused by exposure to environmental pollutants (McCarthy, 1992) and provide an 'early warning system' of the response in organisms (den Besten, 1998) and are measurable.

Cytochrome P450 (CYP) monooxygenase (MO) isoenzymes metabolise endogenous substrates, as well as xenobiotics including drugs and environmental contaminants (Waxman, 1999), rendering them more hydrophilic and facilitating their excretion, although some xenobiotics are activated, producing more reactive metabolites. The induction of particular CYPs can be assessed using substrates specific to the CYP isoenzyme of interest. For example 7-ethoxyresorufin is a relatively specific substrate of CYP1A-mediated 7-ethoxyresorufin *O*-deethylase (EROD) activity and has been established in humans, laboratory rodents and fish (e.g. Besselink *et al.*, 1997; Burke *et al.*, 1994; Haasch *et al.*, 1993).

Since Addison and Brodie (1984) first characterised the CYP1A-mediated EROD activity in grey seals (*Halichoerus grypus*), this and other activities have been employed as correlative markers of contaminant levels in the tissue of marine mammals (Goksøyr, 1995; Letcher *et al.*, 1996; Troisi and Mason, 1997; White *et al.*, 1994). Nonetheless this inference of exposure from CYP isoenzyme induction using catalytic studies alone is unreliable (Stegeman and Hahn, 1994). Hence the function and expression of CYP isoenzymes have also been determined using complementary methods. One such method involves the analysis of particular CYPs using selective inhibitors of CYP-mediated MO activities (Mattson *et al.*, 1998; Nyman *et al.*, 2001; van Hezik *et al.*, 2001; Wolkers *et al.*, 1998b).

Another technique is immunochemical detection. So far heterologous antibodies (monoclonal and polyclonal) raised against purified CYP isoenzymes of other vertebrate species have been employed as probes for determining the presence of orthologous CYPs in the tissues of a variety of marine mammals (Fujita *et al.*, 2001; Goksøyr *et al.*, 1989; Goksøyr *et al.*, 1992; Letcher *et al.*, 1996; Wolkers *et al.*, 1999). Furthermore the

preliminary analysis of hepatic mRNA for CYP1A using oligonucleotide probes, specific for rat CYP1A isoenzymes, has been performed (Wolkers *et al.*, 1998b).

However, species-specific probes are required for the further development of CYP isoenzymes as biomarkers of environmental contaminant exposure (Peters *et al.*, 1999) in marine mammals. Furthermore base-line and background biochemical and molecular data for CYP isoenzymes and their regulation by environmental contaminants is necessary to further characterise the cause and effect links between these contaminants and observed toxicities and pathologies.

1.2. Exposure of Marine Mammals to Environmental Contaminants

Initial studies that indicated the exposure of marine mammals to environmental contaminants occurred following reports of decreased reproductive success and high DDT concentrations in the tissues of seabirds. Koeman and Van Genderen (1966) first reported the presence of the organochlorine pesticides such as DDT and dieldrin in the tissues of harbour seals (*Phoca vitulina*). Furthermore, Jensen (1966) reported the presence of PCBs in the tissues of humans, pike and eagles. Soon after Jensen and colleagues (1969) detected traces of PCBs in addition to DDT in grey seals, harbour seals and ringed seals (*Phoca hispida*) from Swedish waters. Prior to these studies, marine mammals were generally not included in such investigations, since there was a general belief that organisms in the open ocean would not be affected by pollution originating from inland sources (Aguilar and Borrell, 1996).

Since then, a large data collection of the total levels of environmental contaminants in the blubber of many marine mammals from a variety of geographical locations has been established. Levels of organochlorines such as PCBs, DDT, chlordanes, HCH and HCB were greater in the Northern hemisphere compared with the Southern hemisphere

(Kemper *et al.*, 1994; Kleivane and Skaare, 1998). Furthermore an increasing PCB concentration gradient was indicated from west to east of the Arctic region reflecting the contaminant input from Northern and Western Europe (Muir *et al.*, 1999; Skaare, 1995). PCB concentrations were greater in harp seals (*Phoca groenlandica*) from the Barents Sea compared with conspecifics from the 'West Ice' region, east of Greenland. Increasing PCB levels were also observed from north to south within the Northern Hemisphere (Skaare, 1995). PCB levels in male Beluga whales (*Delphinapterus leucas*) from Hudson Bay in 1994 were significantly lower than the levels of the same PCBs measured in male belugas from St Lawrence Estuary sampled in 1993/1994 (Muir *et al.*, 1996b).

The majority of studies have measured environmental contaminant levels in the blubber since this tissue contains most (~90%) of the total body lipids (Beck *et al.*, 1994; Worthy and Lavinge, 1987). However, organochlorines have been detected in other tissues including brain, liver, kidney and muscle (Bruhn *et al.*, 1995; Gauthier *et al.*, 1998; Law and Whinnett, 1992; Schantz *et al.*, 1993). Absolute concentrations of PCBs were measured in several tissues of male ringed seals and were found to be in the following order: Blubber > liver > kidney (Daelemans *et al.*, 1993). However, the PCB congeners found in the different organs of harbour and ringed seals were virtually identical due to the dynamic balance between the blood and organs/tissues (Boon *et al.*, 1994; Boon *et al.*, 1992; Daelemans *et al.*, 1993; Moriarty, 1983).

Several ecological and biological parameters such as species, age, diet and nutritional status, and reproductive status can affect the observed burdens of environmental contaminants (Boon *et al.*, 1992; Espeland *et al.*, 1997). Furthermore individual and species-specific metabolic capabilities can also affect the degree or total

biotransformation and elimination of environmental contaminants and thus the total body burden and exposure. The metabolism of environmental contaminants, in particular PCBs, will be discussed in section 1.4.

Inter-specific differences occur as a consequence of dietary differences. For example krill-eating mysticetes (baleen) whales often have much lower burdens of environmental contaminants than odontocete (toothed) whales, and seals that tend to feed on prey from higher trophic levels (Muir *et al.*, 1999). Differences in environmental contaminant levels between odontocetes and seals can be a consequence of the choice and source of prey and the degree of the prey's contamination. Fish such as the Greenland halibut (*Reinhardtius hippoglossoides*) are predatory, bottom feeding deep sea fish (800 – 2000m) that have relatively fatty muscle compared to whitefish, char and sculpins (Berg *et al.*, 1996; Muir *et al.*, 1999) and therefore would accumulate larger concentrations of hydrophobic contaminants. Furthermore marine mammals feeding on shallow pelagic fish, such as arctic cod (*Boreogadus saida*), compared with those that prey on deep sea fish, can be distinguished in their environmental contaminant levels. For example, deep sea fish contain greater concentrations of higher chlorinated organochlorinated compounds and lower concentrations of the more water soluble pesticides such as HCH, because of the former are preferentially absorbed to sinking particles and the latter is mainly dissolved in the water column (Macdonald *et al.*, 2000). In addition prey resident in more polluted coastal areas are more likely to bioaccumulate larger burdens of contaminants than more remote and open ocean species.

Seasonal variations of environmental contaminant concentrations in marine mammals are reflected in dramatic changes in the thickness of blubber. There was a negative correlation between blubber thickness and circulating environmental contaminants

during the breeding and moulting seasons in both adult male and female harp seals (Kleivane *et al.*, 1995a). Environmental contaminants in the blubber are probably only mobilised when a seal is in negative energy balance such as during pup rearing, breeding and moulting seasons and when the animal is in ill-health (Hall *et al.*, 1992). This suggests that the comparison of environmental contaminant concentrations between individuals and species during different stages of the annual cycle would be inaccurate. Therefore comparing blubber burdens (PCB concentrations based on total blubber lipid mass, rather than lipid weight alone), which do not fluctuate throughout the year would enable such comparison (Addison and Stobo, 1993).

Sex, age and reproductive status can account for intra-specific differences in environmental contaminant levels. The concentrations of persistent environmental contaminants are generally greater in sexually mature males than sexually mature females, in both seals and cetaceans (Addison and Smith, 1974; Larsen, 1995). The burdens of environmental contaminants continue to increase with age in males. This increase is also observed in females until they reach sexual maturity, where a large proportion of the females body burden is offloaded to their offspring as a consequence of gestational and lactational transfer (Addison and Brodie, 1987; Addison and Stobo, 1993; Beckmen *et al.*, 1999; Hall *et al.*, 1999; Tanabe *et al.*, 1982; Wells *et al.*, 1994). However, Wolkers and colleagues (1998a) did not observe any sex differences in Svalbard ringed seals, suggesting that the females continue to feed throughout the breeding period.

Temporal trends in environmental contaminant concentrations, in particular PCBs and DDT have been observed in the tissues of marine mammals. A notable decline in the concentrations of PCBs and DDT were observed between 1970s and 1980s as a

consequence of the prohibition in manufacture and use of these organochlorines (OCs) in the western world during the early 1970s. However, not such a notable decrease in these contaminants was recorded between the 1980s and 1990s (Addison and Smith, 1998; Muir *et al.*, 1999; Muir *et al.*, 1996b). Temporal declines in contaminant levels in marine mammals from remote areas may now be very much slower due to the high load of environmental contaminants such as PCBs, DDT(E) in the marine environment and potentially the continued deposition of contaminants by long range atmospheric transport (Loganathan *et al.*, 1990; Macdonald *et al.*, 2000; Tanabe *et al.*, 1988). Furthermore a reduction in the contaminant levels in oceans connected with developing countries will be equally slow, since pesticides, such as DDT continue to be used in large quantities in tropical third world countries (Dekock *et al.*, 1994; Mowbray, 1986).

During the last ten years, investigations have increasingly focused on the exposure of marine mammals to 'new' contaminants such as PCDDs, PCDFs and PBDEs (Addison *et al.*, 1999; Berggren *et al.*, 1999; Jimenez *et al.*, 1999; Koistinen *et al.*, 1997; Lindstrom *et al.*, 1999; Oehme *et al.*, 1995; vanScheppingen *et al.*, 1996). PCDDs and PCDFs are found to be at lower concentrations in marine mammal tissues compared with sum of the PCBs (Σ PCBs) and DDT (Σ DDT). This is similar to PAHs, which may exert their effects on marine mammal physiology as a consequence of metabolism. Subsequently the majority of these chemicals are excreted as hydrophilic metabolites and not bioaccumulated to any great extent, thus the tissue concentrations of these contaminants would not reflect the animals exposure.

PBDEs were found at high concentrations in the blubber of whitebeaked dolphins (*Lagenorhynchus albirostris*) ($>7\mu\text{g}\cdot\text{g}^{-1}$ lipid) and harbour seals ($>1\mu\text{g}\cdot\text{g}^{-1}$ lipid) (de Boer *et al.*, 1998). These marine mammals had fed in the North, and Wadden Seas.

Furthermore PBDEs had been incorporated into the deep sea food web since the presence of these contaminants were detected in sperm whales (*Physeter macrocephalus*) (de Boer *et al.*, 1998).

In addition to the parent compounds, the PCB metabolites, PCB-methyl sulphonyls (MSFs), DDE-MSFs and hydroxy-PCBs (OH-PCB) have been detected in a variety of wildlife, including marine mammals (Bergman *et al.*, 1992; Haraguchi *et al.*, 1994; Troisi *et al.*, 1998). Some PCB-MSFs are persistent in tissues, particularly the blubber/fat and the liver, as a consequence of their lipophilicity, resistance to further metabolism and protein binding properties (Harguchi *et al.*, 1994; Letcher *et al.*, 2000).

PCB-methyl sulphone metabolites were detected in the blubber of 6 cetacean species: harbour porpoise (*Phocoena phocoena*), striped dolphin (*Stenella coeruleoalba*), Risso's dolphin (*Grampus griseus*), common dolphin (*Delphinus delphis*), pilot whale (*Globicephalus melas*) and the white sided dolphin (*Lagenorhynchus acutus*) (Troisi *et al.*, 1998). These metabolites were also extracted at μg levels on lipid weight basis from the uteri and lungs of grey seals and striped dolphins, where they may exert toxicological effects at a molecular level (Troisi *et al.*, 2000).

In contrast, many hydroxy-PCBs are highly susceptible to conjugation and are subsequently excreted. However, certain OH-PCBs including PCB-122 and -187 have been detected at stable concentrations in the blood of grey seal, human and rats (Bergman *et al.*, 1994). The toxic effects of these metabolites are discussed in the following section (section 1.3).

1.3. Implications of the exposure to environmental contaminants

Reproductive and immunotoxic dysfunctions, prevalence of tumours, mass mortalities and endocrine imbalances, in marine mammals have raised questions about a possible contributory role of environmental contaminants that have accumulated in the food chain (De Guise *et al.*, 1995).

Several studies have documented pathologies in the endocrine/reproductive and immune systems and due to the frequently high concentrations of PCBs and other environmental contaminants, have related these abnormalities to contaminant exposure. Recent field – experimental studies have been performed in harbour seals fed either fish from polluted waters, including the Baltic Sea and the Wadden Sea, or clean fish from the Atlantic (Brouwer *et al.*, 1989; Reijnders, 1986; Ross *et al.*, 1996). Reproductive and immune function parameters were investigated in the two groups.

Furthermore, experiments using laboratory mammals has enabled investigations into the disruption of these systems by environmental contaminants, that can not be performed in seals and cetaceans due to ethical and logistical considerations. However, a definitive cause-effect link remains to be established between the observed pathologies and particular environmental contaminant exposure.

1.3.1. Reproduction

Investigations into the decline of several marine mammal populations in the 1970's and 1980's revealed a decrease in their reproductive success of these animals. Non-pregnant Baltic ringed seals were reported to have higher levels of PCBs than the pregnant females, and over half had enlarged uteri and scar tissue indicative of failed pregnancies (Helle *et al.*, 1976a). Furthermore occlusions and stenosis of the uteri from ringed and

grey seals were attributed to the reduced reproductive success in these species (Helle *et al.*, 1976b). However, these uterine pathologies were not correlated to PCBs. There was no statistical difference between the PCB levels in non-pregnant females with physiologically normal uteri and those with abnormalities (Reijnders, 1980). In contrast premature pups were born to Californian sea lions (*Zalophus californianus*) in the 1970s, were under-developed, having no fur and / or lacked motor coordination and had poor respiratory function (DeLong *et al.*, 1973). These pupping females and their neonates had significantly higher levels of contaminants in their tissues including blubber, liver and brain, than full-term females and pups.

St Lawrence Beluga whales are another population that exhibited reduced reproductive recruitment and reproductive abnormalities. Female belugas, in addition to low pregnancy rates, had little ongoing ovarian activity when compared with arctic belugas (De Guise *et al.*, 1995). Furthermore an adult hermaphrodite was found in the St Lawrence estuary. These abnormalities may be related to the oestrogenic effects of certain contaminants (De Guise *et al.*, 1994; 1995).

Against this background, a feeding study on harbour seals in which females were fed contaminated fish showed the disruption of a plasma steroid hormone. Exposed females had lower plasma concentrations of 17β -oestradiol after implantation, corresponding with a decreased reproductive success, compared to unexposed females (Reijnders, 1986). Furthermore in another study, testosterone plasma levels were negatively correlated with blubber DDE concentrations in free-ranging adult male Dall's porpoises (*Phocoenoides dalli*) (Subramanian *et al.*, 1987). This suggested an increased rate of steroid metabolism by PCB or DDE mediated cytochrome P450 induction. However, a decrease in plasma concentration of steroid hormone would initiate the negative feed

back mechanisms and the subsequent compensatory production of the depleted hormone, unless the metabolic-pathway was inhibited.

Alternatively the reduced reproductive success and altered development may involve changes in steroid hormone receptor concentrations or ligand binding. Disruption of the reproductive process was thought to occur post-ovulation, possibly around the sensitive implantation phase in exposed harbour seals (Reijnders, 1986). Indeed embryo resorption in PCB-fed mink occurred after implantation as determined by the unequal number of kits and implantation sites at parturition (Backlin and Bergman, 1992).

Prior to implantation, the uterus is primed to bind progesterone due to oestrogen-mediated up-regulation of progesterone receptors (Johnson and Everitt, 1995). However, the chlorobiphenyl (CB)-169 - induced litter failure in mink did not alter the progesterone or oestrogen receptor concentrations, rather this PCB congener reduced the binding affinity of the uterine progesterone receptor (Patnode and Curtis, 1994). Recently a uteroglobin -like protein was characterised by immunoblotting from the uteri of Baltic grey and ringed seals (Troisi *et al.*, 2000). Uteroglobin, a progesterone binding protein found in the uterus, was involved in the increased cell division in cultured mink embryos (Daniel and Krishnan, 1969 in Patnode and Curtis, 1994). Reductions in uteroglobin or other progesterone/progesterone receptor -mediated embryonic growth factors may be responsible for PCB-mediated growth impairment, indicating a potential mechanism for embryotoxicity and subsequent decreased reproductive success in marine mammals.

An alteration in the reproductive development of male rats was demonstrated to be due to potent binding of *p,p*-DDE to the androgen receptor (AR), subsequently inhibiting

androgen-induced AR-transcriptional activity (Kelce *et al.*, 1995). The rat's serum testosterone levels were not however, reduced.

1.3.2. Metabolic hormone disruption

Disruption of other areas of the endocrine system was also reported in PCB-fed harbour seals, where reductions in plasma retinol, free and bound thyroxine and triiodothyronine were observed (Brouwer *et al.*, 1989). The chlorine substitution patterns of OH-PCBs, e.g. chlorine atoms on the carbon atoms adjacent to the para OH group and ≥ 1 chlorine on the para position of the non-OH containing phenyl ring, resemble the iodine substitution pattern of thyroxine, the endogenous substrate of transthyretin. Reductions in retinol and thyroid hormones were demonstrated in rats due to the binding of hydroxy-CB-77 to the thyroxine binding protein, transthyretin (Brouwer, 1987 in Brouwer *et al.*, 1989). Furthermore a decline in the plasma retinol concentration was observed in experimentally fed harbour seals (De Swart *et al.*, 1996). Both retinol and thyroid hormones play important functions in growth and development and in the immune system, suggesting that alterations in these hormones could lead to decreased reproductive success, poor development and increased susceptibility to infections.

Grey and harbour seals from the Baltic were reported to suffer from a disease syndrome may be caused by an increased exposure to glucocorticoid hormones (hyperadrenocorticism) (Bergman and Olsson, 1985). Skull-bone lesions, characterised as an osteoporosis, are one of the pathologies exhibited in this disease syndrome (Bergman *et al.*, 1992; Mortensen *et al.*, 1992). Certain PCB-MSFs antagonistically bound to the human and mouse glucocorticoid receptor (GR) *in vitro*, and similar to the synthetic GR inhibitor, RU486, may increase cortisol and adrenocorticotrophic hormone (ACTH) plasma levels due to a feed back mechanism (Johansson *et al.*, 1998).

Furthermore the MSF metabolite of DDE covalently bound to a protein, possibly the mitochondrial cytochrome P45011B, in mouse adrenal cortex caused a reduction in plasma corticosterone concentration (Brandt *et al.*, 1992). A negative feed back mechanism may however, subsequently increase the level of corticosterone via another physiological pathway.

1.3.3. Immune system dysfunction

During the 1988 epizootic, approximately 18,000 seals died in Western Europe from a viral disease similar to canine distemper virus, that was later named phocine distemper virus (PDV) (Dietz *et al.*, 1989; Osterhaus and Vedder, 1988). Furthermore thousands of seals from Lake Baikal died during this period from a related distemper virus. This was probably canine distemper virus (CDV) from escaped, vaccinated dogs (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989). High burdens of environmental contaminants in marine mammals may contribute to the scale of epizootics because of the potential immunosuppressive effects of PCBs (Hall *et al.*, 1992; Ross *et al.*, 1996).

Reports have suggested PCBs are immunosuppressive to marine mammals. For example, harbour porpoises that had died of infectious diseases had higher blubber concentrations of PCBs compared with conspecifics that died due to physical trauma were observed (Jepson *et al.*, 1999). The significantly higher PCB concentrations were found to be irrespective of condition. Furthermore a negative correlation between lymphocyte function, measured by the cells' response to mitogens, concanavalin A and phytohaemagglutinin, and polyhalogenated hydrocarbon concentrations were found in free-ranging bottle-nosed dolphins (Lahvis *et al.*, 1995).

A semi-field study further provided the evidence for the immunosuppressive nature of PCBs, in marine mammals (Ross *et al.*, 1996; Ross, 1996). Harbour seals fed contaminated fish had altered immune function parameters, in particular decreased levels of natural cell killer activity, mitogen –induced lymphocyte proliferative responses, mixed lymphocyte reactions and antigen-specific lymphocyte proliferative responses. The immune functions of rats fed with equivalent doses of the same contaminants as in the Baltic fish fed to seals were not altered, thus the authors concluded that harbour seals may be more sensitive than rats to the immunotoxic effects of contaminants (Ross *et al.*, 1996). So far the mechanism by which environmental contaminants mediate their immunosuppressive effect in marine mammals has not been elucidated.

1.3.4. Tumourogenesis

A high prevalence of tumours has been found in beluga whales from the St Lawrence estuary, that have high tissue contaminant burdens, particularly benzo(a)pyrene (B(a)P) (De Guise *et al.*, 1994). B(a)P and other carcinogenic contaminants may exert their effects through CYP1A-mediated metabolism, producing carcinogenic intermediates. Furthermore PCBs, of which there are high concentrations in the beluga whale, act as promoters of tumours by initiating hyperplasia, an important event in carcinogenesis (De Guise *et al.*, 1994; De Guise *et al.*, 1995).

The observational and experimental studies discussed here provide evidence that exposure of marine mammals to environmental contaminants is affecting the health of these animals. However, many of the mechanisms by which the ranges of effects occur, still remain unidentified.

1.4. Metabolism of Environmental Contaminants

1.4.1. Biotransformation

Biotransformation is the conversion of lipophilic organic molecules into water soluble compounds by enzymes in the liver and other tissues, facilitating their subsequent elimination from the organism. These organic molecules include endogenous substrates such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, retinoids and lipid hydroperoxides and exogenous molecules including drugs, environmental contaminants and natural organic products (Nelson *et al.*, 1996).

The lipophilic environmental contaminants enter marine mammals predominantly through the ingestion of contaminated prey. The absorption of these lipophilic substances involves diffusive transfer across the epithelial membrane of the gut and subsequent distribution to the liver and extrahepatic tissues via circulation. Many highly lipophilic environmental contaminants cannot be biotransformed and therefore are bioaccumulated within the body's lipophilic tissues, rather than excreted.

Biotransformation of other lipophilic compounds occurs by two pathways, which usually leads to the detoxification and subsequent excretion of the compound. The first of these, phase I, includes oxidation, reduction and hydroxylation reactions. These alter the parent compound by the addition of a polar functional group, e.g. -OH, -COOH, -NH₂. Oxidation is the most important of the phase I reactions, of which the cytochrome -P450 associated monooxygenase system (MO) is the central catalyst for the biotransformation of organic lipophilic molecules, such as environmental contaminants (Boon *et al.*, 1992; Timbrell, 1991).

The introduction of a polar group to a contaminant may provide it with a sufficiently hydrophilic character for rapid excretion. For most substances, however, this is not the case and a subsequent phase II reaction is required. The cytosolic phase II enzymes include glucuronyltransferase, sulfotransferase and glutathione-S-transferase. These reactions conjugate a hydrophilic endogenous metabolic intermediate (e.g. glucuronic acid, sulphate, glycine or glutathione) with the polar group of the phase I metabolite and render the compound hydrophilic and readily excretable (Kedderis, 1990; Timbrell, 1991).

Although primarily a pathway of detoxification, the biotransformation of a parent compound may result in the production of a more toxic and reactive metabolite (Kedderis, 1990; Stegeman and Hahn, 1994). This may occur via a number of ways, e.g. the covalent bonding of oxygenated intermediates to biomacromolecules such as proteins, including the CYP itself, and DNA (reviewed in Nebert and Gonzalez, 1987); see section 1.3), leading to mutations and cellular toxicity (reviewed in Stegeman and Hahn, 1994). This pathway has been shown in many studies to correlate with chemical carcinogenesis, mutagenesis, drug toxicity and teratogenesis (Nebert and Gonzalez, 1987). An imbalance between the rate of biotransformation and the rate of detoxification whereby the former exceeds the latter may lead to an excessive production of these reactive intermediates. Consequently, the non-toxic pathway becomes saturated therefore increasing the activity of another minor, but more toxic pathway (Timbrell, 1991), producing, for example, epoxides, free radicals, acid chlorides and N-hydroxy derivatives.

1.4.2. Cytochrome P450 and the associated Monooxygenase System

Cytochrome P450s (CYP) are haeme-thiolate proteins that catalyse the monooxygenation of a diversity of endogenous and exogenous organic molecules (Stegeman and Livingstone, 1998). They are located most abundantly on the membrane of the smooth endoplasmic reticulum in the liver, but are also found in substantial quantities in extrahepatic tissues and organs, such as skin and the endothelial lining of blood vessels (Hyyti *et al.*, 2001; Kedderis, 1990).

The CYP-MO system comprises several components, the heme-protein CYP occupies the key position and two flavoproteins, NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase, which play a role in producing reducing equivalents donated by NADPH and NADH, respectively (Garvish, 1999). In a reconstituted system, three major components are required for activity: CYP, NADPH-cytochrome P450 reductase and phosphatidylcholine (Lu *et al.*, 1969 in Kedderis, 1990). This indicates that the MO-system can function with NADPH alone.

The CYP-MO system is involved in the general phase I oxidative reaction as outlined in the following equation:

where RH is the substrate and ROH is the hydroxylated product.

The catalytic cycle of CYP activity is depicted in Fig. 1.1. (A) The first step is the



binding of the substrate to the oxidised (Fe^{3+}) form of CYP. (B) The iron component of CYP is then reduced (Fe^{3+} to Fe^{2+}) by the transfer of an electron to the substrate-CYP complex via the flavoprotein, NADPH-cytochrome P450 reductase (FP_1). (C) Molecular

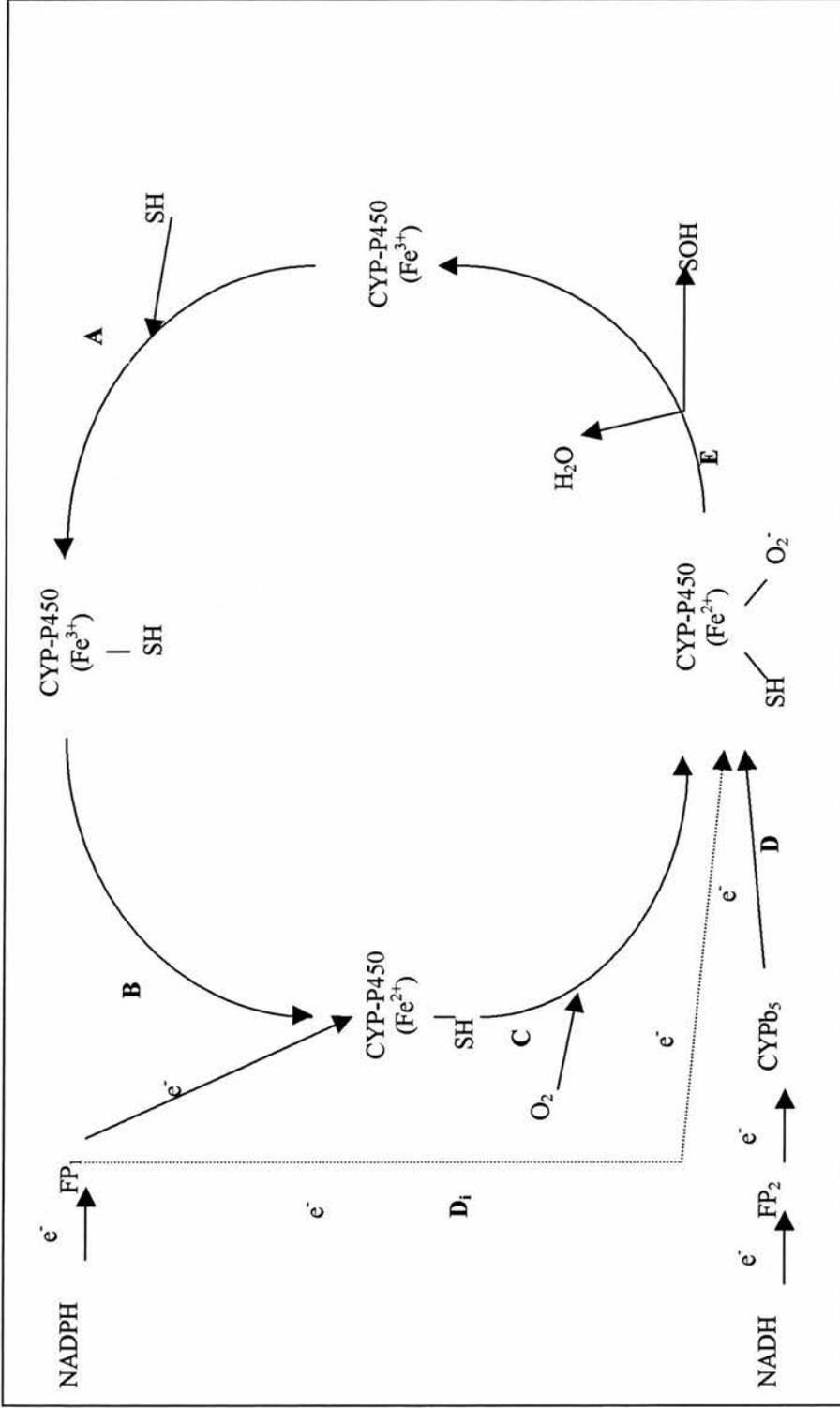


Fig. 1.1 A schematic representation of the cytochrome P450 catalytic cycle modified from Timbrell, 1991. SH is the substrate, SOH is the hydroxylated substrate, FP₁ is NADPH cytochrome P450 reductase; FP₂ is NADH cytochrome b₅ reductase. A-E refer to the stages of the catalytic pathway outlined in the text.

oxygen (O₂) is subsequently incorporated into the reduced complex. (D) The addition of a second electron, donated by NADH and transferred by the second flavoprotein, NADH-cytochrome b₅ reductase (FP₂) and cytochrome b₅, cleaves the O – O bond with the concurrent incorporation of one of the oxygen atoms into a molecule of water and the formation of a reactive iron-oxo (binding of oxygen to an iron species). The second electron may also be donated via NADPH-cytochrome P450 reductase (D₁) resulting in the system functioning with NADPH alone. (E) The oxygen is transferred from the iron-oxo species to the bound substrate, followed by dissociation of the product (SOH). The reoxidised CYP (Fe³⁺) is then available to participate in another catalytic cycle (reviewed in Groves and Han, 1995; Kedderis, 1990; Stegeman and Hahn, 1994; Timbrell, 1991).

Cytochrome P450 isoenzymes are present in all the organisms from prokaryotes, such as bacteria to eukaryotes including mammals, that have been analysed to date and are within the molecular weight range of 45- 60 kDa (Nelson *et al.*, 1996). By 1996, there were 481 individual CYP isoenzymes, each classified into one of 74 CYP gene families. The CYP protein sequences within a given gene family share >40% identity. Furthermore some CYP gene families are subdivided into sub-families, whereby CYP protein sequences have >55% identity with each other, although exceptions to these rules do occur (Nebert and Nelson, 1991).

The latest count has revealed that mammals contain seventeen distinct CYP gene families that together code for between 50 and 80 individual CYP genes in any given individual (Nelson, 1999). The CYP gene families one to four code for enzymes that metabolise environmental contaminants, however almost all the expressed enzymes are involved in endogenous pathways (Nelson, 1999). No endogenous substrate is yet

known for CYP1A isoenzymes, however they are thought to participate in oxidative stress, cell cycle control and apoptosis (Nebert *et al.*, 2000). These CYP enzyme families one to four catalyse many activities, with much overlap, and there are few substrates that are known to be exclusive to just one protein (Nebert and Gonzalez, 1987; Stegeman and Hahn, 1994).

CYP isoenzymes from these four CYP families have been demonstrated in several seal and cetacean species using indirect methods such as catalytic and immunochemical detection, and inhibition studies (See section 1.6). Only very recently have cDNA from the CYP1 family been isolated and identified in several marine mammal species (Godard *et al.*, 2000; Teramitsu *et al.*, 2000), This thesis: Chapter five).

1.4.3. Regulation and induction of Xenobiotic metabolising Cytochrome P450 isoenzymes

Cytochrome P450 gene families, including gene CYP families 1- 4, are regulated by exogenous substrates, such as drugs and environmental contaminants and by endogenous factors including sex steroids, glucocorticoids, growth hormones, insulin and inflammatory cytokines. The physiological regulation of CYPs is less well documented. The differential regulation of CYPs occurs between sexes, during developmental stages, nutritional states, reproductive cycles, stress and disease states (Morgan *et al.*, 1998). Sex-specific CYPs have been reported in mammals, for example members of the CYP2 and CYP3 families are gender-dependent rat CYPs, in that CYP2C11, -2A2 and -3A2 are male-specific where as CYP2C12, -2A1 and -2C7 are female-specific or -dominant CYPs (reviewed in Waxman and Chang, 1995). This differential expression is a consequence of sex-dependent secretion of growth hormone. During stress and fasting an increase in the circulating glucagon/insulin ratio have been documented (common to diabetes and inflammation) and these modulate the down-

regulation of CYP2C11 (Morgan *et al.*, 1998). Furthermore the down regulation or inhibition of a variety of induced CYPs, including CYP1A1, -2B, -3A and -4A have been shown by cytokines and endogenous hormones such as tumour necrosis factor- α (TNF- α), interleukin-1 β (IL- β), IL-6, and transforming growth factor- β (TGF- β) (Abdel-Razzak *et al.*, 1994; Milosevic *et al.*, 1999; Morgan, 1997; Paton and Renton, 1998).

Effects of environmental contaminants on the regulation of CYPs during particular stages of development, reproductive cycle, during times of stress, fasting and disease may be considerably modulated by altered endogenous factors (see section 1.3. (Morgan *et al.*, 1998).

The majority of ligands that transcriptionally induce CYP1 – 4, activate expression via one of four receptor-based mechanisms. Inducers of the CYP1 family of genes (CYP1A1, -1A2 and -1B1) occur via the well-documented aryl hydrocarbon receptor (AhR) (Hakkola *et al.*, 1997; Nebert *et al.*, 2000). The AhR resides in the cytoplasm in a complex with two heat shock proteins of 90kDa (hsp90) to maintain conformation to facilitate subsequent ligand binding. On binding of an AhR ligand, such as 2,3,7,8-TCDD, 3-MC and planar PCBs, the AhR translocates to the nucleus. Subsequently the hsp90 proteins dissociate from the AhR, and the ligand bound AhR forms a heterodimer with Arnt, allowing the binding with the dioxin responsive element (DRE) located upstream of the target CYP1 gene, and transactivation leading to transcription of the target gene (Delescluse *et al.*, 2000; Hahn, 1998; Stegeman and Hahn, 1994; Waxman, 1999; Whitlock *et al.*, 1996). The partial sequences of the AhR have been identified in beluga and white-sided dolphin by cDNA cloning (Hahn, 1998; Jensen and Hahn, 2001).

CYP2B, -3A and -4A genes are induced by ligand binding to 'orphan' nuclear receptors, members of the large nuclear hormone receptors, which includes the steroid/retinoid/thyroid receptors (Moore and Kliewer, 2000). Environmental contaminants that resemble phenobarbital (PB) such as non-planar PCBs induce CYP2B genes via the nuclear receptor designated the constitutive androstane receptor (CAR) (Forman *et al.*, 1998). Ligand binding may cause the dissociation of regulating steroids from the PB responsive enhancer (PBRE) bound heterodimer, CAR-retinoid X receptor (RXR) and induce the transcription of down stream target CYP2B gene(s) (Forman *et al.*, 1998; Waxman, 1999).

Endogenous ligands (steroid hormones and bile acids (CYP3A), fatty acids, arachidonic acid and other eicosinoids (CYP4A) and xenobiotics including organochlorine pesticides (CYP3A), and plasticisers (CYP4A) and certain PCBs (CYP3A and -4A) are ligands of the CYP3A and CYP4A gene families (Moore and Kliewer, 2000; Schuetz *et al.*, 1998). These ligands regulate their respective CYP3A or -4A gene(s) via the pregnane x receptor (PXR) and peroxisome proliferator activated receptor- α (PPAR- α), respectively (Moore and Kliewer, 2000; Waxman, 1999). Both the receptors belong to the family of organ nuclear receptors, similar to CAR and form a heterodimer with RXR after binding of the specific ligand. The ligand - bound PXR receptor binds to the DNA-binding region, dexamethasone-responsive regulatory element (DRRE) and the ligand-bound PPAR- α binds to the peroxisome proliferator chemical response element (PPRE) and induces the transcription of the target CYP3A and -4A genes, respectively (Waxman, 1999). CAR, PXR and PPAR- α - like nuclear receptors remain to be identified in marine mammal tissues.

1.5. Physicochemical properties of organochlorines and their interactions with cytochrome P450 isoenzymes

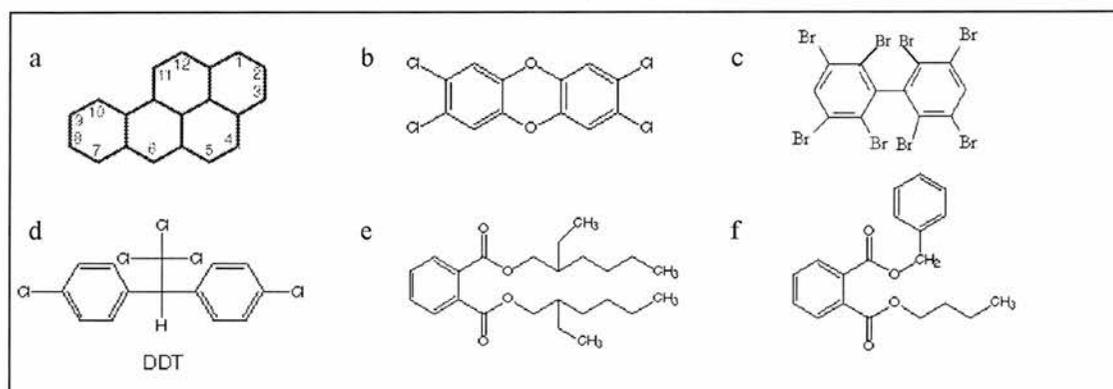
Many environmental contaminants are substrates of CYP isoenzymes and are able to act as inducers and inhibitors of CYP genes/isoenzymes and therefore able to respectively up- or down-regulate the corresponding CYP isoenzyme expression. This initiates their subsequent metabolism and that of endogenous substrates (Besselink *et al.*, 1998; Gooch *et al.*, 1989; Huang and Gibson, 1992; Huuskonen *et al.*, 1996; Murk *et al.*, 1994; White *et al.*, 2000). Also in combination certain contaminants can produce different effects such as synergistic or suppressive effects, compared to the single compound alone (Clemons *et al.*, 1998; Connor *et al.*, 1995).

The environmental contaminants that organisms, including marine mammals are exposed to include a wide diversity of chemical structures, many of which are able to induce CYP expression. Table 1.1. presents an example of the variety of CYP-acting compounds found in environmental samples. Since the experimental part of this thesis has concentrated on PCBs in marine mammals, the interaction of these compounds with CYPs will be focused on in this section. Other environmental contaminants will be mentioned for comparison with the interaction of PCBs and CYPs.

The 209 PCB congeners, which are classified by their chlorine content have comparable but distinctly different physicochemical and ecotoxicological properties (Ballschmiter and Zell, 1980; Safe *et al.*, 1985). Boon and colleagues (1992) have postulated that the metabolism of a chlorobiphenyl (CB) congener depends on the configuration of the congener, the positions of vicinal hydrogen, and chlorine atoms. Hence groups of PCB congeners are often, and will be here, referred collectively as mono-, di-, tri-, or tetra-

Environmental contaminant group	Example and structure	Target CYP isoform
Polyaromatic hydrocarbons (PAHs)	Benzo(a)pyrene ^a	CYP1A
Dioxins	2,3,7,8-TCDD ^b	CYP1A
Polybrominated diphenyl ethers (PBDE)	Octabromo diphenyl ether ^c	CYP1A and CYP2B
Pesticides	DDT ^d	CYP2B and CYP3A
	Chlordane <i>trans</i> -nonachlor	CYP3A
Plasticisers	Phthalates, such as di(2-ethylhexyl)phthalate ^e and butyl benzyl phthalate ^f	CYP4A
Polychlorinated biphenyls (PCBs)	General structure [†]	CYP1A
	CB-118, 114, 105, 156, 189, 167	CYP1A/CYP2B*
	187, 163, 153, 180, 194, 101, 87	CYP2B
	47, 184, 188, 196, 200	CYP3A
	CB-47, -126 [†]	CYP4A

Table 1.1. Examples of contaminants in the environment and their potential target CYP. The target CYPs have often been determined with single congeners, therefore not representative of the mixtures of contaminants that would frequently target a cell. The structures of some of the examples are presented below, corresponding to a letter (X) from a-f. †refer to Fig. 1.2. Data from references: Safe *et al.* (1985), Huang and Gibson, (1992), Connor *et al.* (1995), Schuetz *et al.* (1998) and Zhou *et al.* (2001).



ortho CBs corresponding with zero, one, two, three or four chlorine atoms positioned at the *ortho* position(s) on the biphenyl ring (Fig. 1.2).

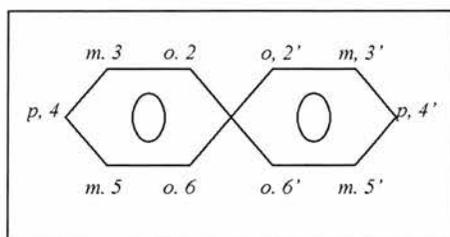


Fig. 1.2. General structure of a PCB molecule. *o*, *m* and *p* refer to *ortho*, *meta* and *para* and are designated a position 2–6 that indicates potential positions for chlorine atoms.

Chlorobiphenyls can be further classified according to their structure-activity relationship (SAR) (Safe *et al.*, 1985; Tanabe *et al.*, 1988). Certain CB congeners show a similar CYP induction as the two drugs, 3-methylcholanthrene (3-MC) and phenobarbital (PB). Pure 3-MC initiates CYP1A expression and pure PB induces CYP2B, whereas a mixture of both 3-MC and PB show a mixed CYP1A/CYP2B induction (reviewed in Boon *et al.*, 1992). Consequently groups of CBs have been classified as pure 3-MC- type inducers, pure PB-type inducers or a mixture of both.

CBs with vicinal hydrogen atoms at the *ortho*-, *meta*- positions and chlorine substitution at both the *para*- positions (Fig. 1.2) such as CB-77, -126 and -169, have similar induction patterns. These non-*ortho* CBs easily assume a planar configuration and are approximate isostereomers of 2,3,7,8-TCDD, the potent synthetic CYP1A inducer. These PCB molecules have also been shown to induce and act as substrates for cytochrome P4501A (CYP1A) e.g. (Besselink *et al.*, 1998; Clemons *et al.*, 1998; Huuskonen *et al.*, 1996; Murk *et al.*, 1994; Safe *et al.*, 1985; White *et al.*, 2000). However, some non-*ortho* CBs, for example CB-15, -37 and -81, as well as various di-*ortho* CBs, i.e. CB-128, -138, -158, -166, -170, exhibit a 3-MC + PB mixed type induction pattern, activating both CYP1A and CYP2B (Safe *et al.*, 1985).

PB-type inducers, such as CB-153 and CB-99 have two chlorines in opposing *ortho* - positions (Safe *et al.*, 1985), rendering the compound in a more globular configuration. These CBs induce cytochrome P4502B (CYP2B) isoforms. Connor and co-workers (1995) demonstrated that the most potent CYP2B inducers in rats were the di- and tri-*ortho* CBs, CB-187 and -163. These congeners had the same or similar chlorine substitution patterns on at least one phenyl ring as the known CYP2B inducers, CB-153 and -101, although the metabolism of CB-153 is a slow process, even in rats (Connor *et al.*, 1995; Safe *et al.*, 1985). Mono-*ortho* CBs tend to be mixed-type inducers (Van Der Burght *et al.*, 1998). All the mono-*ortho* CBs investigated in a previous study (Connor *et al.*, 1995) were shown to be weak CYP2B activators, inducing only low levels of PROD activity. Nevertheless, these CBs did induce CYP2B1 and -2B2 apoprotein as determined by Western blot.

PCBs also affect the induction and expression of CYP3A and CYP4A, two other CYP families of enzymes capable of metabolising environmental contaminants. Five PCB congeners, CB-47, -184, -188, -196 and -200, as well as two organochlorine pesticides (*trans*-nonachlor and chlordane), activated mouse CYP3A23 gene transcription, through binding to a CYP3A receptor, PXR (section 1.4.3.) (Schuetz *et al.*, 1998). Furthermore CB-47 and -126 were shown to have differential effects on CYP4A-mediated laurate 11-, and 12-hydroxylase activities in pre-treated rodent species (Huang and Gibson, 1992). In rat liver microsomes, whilst CB-47 significantly increased both the CYP4A - activities, CB-126, a known CYP1A inducer, significantly reduced these activities. In contrast in guinea pig liver microsomes CB-126 increased both the CYP4A-hydroxylase activities, whereas CB-47 had no effect.

CB-47, as an example, has exerted effects on three of the four CYP-metabolising families. A general rule of the structure activity relationship of PCB congeners can indicate which CYP family might metabolise a particular congener. However, it is difficult to predict how a particular CB congener will affect a number of different CYP families or what their mode of action will be. For example CB-153 is metabolised by CYP2B in rodent species, but it is the most recalcitrant PCB congener, bioaccumulating to be often the individual congener at the highest concentration in marine mammals blubbers.

1.6. Cytochrome P450 diversity in marine mammals and environmental contaminant metabolism

1.6.1. Cytochrome P450s metabolism inferred by contaminant residues in tissues

The cytochrome P450 system in marine mammals is increasingly being investigated, although more so in pinnipeds than cetaceans. Analytical methods and tools such as the metabolic index, a surrogate for the substrate structure activity relationship (SAR) have indicated the presence or absence of specific CYP isoenzymes, in particular CYP1A and -2B (Tanabe *et al.*, 1988). Concomitant catalytic and immunochemical detection have been utilised to characterise these and other CYP isoenzymes in marine mammals (Addison and Brodie, 1984; Boon *et al.*, 2001; Goksøyr, 1995; Goksøyr *et al.*, 1989; Hyyti *et al.*, 2001; Nyman *et al.*, 2000; Troisi and Mason, 1997; White *et al.*, 1994; Wolkers *et al.*, 1998b).

The metabolic index (MI), determined in both pinnipeds and cetaceans, is estimated by comparing the PCB congener pattern between the predator and its prey and has been suggested as a way of determining the biotransformation of an individual CB congener between trophic levels (Boon *et al.*, 1992; Bruhn *et al.*, 1995). The metabolic index is

calculated as $CBx/Cb153_{(predator)}/CBx/Cb153_{(prey)}$. A metabolic index of a particular congener that is equal to or greater than unity is a persistent congener and will accumulate in the predator. In contrast a congener with an metabolic index value <1 will accumulate less, and may be indicative of the metabolism of the congener by the predator (Boon *et al.*, 1997; Bruhn *et al.*, 1995). Bruhn and colleagues (1995) divided individual CB congeners into groups according to their structure-activity relationships. Similar SAR groups of individual CB congeners have also been described in other studies (Boon *et al.*, 1994; Boon *et al.*, 1997; Tanabe *et al.*, 1988). Boon and co-workers (1994; 1997) demonstrated that only PCB congeners with vicinal hydrogens in the *meta* (*m*)- and *para* (*p*)- positions and which have two or more *ortho* (*o*)-chlorines (group IV and V) or with vicinal hydrogens in the *o*-, *m*- positions with a maximum of one *ortho*-chlorine (group III) are metabolised in seals and cetaceans. It is postulated that these particular groups are biotransformed by CYP2B and CYP1A subfamilies respectively.

In Svalbard ringed seals, MI values for groups III, IV and V were less than 1 suggesting CYP1A and -2B activities (Wolkers *et al.*, 1998a). The other two groups of congeners, I and II, containing either no vicinal hydrogens or *o*-, *m*- vicinal hydrogens with more than two *ortho*-chlorines, showed MI values between 0.5 and 1.6 suggesting slow metabolism and even accumulation of these particular congeners, respectively. In contrast to seals, Tanabe and colleagues (1988) found from the metabolic index method, that small cetaceans did not have the capability to metabolise CB congeners with *m*-, *p*- vicinal hydrogens and two or more *ortho*-chlorine. Bruhn and co-workers (1995b) disagreed, reporting MI values <1 for CB congeners with *m*-,*p*- vicinal hydrogens and two or more *ortho*-chlorines (group IV and V) in the harbour porpoise suggesting CYP2B activity in these cetaceans. Furthermore, methyl sulphone metabolites of precursor-PCBs with vicinal hydrogen atoms in the *m*-, *p*- positions (groups IV and V)

were present in the blubber of beluga whales (Letcher *et al.*, 2000). These metabolites were unlikely to have accumulated from their prey species due to the very low CYP2B-mediated methyl sulphone producing capacity, therefore suggesting that the beluga whale has CYP2B-like mediating activities. For this discrepancy to be resolved, methods directly studying the hepatic cytochrome P450 system are now being employed (Nyman *et al.*, 2001; Wolkers *et al.*, 1998b).

1.6.2. Catalytic Detection of Cytochrome P450 Activity

Goksøyr and co-workers (1989) initially characterised the hepatic microsomal cytochrome P450 system of the minke whale (*Balaenoptera acutorostrata*) using an associated monooxygenase (MO), 7-ethoxyresorufin *O*-deethylase (EROD) method and Addison and colleagues (1984; 1986) first studied CYP-MO activities in the grey and harbour seals. EROD, a relatively specific MO of CYP1A is used in *in vitro* microsomal preparations, catalyses the *O*-deethylation of 7-ethoxyresorufin to its fluorescent product, resorufin. The product has distinctive excitation and emission wavelengths (530nm and 585nm respectively) enabling the activity of CYP1A to be indirectly quantified using a fluorimeter (Prough *et al.*, 1978). Other model reactions for CYP1A include aryl hydrocarbon hydroxylase (AHH), ethoxycoumarin *O*-deethylase (ECOD), whereas pentoxyresorufin *O*-deethylase (PROD) is associated with CYP2B, testosterone 6 β -hydroxylase (T6H) with CYP3A and laurate 11- and 12- hydroxylase with CYP4A (Burke *et al.*, 1994; Gibson *et al.*, 1983; Prough *et al.*, 1978; Wood *et al.*, 1983).

Some of these reactions have been employed as surrogates to detect the corresponding CYP isoenzyme in marine mammals (Goksøyr *et al.*, 1989; Troisi and Mason, 1997; Wolkers *et al.*, 1998b). The activities of the particular cytochrome P450 family investigated can then be correlated with relative concentrations of environmental

contaminant inducers such as individual CB congeners and total concentration of PCBs, DDT, DDE and other CYP metabolising contaminants in the blubber (Troisi and Mason, 1997; White *et al.*, 1994; Wolkers *et al.*, 1998a).

In certain species, differences in hepatic CYP-MO activities between sexes were demonstrated. In particular male beluga whales exhibit higher EROD activities than females (413 ± 263 and 94 ± 84 pmol.min⁻¹.mg⁻¹, respectively) and reflected their non-*ortho* and mono-*ortho* CB blubber burdens (White *et al.*, 1994). Furthermore significantly greater activities of PROD (CYP2B) and AHH (CYP1A) were observed in male than female beluga whales. In contrast the female harbour seal exhibits greater EROD activity than the male (28 ± 17 and 8.2 pmol. min⁻¹.mg protein, respectively) (Addison *et al.*, 1986) and no sex difference was reported in grey and harp seals (Addison and Brodie, 1984; Goksøyr *et al.*, 1992).

Characterisation of the CYP isoenzymes' metabolic response using model substrates such as 7-ethoxyresorufin and 7-pentoxyresoufin has been carried out using CYP-specific inhibitors. Chemical inhibition of ringed seals hepatic EROD and PROD activities with the CYP1A1-specific inhibitor, α -naphthoflavone completely inhibited the EROD (100%) and less so PROD (65-85%) activities (Mattson *et al.*, 1998). Evidence for the presence of the CYP1A2 isoenzyme was also provided by the partial inhibition (70-81%) of EROD activity in ringed seals. The presence of CYP1A1 and less so -1A2 in pilot whales was inferred from the immunoinhibition of hepatic EROD and AHH activities using anti-rat CYP1A1 and -1A2 antibodies (Watanabe *et al.*, 1989). The testosterone 6 β -hydroxylase activities in harp seals suggested the presence of CYP3A isoenzyme(s), which was verified by the 80% inhibition of this activity by the CYP3A-specific inhibitor, ketoconazole (Wolkers *et al.*, 1999).

Recently *in vitro* assays have been employed to investigate the biotransformation of PCB and toxaphene® congeners in marine mammal liver microsomes. Species differences were observed in the rate of biotransformation of CB-77, a coplanar PCB, which was higher in beluga whales compared to pilot whales (White *et al.*, 2000). In the same study, however, the biotransformation of CB-52, a di-*ortho* CB, was similar in both species. No species difference in the rate of biotransformation of CB-77 was observed in harbour seal and harbour porpoise liver microsomes (Murk *et al.*, 1994). Inhibition studies with α -naphthoflavone indicated that the metabolism of CB-77 was partially catalysed by CYP1A in beluga whale liver microsomes and the metabolism of the coplanar CB-15 was inhibited by >70% with ellipticine, a CYP1A1/2 inhibitor, in grey seal liver microsomes (van Hezik *et al.*, 2001; White *et al.*, 2000). In contrast the biotransformation of di-*ortho* CB-52 in the beluga was not significantly affected by the CYP1A-specific inhibitor α -naphthoflavone (White *et al.*, 2000).

The chlorobornane congeners, CHB-26 and -50 were highly persistent and not biotransformed in the liver microsomes of harbour and grey seals, and whitebeaked dolphin and harbour porpoise (Boon *et al.*, 1998; van Hezik *et al.*, 2001). However, where-as the two seal species could metabolise CHB-32 and -62, the whitebeaked dolphin and harbour porpoise were only able to convert CHB-32 to its metabolites. (Boon *et al.*, 1998; van Hezik *et al.*, 2001). Sperm whale liver microsomes were unable to metabolise either of these CHB congeners (Boon *et al.*, 1998; 2001). The presence of particular CYP isoenzyme(s) involved in the metabolism of the CHB congeners had differential regio- and entantio-selectivities in the seal and cetacean species (Murk *et al.*, 1994; van Hezik *et al.*, 2001; White *et al.*, 1994). As a consequence different CHB congeners were metabolised, and discrete metabolites were produced.

1.6.3. Immunochemical Detection of Cytochrome P450s

In addition to the catalytic detection of specific CYP P450 isoforms and their activity, immunochemical detection using heterologous antibodies has been used to probe for the presence of CYP isoforms. So far immunoreactive proteins resembling CYP1A, -2B, -3A and -4A have all been identified in seal and cetacean liver microsomes.

Since the mammalian CYP1A sequences are well conserved (Nelson, 1999), the detection of these proteins in a variety of marine mammals species has been documented. For example CYP1A apoprotein(s) have been detected in the liver microsomes of minke, beluga and sperm whales, harbour porpoises and white beaked dolphins, harp, harbour and hooded seals (Boon *et al.*, 2001; Goksøyr *et al.*, 1989; Goksøyr *et al.*, 1992; White *et al.*, 1994). The size of the immunoreactive proteins recognised in the immunoblots varied from 51-57kDa between species.

Immunohistochemical techniques have been employed to assess the distribution of CYP1A isoenzymes in many tissues from grey seal. The highest levels were found in the liver and the endothelial cells of the capillaries and large blood vessels in the liver and other organs (Hyyti *et al.*, 2001). Furthermore the highest concentration of CYP1A was observed in the Baltic grey seals compared with conspecifics from Sable Island, Canada, reflecting their respective exposure to environmental contaminants. CYP1A distribution was also investigated using immunohistochemistry in skin samples from Atlantic right whales (Moore, 1998). CYP1A was concentrated in the endothelial cells, and the concentration of this isoenzyme corresponded with the exposure of these animals to PAHs. In particular greater concentrations of CYP1A correlated with lower amounts of PAHs found in the blubber, reflecting the metabolism of the PAHs.

Preliminary studies have demonstrated the presence of CYP3A and -4A isoenzymes in several marine mammal species recognised by an anti-rat CYP3A and -4A antibodies, respectively (Goksøyr, unpublished results in Goksøyr, 1995). Further evidence was provided for the existence of these two isoenzymes in harbour seals and three cetacean species, sperm whales, whitebeaked dolphin and harbour porpoise (Boon *et al.*, 2001). More recently CYP1A, -2A, -2C, -2D, -2E and -3A immunoreactive proteins have been identified in grey seals (Nyman *et al.*, 2000; 2001).

Unequivocal evidence for the presence of CYP2B, however, is not so apparent. Based on the blubber PCB congener patterns, it was postulated that marine mammals/cetaceans do not possess the CYP2B isoenzyme (Tanabe *et al.*, 1988). A number of investigations lend support to this claim, as immunoreactive proteins to a variety of rodent CYP2B antibodies, were not detected in several marine mammal species, including minke and sperm whale, harbour porpoise, grey and ringed seals (Boon *et al.*, 2001; Goksøyr *et al.*, 1989; Mattson *et al.*, 1998; Nyman *et al.*, 2001; White *et al.*, 1994). However, immunoreactive proteins recognised by other heterologous CYP2B antibodies, such as anti-rabbit CYP2B4, anti-dog CYP2B and anti-rat CYP2B, have been identified in beluga whale, harbour porpoise, pilot whale harp, hooded and ringed seals (Goksøyr *et al.*, 1992; Hummert *et al.*, 1995; White *et al.*, 1994; White *et al.*, 2000; Wolkers *et al.*, 1999; Wolkers *et al.*, 1998b).

The investigations of CYP isoenzymes in marine mammals are increasingly employing immunochemical detection. However, since the species-differences in CYP primary structure and the epitope(s) of the antibodies are unknown, the absence of an immunoreacting protein(s) to an antibody may not accurately reflect the CYP isoenzyme content/diversity of the target tissue. This suggests the need for homologous

antibodies to be employed in the further characterisation of CYPs and their continued use as biomarkers (Goksøyr, 1995). Nonetheless, the information collected to date will contribute to a thorough knowledge of the molecular and biochemical properties of marine mammal CYPs that are required for subsequent investigations of the adverse effects of environmental contaminants.

1.7. Biomarkers of environmental contaminants exposure

A biomarker is a molecular, biochemical or cellular change that occurs in response to environmental contaminant exposure of an organism, beyond a certain threshold (McCarthy, 1992). Biomarkers may be applied for screening, as diagnostic tools, as parameters of trend analysis or as predictive markers, for subsequent analysis of an organisms' exposure (den Besten, 1998). Screening or early warning biomarkers include CYP-MO activities and CYP apoprotein levels which, in combination with residue analyses, may serve as a biomarker of exposure. In contrast a suite of clinical biochemical biomarkers may serve as diagnostic tools of a particular effect of environmental contaminants. All these biomarkers however require validation in the species of interest through obtaining baseline values in reference animals, prior to their use in assessing the risks from contaminant exposure among free-ranging wildlife species.

The majority of tissues used in biomarker studies to date have been obtained in a destructive manner, from local and scientific hunts, strandings and from by-caught animals (Fossi and Marsili, 1997). Obtaining fresh tissue from hunted animals is considered unethical and many countries have ceased to exploit marine mammals as a resource. Furthermore tissue samples from stranded or by-caught animals, unless recently dead (1-2 hours) will be degraded, as a consequence exposure may be under

estimated due to the inactivation of enzyme activities such as CYP-MO and the leaching of environmental contaminants from tissues (Borrell and Aguilar, 1990; Forlin and Andersson, 1985). Troisi and Mason (1997) proposed that CYP P420, the inactive form of CYP P450, could be used as a biomarker of PCB exposure. The summation of P450 and P420 concentrations was similar to those determined in fresh samples and correlated well with PCB concentrations in the liver and blubber of harbour seals.

Tissues such as blood, skin, hair, blubber and faeces can be sampled in a non-destructive manner from pinnipeds that haul-out on land and skin and blubber can be obtained by biopsy dart from free-ranging cetaceans (Fossi and Marsili, 1997). Subsequent biomarker analyses on some of these tissues have been recently developed (Fossi *et al.*, 1992; Fossi *et al.*, 1997b; Marsili *et al.*, 1996; Moore, 1998).

A variety of biomarkers can be obtained from different tissue samples, although blood samples appear to be the most versatile. Clinical biochemical parameters including the measurement of specific enzymes, of which alterations from the baseline, can be indicative of organ damage (Fairbrother, 1994). Biomarkers of DNA damage, such as the presence of micronuclei, sister chromatid exchange and chromosome aberrations can be determined in blood lymphocytes from marine mammals (Gauthier *et al.*, 1999). Furthermore the formation of DNA-adducts in a dose-dependent manner of B(a)P in bottlenose dolphin (*Tursiops truncatus*) kidney epithelial cell line indicates the potential for DNA-adducts to be a biomarker of exposure to B(a)P (Carvan *et al.*, 1995).

Environmental contaminant alteration of reproductive, corticosteroid and thyroid hormones have been demonstrated in marine mammals (section 1.3) and can be indicative of a variety of growth, metabolic, immune or reproductive dysfunctions.

However, since hormones fluctuate on a circadian and/or circannual basis, and by sex and age, and are influenced by stress of handling and blood collection, baseline values for the species of interest must first be established (Fairbrother, 1994). Because such a range of factors can influence these parameters, they are not sufficiently sensitive as stand-alone biomarkers.

CYP-MO, in particular the hydroxylation of B(a)P (BaPMO) activities have been measured in skin biopsies from striped dolphin, fin (*Balaenoptera physalus*) and Atlantic right whales (*Eubalaena glacialis* and *E. australis*), and Southern Californian sea lions (*Otaria flavescens*) (Fossi *et al.*, 1992; Fossi *et al.*, 1997b; Moore, 1998). The BaPMO activities were higher in skin from sea lions inhabiting more polluted waters compared with conspecifics from cleaner waters (Fossi *et al.*, 1997b). A preliminary study has indicated that skin BaPMO activity was 1/5 of the liver BaPMO activity in the same animal (Fossi *et al.*, 1997b). Further investigations are required to determine if the skin CYP-MO activities accurately reflect exposure to environmental contaminants.

EROD and AHH activities have also been used to detect the induction of CYP1A in human hair roots following the application of crude coal tar (PAHs) (Merk *et al.*, 1985; 1987), and may be applied to pinnipeds captured during haul-out. Collection of faeces from known individuals could be analysed for porphyrins, which are the products of altered haeme biosynthesis. Hahn and colleagues (1996) demonstrated porphyrin production in fish hepatoma cell line following exposure to certain planar PCB congeners and 2,3,7,8-TCDD.

This early work has shown promising results, which indicate that non-destructive biomarkers can be used to assess the exposure of healthy, free-ranging marine

mammals. These will be much more representative of the live population and will allow more appropriate and relevant risk assessments to be carried out and management options to be considered. This is the way of the future for biomarkers and is the focus and drive for much of the work in this thesis

1.8. Summary

Lipophilic contaminants are now ubiquitous in the environment and bioaccumulate to sometimes high levels in the lipid tissues of marine mammals. The pharmacokinetics of PCBs enables their transfer to subsequent generations during gestation and lactation and often prevents their metabolism and excretion by the detoxification CYP-MO system. The first aim of this thesis was to investigate CYP1A and CYP2B activities and apoproteins as biomarkers of PCB exposure in several marine mammal species. Chapter two investigates the patterns of PCB accumulation and the generational transfer of certain PCB congeners in two species of Arctic phocid seal. In conjunction with this contaminant data, the CYP-MO activities and apoprotein levels were determined and their relationship with both the concentrations of the mono-*ortho* CBs and the total 2,3,7,8-TCDD equivalents (Σ TEQ) of these congeners was investigated. Furthermore the capacity of developing pups for metabolising PCBs compared with their mothers was investigated, by examining CYP activities and expression.

Differences in the concentrations of PCBs and CYP activities and expression are frequently observed between species. Chapter three presents a preliminary investigation into the CYP-MO system of a harbour porpoise and a grey seal and again examines the levels of concomitant blubber PCBs in these two species. Most of the studies on CYP isoenzymes have immediately stored the liver samples in liquid nitrogen, prior to the preparation of microsomal suspensions which may take up to several weeks. Chapter

four aimed to determine the effect of prolonged frozen (-196°C) storage of marine mammal liver samples on CYP-MO activities. This research presents the first study documenting the effects of storing marine mammal liver in liquid nitrogen on CYP-MO activities. Furthermore the viability of partially preparing the liver samples in a glycerol buffer, in the field, before storing in liquid nitrogen was investigated.

The third aim of this research project was to determine the sequence of CYP1A from marine mammals and to produce species-specific CYP1A probe(s) for development of CYP1A as a biomarker for exposure to particular groups of environmental contaminants. A comparative analysis of CYP1A1 and -1A2 cDNA in two seal species and their deduced amino acid sequences are presented in Chapter five. Chapter six presents the first anti-peptide antibodies designed to be specific for seals, based on comparative mammalian CYP1A1 sequence data and the deduced sequence data in Chapter five.

Finally, non-destructive biomarkers need to be developed in order to monitor exposure of healthy, free-ranging populations of marine mammals to environmental contaminants. The final aim of this thesis was to develop such a biomarker using skin tissue from seals. Chapter seven presents the initial development of the CYP1A-mediated EROD activities in seal skin samples, based on a microassay. The final discussion of my research project and the directions that should be taken in the future are presented in Chapter eight.

2 Activity and expression of cytochrome P450 isoenzymes in relation to organochlorines in two Arctic species: Harp seal (*Phoca groenlandica*) and hooded seal (*Cystophora cristata*), with particular reference to mother – pup pairs.

2.1. Introduction

Environmental contaminants, including polychlorinated biphenyls (PCBs) are found at relatively high levels in the Arctic marine environment, a non-industrialised area. Contaminants are transported to the Arctic in the gas phase of the troposphere, on particles and via the oceans currents (Macdonald *et al.*, 2000). The Arctic may therefore be a principal global sink for environmental contaminants, as these pollutants have a tendency to be transported from warmer to colder temperatures, where they partition into the water column (Muir *et al.*, 1999; Norstrom and Muir, 1994 and references within).

Many environmental contaminants, particularly PCBs, have a low water solubility and a high lipid – water partitioning coefficient (Mackay, 1982). These lipid soluble contaminants efficiently accumulate in particulated organic matter and in the tissues of organisms, reaching the highest levels in the thick subcutaneous layer of blubber in marine mammals (Boon *et al.*, 1992).

Harp seals (*Phoca groenlandica*) and hooded seals (*Cystophora cristata*) are predominantly found in the Arctic, breeding on ice floes in regions such as West Ice (Bonner, 1989). The seals from the present study were sampled from the West Ice region during the 2000 breeding season. The lactation periods of these two species are more condensed than other seal species. In particular the hooded seal lactates for, on average, 4 days, compared to the 12 days of harp seals (Bowen *et al.*, 1985; Oftedal *et al.*, 1993; 1996). The abbreviated lactation periods of these seals are consistent with the

unstable nature of the ice (Bonner, 1989; Bowen *et al.*, 1985). Furthermore the hooded seal neonates are at a more advanced stage of development of any other seal species, including the harp seal.

During gestation and lactation the maternal fat depot is metabolised releasing contaminants back into circulation. The transfer of organochlorines and polyaromatic hydrocarbons from mother to pup occurs during lactation via the lipid-enriched milk, resulting in pollutants circulating in the pups system, prior to deposition in the blubber. Generation transfer of PCBs and other contaminants from the female to her pup has been observed in a number of seal species, including harp and hooded seals that were sampled from the West Ice region during 1990 (e.g. Addison and Brodie, 1987; Addison and Smith, 1998; Espeland *et al.*, 1997; Green *et al.*, 1996; Pomeroy *et al.*, 1996). In particular, first born of several animals, fin whales (*Balaenoptera physalus*), Steller's sea lion (*Eumetopias jubatus*) and humans, were exposed to higher concentrations of organochlorines than subsequent offspring (Aguilar and Borrell, 1994; Lee *et al.*, 1996; Rogan *et al.*, 1986).

Several studies have determined the concentration and blubber burdens of PCBs and other organochlorines in harp seals from the Canadian and Norwegian Arctic (Beck *et al.*, 1994; Oehme *et al.*, 1995; Ronald *et al.*, 1984; Wolkers *et al.*, 2000; Wolkers *et al.*, 1999; Zitko *et al.*, 1998). However, very few studies have investigated the exposure of hooded seals, or harp seals from West Ice and those that have suggesting CYP2B activity in these cetaceans. record exposure of the animals during 1990, some ten years ago (Espeland *et al.*, 1997; Goksøyr, 1995; Skaare, 1995). This study has investigated changes in exposure and uptake of PCBs during a time following legislation banning the

production and use of PCBs, suggesting reduced inputs of these contaminants into the environment.

The main excretion pathway of environmental contaminants, apart from during lactation in female animals, occurs predominantly via the hepatic detoxification system. Cytochrome P450 (CYP) isoenzymes, particularly families 1-4, are involved in the first phase of detoxification of these organic environmental contaminants (Stegeman and Hahn, 1994).

Several studies have investigated the induction and expression of different CYP isoenzymes in the liver of marine mammals that were environmentally exposed to environmental contaminants (e.g. Addison and Brodie, 1984; Hyyti *et al.*, 2001; Mattson *et al.*, 1998; Murk *et al.*, 1994; Nyman *et al.*, 2000; Nyman *et al.*, 2001; Troisi and Mason, 1997; van Hezik *et al.*, 2001; Wolkers *et al.*, 1999). However, only one study of hooded seals and two studies of harp seal CYP induction and expression have previously been performed (Goksøyr *et al.*, 1992; Wolkers *et al.*, 1999). Of these only one study investigated the CYP induction of pups of the two species (Goksøyr *et al.*, 1992). Pre-weaned pups are exposed to large concentrations of environmental contaminants via the maternal milk and consequently the pups own detoxification system, which includes the CYP isoenzymes, are targeted. Knowledge of how the pups can deal with exposure to environmental contaminants requires understanding of the CYP isoenzyme system.

The induction of CYP isoenzymes has been measured using specific CYP-mediated monooxygenase (MO) activities, such as CYP1A – mediated EROD and PROD in fish (Huuskonen and Lindstromseppa, 1995; Machala *et al.*, 1997), humans and rodents

(Burke *et al.*, 1994) as well as seals (e.g. Goksøyr, 1995; Goksøyr *et al.*, 1992; Mattson *et al.*, 1998). Further characterisation of the CYP isoenzymes in marine mammals has been determined by immunodetection using heterologous antibodies (Boon *et al.*, 2001; Goksøyr *et al.*, 1989; Goksøyr *et al.*, 1992; Hyyti *et al.*, 2001; Mattson *et al.*, 1998; White *et al.*, 1994; Wolkers *et al.*, 1999).

Molecular, biochemical and cellular changes that occur in response to environmental contaminants can be used as biomarkers of such exposure (McCarthy, 1992). Biomarkers, such as the induction of CYP isoenzymes, can serve as an early warning system of the response in organisms (den Besten, 1998). Differential CYP induction and expression have been observed between species, therefore in order to use CYPs as biomarkers, knowledge of the CYP system in the species of interest is required.

The aim of this study was to investigate the levels of PCBs in harp and hooded seals and compare them with the levels of PCBs in these two species from the same region sampled in 1990. Furthermore the generation transfer of PCBs from mother to pup in both species was investigated and the induction of CYP1A and CYP2B in harp and hooded seal adults and pups was examined. The expression of CYPs in pups was examined and compared with their respective mothers to investigate the capability of the developing pups to metabolise PCBs that target particular CYPs. The sum of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (-TCDD) toxic equivalents (Σ TEQ) values were calculated for certain PCB congeners and the relationship with CYP content and activities were investigated as a method for biomonitoring.

2.2. Materials and Methods

Chemicals. 7-ethoxyresorufin, 7-pentoxyresorufin and their product, resorufin, phenazine ethosulphate (PES) and the pre-stained molecular weight markers (SDS-7B) were all purchased from Sigma Chemicals, UK. Coomassie Brilliant Blue R250 was bought from Biorad, Germany. The primary antibodies, anti-trout CYP1A1 and anti-rat CYP2B were kind gifts from Dr R. Addison, Institute of Oceanic Sciences, Canada and Dr S. Bandiera, University of British Columbia, Vancouver, respectively. The enzyme conjugated secondary antibody, anti-rabbit horseradish peroxidase (HRP) was purchased from the Scottish Antibody Production Unit (SAPU, Diagnostics Scotland, UK). All the chemicals were of analytical grade.

2.2.1. Samples. Liver samples from two adult female hooded seals (*Cystophora cristata*) and their two pups, and three adult female harp seals (*Phoca groenlandica*) and their three pups were taken from West Ice, about 60 miles east of Greenland, during March 2000. In addition one adult female hooded seal and four unrelated hooded seal pup livers and three unrelated harp seal pup livers were also collected. The tissues collected were a surplus of samples taken by the Department of Arctic Biology, Tromsø. Within 10 minutes of death, liver samples were placed immediately on ice, prior to preparation of the post-nuclear pellet. Body length was measured to the nearest centimetre and body mass was measured to the nearest 0.5 Kg. The blubber thickness was measured at the thickest point, ventrally. Blubber mass (kg) was calculated from the following formula (Ryg *et al.*, 1990):

% Blubber mass

$$= 4.44 + (5693 \times \text{SQRT} ((\text{Length (m)}/\text{Body mass (kg)}) \times \text{Blubber thickness (m)})),$$

where 4.44 and 5693 are constants for phocid seals. Data of biological parameters of the 18 seals are presented in Table 2.1. Samples of blubber (~20g) were excised from the

Species	Identification	Sex	Age	Weight (kg)	Length (m)	Blubber mass (Kg)
Hooded seal	CcMa	F	Adult	118	1.6	35.54
	CcMb	F	Adult	179	1.87	54.53
	CcA1	F	Adult	143	1.63	45.22
	CcPa	F	Pup	50.5	1.07	23.17
	CcPb	M	Pup	20	0.8	9.71
	CcP1	M	Pup	47	0.99	17.13
	CcP2	M	Pup	35	1.06	17.06
	CcP3	F	Pup	25	0.98	12.95
	CcP4	F	Pup	35.5	1.15	15.76
	PgMa	F	Adult	89.5	1.74	35.75
	PgMb	F	Adult	104	1.63	45.33
	PgMc	F	Adult	97.5	1.56	45.87
	PgPa	M	Pup	28.5	0.89	13.80
	PgPc	M	Pup	28	0.97	13.59
PgPb	F	Pup	29.5	1.02	15.28	
Harp seal	PgP1	F	Pup	11	0.84	5.96
	PgP2	F	Pup	24	0.92	11.91
	PgP3	M	Pup	22	0.88	11.07

Table 2.1. Biological data for hooded (Cc) and harp (Pg) seals. Mother -pup pairings are indicated by M or P followed by a letter. Unrelated adults and pups are referred to as A or P followed by a number. Blubber mass (Kg) was calculated using the formula from Ryg *et al.*, 1990, presented in the materials and methods section 2.2.1.

below the sternum, wrapped in foil and immediately stored at -20°C until preparation for PCB analysis.

2.2.2. Preparation of liver samples. Liver samples were prepared according to Ozols (1990) with the following modifications. The seal liver samples ($\sim 10\text{g}$) were macerated using scissors. Subsamples of liver ($\sim 3.5\text{g}$) were initially homogenised in one volume of store buffer (0.1M Tris - acetate, pH 7.4 containing 0.4mM PMSF, 1.0mM EDTA, 0.1mM DTT and 20% glycerol) using a blade homogeniser at 3000rpm for 2x 10s. The resulting crude suspension was then further homogenised for 2x 5 passes using a Potter-Elvehjem homogeniser, to attain a homogenous suspension. The homogenate was stored on ice for one minute between homogenisations to prevent an increase in temperature and was subsequently maintained at 4°C .

The sub-sampled homogenates were filtered through two layers of wet muslin to remove connective and fatty tissue. The muslin was rinsed with a further two volumes of store buffer. Subsequently the filtrates were centrifuged in a pre-cooled (4°C) Centra-3R centrifuge at 700g for 15 minutes, to remove nuclear material and other debris. The post-nuclear supernatant was divided into cryovials and stored in liquid nitrogen until further preparation. Glycerol was added to the store buffer to protect against enzyme inactivation. The addition of 20% glycerol to the final store buffer containing liver microsomes prevented the degradation of cytochrome P450 and UDP-glucuronyl transferase enzyme activities in fish, during storage at -80°C (Forlin and Andersson, 1985; This Thesis: Chapter four).

On return to the laboratory, microsomal suspensions were prepared from the post-nuclear supernatants. The post-nuclear supernatants were thawed on ice and transferred

into centrifuge tubes. The homogenate was then centrifuged in a Beckman JA-14 centrifuge with a JA-20 fixed angle rotor at 10,000g for 30 minutes to sediment the mitochondria. Filtration of the resulting supernatant through four layers of muslin removed lipids and fibrous debris prior to its transfer to Beckman ultracentrifuge tubes. The microsomal fraction was attained after differential centrifugation of the supernatant in a pre-cooled Beckman L-60 Ultracentrifuge with 42.1 rotor at 105,000g for 90 minutes.

The microsomal pellet was washed once in wash buffer (0.05M Tris-HCl, pH 7.4 containing 0.15M KCl) to remove haemoglobin and other adsorbed proteins (Gibson and Schenkman, 1978). The microsomal pellet was dispersed using the homogeniser and a spatula in 2mL wash buffer/ tube prior to the wash in a further 20mL/ tube. Re-sedimentation of the microsomal fraction occurred after ultracentrifugation at 105,000g for 60 minutes. Finally the microsomal fraction was resuspended in store buffer containing 20% glycerol to a protein concentration of 25mg/mL and stored in 1mL aliquots at -70°C.

2.2.3. Total Protein Concentration. Total protein concentration of the microsomal fractions was determined using a modified procedure of the Bradford assay (Stoscheck, 1990). This method has been shown to be more accurate than the Lowry's method, which is subject to interference by a number of compounds such as potassium and magnesium ions, EDTA, Tris, thiol reagents and carbohydrates that are likely to be present in the fraction and the suspension buffer. The Biuret assay is also relatively insensitive (Bradford, 1976).

Microsomal protein fractions were prepared as previously described and diluted 10-, 20- and 40- fold in wash buffer. Dilutions of microsomal protein were necessary to ensure that the absorbance was < 1.0 a.u. since above 1.0 a.u. the standard curve became non-linear (Bradford, 1976). Bovine serum albumin (BSA) was used for the standard curve at concentrations between 0 and 1mg/mL at 0.2 increments.

Briefly, 5 μ L 10M NaOH was added to 5 μ L of either microsomal protein at 10-, 20- and 40-fold dilutions or 5 μ L wash buffer for the blank into their respective wells in microtitre plates. The addition of NaOH solubilises membrane bound protein and is necessary to prevent underestimation of the total protein concentration (Stoscheck, 1990).

Finally, 200 μ L of the Bio-Rad protein assay dye at 1 in 5 dilution was added to all the wells and the colour of the Coomassie Brilliant Blue G-250 dye from red to blue form, which occurs on binding to protein, was allowed to develop. After 5 minutes the absorbance at 570nm was measured against the protein blank on a Dynatek MR 5000 plate reader (Guernsey Channel Islands, UK). Subsequently, the relative concentration of protein in the microsomal fractions was calculated using the equation derived from the standard curve (absorbance vs. concentration of BSA in mg/mL).

2.2.4. Spectrophotometric determination of the concentration of cytochrome P450.

The concentration of cytochrome P450 was determined according to the method of Johannesen and DePierre (1978). The method was based on the difference spectrum of dithionite-reduced carbon monoxide (CO)-complexed microsomes minus non-reduced CO-complexed microsomes. This method was not particularly sensitive for detecting individual CYP isoenzymes, as increases in individual CYP isoform concentration may not affect the overall CYP concentration as determined spectrophotometrically.

However, the total concentration of CYP was used to express CYP1A-associated EROD activity per nmol of total CYPs present in the sample, independently of protein concentration and therefore effects of protein degradation. Measurement of this cytochrome may be interfered by several contaminants that have similar absorption maxima. Haemoglobin and methaemoglobin, in particular are present in high concentrations in diving mammals, and have absorption maxima at 424nm. The presence of cytochrome b5, which has an absorption maximum at 425nm, may obscure the peak at 450nm.

Partial degradation of liver samples may also cause the solubilisation of subcellular components, such as mitochondria, thus releasing cytochrome c (absorption maximum at 420nm) and cytochrome oxidase which have absorption minimum at 445nm, and could cause a shift in the 450nm peak. Reduction of these contaminants in both the sample and reference cuvettes, without reducing the cytochrome P450, enables the measurement of this cytochrome alone (Johannesen and DePierre, 1978).

Initially, CO was bubbled through the microsomal protein (25mg/mL) at a rate of ~1 bubble/ second for 3 min. Both the sample and reference cuvettes (optical glass, 1cm pathlength, Hellma, Germany) contained 910 μ L 100mM Tris buffer, pH 7.5, 50 μ L CO-complexed microsomal protein, 10 μ L 25mM ascorbate, 10 μ L 0.25mM Phenazine ethosulphate and 10 μ L NADH. The assay was subjected to spectrophotometric analysis in a Uvikon 923 double beam spectrophotometer (Bio-tek, Kontron Instruments) between 400nm and 500nm and the baseline was recorded. The addition of 10 μ L 100mM sodium dithionite to the sample cuvette reduced the cytochrome P450 and the difference spectrum was recorded between 400 and 500nm. The concentration of P450 was calculated from the change in absorption between 450nm and 490nm and the molar

extinction coefficient (Σ), $105\text{mM}^{-1}\cdot\text{cm}^{-1}$, using the Beer-Lamberts relationship, concentration = absorbance/ Σ .

2.2.5. EROD and PROD Assays. Reagents: Substrates and products: Stock solutions of the substrates, 1mM 7-pentoxoresorufin and 7-ethoxoresorufin and the product, 0.145mM resorufin were prepared in DMSO and stored at -20°C until required.

The enzyme activities of the cytochrome P4501A- mediated monooxygenases (MO), 7-ethoxoresorufin-*O*-deethylase (EROD) and 7-pentoxoresorufin-*O*- deethylase (PROD), respectively, were determined using the methods of Prough and colleagues (1978) and Nilsen and co-workers (1998). The protocol and technique were checked using quality control flounder liver post-mitochondrial supernatant (S9 fraction; CEFAS, UK) in the EROD assay. The specific activity obtained was $49.1 \pm 0.05 \text{ pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein compared to $55 \text{ pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein determined by the CEFAS laboratory, indicating that the assay was accurate. The limit of detection of resorufin was highly sensitive at $0.01 \text{ pmol resorufin /min}$.

The EROD assay contained a final concentration of 0.1mg/mL microsomal protein, $1\mu\text{L}$ 1mM 7-ethoxoresorufin, in a final volume of 1mL made up with 0.1M Tris-HCl buffer, pH 7.8. The PROD assay contained a final concentration of 0.2mg/mL microsomal protein, $3.2\mu\text{L}$ 1mM 7-pentoxoresorufin, made up to a final volume of 1mL with pre-warmed 0.1M Tris-HCl buffer, pH 7.8.

Prior to recording the baseline, the assay contents were mixed by gentle vortexing and incubated for 2 min at 37°C , then transferred to pre-warmed fluorimetric quartz cuvettes (capacity - 1.0mL, pathlength 1cm, Hellma, Germany).

Subsequently, the reaction mixture was subjected to fluorimetric spectrophotometric analysis in a Perkin-Elmer Fluorimetry Spectrometer. The product, resorufin has specific excitation (ex) and emission (em) wavelengths (λ): λ_{ex} 530nm and λ_{em} 585nm that were pre-checked using the scanning programme on the Perkin –Elmer fluorimeter.

Initially the baseline was recorded for 1 min prior to initiating the reaction with 10 μ L 10mM NADPH (prepared fresh) and measuring the increase in fluorescence over 4 min. The reaction was internally calibrated by the addition of 10 μ L 30 μ M resorufin. The concentration of the product formed over time from the concentration of microsomal protein initially added and was quantified by the following calculation (Nilsen *et al.*, 1998):

$$\text{nmol product formed. min}^{-1} \cdot \text{mg}^{-1} \text{ protein} = \frac{F_{\text{s}}}{\text{min}} \times \frac{R}{F_{\text{r}}} \times \frac{1}{C_{\text{s}}} \times \frac{1}{V_{\text{s}}}$$

Whereby, F_{s}/min = the increase in sample fluorescence / minute; R = the amount of product added as internal calibration (nmol); Fr = the increase in fluorescence of product standard; Vs = sample volume (mL); Cs = Protein concentration of sample (mg. mL⁻¹). A standard curve of the purified product resorufin was constructed in order to check the addition of internal resorufin standard (Fig. 2.1.).

The complete reaction was set up as previously described, with the substitution of heat-inactivated microsomes (100°C for 5 min) for the microsomal protein. Resorufin was added to 0, 0.1, 0.25, 0.5 and 1.0 nmol in the assay and the intensity of fluorescence emitted was recorded. The intensities obtained from resorufin added internally compared with the standard curve were similar.

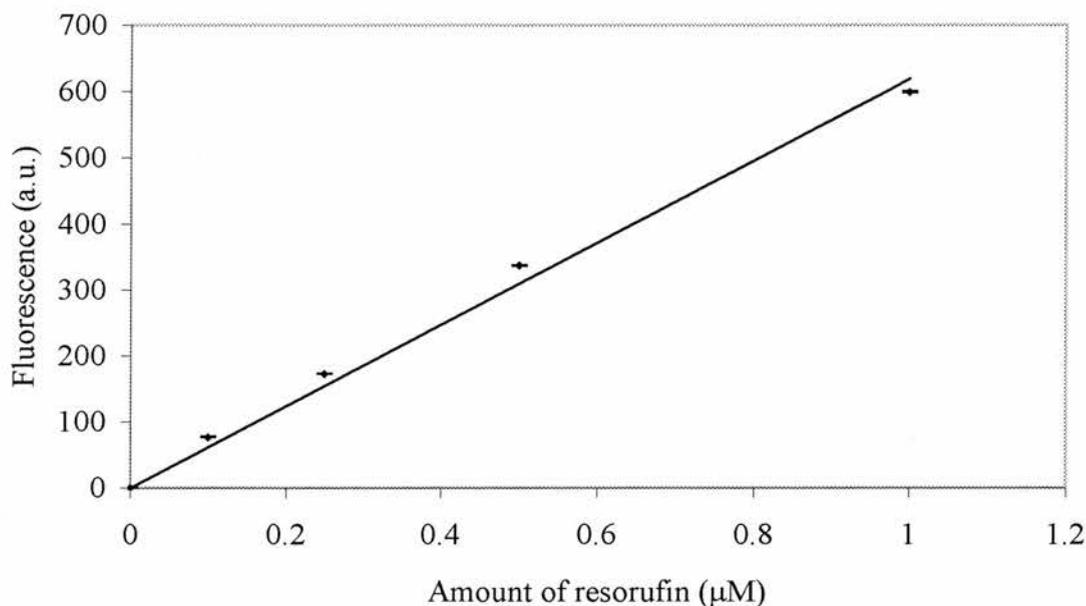


Fig. 2.1. A standard curve of purified resorufin in the EROD assay mixture with heat-inactivated microsomes. $R^2 = 0.9923$.

2.2.6. SDS-PAGE and immunochemical detection.

2.2.6.a. SDS-PAGE. Microsomal samples were initially solubilised in 5µL (per 30µL sample) reducing solution (3 parts magic mix: 20% SDS, 1M Tris, pH 6.9, 25% glycerol, 10mg bromophenol blue to 1 part mercaptoethanol) and boiled in a heating block for 2 minutes prior to loading into the stacking gel. Microsomal proteins, including cytochrome P450 isoenzymes, were separated by SDS-PAGE on slab gels according to Laemmli (1970). Concentrations of microsomal samples and % SDS-PAGE gels used are provided in figure legends.

2.2.6.b. Visualisation of the protein bands was by Coomassie staining (2.5g Coomassie Brilliant Blue R250, 450mL methanol, 450mL water and 100mL acetic acid) and subsequent destaining of the background in destain (500mL methanol, 150mL acetic acid to a final volume of 2L with water). Polyacrylamide gels for transfer of separated protein bands on to polyvinylidene difluoride (PVDF) blotting membranes (Electran[®], BDH, UK) and subsequent immunodetection, were not stained prior to transfer.

2.2.6.c. Wet blotting of protein bands to PVDF membrane. At the end of the electrophoresis run, the polyacrylamide gels for immunochemical detection, were washed 3 times each for 4 minutes in transfer buffer (10mM CAPS, 10% Methanol, pH 11.0 adjusted with 5M NaOH). The PVDF membrane was activated in methanol then washed in the transfer buffer for 4 minutes. Blotter papers and fibre pads were also pre-soaked in transfer buffer. The 'transfer sandwich' was prepared in transfer buffer with 2 fibre pads enclosing the blotting paper, PVDF membrane and acrylamide gel. Successful transfer of the proteins from the gel to the PVDF membrane was accomplished in the tank electro-transfer unit (Biorad, Germany) at 300mA constant current for 2 hours. A cooling container was placed in the tank to maintain low temperatures and was changed after 1 hour.

2.2.6.d. Immunochemical Detection. The transferred protein bands were detected on the PVDF membrane by immunoblotting using the method of Okita and co-workers (1997) with modifications.

Detection of CYP1A. At the end of the transfer procedure, the membrane was blocked with 20mL blocking buffer (PBS, 5% (w/v) nonfat milk powder, 0.1% Tween-20) for 2 hours on a rotating platform. Subsequently the membrane was incubated with the anti-trout CYP1A1 peptide antibody at 1 in 20,000 dilution in blocking buffer over night.

The non-specifically bound primary antibody was washed for 10 minutes from the membrane with 2x 20mL blocking buffer. The membrane was rinsed and transferred into clean containers between each wash. The secondary antibody, conjugated to horseradish peroxidase (HRP), was used to detect the protein bands bound to the primary antibody. Anti-rabbit HRP antibody was used as the secondary antibody at a 1

in 5000 dilution, in 20 mL of blocking buffer and was incubated on a rotating platform for 2 hours at room temperature.

Subsequently non-specifically bound secondary antibody was removed from the membrane by washing with 2x 20mL blocking buffer, followed by 3x 20mL PBS containing 0.1% Tween-20. The protein bands were detected by enhanced chemiluminescence (ECL) and visualised by autoradiography films (Fuji medical x-ray films, Fuji Photo Film Co. LTD, Japan). The intensities of the bands were determined using GelDoc 1000 analysis system and software (Multianalyst, version 1.02, 1997, Bio-Rad, Germany).

Detection of CYP2B. CYP2B protein bands were detected essentially as described for CYP1A, with the following modifications. The initial blocking occurred for 10 minutes, followed by incubation of the PVDF membrane with anti-rat CYP2B antibody at 1 in 860 dilution in 20mL of blocking buffer for 2 hours. The same secondary antibody was used and detection and visualisation of the protein bands were identical to that described for CYP1A.

The detection of specific proteins was highly sensitive (<0.7pmol) and the degree of accuracy was relatively good, reacting with only one or two bands in most cases, although the antibodies used were raised against a homologous protein from other vertebrate species.

2.2.7.a. PCB analyses. Dr. N. Green (University of Lancaster, UK) determined PCB analysis and lipid content of the blubber as previously described (Pomeroy *et al.*, 1996). Briefly the samples were allowed to thaw to room temperature and a subsample was

excised from the centre of the sample, in order to avoid tissue that had been in contact with the air. The subsample was weighed into an extraction thimble containing anhydrous sodium sulphate. Aliquots were spiked with standard amounts of seven $^{13}\text{C}_{12}$ -labelled CB congeners (IUPAC numbers CB-28, -52, -101, -138, -153, -180, -209) (Ballschmiter and Zell, 1980). Each sample was transferred to a pre-cleaned Buchi 810 soxhlet apparatus and extracted with hexane for 16 hours. A known volume of the extract was transferred to a pre-weighed vial for the determination of lipid content. The solvent was evaporated under a stream of dry nitrogen and the lipid content of the blubber was determined by mass difference.

2.2.7.b. Chlorobiphenyl (CB) separation and quantification. The remaining portion of the extract was cleaned up by a two step adsorption chromatography procedure. The extract was transferred onto a 20mm ID column packed from bottom to top with 0.5g sodium sulphate, 1g silica gel, 2g 33% 1N NaOH_{aq} /silica gel, 1g silica gel, 4g 46% CH_2SO_4 /silica gel, 1g silica gel and 0.5g sodium sulphate and the adsorbent was washed with 20mL hexane, which was then discarded. The PCBs were eluted from the column with 110mL hexane. The eluate was concentrated under reduced pressure of N_2 at 40°C.

The eluate was further cleaned on a 10mm column packed with 4.5g Alumina 1 Super B (ICN Biomedical) and topped with a plug of sodium sulphate. The adsorbent was rinsed with 20mL hexane before applying the eluate, which was discarded. The sample was applied to the column and eluted with 15mL 7% DCM/hexane. The eluate was concentrated in volume at 40°C under a gentle stream of nitrogen. The clean extract was transferred to a GC vial and an injection of standard $^{13}\text{C}_{12}$ -labelled PCB #141 was added. Subsequently the extract was reduced to a final volume of 15 μL .

Twenty-seven CB congeners (nineteen CB congeners and four co-eluted CB congeners) (IUPAC numbers CB-95, CB-101/90, CB-99, CB-87/115, CB-110, CB-118, CB-114, CB-105, CB-151, CB-149, CB-153/132, CB-141, CB-138, CB-156, CB-157, CB-187, CB-183, CB-174, CB-180, CB-170/190, CB-189, CB-203, CB-194) were isolated by automated splitless injection, using a Hewlett Packard 6890 gas chromatograph (GC). The GC was set up to inject 1 μ L of the final extract onto a 30m Hewlett Packard 5- ms capillary column. The GC was coupled to a Micromass Autospec Ultima high resolution mass spectrometer, tuned to 2500 tuning power, operating in SIM. Quantification of the PCBs was by isotope dilution and recoveries were corrected for on the basis of the seven surrogate standards (mean recoveries of $^{13}\text{C}_{12}$ -labelled internal standards were CB-28: 71% recovery; CB-52 78% recovery; CB-101: 79% recovery; CB-153: 99% recovery; CB-138: 105% recovery; CB-180: 115% recovery; CB-209: 122% recovery).

2.2.7.c. Quality assurance of chemical analyses. Three laboratory blanks were run with the eighteen blubber samples and detection limits were derived from the mean levels in the blanks plus three times their standard deviations. The blubber concentrations were not blank corrected, however were at least 10 times higher than the detection limits for all reported congeners. The blubber samples were run in two batches. One blubber sample was analysed in triplicate with the first batch and a fourth time with the second batch in order to ensure consistency of analytical methods.

2.2.8. Statistical analyses. The data was tested for normal distribution using the Kolmogorov-Smirnov one sample test for each species. Comparison between mother-pup pairs was carried out using pairwise t-test for two sample means, after log transformation of the individual data. Correlation between different catalytic activities and between activities and PCB levels were calculated for each species after log

transformation of the individual data. All other statistical analyses were performed using the non-parametric, Kruskal-Wallis test. The level of significance, in all cases, was pre-set to $p \leq 0.05$.

Principal component analysis (PCA) was performed on the data of 27 CBs expressed as the % of the total PCBs, to reduce the data into a set of independent composite factors. The factors within the PCA were computed from the correlation matrix of varimax rotated data and expressed the contaminant patterns within both seal species. The number of factors that defined the contaminant patterns was determined from the % variance and the scree plot. Statistical analyses were performed using SYSTAT (version 9, SPSS Inc., 1999).

2.3. Results

2.3.1. PCB Concentrations and burdens

2.3.1.a. Inter-specific comparison: PCB concentrations. Blubber concentrations of PCBs (Σ PCBs, sum of 27 CB congeners) in harp and hooded seals ranged between 212.09 – 822.44ng.g⁻¹ lipid and 388.99 – 2854.23ng.g⁻¹ lipid, respectively (Table 2.2). The geometric mean PCB concentrations were very similar to the arithmetic mean (Table 2.2) and were significantly higher in the hooded seal adults and pups compared to harp seals (For the adults, $H = 3.857$, $df = 1$, $p = 0.05$ and for pups, $H = 8.308$, $df = 1$, $p = 0.004$). The adults of each species also contained significantly higher PCB concentrations compared to the pups of the same species, hooded seal adults > hooded seal pups and harp seal adults > harp seal pups (For both, $H = 5.4$, $df = 1$, $p = 0.02$).

2.3.1.b. Inter-specific comparison: Body burdens. The body burdens were calculated using the following equation:

Species: Groups of PCB congener	Parameter	Harp			Hood			
		Adult (R) n=3	Pup (R) n=3	Pup (UR) n=3	Adult (R) n=2	Pup (R) n=2	Adult (UR) n=1	Pup (UR) n=4
Σ Mono- <i>ortho</i> CBs (ng/g lipid)	Mean \pm SE	65.81 \pm 16.98	42.01 \pm 5.75	45.95 \pm 1.77	84.53 \pm 29.39	37.56 \pm 7.30	75.71	48.74 \pm 3.34
	Median	75.70	44.09	44.73	84.53	37.56		46.09
	Range	32.72 - 88.99	31.17 - 50.77	43.67 - 49.45	55.14 - 113.93	30.26 - 44.86		44.11 - 58.67
Σ Di- <i>ortho</i> CBs (ng/g lipid)	Mean \pm SE	557.03 \pm 110.21	223.98 \pm 26.83	278.79 \pm 2.68	1957.75 \pm 642.19	505.80 \pm 166.34	980.58	621.10 \pm 73.09
	Median	623.48	242.5	279.82	1957.75	505.804		602.03
	Range	341.81 - 705.81	171.11 - 258.33	273.71 - 282.84	1315.55 - 2599.94	339.47 - 672.14		495.89 - 784.44
Σ Mixed ^a (ng/g lipid)	Mean \pm SE	38.26 \pm 7.13	13.20 \pm 1.74	14.58 \pm 0.29	114.84 \pm 25.53	26.52 \pm 7.27	53.83	31.56 \pm 4.4
	Median	40.93	14.21	14.82	114.84	26.52		31.14
	Range	24.80 - 49.05	9.82 - 15.57	13.99 - 14.93	89.31 - 140.36	19.25 - 33.79		23.19 - 40.77
Σ PCBs (ng/g lipid)	Mean \pm SE	661.10 \pm 132.06	279.19 \pm 33.62	339.32 \pm 1.58	2157.12 \pm 697.11	569.89 \pm 180.90	1110.12	701.40 \pm 80.13
	Median	761.53	308.85	338.42	2157.12	569.89		678.32
	Range	399.32 - 822.44	212.09 - 316.63	337.15 - 342.39	1460.00 - 2854.23	388.99 - 750.79		565.07 - 883.88
	G. mean	630.05	274.75	339.31	2041.37	540.41		687.80

Table 2.2. Mean \pm standard error (SE), median and range of the concentration of blubber PCB congeners divided into three groups, mono-*ortho* CBs, di-*ortho* CBs and mixed^a mono- and di-*ortho* CBs, and total PCB concentration. The mixed CBs are a consequence of difficulty in separation of four CBs. (R) represents related adults and pups; (UR) represents unrelated animals that were measured for PCB concentration. Mean \pm SE is the arithmetic mean, whereas G. mean refers to the geometric mean.

$$\text{PCB burden (g)} = (\text{PCB concentration}) \times (\text{concentration of lipid}) \times (\text{blubber weight})$$

$$\text{(g. g}^{-1} \text{ lipid)} \qquad \text{(g. g}^{-1} \text{ blubber)} \qquad \text{(g)}$$

The burdens of the PCBs for harp and hooded seals are presented in Table 2.3 and ranged between 0.22 – 24.18mg and 5.67 – 76.33mg, respectively. Again, the geometric means were very similar to the arithmetic means. The hooded seals had significantly greater burdens of PCBs than the harp seals of comparable age range (adults, $H = 3.857$, $df = 1$, $p = 0.05$ and pups, $H = 5.769$, $df = 1$, $p = 0.016$). The adults of each species also had significantly higher PCB burden than the pups of the same species, hooded seal adults > hooded seal pups and harp seal adults > harp seal pups (For both species, $H = 5.4$, $df = 1$, $p = 0.02$).

The total concentration and burdens of mono-*ortho* chlorobiphenyls (CBs) in adult seals were very low in comparison to the di-*ortho* CBs, which made up the majority of the total PCBs measured in the blubber. The most prominent individual CBs included the mono-*ortho* CB-118, -105, -110 and -156 and the di-*ortho* CB-153/132, -138, -180, -99, in both the harp and hooded seals. The four prominent mono-*ortho* CBs comprised only 12% and 6% of the total PCB burdens, compared to 66% and 72% of the total PCB burdens for the four di-*ortho* CBs in harp and hooded seals, respectively.

2.3.1.c. Inter-specific variation of PCB patterns. The variation between contaminant patterns between hooded and harp seals was examined by principal component analysis (PCA). Three factors were generated by PCA and explained 90.52% of the total variability. The first two factors combined explained 80.54% of this variability and were used to plot factor scores for each individual of both species (Fig. 2.2).

Group of PCB congeners	Species: Parameter	Harp			Hooded			
		Adult (R) n=3	Pup (R) n=3	Pup (UR) n=3	Adult (R) n=2	Pup (R) n=2	Adult (UR) n=1	Pup (UR) n=4
Σ Mono- <i>ortho</i> CBs (mg)	Mean \pm SE	2.47 \pm 0.56	0.51 \pm 0.05	0.25 \pm 0.13	3.02 \pm 0.59	0.54 \pm 0.38	2.93	0.65 \pm 0.08
	Median	2.89	0.51	0.38	3.02	0.54		0.68
	Range	1.36 – 3.17	0.42 – 0.59	1.8x10 ⁻⁴ -0.38	2.43 – 3.62	0.16 – 0.93		0.43 – 0.79
Σ Di- <i>ortho</i> CBs (mg)	Mean \pm SE	21.34 \pm 4.46	2.71 \pm 0.19	1.51 \pm 0.76	70.24 \pm 12.28	7.86 \pm 6.06	37.96	8.04 \pm 0.86
	Median	20.21	2.84	2.12	70.24	7.86		7.56
	Range	14.24 – 29.56	2.33 – 2.97	0.0011 – 2.41	57.96 – 82.51	1.80 – 13.92		6.53 – 10.50
Σ Mixed ^a (mg)	Mean \pm SE	1.45 \pm 0.21	0.16 \pm 0.01	0.08 \pm 0.04	4.19 \pm 0.26	0.40 \pm 0.30	2.08	0.41 \pm 0.05
	Median	1.59	0.16	0.11	4.19	0.40		0.37
	Range	1.03 – 1.71	0.13 – 0.18	6x10 ⁻⁵ – 0.13	3.93 – 4.45	0.10 – 0.70		0.35 – 0.55
Σ PCBs (mg)	Mean \pm SE	25.25 \pm 5.15	3.38 \pm 0.25	1.84 \pm 0.92	77.45 \pm 13.13	8.81 \pm 6.74	42.98	9.09 \pm 0.96
	Median	24.69	3.61	2.61	77.45	8.81		8.61
	Range	16.63 – 34.44	2.89 – 3.64	0.0014 – 2.91	64.33 – 90.58	2.07 – 15.55		7.31 – 11.83
	G. mean	24.18	3.36	0.22	76.33	5.67		8.95

Table 2.3. Mean \pm standard error (SE), median and range of the body burdens of PCB congeners (mg) divided into three groups, mono-*ortho* CBs, di-*ortho* CBs and mixed^a mono- and di-*ortho* CBs, and body burden expressed in mg Σ PCB congener. The mixed CBs are a consequence of difficulty in separation of four CBs. (R) represents related adults and pups; (UR) represents unrelated animals that were measured for PCB burden in the blubber. Mean \pm SE is the arithmetic mean, whereas G. mean refers to the geometric mean.

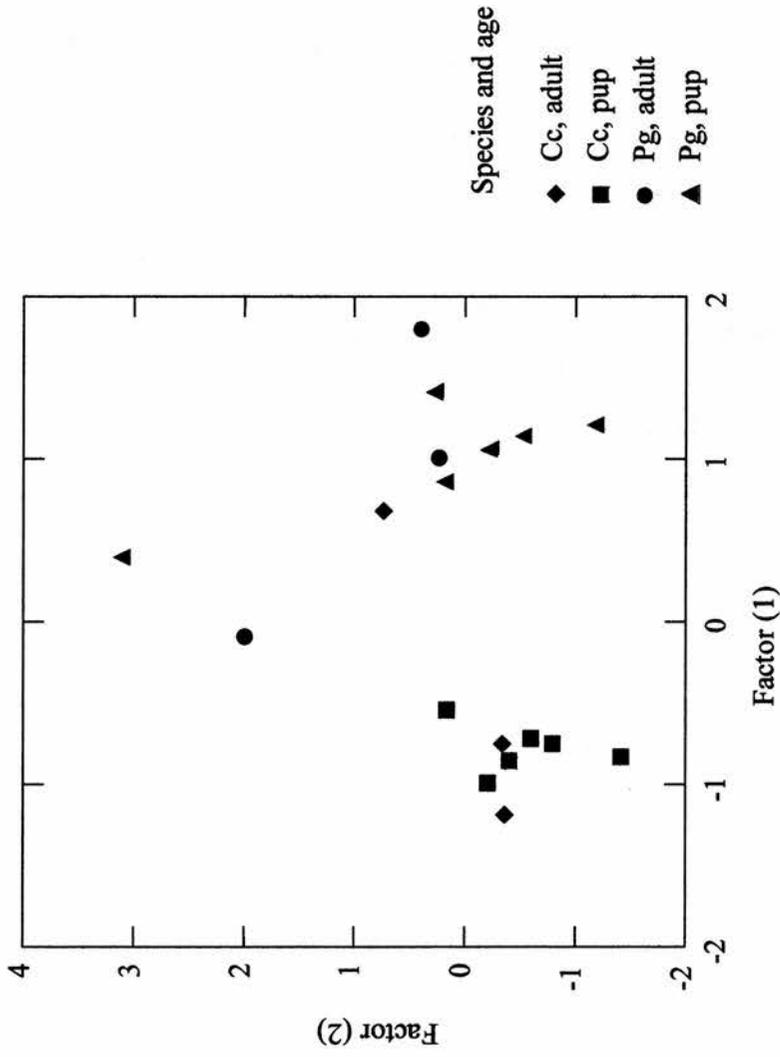


Fig.2.2. Factor plot of principal components one and two (Factor 1 and 2, respectively) of the correlation of the % of each CB to the total PCBs in each individual animal. Cc, *Cryostaphora cristata* (Hooded seal), Pg, *Phoca groenlandica* (Harp seal). Age is by category.

The factor score plots (Fig. 2.2) show the factors were separated between species, but not age group (adult or pup). Separation of the two species occurred because of the higher proportion of CBs-138, -141, -174, -170/190, -149 and -114 in the hooded seals than the harp seals. Whereas the harp seals have a higher proportion of the CBs-156, -157, 87/115, -110, -99, -203, -194 and -183. Two harp seals (PgMc and PgPc) showed higher proportion than the other harp seals and hooded seals of CBs-118, -105 and -151.

2.3.2.a. Intra-specific comparison of PCB levels: Mother-pup pairs.

The individual congeners were plotted as the % of total PCB concentration to compare the profiles between the mother and her respective pup for the mean of the three harp seal mother - pup pairs and the mean of both hooded seal mother – pup pairs (Fig.2.3.a and b). In all cases, the concentrations of the lower chlorinated CBs (CB-95, -101/90, -99, -87/115, -110, -118, -114, -105, -151, -149, -141, -157 and -174) were higher in the pup than the mother, and were proportionally represented. In addition, PgPa, PgPb, CcPb had higher %CB-138 of Σ PCBs and CcPa had higher %CB-153/132 of Σ PCBs than the respective mother.

The harp and hooded seal pups all exhibited an identical pattern of mono-*ortho* CBs in their blubber, in decreasing concentrations CB-118 > CB-105 > CB-110 > CB-156 > CB-157 > CB-114 > CB-189. The three most concentrated and the five least concentrated di-*ortho* CBs were present in an identical pattern in both species, in decreasing concentrations, CB- 153/132 > CB-138 > CB-99 and the least concentrated, CB-141 > CB-151 > CB-203 > CB-194 > CB-174. However, the remaining CBs were in a variety of combinations between the two extremes.

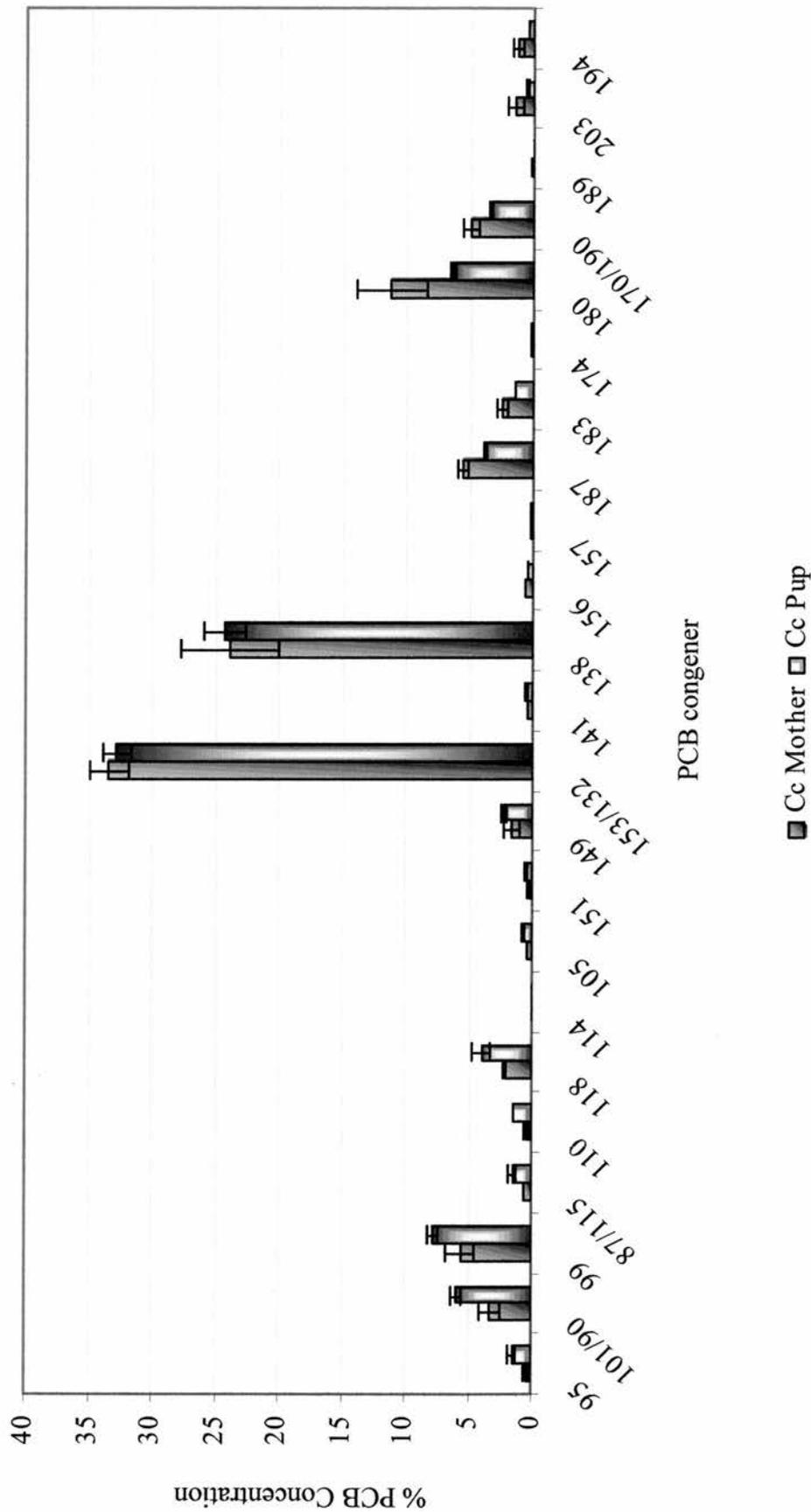


Fig. 2.3.a. Comparison of Hooded seal (Cc, *Cystophora cristata*) mother and pup congener patterns from the blubber. The % PCB concentration is the mean \pm 1 standard error for mothers CcMa and CcMb and their respective pups CcPa and CcPb. For identification of individuals refer to Table 2.1. PCB congeners are shown as IUPAC numbers. Missing peaks for certain congeners are due to very small % contribution to Σ PCB, similarly the error bars are apparently missing for some peaks when the error was extremely small.

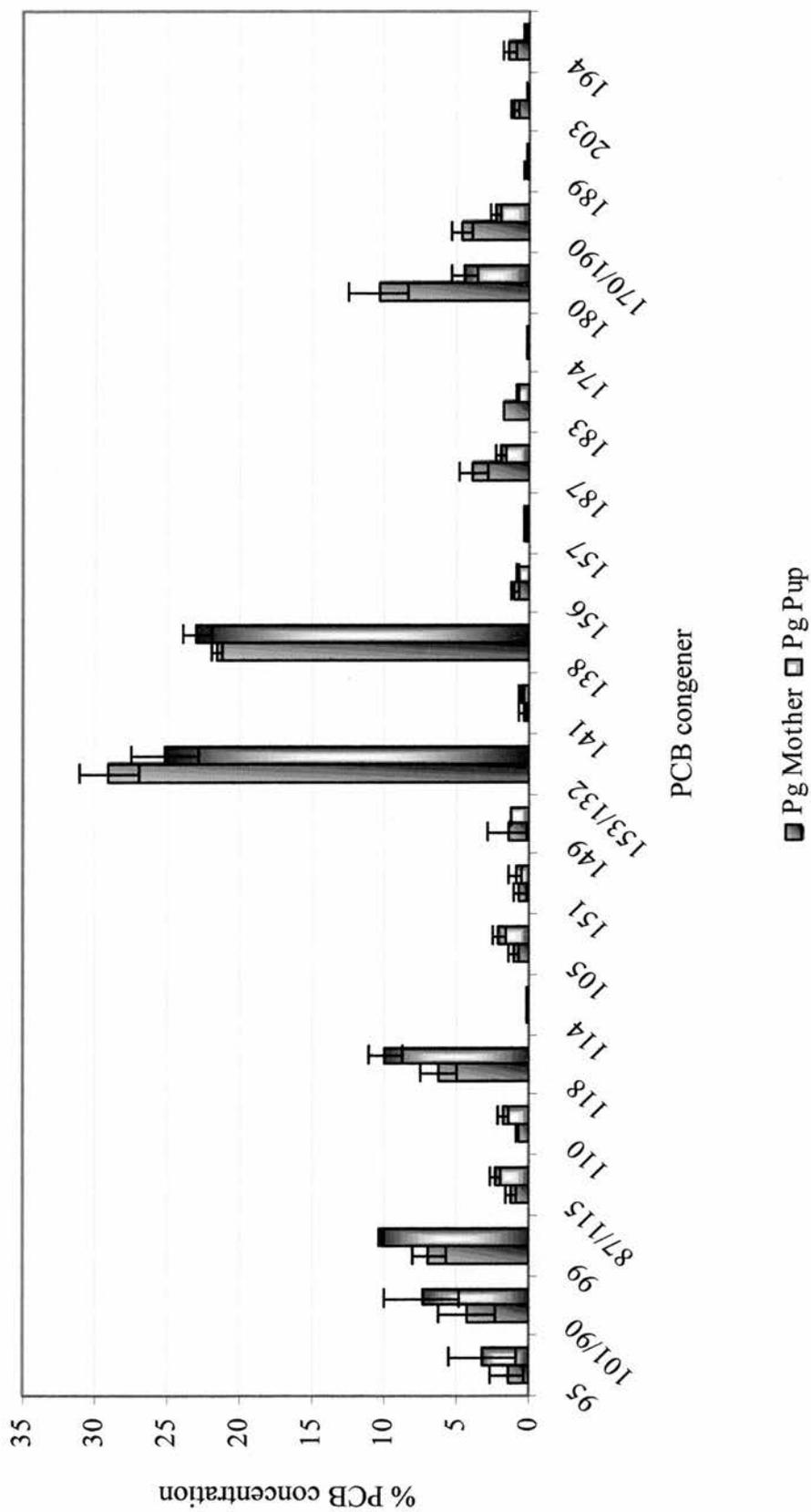


Fig. 2.3.b. Comparison of harp seal (Pg, *Phoca groenlandica*) mother and pup congener patterns from the blubber. The % PCB concentration is the mean \pm 1 standard error for mothers PgMa, PgMb and PgMc and their respective pups PgPa, PgPb and PgPc. For additional information refer to legend of Fig. 2.2.a.

2.3.2.b. Transfer of CB congeners from mother to pup. The relative transfer ratios of the CBs from mother to pup for each species were calculated in order to investigate the pattern of transfer of the CBs in relation to the number of chlorines present on the biphenyl molecule. These ratios were calculated assuming that the pup only received CBs from its mother, the mother transfers all of the mobilised CBs from the blubber to her pup and the pup did not metabolise and excrete a significant amount of the transferred CBs.

The relative transfer ratios for the mother-pup pairs of each species are presented in Fig. 2.4.a and b. The ratios were calculated by the % contribution of each CB congener to Σ PCB in blubber of pup divided by the % contribution of each CB congener to Σ PCB in blubber of mother. The ratios for each congener were divided into their homologue groups according to the number of chlorine atoms present on the biphenyl ring.

The relative transfer ratio was inversely proportional to the number of chlorines the congener contained (Fig. 2.4.a and b). The transfer of pentaCBs was much greater than the higher chlorinated CBs, hexaCBs > heptaCBs > octaCBs for both the harp and hooded seals. Interestingly, the relative transfer ratio varies for CBs within a homologue group and may be explained by their division into different metabolic groups (I to V; refer to section 1.6.1). Both CB151, a hexa-homologue and CB174, a hepta-homologue, are followed by a dramatic decrease in the relative transfer ratio. The CBs that follow 151 and 174, are PCBs that belong to group I, and are very persistent congeners and are generally considered not be metabolised by marine mammals.

2.3.2.c. Intra-specific variation of PCB patterns. The intra-specific variation of the PCB patterns was investigated by PCA for either harp or hooded seals (Fig. 2.5.1 and

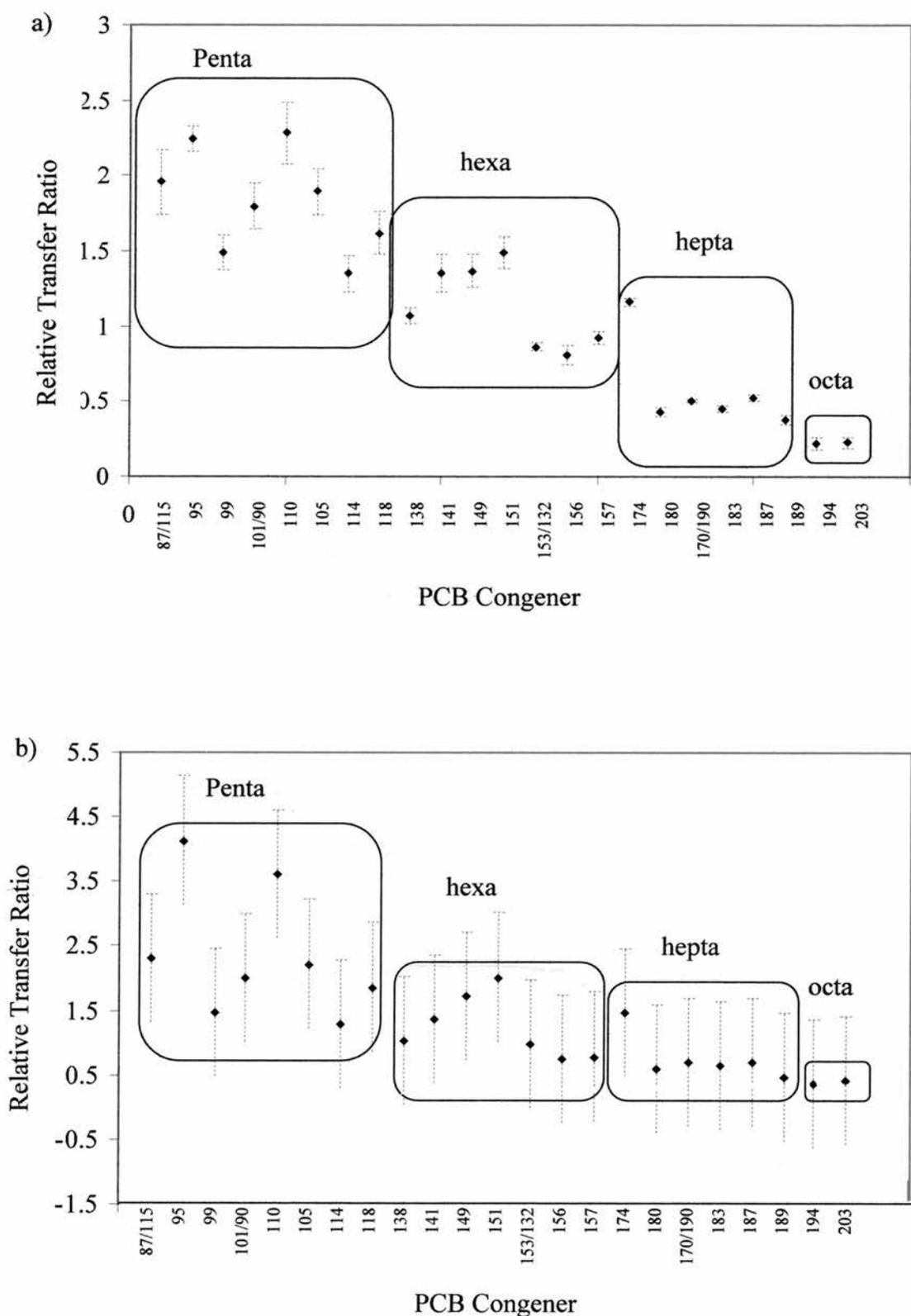


Fig. 2.4. Relative transfer ratio of individual CBs (IUPAC number) from mother to pup (a) harp seals (b) hooded seals. CBs are boxed into their homologue groups, penta-, hexa-, hepta- and octa-CBs, according to the number of chlorines present on the biphenyl ring. Data points represent the mean \pm 1 standard error.

2). Three factors were generated in the PCA of the harp seal data and explained the 92.73% of the total variability. The first two factors combined explained 86.10% of the variability. Four factors were generated in the PCA of the hooded seal data and contributed 94.65% of the total variability. The first two factors responsible for 81.64% of this variability.

Figs 2.5.1 (a and b) and 2.5.2.(a and b) show the plots of the loadings and scores of the factors for the harp and hooded seals, respectively. In both the harp and hooded seals, the adults were separated from the pups. The adult harp seals were predominantly separated from the pups by CBs 110, -183, -187, -174, -101/90, -105, -114, -153/132, whereas the pups had higher proportions of CBs-194, -141, -138, -156, -157, -118, -99 and -87/115. One exception was the pup (PgPc) that tended toward the adult group on the factor score plot. This may be because of the higher proportion of CB-110 in this pup compared to the other pups.

The adult hooded seals had higher proportions of CBs such as CB-203, -189, -110, -187, -151, whereas the pups had higher proportions of CB-156, -157 and some of the higher chlorinated CBs such as CB-183, -194, -170/190. In particular the pups contained a very high proportion of CB-149 compared to the adults.

2.3.3. Total Cytochrome P450 Content, EROD and PROD activities.

Total cytochrome P450 (CYP) content and the EROD and PROD activities are presented in Table 2.4 for all nine individuals from both the hooded and harp seals. The means \pm 1 standard error (SE) for the groups of seals are presented in Table 2.5.

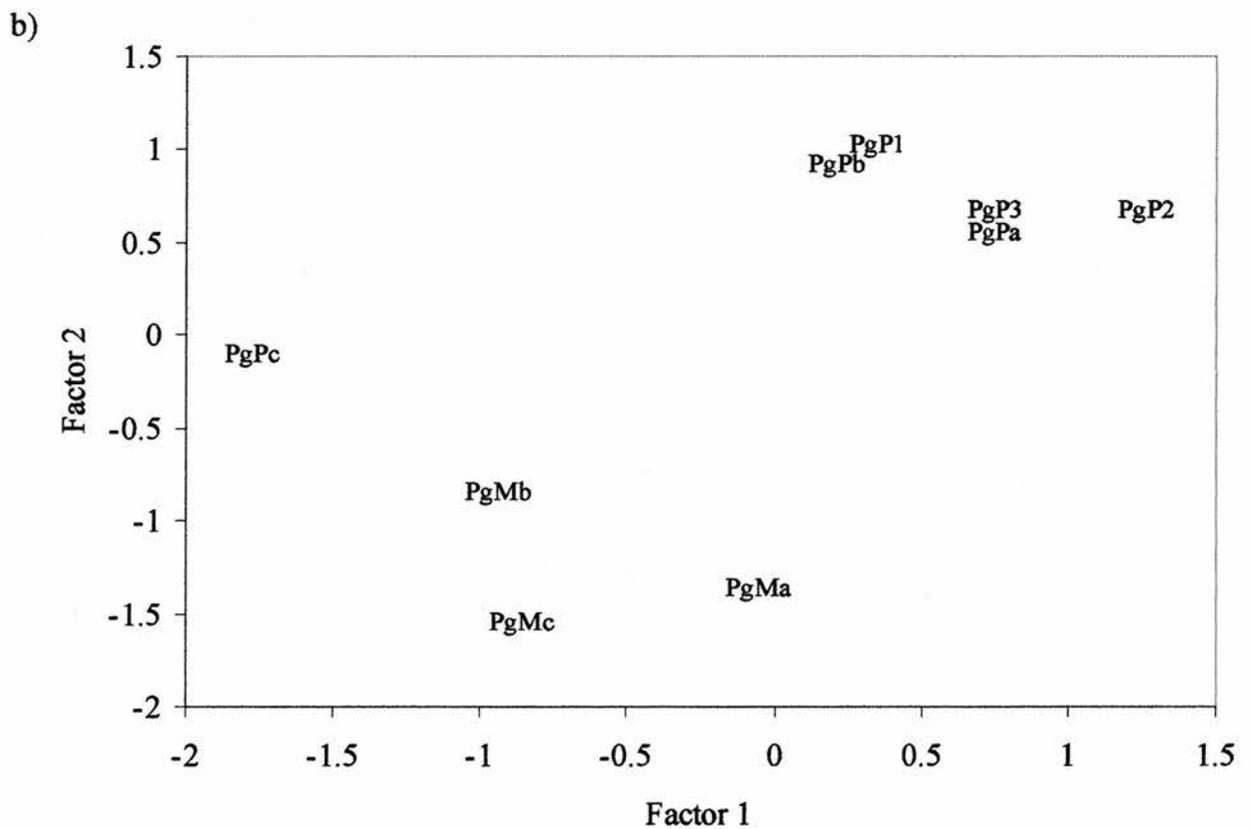
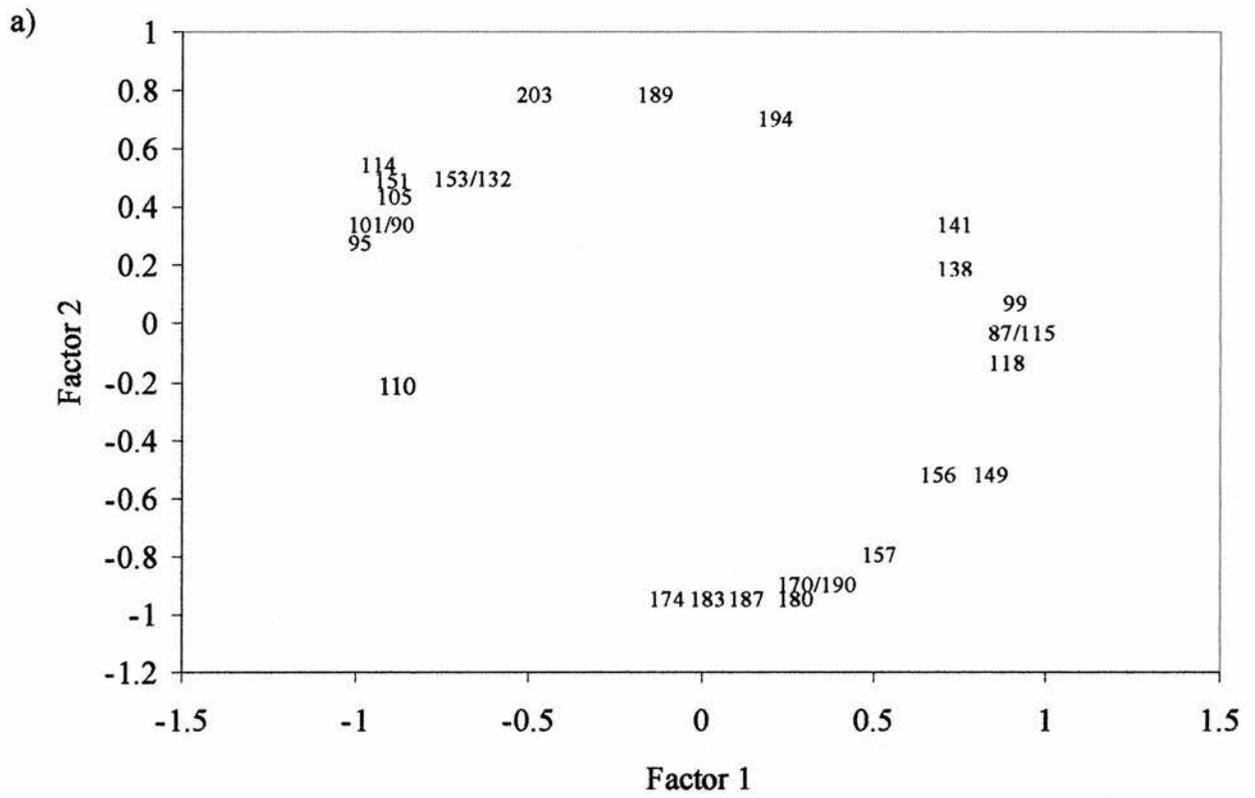


Fig. 2.5.1. a) Factor loadings and b) factor score plots generated by PCA for harp seals. For abbreviations refer to Table 2.1.

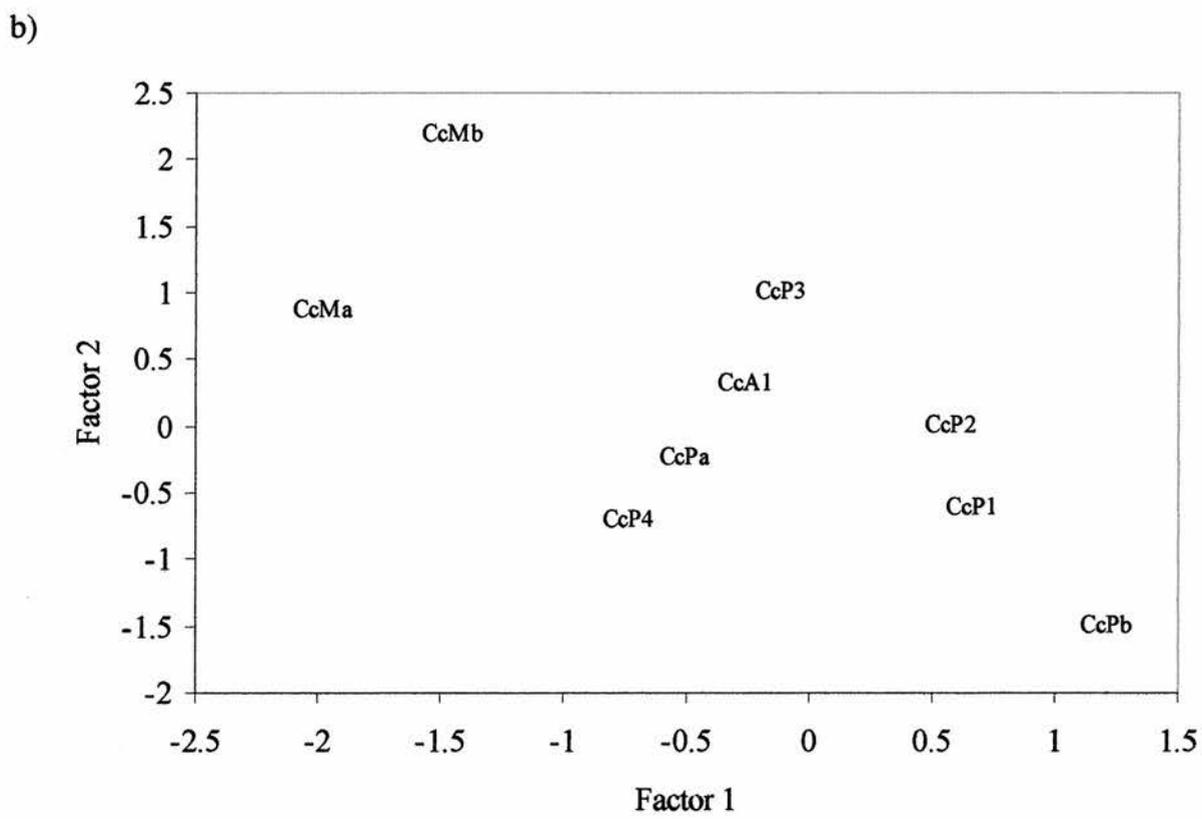
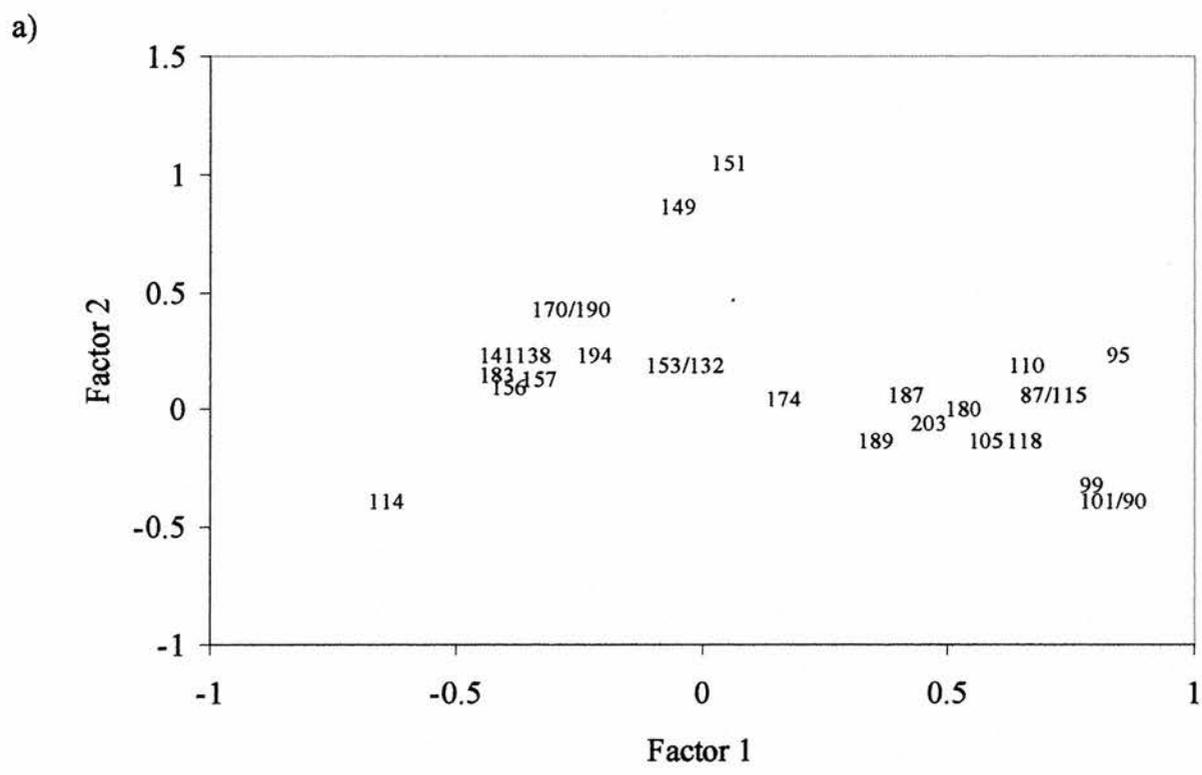


Fig. 2.5.2. a) Factor loadings plot and b) factor scores plot generated by PCA for hooded seals. For abbreviations refer to Table 2.1.

Species	Identification	Total CYP content ^a	EROD Specific Activity ^b	EROD Turnover rate ^c	PROD Specific Activity ^b	PROD Turnover rate ^c
Hood	CcMa	0.25	2.22	12.68	0.09	0.37
Hood	CcMb	0.66	8.44	22.99	0.41	0.62
Hood	CcAl	0.71	12.51	51.05	0.64	0.88
Hood	CcPa	0.79	14.86	38.31	0.84	1.06
Hood	CcPb	0.29	5.84	24.07	0.31	1.09
Hood	CcP1	0.46	4.31	9.42	0.12	0.26
Hood	CcP2	0.59	9.99	10.71	0.17	0.30
Hood	CcP3	0.62	1.99	6.03	0.21	0.33
Hood	CcP4	0.41	5.11	18.11	0.29	0.70
Harp	PgMa	0.55	3.81	14.36	1.30	2.37
Harp	PgMb	0.54	3.81	8.01	1.15	2.11
Harp	PgMc	0.39	2.40	15.20	0.20	0.52
Harp	PgPa	0.29	1.87	14.78	0.23	0.79
Harp	PgPb	0.36	2.70	16.30	4.43	3.53
Harp	PgPc	0.30	1.98	14.22	0.78	2.17
Harp	PgP1	0.20	5.88	38.15	0.18	0.90
Harp	PgP2	0.34	5.53	21.03	0.49	1.44
Harp	PgP3	0.28	6.03	32.91	0.56	2.01

Table 2.4. Total cytochrome P450 content and CYP-monoxygenase (MO) activities in harp and hooded seals. Cc, refers to hooded seal and Pg indicates harp seal. For identification see Table 2.1. ^a, total CYP content expressed as nmol P450.mg⁻¹ protein; ^b, specific activity expressed as pmol resorufin.min⁻¹.mg⁻¹ microsomal protein; ^c, rate of turnover expressed as pmol resorufin.min⁻¹.nmol⁻¹ P450.

	Harp seals		Hooded seals	
	Mothers	Pups	Mothers	Pups
Total CYP Content^a	0.49 ± 0.05*	0.32 ± 0.02	0.45 ± 0.21	0.54 ± 0.25
EROD Specific Activity^b	3.34 ± 0.47	2.18 ± 0.26	5.33 ± 3.11	10.35 ± 4.51
EROD Turnover Rate^c	12.52 ± 2.27*	15.10 ± 0.62	17.83 ± 5.16	31.19 ± 7.12
PROD Specific Activity^b	0.89 ± 0.34	1.81 ± 1.32	0.25 ± 0.16	0.57 ± 0.26
PROD Turnover Rate^c	1.67 ± 0.58	2.16 ± 0.79	0.50 ± 0.12	1.07 ± 0.02

Table 2.5. Mean ± 1 standard error of total cytochrome P450 content, EROD and PROD activities in mother and pups of harp and hooded seals. ^a, total content expressed as nmol P450.mg⁻¹ protein; ^b, specific activity expressed as pmol resorufin.min⁻¹.mg⁻¹ protein; ^c, rate of turnover expressed as pmol resorufin.min⁻¹.nmol⁻¹ P450. Statistically significant differences between age categories intra-specifically are denoted by asterisks (* $p < 0.01$).

2.3.3.a. Inter-specific comparison. Total CYP content (nmol P450.mg⁻¹ protein) was significantly different between hooded and harp seals. The hooded seals displayed a significantly higher EROD specific activities than harp seals ($H = 7.33$, $df = 1$, $p = 0.017$). However, EROD as a rate of turnover (pmol product.min⁻¹.nmol P450⁻¹) was very similar between the two species.

In contrast to EROD, PROD specific activity and rate of PROD turnover were significantly higher in the harp seals than the hooded seals ($H = 6.907$, $df = 1$, $p = 0.009$ and $H = 19.24$, $df = 1$, $p < 0.001$, respectively).

2.3.3.a. (i). Inter-specific comparison: Age and gender category comparison.

1) Total CYP content.

No significant difference was observed between the adults of the two species, however total CYP content was significantly higher in the hooded seal pups than the harp seal pups ($H = 5.026$, $df = 1$, $p = 0.025$). The difference probably occurred because of the significant difference between female pups from both species ($H = 3.857$, $df = 1$, $p = 0.05$) compared to no significant difference between the male pups.

2) EROD Activities.

No significant difference between the EROD specific activities of adult hooded and harp seals was observed. Furthermore, the hooded and harp seal pups EROD specific activities were very similar. However, when the seal pups were categorised by gender, a difference was observed between the male pups of the two species, where the male hooded seal pups had a significantly higher specific EROD activities than the male harp seal pups ($H = 4.306$, $df = 1$, $p = 0.038$).

The rate of EROD turnover was significantly different for the adults of the two species (adult hooded seals > adult harp seals; $H = 5.467$, $df = 1$, $p = 0.019$). No significant difference was observed inter-specifically for the pups.

3) PROD Activities.

Adult harp seals had significantly higher specific activity and rate of PROD turnover than the adult hooded seals ($H = 4.306$, $df = 1$, $p = 0.038$ and $H = 5.070$, $df = 1$, $p = 0.024$, respectively). However, only the rate of PROD turnover was significantly higher in the harp seal pups than the hooded seal pups ($H = 12.876$, $df = 1$, $p < 0.001$), compared with PROD specific activity. The lack of any significant difference between the pups of the two species in PROD specific activity was probably because of no significant difference between the female harp and hooded seal pups. In contrast, PROD specific activity and rate of PROD turnover were significantly higher in the male harp pups than the male hooded seal pups ($H = 5.672$, $df = 1$, $p = 0.017$ and $H = 6.188$, $df = 1$, $p = 0.013$, respectively).

2.3.3.b. Intra-specific comparison. Adult (female) harp seals had a significantly higher rate of EROD turnover compared to harp seal pups of both genders (with male pups, $H = 3.947$, $df = 1$, $p = 0.047$ and with female pups, $H = 6.786$, $df = 1$, $p = 0.009$). There were no intra-specific differences within the hooded seals for both EROD specific activity and the rate of EROD turnover. Furthermore, there were no intra-specific differences in PROD activities (specific activity or rate of turnover) for both the hooded and harp seal species.

2.3.4. CYP content, EROD and PROD activities: Mother – pup pairs.

Total CYP content was significantly higher in the harp seal mothers compared with their respective pups ($p = 0.0077$ two-tail, $t = 11.31$, $df = 2$). In contrast, there was no statistical difference between the hooded seal mother – pup pairs.

Comparison of the EROD and PROD specific activities and rates of turnover, the hooded seal pups had very similar activities to their respective mothers (Table 2.5). However, in the harp seals, the pups had less EROD and PROD activities compared to their mothers, although this difference was not significant.

2.3.5. Immunochemical detection of CYP1A – like and CYP2B – like proteins.

2.3.5.a. Inter-specific comparison. Fig. 2.6. shows the results of the Western blotting with hepatic microsomes from selected hooded seal (a) and harp seal (b) samples using a anti-trout CYP1A1 anti-peptide antibody (Fig. 2.6.1.) and a polyclonal anti-rat CYP2B antibody (Fig. 2.6.2.). As a reference, purified rat hepatic CYP1A1 (Fig. 2.6.1a and b, lane 8) and phenobarbital induced rat hepatic microsomes (Fig.2.6.2.a and b, lanes 1 and 9) were included. A single distinct band was detected in both the reference lanes, with the respective antibodies. A second band of higher molecular weight was detected in the PB – induced rat microsomes, and may be non-specific reaction with the CYP2B antibody (not shown).

(1) CYP1A- like protein. A distinct protein band was recognised by the anti-trout CYP1A anti-peptide antibody in all six harp seals and four hooded seals (Fig. 2.6.1a and b). The band corresponded to a molecular weight of approximately 54kDa, and was just below that of the purified rat CYP1A1 (lane 8; 55kDa).

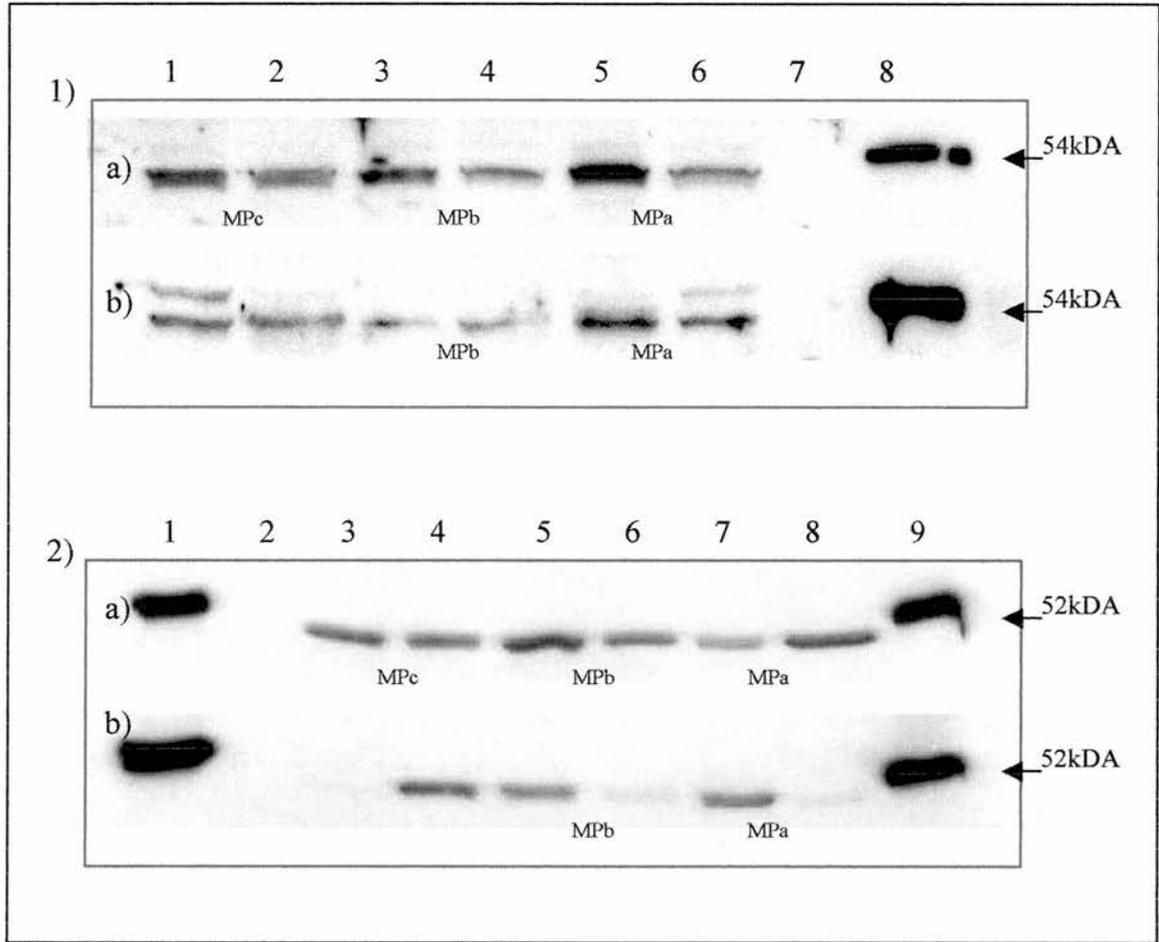


Fig. 2.6. Immunoblots of (a) harp seal and (b) hooded seal hepatic microsomes separated in a 10% polyacrylamide gel by SDS-PAGE, using polyclonal antibodies against (1) anti-trout CYP1A1 and (2) anti-rat CYP2B.

- 1) In each of lanes 1-6, 40 μ g protein was applied. (Lanes 1, 3, 5) pups and (lanes 2, 4, 6) adult females. (Lane 8) 4.74 μ mol purified rat hepatic CYP1A1. (Lane 7) intentionally blank.
- 2) In each of lanes 3-8, 30 μ g protein was applied. (Lanes 4, 6, 8) pups and (lanes 3, 5, 7) adult females. (Lanes 1 and 9) 0.748 μ mol phenobarbital induced rat hepatic microsomes. (Lane 2) intentionally blank.

MPx, where x is a letter, denotes the mother – pup pairs. The MPx is placed in the middle of the bands from the pair. For abbreviations refer to Table 2.1.

The anti-trout CYP1A1 antibody, also detected a much less prominent second protein that had a lower electrophoretic mobility in two of the hooded seals (Fig. 2.6.1a, lanes 5 and 6). This protein band corresponded to the standard at 55kDa and was detected in 5 out of the 6 harp seals (Fig. 2.6.1b, lanes 1, 3, 4, 5 and 6).

A 62kDa protein band reacted with the CYP1A1 antibody in 5 hooded seals, at varying concentrations and very prominently in all 6 harp seals. Since the protein was out of the molecular weight range for CYPs, it was thought to be not related to CYPs and probably a non-specific cross-reaction with the anti-trout CYP1A1 antibody. However, the identity of the 62kDa protein is unknown (not shown), but may be a serum protein (Refer to Chapter six, this thesis).

(2) CYP2B-like protein. The same twelve animals, six harps and six hooded seals, were also probed for the presence of a CYP2B – like protein using an anti-rat CYP2B antibody (Fig. 2.6.2a and b). A single 52kDa protein band was detected in all six harps, however only five hooded seal samples reacted with the anti-rat CYP2B antibody, and of these only three exhibited concentrations at a similar level to the harp seals. The seal protein bands corresponded with a distinct protein band in the liver microsomes from phenobarbital induced rat at a molecular weight slightly higher than 52kDa.

2.3.5.b. Intra-specific comparison of CYP1A-like and CYP2B-like proteins: Mother - pup pairs. Three harp seal mother –pup pairs (Fig. 2.6.1b, lanes 1 and 2, 3 and 4, 5 and 6) and two hooded seals mother – pup pairs (Fig. 2.6.1a, lanes 3 and 4, 5 and 6) reacted with the anti-trout CYP1A1 antibody. The harp seal pups exhibited a higher level of apoprotein compared to their respective mother, with pup in lane 5 (Fig. 2.6.1.b) displaying the greatest concentration, whereas the hooded seal mother – pup pairs

exhibited similar levels of CYP1A. In contrast, the harp seal mother-pup pairs had similar levels of CYP2B-like apoprotein (Fig. 2.6.2b, lanes 3 and 4, 5 and 6, 7 and 8), however, the hooded seal mothers had much less CYP2B – like protein compared to their pups (Fig. 2.6.2a, lanes 5 and 6, 7 and 8).

2.3.6. CYP activities and relative CYP1A and CYP2B concentrations.

The relative concentrations of the seal protein bands were determined using optical density (methods section 2.2.6). No correlation was observed between the EROD or PROD activities with CYP1A or CYP2B relative concentration for harp seals. Furthermore, there was no correlation between EROD or PROD with CYP1A, and PROD with CYP2B in the hooded seals. However, a significant correlation ($p < 0.05$, $R = 0.849$) occurred between EROD and relative concentration of hooded seal CYP2B (data not shown). The sample size was too small to investigate relationship between the EROD and PROD activities and CYP1A and –2B relative concentration between either the adults or the pups on their own.

2.3.7. TEQ, PCB concentrations and CYP activities.

The 2,3,7,8-TCDD toxic equivalents (TEQ) for 5 CB congeners (CB-118, -105, -114, -156 and -158) expressed per lipid weight were calculated for harp and hooded seals using the published toxic equivalent factors (TEFs) (Van den Berg *et al.*, 1998). The sum of the 5 CBs ($\Sigma 5\text{TEQ}$) for each individual of both species are presented in Fig. 2.7. The range of the $\Sigma 5\text{TEQs}$ were comparable for both harp and hooded seal pups (0.004-0.007 ng.g^{-1} lipid and 0.004-0.008 ng.g^{-1} lipid, respectively). The adults of both species had greater $\Sigma 5\text{TEQs}$ than the pups, however were similar to each other (adult harp seals: 0.005 – 0.014 ng.g^{-1} lipid and adult hooded seals: 0.01 – 0.019 ng.g^{-1} lipid).

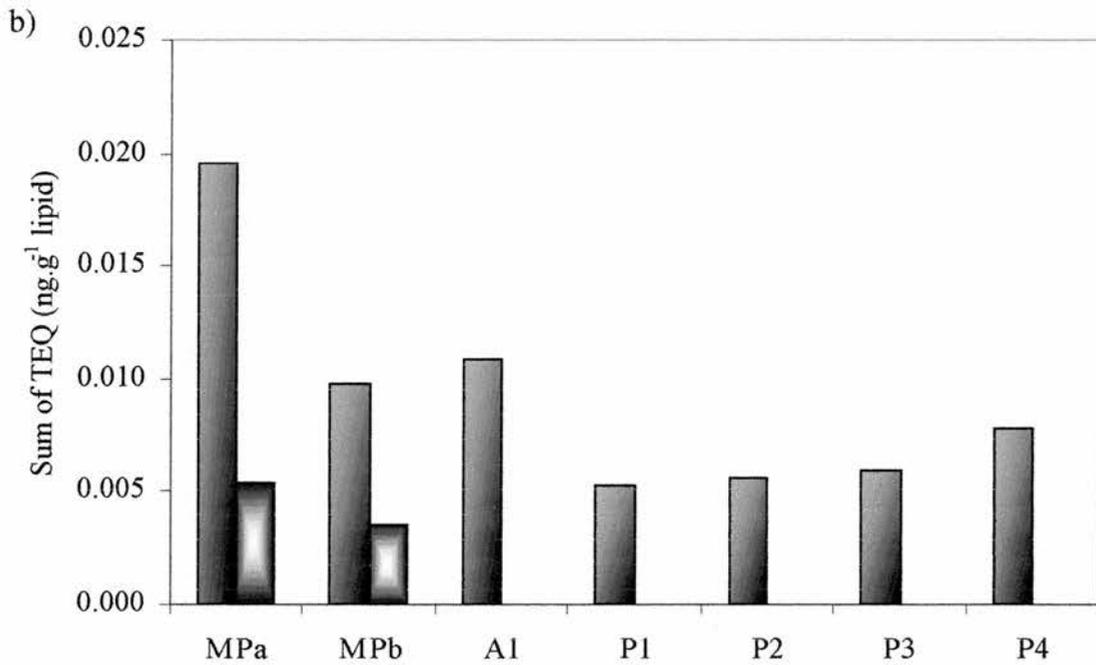
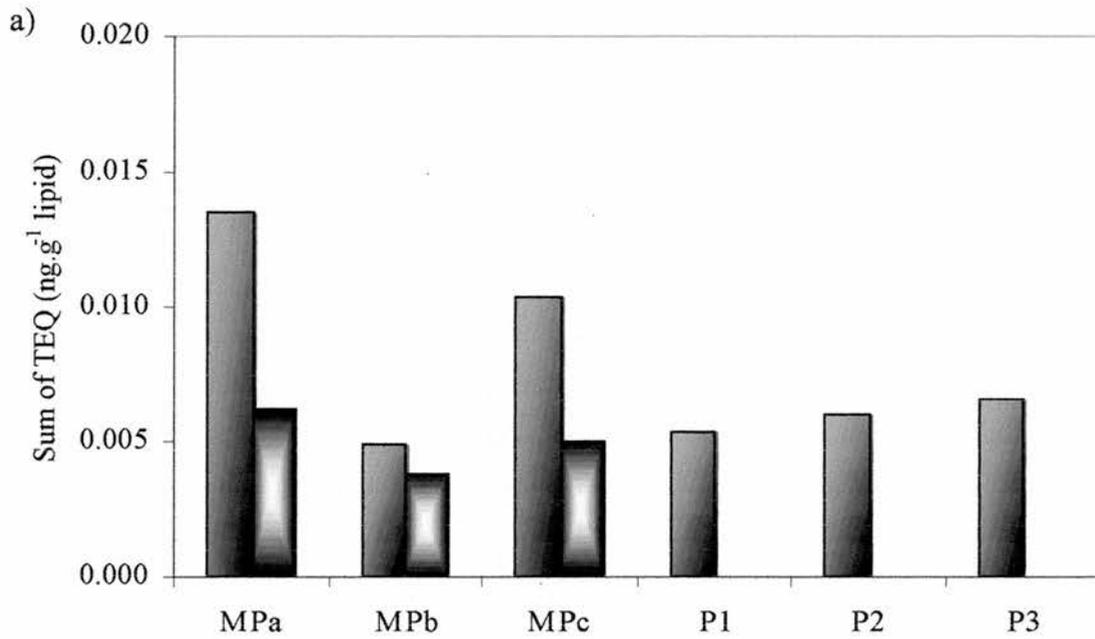


Fig. 2.7. Σ TEQ in a) harp seals and b) hooded seals. MP refers to mother-pup pairs in each species (e.g. in (a) MPa denotes PgMa and PgPa or in (b) CcMa and CcPa). For abbreviations refer to Table 2.1.

The % of each of the 5 CB congeners to the Σ TEQ were also calculated for the nine individuals from the harp and hooded seals. The % TEQs of the 5 CBs is presented as the mean \pm 1 standard error for the adults and pups of each species (Fig. 2.8). The % TEQ for CB-118 contributed \sim 50% of the Σ TEQ for both harp (45.1 – 60.2%) and hooded (40.9 – 48.9%) seal pups and adult harp seals (40.8 – 48.9%). In contrast the % TEQ of CB-156 (40.0 – 48.9%) contributed more to the Σ TEQ than CB-118 (31.1% - 38.2%) on adult hooded seals.

The rank order of the 5 CBs with decreasing % contribution to the Σ TEQ CB118 > CB-156 > CB-105 = CB-157 > CB-114 in harp seals and hooded seal pups. In the adult hooded seals, CB-156 replaced CB-118, and CB-157 contributed a greater % to Σ TEQ than CB-105.

CYP1A-associated EROD and PROD activities were plotted against the sum of TEQ for five CYP1A-inducing CBs (CB-105,-114,-118,-156 and -157), however no correlation was observed for the harp or hooded seals. In addition, there was no correlation between the EROD and PROD activities and the TEQ for the individual CBs, or with the concentrations of the individual CBs.

2.4. Discussion

This chapter has investigated the levels of cytochrome P450 and the analysed 27 PCB congeners in the blubber of hooded and harp seals. The relationship between CYP1A associated EROD and PROD activities in mother – pup pairs, which previously have not been investigated and the PCB levels transferred from mother to her pup have also been examined to investigate the potential capability of adults and developing pups to metabolise PCBs that target particular CYPs.

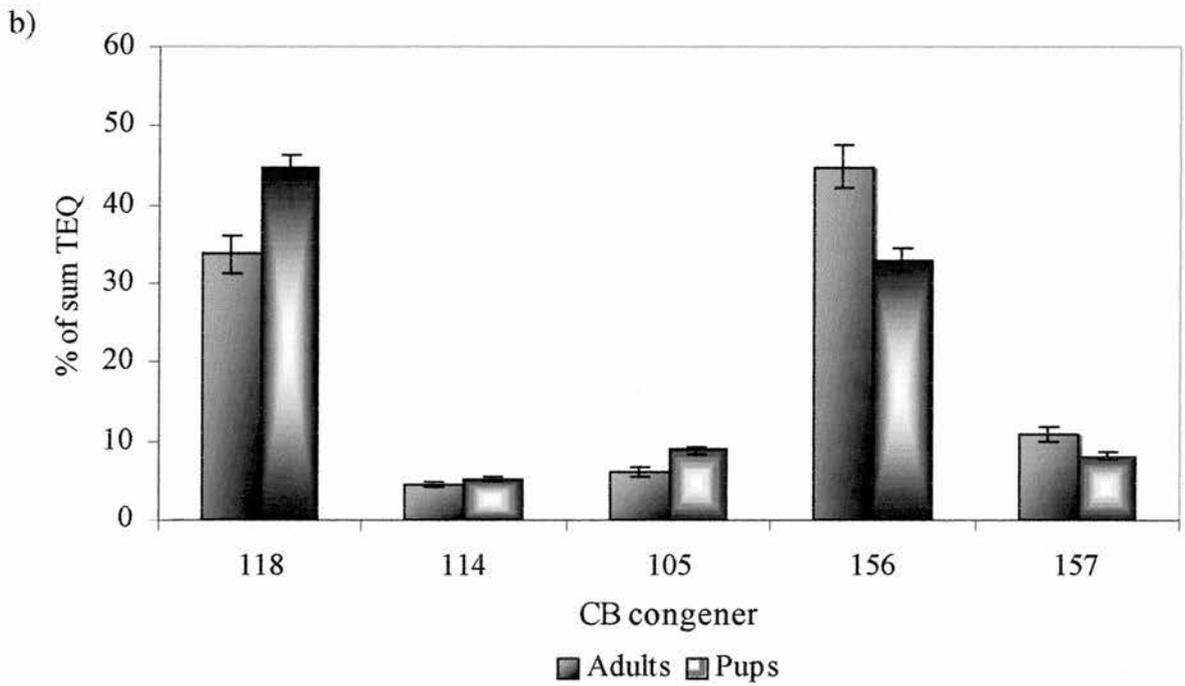
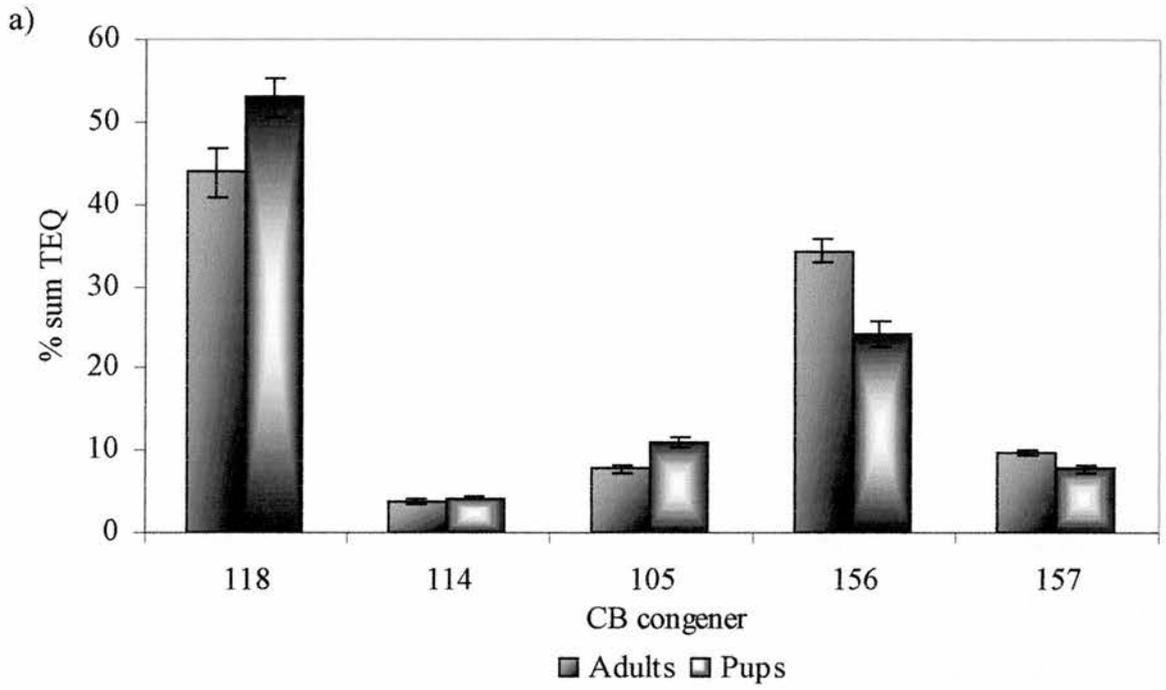


Fig. 2.8. % TEQ for the five individual PCB congeners displayed as the mean \pm 1 standard error for the adults and pups of (a) harp seals and (b) hooded seals.

General aspects. The concentration, burdens and patterns of PCBs in seals are influenced by many biological factors such as age, sex, reproductive status (number of previous offspring) and season (nutritional, reproductive, feeding habits) (reviewed in Boon *et al.*, 1992). An individual's capacity to metabolise PCBs through the detoxification system (primarily CYP isoenzymes) may also have an effect on the levels and patterns of PCBs. Furthermore several physiological and biological factors are known to influence the expression of CYP isoenzymes in experimental animals. Such factors include sex, stage of development, age, endogenous factors, such as hormones and cytokines and exogenous factors, including PCBs, polyaromatic hydrocarbons (PAHs) (reviewed in Stegeman and Hahn, 1994). An individual's capacity to metabolise certain foreign chemicals may also vary.

2.4.1. PCB concentrations and burdens.

2.4.1.a. Inter-specific comparison: PCB concentrations. Twenty-seven PCB congeners from the blubber of harp and hooded seals were analysed. The total PCB concentrations (sum of 27 congeners, denoted $\Sigma 27\text{PCB}$), expressed as ng.g^{-1} lipid weight, were significantly higher in hooded seals of both age categories (adult and pup) than the corresponding age category in harp seals. This was consistent with a previous study where hooded seals had greater PCB concentrations than harp seals (Espeland *et al.*, 1997).

The hooded and harp seal $\Sigma 27\text{PCB}$ concentrations (ng.g^{-1} lipid) were lower than conspecifics caught during March/April 1990, in West Ice (Espeland *et al.*, 1997). In particular, the $\Sigma 27\text{PCB}$ concentrations were substantially lower, by a factor of 10, in the hooded and harp seal pups from the present study compared to those from the previous study. Certain PCB congeners bioaccumulate in organisms more than other PCBs,

therefore the different CB congeners measured in the two studies may have contributed to the observed difference in the total PCB concentrations. Furthermore the time difference of ten years between the two studies could potentially play a role in the decreased Σ PCB concentrations observed in the present study. However, the seals in the present study had thinner blubber layers to the corresponding level in the previous study. This may indicate a change in feeding habits of the harp and hooded seals since 1990. However, an exception was observed with harp seal pups since they had comparable blubber thickness.

Harp seals are known to feed on pelagic fish such as capelin (*Mallotus villosus*), herring (*Clupea harengus*) and polar cod (*Boreogadus saida*) and a pelagic amphipod *Parathermisto libellula* (Lindstrom *et al.*, 1998), whereas hooded seals tend to feed on deeper species such as the greenland halibut (*Reinhardtus hippogloides*), redfish (*Sebastes marinus*) and squid (*Gonatus farbricii*) (Bonner, 1989; Espeland *et al.*, 1997).

However, in a recent report the observed diets of harp and hooded seals feeding between Greenland and Svalbard during the autumn, were comprised of relatively few species. The pelagic amphipod, *Parathermisto libellula*, squid (*Gonatus fabircii*) and polar cod (*Boreogadus saida*) constituted 85 – 98% (calculated biomass) of the diet in hooded seals and 95% in harp seals (Potelov *et al.*, 2000), suggesting a change in diet during this time of year. The age and gender structure and size of the sample, as well as the exact locations of the two species when caught were not mentioned in the report. However, the data implies that during the autumn, between Svalbard and Greenland, these two species were feeding within the same niche. Since in the present study the PCB concentrations were significantly higher in the hooded seals than the harp seals,

the two seal species may have foraged within the same niche although in different areas, and / or the two species feed in other niches throughout the rest of the year.

Σ 27PCB concentrations in harp seals (this study) were comparable with the same species from another study also from the West Ice region in 1990 (Skaare, 1995). In contrast Σ 27PCB concentrations were lower in hooded and harp seals from West Ice (this study) compared to the corresponding species from East Ice during 1993.

Geographical gradients in Σ PCB concentrations have been observed, increasing from West to East, and North to South in the Northern Hemisphere. For example, harp seals from west ice had lower Σ PCB concentrations than the same species from East Ice, which may reflect the higher amount of pollution in the European Arctic compared to the region around Greenland (Skaare, 1995). Harp seals were also reported to have higher Σ PCB concentrations in the Barents sea compared to conspecifics near Svalbard (Wolkers *et al.*, 2000).

Substantially higher Σ PCB concentrations were observed in harp seals from the Svalbard (2,093 – 20,382ng.g⁻¹ lipid) (Kleivane *et al.*, 2000) compared to both seal species from this study. The difference in Σ PCB concentrations may be a result of different geographical locations between the studies. Differences in analytical techniques between laboratories make comparisons between published data sets difficult. However, the harp seals from the Svalbard were sampled during the moulting season in late May/June when the animals were lean as a result of fasting.

2.4.1.b. Inter-specific comparisons: PCB Blubber Burdens. Total PCB concentrations were found to be inversely related to blubber thickness (Addison and Smith, 1974;

Addison and Stobo, 1993; Kleivane *et al.*, 1995a) and therefore may be poor indicators for comparing individuals, inter-specific comparison and conspecific comparison during different stages of the annual cycle. In contrast, Addison and Stobo (1993) observed constant body burdens in grey seals (*Halichoerus grypus*) with fluctuating blubber thickness throughout their first year of life.

Body burdens of PCBs (mg) were calculated for the harp and hooded seals from the present study, based on the concentration of PCBs and lipid of the whole blubber mass (Results, 2.3.1.b). The blubber represented 30 – 51% and 40 – 54% of the total body mass in hooded and harp seals, respectively, and comprised between 81 and 92% of lipid in the adult seals. A lower % lipid was observed in the younger seal pups of both species. Furthermore, the lowest % lipid (54.7%) corresponded with the new born hooded seal (CcPb).

Seal pups during the nursing period deposit a subcutaneous layer of lipid (blubber) of which the triacylglycerol content increases substantially in the first few days of neonatal development to levels observed in adult blubber (Bailey *et al.*, 1980). The observed increase in triacylglycerol content may correspond to the increase in % of extractable lipid. The differences in lipid concentration in the blubber of each individual were accounted for in the calculation of the body burdens. Furthermore the majority of the bodies lipids were estimated to be in the blubber with only 10% in other tissues of harp seals (Beck *et al.*, 1994) and virtually all the body burdens of PCBs were assumed to be contained within the blubber lipids (Addison and Stobo, 1993).

Similar to the Σ PCB concentrations, the total PCB body burdens (mg) were significantly greater in hooded seals compared with harp seals. The total body burdens

of PCBs (mg) for the harp seals from this study were at the lower end of the range of the PCB burdens for harp seals from Canada (Beck *et al.*, 1994). This study was the first to document the PCB body burdens, calculated as described in section 2.3.1.b., in hooded seals. Comparing the hooded seals from this study with the harp seals from Canada, the burdens were similar: hooded seals (this study) contained PCB burdens 77.45 ± 13.13 mg and harp seals (Beck *et al.*, 1994) were 76.27 ± 42.53 mg.

Furthermore PCB body burdens for harp and hooded seal pups have not been reported before. However, grey seal new born pups had Σ PCB burdens between 0.5 and 0.8 mg (Addison and Stobo, 1993) that were consistent with the Σ mono-CB burdens in the harp and hooded seal pups. However, the Σ PCB burdens in the harp and hooded seal pups were greater than the grey seal pups. The higher burdens in the pups from this study may reflect the higher burdens in the maternal blubber, compared to the burdens in the grey seal maternal blubber (See section 2.4.2.a. for further discussion of transfer of PCBs from maternal blubber to pup blubber) and since the harp and hooded seal pups, except one individual, were not newborn animals will have accumulated larger body burdens.

2.4.1.c. Inter-specific variation of PCB pattern. Mono-ortho CB burdens only contributed 12% and 6% of the total PCB burdens in harp and hooded seals, respectively. In contrast the burdens of di-ortho CBs, -153/132, -138, -180 and -99, comprised 66% and 72% of Σ PCB burden in harp and hooded seals, respectively. The contribution of the most prominent di-ortho CBs was consistent with other studies. For example, >70% of Σ PCBs resulted from the sum of the concentrations of CBs-153, -138, -99, -180 and -101 in ringed seals (*Phoca hispida*) (Wolkers *et al.*, 1998b) and CB-153 and -138, together contributed 45% of Σ PCBs in harp seals (Skaare, 1995).

Mono-*ortho* CBs, such as CB-118, -105, -156 and -157 are known to be more easily metabolised than di-*ortho* CBs in rats (Safe *et al.*, 1985). Concentrations of mono-*ortho* CBs in predators such as seals, cetaceans and otters, were found to be less compared to the model food source, and the contribution of di-*ortho* CBs (Boon *et al.*, 1997). This was indicative of more favourable biotransformation of mono-*ortho* CBs rather than di-*ortho* CBs.

The contaminant pattern varied between the species as determined by PCA (Fig. 2.1). Factor scores of the hooded and harp seals indicate a distinct separation of the two species, because of higher proportion of CBs-138, -141, -174, -170/190, -149 and -114 in hooded seals compared to the higher proportion of CBs-156, -157, -87/115, -110, -99, -203, -194 and -183 in harp seals.

Boon *et al.* (1994; 1997) proposed a pharmacokinetic model to assess the metabolic capacity of marine mammals to metabolise CB congeners. CB congeners with no vicinal hydrogen atoms (Group I) and congeners with vicinal hydrogens in the *ortho*- and *meta*- positions and ≥ 2 *ortho* chlorine atoms (group II) were very resistant to metabolism. However, CB congeners with vicinal hydrogen atoms in the *ortho*- and *meta*- positions and in combination with ≤ 1 *ortho* Chlorine (group III) were more easily metabolised by both seals and cetaceans. CB congeners with vicinal hydrogen atoms in the *meta*- and *para*- positions and in combination with either 2 *ortho* chlorines (group IV) or ≥ 3 *ortho* chlorines (group V) were metabolised in seals but not cetaceans (Boon *et al.*, 1994; Boon *et al.*, 1997; Tanabe *et al.*, 1988). However, methyl sulphone metabolites of CB congeners from groups IV and V were measured in the blubber of beluga whales (Letcher *et al.*, 2000) and CBs with *m*-, *p*-, vicinal hydrogen atoms were

metabolised in harbour porpoises' compared with their prey species (Duinker *et al.*, 1989). Contrary to belief, this suggests that cetacean species are capable of metabolising group IV and V PCB congeners.

Groups I, II, IV and V were equally represented in hooded and harp seals, although by different normalised concentrations of the congeners in the two species. This suggests that the metabolic systems responsible for detoxifying these congeners were similar in the two species. The difference in normalised concentrations of the CBs that constitute the four groups may reflect inter-specific dietary and habitual differences in prey species, depth and range of feeding ground.

However, a higher proportion of group III CB congeners were observed in harp seals indicating that they have a reduced capacity to metabolise group III CB congeners compared to hooded seals. The lower group III CB congeners in hooded seals may correspond to the higher CYP1A-EROD activities of these seals (hooded seals > harp seals, $p < 0.05$). However, Boon *et al.* (1997) argued that the degree of metabolism influences the PCB pattern and is proportional to the total PCB concentration in the animal. The hooded seals in the present study had higher levels of PCBs with corresponding lower proportion of group III CB congeners, suggesting greater biotransformation due to slightly higher CYP1A activity. Harp seals may feed during lactation, although do not require the extra energy gained, whereas hooded seals are known to fast during this period (Lydersen and Kovacs, 1996; Mellish *et al.*, 1999). This would have provided a new input of environmental contaminants, including group III congeners into the harp seal circulation compared with hooded seals that in contrast may have further metabolised the store of group III congeners.

2.4.2. Comparison of PCB levels in mother – pup pairs.

Pups receive a large energy store from their mothers during the lactation period. A consistent ~50% of the milk is lipid in hooded seals, and 30% increasing to 50% by the end of lactation in harp seals (Debier *et al.*, 1999). This enables the 76% of the daily weight gain of fat in hooded seal pups (Lydersen *et al.*, 1997) and 50% in harp seals (Lydersen and Kovacs, 1996).

Since harp seals feed opportunistically, i.e. some feed and others fast, and hooded seals fast during lactation (Lydersen and Kovacs, 1996; Mellish *et al.*, 1999), the majority of the lipid in the milk comes from blubber reserves, mobilised due to hormonal changes associated with increased energy demand (Stryer, 1995; Vander *et al.*, 1994). Along with the mobilisation of lipid, is the release of less chlorinated PCBs into the adult female's circulation, where it becomes available for transfer to the pup via the milk (Addison and Brodie, 1987).

New born hooded seals have ~14% fat (Ofteidal *et al.*, 1993) consistent with their more advanced state of development and the extremely short lactation period of 4 days. In contrast new born harp seals were born with ~5% fat (Worthy and Lavigne, 1983) and are nursed for on average 12 days. The presence of fat deposited *in utero* is indicative of the potential trans-placental transfer of PCBs. However, *in utero* transfer of PCBs was observed to be a minor route of exposure in harp seals (Jones *et al.*, 1976) and hooded seals (unpublished observations, Ronald *et al.*, 1984), therefore pups ingest the majority of their PCB burden via the milk.

2.4.2.a. Transfer of PCBs. The CB profiles in maternal and pup blubber for 3 harp seal mother – pup pairs and 2 hooded seal mother – pup pairs were presented in Fig. 2.3. The

individual congeners in the mother and pup were at relative concentrations and were similar to mother and pup pairs from the same region (Espeland *et al.*, 1997). Furthermore, some of the lower chlorinated CBs were found at higher concentrations in the pup compared to the mother. All five pups from the harp and hooded seals had higher %CBx/ Σ PCB of CB-95, -101/90, -99, -87/115, -110, -118, -114, -105, -151, -149, -141, -157 and -174 compared to their respective mother. In addition, PgPa, PgPb and CcPb had higher %CB-138 of Σ PCBs and CcPa had higher %CB-153/132 of Σ PCBs than the respective mothers. In a previous study, harp and hooded seal pups contained a higher concentration of CB-99 and harp seal pups had higher concentration of CB-153 than the respective mother (Espeland *et al.*, 1997).

An identical pattern of certain mono-*ortho* CBs and an almost identical pattern of di-*ortho* CBs were exhibited in the harp and hooded seal pup blubber in the present study, suggesting a common mechanism in both seal species for the selective transfer of particular PCB congeners. Total PCB concentrations in milk of grey seals were much lower compared to the blubber (Green *et al.*, 1996). This confirmed selective barriers were inhibiting the simultaneous release of certain PCBs with lipid from the blubber, and in the milk at the mammary gland (Addison and Brodie, 1987; Espeland *et al.*, 1997).

A model of equilibrium partitioning (Matthews and Dedrick, 1984) may explain the selective transfer of PCBs during lactation in seals. Transfer efficiencies of certain CB congeners from blubber to milk were influenced by the number and position of chlorine atoms on a biphenyl molecule (Green, 1997). Furthermore, the polarity of CB congeners decreased with increasing number of chlorine atoms (Matthews and Dedrick, 1984). Addison and Brodie (1987) observed the partition of lower chlorinated PCB congeners

at higher relative concentrations into the maternal circulation, corresponding with lower lipid solubility.

The transfer of CBs divided into their homologue groups were investigated as a ratio of the % contribution of CBx to Σ PCB in pup blubber divided by that in the maternal blubber (Fig. 2.4.). The mono-*ortho* CBs in the present study consisted mainly of penta-CBs, the least chlorinated CBs measured. The transfer ratio of CBs from mother to pup was inversely proportional to the number of chlorines on the biphenyl ring. Hence, the penta-CBs exhibited higher ratios compared to the hexa-, hepta and octa-CBs in the harp and hooded seals. The larger standard error in the relative transfer ratios of the hooded seals may be due to one of the two hooded seal pups was a newborn.

The variation of the relative transfer ratio of CB congeners within homologue groups was related to the metabolic groups. This suggests that both the number of chlorines and the position of the chlorines on the biphenyl rings influences the transfer from mother to pup in harp and hooded seals. Furthermore, this pattern was detected by comparing PCB congeners from the blubber of mother and pup, rather than blubber to milk (Green, 1997). Methyl-sulphone metabolites of PCBs were also transferred from mother to pup during lactation, however, in contrast to the precursor PCBs, no selective transfer was observed of the metabolites of the different PCB congeners (Green, 1997).

2.4.2.b. Intra-specific variation in PCB patterns. The intra-specific contaminant patterns were examined by PCA (Fig.2.5). Factor scores of the hooded and harp seals indicated a separation of the mothers and pups. Adult harp seals had higher proportions of CBs 110, -183, -187, -174, -101/90, -105, -114, -153/132, whereas the pups had higher proportions of CBs -194, -141, -138, -156, -157, -118, -99 and -87/115 (Fig.

2.5.1). One exception was observed, where harp seal pup, PgPc, tended toward the adult group, probably because of higher proportion of CB-110 than the other pups.

The factor scores of hooded seals showed that adult hooded seals had higher proportions of the more resistant CBs such as CB-203, -189, -110, -187, -151, whereas the pups had higher proportions of CB-156, -157 and some of the higher chlorinated CBs such as CB-183, -194, -170/190 (Fig. 2.5.2). In particular the pups contained a very high proportion of CB-149 compared to the adults.

Since lower chlorinated PCBs are more polar, they are transferred more efficiently from maternal blubber to pup blubber, however the presence of higher chlorinated CBs in the pups blubber may reflect the large burden of these CBs in the maternal blubber that were able to be transferred. Hooded seal pup (CcPb) was marginally separated from the other pups and was further separated from its mother (CcMb) than the pup (CcPa) from its mother (CcMa). This may be a result of pup CcPb was a new born and its blubber may only contain those CBs transferred *in utero* and via the milk in the first feed(s).

2.4.3. Cytochrome P450 content, EROD and PROD activities.

Total cytochrome P450 (CYP) contents for the hooded and harp seals, 0.53 ± 0.0063 nmol.mg⁻¹ protein and 0.36 ± 0.0039 nmol P450.mg⁻¹ protein, respectively, were comparable to previous studies in these two species (Goksøyr, 1989) and harbour seals (*Phoca vitulina*) (Addison *et al.*, 1986; Troisi and Mason, 1997). The hooded seal CYP content was higher in the present study, although not significantly, than the harp seals.

EROD has been characterised as a model substrate for CYP1A in rats, humans and fish (Besselink *et al.*, 1998; Burke *et al.*, 1994). PROD, in PB-induced rat liver microsomes,

was predominantly metabolised by CYP2B, however in 3-methylcholanthrene (3-MC) treated rats, PROD was predominantly metabolised by CYP1A (Burke *et al.*, 1994). Furthermore, PROD was metabolised by CYP1A isoenzymes in ringed and grey seal liver microsomes, where PROD activity correlated to EROD activity and had a similar K_m to EROD in kinetic studies (Mattson *et al.*, 1998; Nyman *et al.*, 2000). Therefore in this study, it was assumed that EROD and PROD were metabolised by CYP1A in harp and hooded seals.

Hooded seals had significantly higher EROD specific activities than harp seals, which were reflected by the pups of the two species, where the male hooded seal pups had greater EROD specific activities than the male harp seal pups. In contrast, PROD specific activities were significantly higher in harp seals compared to hooded seals and was reflected in the adults and the male pups. Female harp and hooded seal pups had similar PROD specific activities.

EROD and PROD activities in the harp and hooded seals from this study were similar compared to a previous study of these two species from the Arctic, although the activities were determined in 10,000g supernatant protein (Goksøyr, *et al.*, 1992). However, the EROD activities from the harp and hooded seal pups from the present study were lower compared to the activities from phenobarbital induced seal pups, although the sample size in both cases was small. In contrast with the animals in this study, the EROD activities were not detectable in untreated hooded seal pups and PROD activity was not observed in harp or hooded seal pups, only adults of these two species showed notable PROD levels in the previous study (Goksøyr, 1992).

Wolkers and colleagues (1999) observed EROD activities in harp seals (0.449 nmol.min⁻¹.mg⁻¹ protein) from the Barents Sea that were between 30 and 200 fold greater than the harp seals in this study (3.78 ± 0.56 pmol.min⁻¹.mg⁻¹ protein). Furthermore these activities were lower than harbour, ringed and grey seals that have previously been investigated (Nyman *et al.*, 2000; Troisi and Mason, 1997).

A geographical gradient in CYP1A-associated EROD activities has been observed in ringed and grey seals (Hyyti *et al.*, 2001; Nyman *et al.*, 2001). Arctic populations of the same species had significantly lower CYP1A expression and EROD activities compared to populations in the Baltic, based on the latter region was more polluted. Such a geographical gradient related to pollution may also explain the much lower EROD and PROD activities in measured in the two seal species from the present study.

However, comparisons between studies should be made with caution since the different seasons in which the animals were taken, may also explain the differences in the enzyme activities from one study to the next. In addition, any small differences in the activities may represent inter-laboratory differences in technique and methodology.

CYP-MO activities recalculated as rate of turnover (pmol.min⁻¹.nmol⁻¹ P450) rather than per unit protein concentration enabled the EROD and PROD activities to be expressed independently of protein content and effects of enzyme deactivation (Troisi and Mason, 1997). EROD activity as rate of turnover was very similar in both the hooded and harp seals, whereas the rate of turnover of PROD was significantly higher in the harp seals ($p = 0.007$). This apparent difference was a consequence of the difference between the pups of the two species. A high protein content compared to a low CYP1A content in the liver could obscure any differences in activities when expressed per unit protein.

The inter-specific difference in EROD activities may indicate that the harp seals CYP1A isoenzyme whilst having a greater specificity for PROD substrate, 7-pentoxoresorufin, has a lesser specificity for the EROD substrate, 7-ethoxoresorufin, compared to hooded seals.

2.4.4. CYP content, EROD and PROD activities: Mother – pup pairs.

Harp seal mothers had significantly greater total CYP content compared to their respective pups, however no such relationship was observed between the hooded seal mother - pup pairs. The EROD and PROD activities in hooded seal liver microsomes were very similar in the females and their respective pups, suggesting that the CYP1A isoenzyme induction was comparable between developing young and the adult. However, the harp seal pups appeared to have less EROD and PROD activities than the maternal CYP-MO activities. Hooded seal neonates are at a more advanced state of development compared to the corresponding harp seals (see section 2.4.2). The lower EROD and PROD activities in harp seal pups may be indicative of the earlier stage of development compared to the hooded seal pups. However, in rodents and human livers, CYP1A1 and -1A2 are expressed very early in development (Rich and Boobis, 1997).

Although hooded seals had greater body burdens of total PCBs, the burdens of mono-*ortho* CBs were lower compared with the harp seals. Hooded seal neonates may have already received greater burdens of CYP1A- type congeners via transplacental transfer since the neonates have a layer of blubber compared to virtually no blubber layer in harp seal neonates. A greater induction of the CYP1A isoenzymes may have occurred pre-natally rendering the levels similar to that of the maternal hooded seal CYP1A activity. However, in both cases, the presence of *in vitro* CYP1A-MO activities suggests

that these pups are potentially capable of *in vivo* biotransformation of certain environmental contaminants that target these CYP isoenzymes.

The CYP – MO relationship between lactating females and their pups has, so far, not been investigated, however one other study has provided CYP-MO activities for female adults and pups. It is unknown whether the adult females and pups are related in these studies. For example, EROD activities were reported in harp seal pups, that unlike the present study, were comparable to the adult female harp seals (Goksøyr *et al.*, 1992). In contrast PROD activities in harp seal pups and EROD and PROD activities in hooded seal pups were unable to be detected, compared to the adult female seals. This could be a result of the apparent dilution of activity caused by measuring CYP associated MO activities in the 10,000g supernatants rather than liver microsomes.

2.4.5. Immunochemical detection of CYP1A and CYP2B proteins.

2.4.5.a. Inter-specific comparison. This study confirms the presence of proteins that closely resemble CYP1A and CYP2B in harp and hooded seal liver microsomes (Fig. 2.5) that were analogous to previous studies (Goksøyr *et al.*, 1992; Hyyti *et al.*, 2001; Mattson *et al.*, 1998; Nyman *et al.*, 2000; Wolkers *et al.*, 1998b).

Using anti-trout CYP1A1 anti-peptide antibody, a distinct band at 54kDa in both harp and hooded seals, and a weaker band at 55kDa, that was only present in a few of the individuals analysed, were detected. In comparison, Goksøyr and colleagues (1992) reported two protein bands also detected by a fish CYP antibody, anti-cod CYP1A, one at 54kDa, however the other was at a lower molecular weight of 52kDa.

A third band was also detected by the anti-trout CYP1A1 antibody, at a molecular weight (62kDa) that does not correspond to the molecular weight range for CYP isoenzymes. Similar bands at molecular weights higher than the top of the range for CYP isoenzymes have been detected by monoclonal antibodies in microsomal preparations in various species (Gelboin and Freidman, 1985). The identification of these bands are unknown, however are thought to be non-specific and not related to CYPs.

The anti-rat CYP2B antibody recognised a single 52kDa protein in the two species in the present study. However, the CYP2B-like protein bands were stronger in harp seals compared to the hooded seals, where only three seals reacted with the CYP2B antibody at a similar degree as the harp seals. This suggests that the epitope of the CYP2B antibody and the CYP2B-like protein in the seal samples have a common conserved peptide sequence.

Similar CYP2B-like bands at ~52kDa have been recognised in other studies of harp and hooded seals (Goksøyr, 1995), ringed seals (Wolkers *et al.*, 1998b) and harbour seals (Boon *et al.*, 2001). In contrast, no CYP2B-like proteins were detected in ringed seals in another study using an anti-rat CYP2B1 monoclonal antibody (Mattson *et al.*, 1998). No reaction with the antibody may suggest that there was no common peptide sequence in the rat CYP2B used to prepare the antibody and the corresponding seal CYP.

2.4.5.b. Intra-specific comparison: CYP1A and CYP2B relative levels in mother – pup pairs. In mother – pup pairs, CYP1A and CYP2B were at comparable levels in the hooded and harp seals, respectively. However, a greater relative concentration of CYP1A was observed in harp seal pups. The relative concentration of CYP2B was

higher in one hooded seal female (CcMb) compared to her pup (CcPb), whereas CYP2B concentration was lower in the other female (CcMa) compared to her pup (CcPa).

Endogenous factors may be responsible for the differences in CYP1A and CYP2B expression between the species and the mothers and their pups during a physiologically complex stage of the annual cycle. CYP isoenzyme expression is regulated by numerous endogenous factors. For example steroid hormones, androgens, oestrogens and thyroid hormones (triiodothyronin and thyroxine), regulate the postnatal expression of sex-specific CYP isoenzymes such as various members of the CYP2C subfamily, CYP3A2 and possibly CYP4A2 (reviewed in Waxman *et al.*, 1985). Endogenous substrates of CYP1A isoenzymes are currently unknown, however a role for CYP1A may involve cell cycle control, apoptosis and oxidative stress response (Nebert *et al.*, 2000). Other CYP isoenzymes have been implicated in the metabolism of 17β - oestradiol (CYP1A2) in rats (Dannan *et al.*, 1986) and the hydroxylation of progesterone to the 6β (CYP1A1 and CYP3A) and 16α (CYP2B) metabolites in rat, human and dog (Swinney, 1990).

The expression of CYP2B-like isoenzymes in these seals may be due to induction by PB-type PCBs and/or PCB metabolites. 3-methyl sulphone (MSF) PCB metabolites were present in the blubber of grey, harbour and ringed seals (Haraguchi *et al.*, 1992) and the retention of high concentrations of a few 3-MSF PCBs was observed in the livers of mink, otters and grey seal (Bergman *et al.*, 1994; Haraguchi *et al.*, 1994). In particular 3-MSF metabolites of PCBs 70, -87, -101 and -141 were potent PB-type inducers of hepatic CYP2B isoenzymes in rats (Kato *et al.*, 1995). Furthermore these four 3-MSF PCBs were between 34.2 and 684 fold more potent than their precursor PCBs (Kato *et al.*, 1995) and three (3-MSF - 87, -101 and -141) were transferred to grey seal pups during lactation (Green, 1997). Future studies should investigate these

and other PCB metabolites and their relationships with the drug metabolising isoenzymes.

During parturition, lactation and subsequent oestrus of the mature females, or development of the seal pups, numerous physiological changes occur. One or several changes may simultaneously affect the expression of CYP isoenzymes. Furthermore the perturbation of endogenous pathways by xenobiotics such as environmental contaminants, and/or the induction of CYPs by metabolites, may ultimately affect the regulation of CYPs.

2.4.6. EROD, PROD and relative CYP contentration.

EROD and PROD activities did not correlate with the relative content of immunoreactive CYP1A or CYP2B in the harp or hooded seals. Furthermore no correlation was observed between EROD and PROD specific activities or turnover rates in either of the two species.

In contrast, a positive correlation between EROD and PROD activities was reported, which suggested a common catalyst in ringed seals (Mattson *et al.*, 1998). Furthermore the induction of CYP1A-mediated EROD in Baltic ringed and grey seals compared to the reference site was reflected in the relative concentrations of CYP1A (Nyman *et al.*, 2000). However, EROD activities were low compared to the relative levels of CYP1A in sperm whales (Boon *et al.*, 2001).

The immunoreactive CYP1A in harp and hooded seals in the present study did not reflect the EROD and PROD activities. The cause of such incongruous patterns can be speculated. Harp seal EROD activity may be low compared to the high level of immunoreactive CYP1A due to the CYP1A isoenzyme may be a less efficient catalyst

for 7-ethoxyresorufin compared to that of the hooded seals (as previously discussed in section 2.4.3). However, the higher EROD activities observed in the hooded seal liver microsomes may be a consequence of another CYP isoenzyme contributing to this activity.

The peptide sequence recognised by the anti-trout CYP1A1 peptide antibody has a high % identity with the harp seal CYP1A1 sequence (Chapter five, this thesis), consistent with the good detection observed in the immunoblot. However, the % identity of the hooded seal CYP1A1 protein sequence with the peptide sequence of the antibody is unknown. Furthermore, a degree of the CYP isoenzymes of interest may be catalytically inactive as a consequence of storage (refer to Chapter four, this thesis) and handling during the preparation of the microsomes. This would result in lower activities compared with the higher CYP levels determined by Western blotting that only requires an intact protein primary sequence.

The lower EROD activity in harp seal liver microsomes may also be a consequence of competitive inhibition of this activity by the presence of particular PCB congeners in the liver microsomes. Inhibition of the CYP isoenzymes would not necessarily interfere with the reaction between an antibody and the epitope on the protein.

Coplanar PCBs, CB-77, -126, -169, the di-*ortho* CB, CB-153 and the commercial mixture, clophen A50 are all potent competitive inhibitors of CYP1A EROD activity in the flounder (*Platichthys flesus*) and rat (Besselink *et al.*, 1998). Haasch and colleagues (1993) showed that EROD activity, *in vitro*, was increased three-fold when β -naphthflavone (β NF) – induced trout liver microsomes were pre-incubated with

NADPH, compared to pre-incubation with both NADPH and substrate, indicating inhibition by residual β -NF.

The PCB burden of the liver was not determined in the present study therefore the influence of CB congeners on the catalytic activity of the CYP isoenzymes can not be ascertained. However, other *in vitro* experiments with liver microsomes from environmentally exposed harp and hooded seals, may determine the degree of competitive inhibition by an integral component, whether endogenous or exogenous, such as CB congeners.

2.4.7. TEQ, PCB concentrations and CYP isoenzymes.

Toxic equivalency factors (TEF) were assigned to PCB congeners and reflect the CYP1A-induction potency mediated by the aryl hydrocarbon (Ah) receptor, relative to that of 2,3,7,8-TCDD (Van den Berg *et al.*, 1998). Toxic equivalents (TEQs) were calculated for five mono-*ortho* CBs (CB-118, -105, -114, -156 and -157) to evaluate their relative potency in harp and hooded seals.

The Σ 5TEQ (sum of 5 CB congeners) for the pups of both species were comparable and were lower, although only by ~ 2 fold, than the Σ 5TEQs calculated for the adult harp and hooded seals. CB-118 contributed between $\sim 40\%$ and $\sim 60\%$ of Σ TEQs in harp and hooded seal pups and harp seal adults. This was consistent with the %TEQ of CB-118 observed in harbour porpoises (*Phocoena phocoena*) (Berggren *et al.*, 1999; Tanabe *et al.*, 1997). CB-156 contributed the highest % to the TEQs ($\sim 50\%$) in the adult hooded seals. This congener was also the greatest contributor to the Σ TEQ in polar bears (*Ursus maritimus*) (Letcher *et al.*, 1996).

CB-118 is relatively less potent as a CYP1A-inducer than CB-156 as the TEF value assigned was ~5 fold greater for CB-156 (0.0005) than CB-118 (0.0001). However, the similar % contribution of these congeners to the Σ 5TEQ in the harp seals and hooded seal pups (CB-118) and adult hooded seals (CB-156), suggests that the higher concentration of the CB-118 congener may elicit a similar overall toxicity as CB-156 in these animals. Furthermore the level of Σ TEQ indicated an efficient transfer of toxic compounds to the pup from maternal blubber, during a developmental stage.

The most reactive CBs are the coplanar congeners, which are substituted at both *para* positions and two or more *meta* positions and are approximate isostereomers of 2,3,7,8-TCDD (Safe *et al.*, 1985). The coplanar CBs were not measured in the present study. However, of the mono-*ortho* CBs measured, CB-118, which was at the highest concentration and burden in the pups blubber, most resembled the coplanar chlorine substitution pattern. Furthermore CB-118 had the highest %TEQ of the Σ TEQs in all the seal pups blubber.

Several mono-*ortho* CBs were implemented in numerous toxicities in rats and mice. CB-118, -105, -156 and -157 caused thymic atrophy in rats (Parkinson *et al.*, 1983) and the administration of CB-105 and -99 to mice and rats resulted in weight loss, oedema, accumulation of liver lipid, extensive hepatic damage and splenic atrophy (reviewed in Safe *et al.*, 1985). Furthermore decreased levels of thyroid hormone were found in harbour seal due to the PCB-77 metabolite binding to the thyroxine protein carrier, transthyretin (TTR) (Brouwer *et al.*, 1989). Potential toxicities and developmental disorders manifesting in seal pups during lactation and the post-weaning fast, when such CBs may exert their effects after metabolism by CYP isoenzymes, are implied.

2.4.8. Immunoreactive CYP and CYP-MO activities as biomarkers of hooded and harp seal exposure to PCBs.

Molecular, biochemical or cellular signals that indicate an animals' exposure to xenobiotics whereby the toxicants have reached a threshold and elicited a response, can be used as biomarkers (McCarthy, 1992). A model for estimating exposure of an individual to environmental contaminants using CYP isoenzymes would aid as a potential biomarker (den Besten, 1998; Payne *et al.*, 1987; Van den Berg *et al.*, 1998) in marine mammals.

CYP1A-associated EROD and PROD activities and content were plotted against the Σ TEQ of five potentially CYP1A-inducing CBs and PCB concentrations to estimate the exposure of the harp and hooded seals to PCB compounds. No correlation was obtained between CYP activities or content and either TEQ or concentrations of PCBs for both harp and hooded seals in the present study. However, this is only representative of the PCBs measured in this study, since other aryl hydrocarbon (Ah) receptor responsive compounds, i.e. co-planar PCBs, dioxins, PAHs, may correlate with CYP1A activities and expression.

The lack of correlation between CYP1A-EROD and PCB concentrations in the present study corresponded with harp seals from the Barents Sea (Wolkers *et al.*, 1999) and West Ice (Goksøy, 1995). In contrast, a correlation between EROD activity and PCB levels was observed in hooded seals (Goksøy, 1995). Furthermore relationships between CYP1A activity and PCB levels in the blubber have been reported for other marine mammals (Letcher *et al.*, 1996; White *et al.*, 1994).

Harp seals in the Barents Sea feed on a large amount of the crustacean *Parathermista libellula*, which contains a high concentration of CYP1A-inducing β -carotene (Wolkers *et al.*, 1999). Furthermore the harp seals had high EROD activities with corresponding low PCB burdens, suggesting that the lack of correlation between CYP content and PCB burdens in harp seal could be a diet related factor.

During parturition/lactation the mobilisation of a huge concentration of PCBs into circulation may cause the down-regulation of CYPs. Furthermore, down-regulation of CYP expression by long term PCB exposure, or an endogenous factor, may also play important roles. For example, continuous long-term exposure (21 days) of trout (*Oncorhynchus mykiss*) to a low concentration (10 μ g/L water) of β -NF produced an elevated level of CYP1A1 mRNA, CYP1A1 apoprotein and EROD activity (Haasch *et al.*, 1993). However, an increase of β NF to 50 μ g/L water caused down-regulation of CYP1A (Haasch *et al.*, 1993). Fasting has affected the levels of CYP2C11 in male rats due to an altered ratio of circulating glucagon/insulin (Donahue *et al.*, 1991), and may have implications for CYP regulation in fasting seals during the lactation period.

Activation of the immune system resulted in the down-regulation of microsomal CYP1A1 activity through the production of cytokines, in cell culture (murine hepatoma (Hepa1) cells), (Paton and Renton, 1998). Large concentrations of circulating PCBs may also potentially overload the normal pathways of CYP-mediated detoxification, so that metabolism occurs through a less optimal route, resulting in the exposure to PCBs not being reflected in the CYP activities or content.

Immunoreactive CYP concentrations and CYP-MO activities have the potential for being effective biomarkers of a seal's exposure to environmental contaminants. However, further characterisation of the hooded and harp seals detoxification system is

required before CYPs can be used as biomarkers of PCB exposure in these species. Furthermore the effect of endogenous factors on CYP isoenzyme expression during particular seasons needs to be determined in order to interpret CYP induction due to xenobiotics.

2.4.9. Summary

This study has investigated the induction of cytochrome P4501A – associated EROD and PROD activity and expression of immunoreactive CYP1A and CYP2B as an early warning system for PCB exposure in harp and hooded seals. The relationship of CYP isoenzymes between mother and pup pairs in both species and the transfer of PCB congeners from maternal blubber to pup blubber were also examined. The following observations were made:

- Hooded seal $\Sigma 27$ PCB concentrations and body burdens were greater than those measured in harp seals.
- *Di-ortho* CBs, compared to mono-ortho CBs, contributed the greatest % of Σ PCB burdens in both species.
- A selective transfer of PCBs was observed from mother to pup, with decreasing relative transfer of penta-, hexa-, hepta- and octa- CBs, in both seal species.
- Hepatic EROD and PROD activities were detected in both harp and hooded seal adults and pups. The EROD activities were greater in hooded seals than harp seals, however the PROD activities were higher in harp seals.

- CYP1A- EROD activities were similar in hooded seal mother and pup pairs, however this activity was lower in the harp seal pups compared to the maternal activity. A greater induction of the CYP1A isoenzyme activity in hooded seal pups may have occurred pre-natally.
- Anti-trout CYP1A1 and anti-rat CYP2B antibodies in both species detected distinct CYP1A-like and CYP2B-like proteins, although the reaction with harp seal liver microsomes was stronger and more consistent than hooded seals.
- A less distinct protein was detected by the anti-trout CYP1A1 antibody in some of the seals from both species that may be another member of the CYP1A subfamily.
- The pups of both species showed levels of immunoreactive CYP1A and of CYP1A-MO activities that were comparable or higher level than the adult females, and harp seal pups had a comparable level of CYP2B too. This indicates the potential of the developing seals to metabolise certain environmental contaminants.
- No correlation between Σ TEQ and the CYP-MO activities or PCB concentrations/burdens and CYP-MO activities, suggests that CYP-MO activities at this stage should not be used as a biomarker for PCBs in harp and hooded seal species taken during the breeding season

3

Cytochrome P450 induction and expression in harbour porpoise and grey seal from the East Coast of Scotland, exposed to environmental PCBs

3.1. Introduction

Environmental contaminants, such as polychlorinated biphenyls (PCBs), are anthropogenic and widely distributed in the marine environment by rivers, ocean currents and atmospheric transport. Highly industrialised countries surround the North Sea, utilising it for commercial purposes, such as shipping, fishing, oil industry, and recreational uses. Industrial waste products enter the sea from direct disposal as waste waters, dumping at sea (until 1993 in UK waters), accidental chemical spillage and leakage from landfill sites and partitioning from the gas phase to the water phase after atmospheric transport (Anon., 2000; Mackay, 1982). Approximately 11 –17 tonnes of PCBs were estimated to enter the North Sea every year prior to 1990 (Klamer *et al.*, 1991), with the highest concentrations found near to the Dutch coast and German Bight.

The North Sea supports populations of harbour porpoise (*Phocoena phocoena*) and grey seal (*Halichoerus grypus*). The most recent relative abundance of harbour porpoise was estimated to be within the range of 260,000 – 450,000 (Hammond *et al.*, 1996). The UK hosts grey seal populations, which haul out in coastal areas. The estimated population size of the North Sea populations of grey seals is 124,300 (SMRU 2001, unpublished observations).

Recent and potential threats to marine mammals include interactions with fisheries through by-catches in nets and competition for fish, disturbance of their natural environment due to commercial and recreational use, disease and pollution (Hall *et al.*,

1992; Hammond *et al.*, 1996; Tregenza *et al.*, 1997). A combination of such interactions may affect the stability of marine mammal populations.

Environmental contaminants have been reported in marine mammal tissues, world wide for several decades and have largely focused on PCBs and the pesticide DDT(E) in blubber (e.g. Hall *et al.*, 1999; Muir *et al.*, 1996a; Norstrom and Muir, 1994; Vetter *et al.*, 1996; Zitko *et al.*, 1998). PCB concentrations in harbour porpoises and grey seals were reported in animals from the European North Sea, the Baltic Sea and regions of the Atlantic (Addison and Brodie, 1987; Addison *et al.*, 1999; Addison and Stobo, 1993; Berggren *et al.*, 1999; Blomkvist *et al.*, 1992; Boon *et al.*, 1994; Bruhn *et al.*, 1995; Bruhn *et al.*, 1999; Skaare, 1995; Vetter *et al.*, 1995; Vetter *et al.*, 1996). However, the majority of the investigations of PCB concentrations from harbour porpoises and grey seals inhabiting UK waters are from animals sampled during the eighties and early nineties, the most recent being 1996 (Green *et al.*, 1996; Jepson *et al.*, 1999; Kuiken *et al.*, 1994; Kuiken *et al.*, 1993; Law *et al.*, 1989; Pomeroy *et al.*, 1996; Smyth *et al.*, 2000; Troisi *et al.*, 1998; Wells *et al.*, 1994).

The comparison of studies can be difficult because PCB concentrations are often given as the total and the individual PCB congener concentrations contributing to the total are not provided. Furthermore PCB concentrations are often the sum of different PCB congeners from one study to the next. However, CB-153 and CB-138 have been reported in the majority of studies and provide the means for inter-study comparison of conspecifics. Observed $\Sigma 2CB$ (sum of CB-153 and -138) concentrations have varied from $1.27\mu\text{g}\cdot\text{g}^{-1}$ lipid to $5.88\mu\text{g}\cdot\text{g}^{-1}$ lipid in harbour porpoises and $\Sigma 2CB$ was reported to be $5.2\mu\text{g}\cdot\text{g}^{-1}$ lipid in grey seals, inhabiting UK waters (Law *et al.*, 1989; Smyth *et al.*, 2000; Wells *et al.*, 1994). Furthermore the $\Sigma 2CB$ concentrations in conspecifics varied

geographically in these two species. The Σ 2CBs in the harbour porpoise from the UK waters are lower than those from the Baltic sea (11.4 – 33 $\mu\text{g.g}^{-1}$ lipid), although higher than the reported Σ 2CB concentrations detected in harbour porpoises from Arctic waters (0.18 $\mu\text{g.g}^{-1}$ lipid) (Berggren *et al.*, 1999; Bruhn *et al.*, 1999). The Σ 2CB concentrations in grey seals inhabiting UK waters were comparable to conspecifics from Sable Island (1.82 – 5.28 $\mu\text{g.g}^{-1}$ lipid), although were lower than the Σ 2CB concentrations reported from grey seals in the St Lawrence Estuary (2.08 – 8.97 $\mu\text{g.g}^{-1}$ lipid). Similar to the UK harbour porpoises, the Σ 2CB concentrations from grey seals in UK waters were higher than grey seals sampled from Iceland (0.22 – 2.48 $\mu\text{g.g}^{-1}$ lipid) (Addison and Brodie, 1987; Addison and Stobo, 1993; Bernt *et al.*, 1999; Vetter *et al.*, 1995). Differences in age, sex, nutritional and reproductive status, year the samples were taken, whether the animals were by-caught or hunted, numbers of PCB congeners analysed and analytical methods, however, can effect the observed PCB concentrations (reviewed in Boon *et al.*, 1992).

Several studies have indicated that pollution, in particular PCBs may be affecting the immune system of marine mammals. For example, a recent study of stranded harbour porpoises showed those, which died from infectious diseases, had higher PCB burdens than those, which died from trauma (Jepson *et al.*, 1999). The authors concluded that “the results of this study show a statistically significant association between high blubber CB levels in harbour porpoises and mortality due to infectious disease that was both highly significant ($F_{1,65} = 12.48$, $P < 0.001$) and not confounded by any other measured variables including age, sex, and nutritional status [ventral blubber thickness and body weight/length ratio]” and suggests that PCBs may be exerting an immunosuppressive effect in this species (Jepson *et al.*, 1999). However, other environmental contaminants, such as PAHs and dioxins (PCDD/Fs), may also play an

immunosuppressive role independent of the PCBs. The phocine distemper virus (PDV) and a strain of canine distemper virus (CDV) outbreaks of 1988-89 were recorded in several seal species (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989), including some grey seals and a harbour porpoise in the UK. Indeed the European PDV mass mortality in harbour seals indicated PCBs may have increased their severity (Hall *et al.*, 1992; Osterhaus *et al.*, 1995). Therefore a method of detecting the biological response of marine mammals to PCBs could serve as an early warning system of such exposure and of potential threats to the animals health.

Inducible cytochrome P450 (CYP) isoenzymes, particularly certain members of families 1-4, metabolise xenobiotics, including PCB congeners (Stegeman and Hahn, 1994). *In vitro* analysis of CYPs using specific model monooxygenases (MO) such as 7-ethoxyresorufin *O*-deethylase (EROD), and immunochemical detection of the CYP proteins enables an indirect measure of PCB exposure in numerous organisms, including several marine mammal species (Fossi *et al.*, 1997a; Haasch *et al.*, 1993; Machala *et al.*, 1997; Troisi and Mason, 1997; White *et al.*, 1994; Whyte *et al.*, 2000).

Catalytic detection of induced CYP1A isoenzymes with the associated monooxygenase, EROD has been characterised in laboratory mammals, fish and humans (Burke *et al.*, 1994; Machala *et al.*, 1997; Murk *et al.*, 1994). EROD activity was observed in a study of grey seals from the Baltic and Sable Island (Hyyti *et al.*, 2001; Nyman *et al.*, 2000). However, EROD activity has only been observed in liver microsomes from a single harbour porpoise (Boon *et al.*, 1994; Murk *et al.*, 1994).

7-Pentoxoresorufin *O*-deethylase (PROD), another CYP-mediated MO, is a selective probe for CYP2B in phenobarbital (PB)-treated rats, however in 3-methylcolanthrene

(3MC)-treated rats, PROD was predominantly catalysed by CYP1A (Burke *et al.*, 1994). In grey seals, PROD activity was inhibited by α -naphthoflavone (α -NF), a CYP1A-specific inhibitor (Nyman *et al.*, 2000). However, PROD activity has not been investigated in harbour porpoises.

So far immunoreactive CYPs resembling CYP1A, 3A and 4A apoproteins have been identified in harbour porpoise liver microsomes (Boon *et al.*, 2001; Goksøyr, 1995) and potential CYP1A, -2A, -2C, -2D, -2E and -3A proteins were observed in grey seals (Nyman *et al.*, 2001). However, the unequivocal presence of a CYP2B isoenzyme in these two species is not so apparent. A CYP2B apoprotein was observed in harbour porpoise liver microsomes in one study (Goksøyr, 1995), however was absent in another investigation (Boon *et al.*, 2001). Furthermore a CYP2B isoenzyme appears to be absent in grey seals (Nyman *et al.*, 2001), despite the identification of an immunoreactive CYP2B apoprotein in several other seal species, including hooded seals (*Cystophora cristata*), harp seal (*Phoca groenlandica*), ringed seals (*Phoca hispida*) and harbour seals (*Phoca vitulina*) (Goksøyr, 1995; van Hezik *et al.*, 2001; Wolkers *et al.*, 1998b). Species differences exist in the regulation and function of CYP isoenzymes (reviewed in Murray *et al.*, 2001; Stegeman and Hahn, 1994; Stegeman and Livingstone, 1998); therefore the characterisation of the CYP isoenzymes that are involved in xenobiotic metabolism in the species of interest is necessary.

CYP activities, the presence of immunoreactive CYPs and PCB concentrations have been reported from marine mammal tissue samples that were obtained from several sources including hunted animals, by-caught and stranded dead animals. Due to the ethical considerations, marine mammal tissues are increasingly being obtained from opportunistic by-catches and strandings e.g. (Berggren *et al.*, 1999; Fossi and Marsili,

1997; Law *et al.*, 1989; Troisi and Mason, 1997). However, the number of animals obtained is often limited to a single animal, or very low numbers of animals at a time.

The study aimed to provide preliminary data on the xenobiotic metabolising system and the exposure to PCBs of a harbour porpoise and a grey seal that were victims of by-catches on the East Coast of Scotland. Very few previous investigations have studied cytochrome P450 isoenzymes in harbour porpoises and grey seals, therefore I aimed to provide further information regarding the presence of immunoreactive CYP1A and -2B apoproteins and the specificity of EROD and PROD for CYP1A-isoenzymes, in these two animals from different species. Furthermore PCB concentrations in harbour porpoise and grey seal blubber were compared with conspecifics from other studies. The value of using tissues from by-caught and stranded animals in xenobiotic studies is also discussed.

3.2. Materials and Methods

Chemicals. α -naphthoflavone was purchased from Sigma. All other reagents were purchased or obtained as described in Chapter two.

3.2.1. Samples.

A male, juvenile (< 2years) harbour porpoise (*Phocoena phocoena*) was caught in a net in St Andrews Bay and appeared healthy. The porpoise had only been dead for a maximum of 2 hours prior to the preparation of microsomal fractions. A sample of liver (~60g) was excised, chopped into small pieces (~5cm³), wrapped in foil and immediately frozen in liquid nitrogen. Body length and girth were measured to the nearest centimetre and were 1.03m and 0.84m, respectively. The body mass was 39Kg and the blubber thickness measured ventrally was 2.1cm.

A mature (>5 years) female grey seal (*Halichoerus grypus*) was caught in a net east of Peterhead, Aberdeen and appeared to be healthy. The grey seal had been dead for 12 hours before subsamples of excised liver were wrapped in foil and frozen in liquid nitrogen until further preparation of the liver microsomal protein.

3.2.2. Preparation of liver microsomes.

The liver subsamples from the harbour porpoise and grey seal were thawed on ice and subsequently prepared as described in Chapter two (section 2.2.2), except for the following modification. The liver samples were not subjected to a centrifugation at 700g, however were immediately centrifuged at 10,000g in a Beckman JA-14 centrifuge for 30 minutes. The rest of the protocol was followed exactly as described in Chapter two.

3.2.3. Total protein content and CYP enzyme activities and immunochemical detection of CYP isoenzymes.

Determination of total protein concentration, EROD and PROD associated CYP activities, SDS-PAGE and immunodetection of the CYP isoenzymes, CYP1A and CYP2B, were conducted according to the procedures described in Chapter two (sections 2.2.3, 2.2.5 and 2.2.6).

3.2.4. Inhibition of CYP enzyme activities.

EROD and PROD activities were performed in triplicate as described in Chapter two (section 2.2.5). These were the 100% values. Inhibition of the each EROD and PROD activities with α -naphthoflavone (α -NF), a CYP1A1 inhibitor (Chang *et al.*, 1994; Wolkers *et al.*, 1999), were performed. Briefly, the EROD inhibition assay contained 0.1mg/mL microsomal protein, 1 μ L 1mM 7-ethoxyresorufin, 10 μ L 50mM α -NF, to a

final volume of 1mL with 0.1M Tris-HCl, pH 7.8. The mixture was gently vortexed and incubated at 37°C for 2 minutes, prior to reading the baseline. The reaction was initiated by the addition of 10µL 10mM NADPH (freshly prepared) to 1mL of the reaction mixture.

3.2.5. PCB contaminant analysis.

PCB analysis and lipid content of the blubber were determined by Dr N. Green (University of Lancaster, UK). The procedure was essentially the same as that described in Chapter two (section 2.2.7), with the following modifications. Twenty-eight PCB congeners (Twenty- three CB congeners and five co-eluted CB congeners), CBs -18/ 17, -31/28, - 49, -52, -61/74, -95, -99, -101, -87/115, -105, -110, -118, -138, --149, -151, -153/132, -158, -156, -157, -167, -170, -174, -180, -183, -187, -194, 199, -203, were extracted from the blubber of the harbour porpoise.

Two CBs, CB-158 and CB-167 were not measured in the grey seal, however an additional ten CBs (8 CBs and 2 co-eluted CBs) were extracted from grey seal blubber including CBs – 22, 44, -41/64, -70, -60/56, -114, -155, -141, -188, -189, resulting in thirty-six CB congeners (twenty-seven CB congeners and nine co-eluted CB congeners). However, of the thirty-six CB congeners extracted from grey seal blubber, five CBs were below the limit of detection (CBs –18/17, -31/28, -22, -41/64 and –60/56).

3.3. Results

3.3.1. EROD and PROD activities and the effect of inhibition.

The liver microsomal EROD activity was higher, by two orders of magnitude, in the harbour porpoise (mean \pm 1 standard error (S.E.) $2.14 \pm 0.05 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1} \text{ protein}$)

than the grey seal (mean \pm 1 S.E., 0.037 ± 0.0036 nmol.min⁻¹.mg⁻¹ protein). However, the PROD activities were very similar in both the harbour porpoise (mean \pm 1 S.E. 0.8 ± 0.025 pmol.min⁻¹.mg⁻¹ protein) and the grey seal (mean \pm 1 S.E. 1.1 ± 0.12 pmol.min⁻¹.mg⁻¹ protein).

The EROD and PROD activities in the harbour porpoise and grey seal from the present study were compared with corresponding activities from the literature on conspecifics and a selection of other marine mammal species (Table 3.1.). The harbour porpoise EROD activity was ~5 fold higher than that in the other single harbour porpoise measured and the other selected cetacean species in Table 3.1. However, the PROD activity in the harbour porpoise was approximately 8 fold lower than the corresponding activity in beluga whale (White *et al.*, 1994). The EROD and PROD activities in the grey seal liver microsomes from the East Coast of Scotland were much lower than conspecifics from the Baltic (Nyman *et al.*, 2000) and harbour seals from the East Coast of England (Troisi and Mason, 1997). In contrast, the PROD activity determined from the grey seal in the present study was comparable to conspecifics from the Baltic and ringed seals from the Svalbard (Wolkers *et al.*, 1998; Nyman *et al.*, 2000).

Fig. 3.1 shows EROD and PROD activities in the presence of the CYP1A specific inhibitor, α -naphthoflavone (α -NF). EROD activity in the presence of 1 μ M α -NF was inhibited by >99% in both the harbour porpoise and grey seal liver microsomes. Furthermore, 1 μ M α -NF inhibited PROD activity by $68.63 \pm 8.04\%$ and $72.98 \pm 19.98\%$ in harbour porpoise and in grey seal, respectively.

Table 3.1. Comparison of the EROD and PROD activities in the harbour porpoise and grey seal from the present study with conspecifics and other marine mammal species from select papers. ^aEROD and PROD activities are presented as nmol.min⁻¹.mg⁻¹ protein. * Activities were determined from post-mitochondrial supernatants and may be 10 fold lower than the relative liver microsomal levels Goksøyr (1995). Liver microsomal protein was used in the activity assays, except *.

Species	Location	Sex	Cause of death	^a EROD activities	^a PROD activities	Reference
Harbour porpoise n = 1	East Coast Scotland	Male (J)	By-catch	2.14	0.0008	Present study
Harbour porpoise n = 1	Texel, Netherlands	Female (A)	Stranded, ill health	0.417	0.027	Murk et al, 1994, Boon et al, 1994
Beluga whale n=8 n = 5	Canadian Arctic	Male Female	Taken	0.413 ± 0.263 0.097 ± 0.084	0.0067 ± 0.0045 0.0014 ± 0.0006	White et al, 1994
Sperm whale n = 2 n = 1	The Hague, NL Rømø, DK	Male (A)	Stranded	0.015 - 0.028	n.d.	Boon et al, 2001
White beaked dolphin n = 1	Texel, NL	Female (A)	Stranded	0.049	n.d.	
Pilot whale n = 8 n = 10	Canadian Arctic	Male Female	Taken	0.067 ± 0.076 0.093 ± 0.182	n.d. n.d.	White et al, 2000
Striped dolphin n = 5	Coast of Japan	n.d.	Taken	0.191 ± 0.015	n.d.	Watanabe et al, 1989
Grey seal n = 1	East Coast Scotland	Female (A)	By-catch	0.037	0.0011	Present study
Grey seal n = 16	Baltic Sea	Male and Female (A)	Taken	1.3 ± 0.5	0.007 ± 0.002	Nyman et al, 2000
Grey seal n = 8	Sable Island, Canada	Male Female	Taken	3.35 ± 4.54* 2.6 ± 2.2*	n.d.	Addison and Smith, 1994
Harbour seal n = 14	East Coast England		Ill health	9.6 ± 10	n.d.	Troisi and Mason, 1997
Harp seal n = 13	Svalbard		Taken	0.449 ± 0.146	n.d.	Wolkers et al, 1999
Ringed seal n = 7 N = 7 N = 14	Svalbard	Male (A) Female (A) Juveniles	Taken	0.154 ± 0.132 0.172 ± 0.107 0.065 ± 0.051	0.003 0.003 0.001	Wolkers et al, 1998

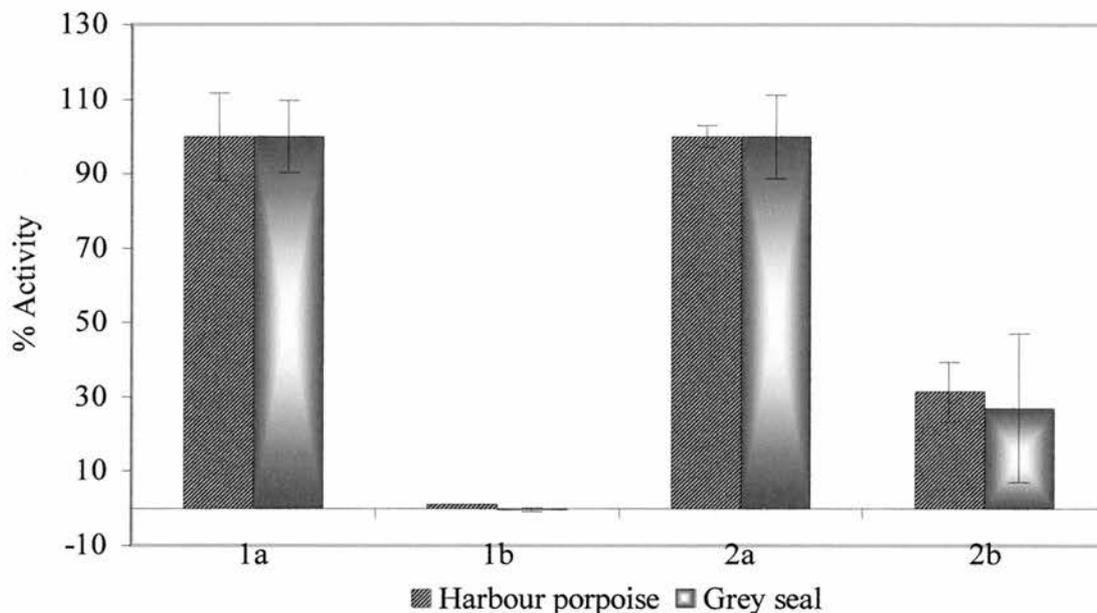


Fig. 3.1. The effect of the specific CYP1A inhibitor, α NF on EROD and PROD activities. (1), EROD activity and (2), PROD activity (a) without inhibitor and (b) with inhibitor in harbour porpoise and grey seal liver microsomes. Standard error is based on the mean of triplicate activities from each the harbour porpoise or the grey seal.

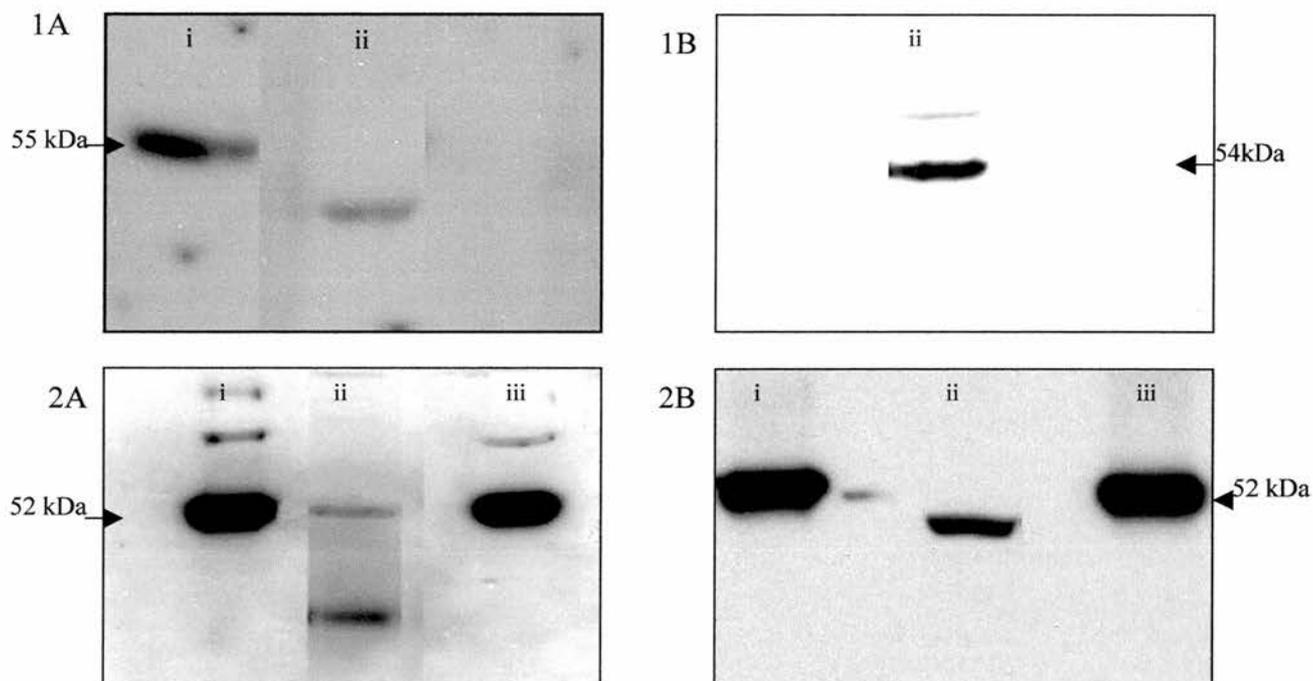


Fig. 3.2. Immunochemical detection of protein bands that reacted with (1) an anti-trout CYP1A1 anti-peptide antibody and (2) an anti-rat CYP2B antibody. Liver microsomes from (A) a harbour porpoise (40 μ g, lane ii) and (B) a grey seal (40 μ g, lane ii) were separated in 10% polyacrylamide by SDS-PAGE. (1Ai) 2.4 μ mol purified rat CYP1A1 and (2A and 2B, i and iii) 0.748 μ mol phenobarbital-induced rat liver microsomes. No standard was available for (1B).

3.3.2 Immunochemical detection of CYP1A and -2B like apoproteins.

Figs. 3.2.1. and 3.2.2. show the immunoblots of harbour porpoise and grey seal liver microsomes using anti-trout anti-peptide CYP1A1 and anti-rat CYP2B antibodies, respectively. A single protein band was recognised by the anti-trout CYP1A antibody in harbour porpoise liver microsomes. The molecular weight of the protein band was lower (~52kDa) than that of the rat purified CYP1A1 standard at ~55kDa (Fig, 3.2.1A). However, the anti-trout CYP1A1 antibody reacted with a distinct ~54kDa band in the liver microsomes of grey seal. The molecular weight was obtained from calibrated molecular weight standards.

The anti-rat CYP2B antibody detected a single protein band that corresponded to the molecular weight of the protein in the phenobarbital (PB)-induced rat liver microsomes (Fig. 3.2.2.A). The lower and more prominent band in harbour porpoise liver microsomes that reacted with the CYP2B antibody may be a degradation product as the liver microsomes used in the immunoblot were prepared from liver that was frozen for 14 days prior to microsomal preparation. In contrast, the anti-rat CYP2B antibody detected a protein of ~51kDa in grey seal liver microsomes. The detection of the CYP1A and CYP2B protein bands in harbour porpoise and grey seal from this study were compared with similar bands in the same species and other cetacean and pinniped species (Table 3.2).

3.3.3. PCB concentrations and relative patterns.

Harbour porpoise and grey seal blubber lipid content were similar (85% and 91.1%, respectively). Twenty-eight and thirty-six PCB congeners were identified in the blubber of the harbour porpoise and grey seal, respectively. The $\Sigma 28$ PCB concentration in the blubber of the harbour porpoise was 4852ng.g⁻¹ lipid and the $\Sigma 36$ PCB concentration in

Table 3.2. Comparison of harbour porpoise and grey seal CYP1A and -2B like proteins with corresponding proteins in conspecifics and other pinniped and cetacean species detected on an immunoblot with the named antibodies. n.d. indicates the band was not detected by the specified antibody.

^a approximate molecular weight (MW) in kDa determined by immunochemical detection

^b polyclonal anti-peptide antibody, donated by Dr R. Addison, Institute of Ocean Sciences, Canada

^c rabbit anti rat CYP2B1/2 polyclonal antibody, donated by Dr S. Bandiera, University of British Columbia, Canada

^d monoclonal mouse anti-rat CYP1A1/2 antibody (Oxford Biomedical Research, USA, Cat. No. PM16)

^e monoclonal rabbit anti-rat CYP1A1 antibody (ECL, Amersham, UK)

^f monoclonal mouse anti-rat CYP2B1/2 antibody (Oxford Biomedical Research, USA, Cat. No. PM20)

^g ECL, Amersham, UK

^h ECL, Amersham, UK

ⁱ monoclonal 1-12-3

^j polyclonal

^k Monoclonal 4-29-5 p4

^L goat anti-rabbit CYP2B4 (Oxford Biomedical Research, USA)

^m Anti-dog CYP2B11

ⁿ Monoclonal 1-7-1

^o Polyclonal anti-peptide antibody

^p Polyclonal (Gentest, MA, USA, Cat. No. 210112)

^q Monoclonal 2-66-3

Species	MW of CYP1A protein ^a	Antibody used in immunodetection	MW of CYP2B protein ^a	Antibody used in immunodetection	Reference
Harbour porpoise	52	Anti-trout CYP1A1 ^b	52	Anti-rat CYP2B1/2 ^c	Present study
Harbour porpoise	52 57 (weak)	Anti-rat CYP1A1/2 ^d Anti-rat CYP1A1 ^e	n.d.	Anti-rat CYP2B1/2 ^f	Boon et al., 2001
Harbour porpoise	54	Anti-rat CYP1A1 ^g	52	Anti-rat CYP2B1/2 ^h	Goksoyr, 1995
Beluga Whale and Pilot whale	53	Anti-scup CYP1A1 ⁱ	n.d.	Anti-scup CYP2B ^j Anti-rat CYP2B1/2 ^k Anti-rabbit CYP2B4 ^L Anti-dog CYP2B11 ^m	White et al., 1994; 2000
Grey seal	54	Anti-trout CYP1A1 ^b	51	Anti-rat CYP2B1/2 ^c	Present study
Grey seal	56	Anti-rat CYP1A1 ⁿ Anti-scup CYP1A1 ⁱ	n.d.	Anti-human CYP2B6 ^o Anti-rat CYP2B1/2 ^c Anti-rat CYP2B1/2 ^p Anti-rat CYP2B1 ^q	Nyman et al., 2000 (CYP1A) Nyman et al., 2001 (CYP2B)
Harp seal Harbour seal Hooded seal Harbour seal	54 55	Anti-rat CYP1A1 ^g Anti-rat CYP1A1/2 ^d	52 53	Anti-rat CYP2B1/2 ^g Anti-rat CYP2B1/2 ^c	Goksoyr, 1995 Boon et al., 2001

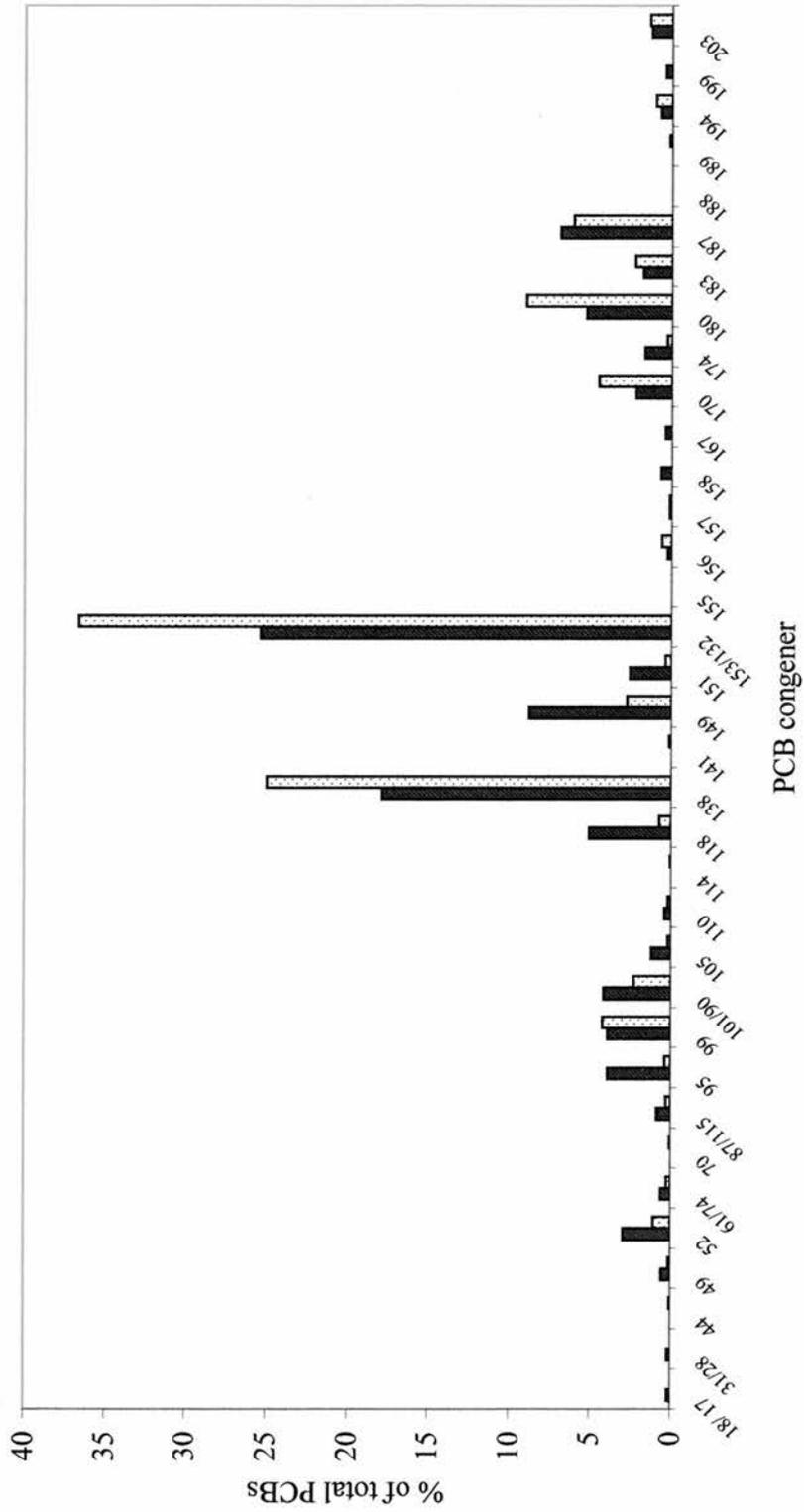
grey seal blubber was 1848.08ng.g⁻¹ lipid. Twenty-six of the PCB congeners were common to both animals and were used for comparison of the PCB concentrations and patterns between the two animals. The harbour porpoise had a greater ΣPCB concentration than the grey seal, when the ΣPCB concentration was re-calculated as a total of the common twenty-six CB congeners (4514ng.g⁻¹ lipid and 1840.22ng.g⁻¹ lipid for the harbour porpoise and grey seal, respectively).

The relative patterns of the CB congeners were calculated as a % of the Σ26CBs in each animal and were similar in the harbour porpoise and grey seal samples (Fig. 3.3.). CB-153 and CB-138 contributed the highest proportion of Σ26CBs in the harbour porpoise (25.7% and 18.1%) and grey seal (36.8% and 25.1%). CB-180 was the next highest contributor to the Σ26CBs, however CB-149 replaced CB-180 in the harbour porpoise.

The twenty-six CB congeners, common to both animals, were classified into one of five metabolic groups (Table 3.3.) according to the position of vicinal hydrogen atoms and number of chlorine substituents on the biphenyl molecule (Boon *et al.*, 1997).

- Group I contained congeners without any vicinal hydrogen atoms.
- Group II congeners had vicinal hydrogen atoms exclusively in the *ortho*- and *meta*- positions in combination with ≥ 2 *ortho* chlorine substituents.
- Group III congeners had vicinal hydrogen atoms in the *ortho*- and *meta*- positions and ≤ 1 *ortho* chlorine.
- Group IV congeners had vicinal hydrogen atoms in the *meta*- and *para*- positions in combination with ≤ 2 *ortho* chlorine substituents.
- Group V contained congeners with vicinal hydrogen atoms in the *meta*- and *para*- positions in combination with ≥ 3 *ortho* chlorine substituents.

The potential for CYP induction of each CB congener is also presented in Table 3.3.



■ Harbour porpoise □ Grey seal

Fig.3.3. Comparison of the harbour porpoise and grey seal blubber PCB congener patterns based on the relative proportion of $CB_x/\Sigma PCB$. PCB congeners are labelled as IUPAC numbers (Ballschmiter and Zell, 1980). Congeners CB-22 and the co-eluted CBs 41/64 and 60/56 were below the level of detection in the grey seal and were not measured in the harbour porpoise so are not presented in the profile.

Metabolic Group	CB congeners
I	153*, 167 ⁺ , 180*, 183*, 189 ⁺ , 194*, 203 nd , 155 nd , 188 nd
II	90 nd , 99 ⁺ , 115 nd , 138 ⁺ , 158 ⁺ , 170 ⁺
III	118 ⁺ , 114 ⁺ , 105 ⁺ , 156 ⁺ , 157 ⁺ , 28 nd , 22 nd , 31 nd , 60 nd /56 nd , 61 nd /74 ^w , 70 ^w
IV	101*, 110 nd , 141 nd , 52 ^w , 18 ^w /17 nd , 41 nd /64 nd , 44 ^w , 49 ^w , 87*
V	95 nd , 151 ^w , 149 nd , 174 nd , 132 nd , 199 nd

Table 3.3. PCB congeners divided into their metabolic groups (I-V) according to the criteria of Boon et al. (1997) and are presented in the text. Individual congeners have the potential to induce CYP isoforms and those that are known are denoted by ⁺, mixed CYP1A/ CYP2B, * CYP2B, nd, no data for these congeners, ^w, weak/ non-inducers of CYP isoforms, sources of data Safe et al. (1985) and reviewed in Boon et al. (1992). Systematic numbering after Ballschmitter and Zell (1980). Pp, harbour porpoise (*Phocoena phocoena*) and Hg, grey seal (*Halichoerus grypus*).

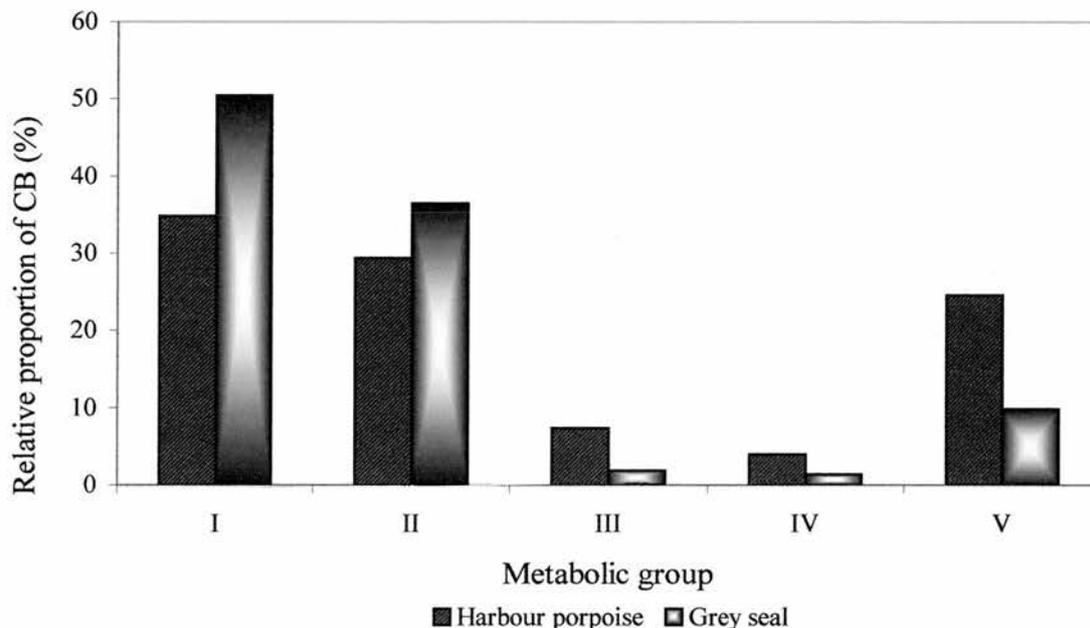


Fig. 3.4. Relative proportion (%) of the sum of 26 CB congeners divided into their metabolic group by the criteria of Boon et al. (1997), see text and Table 3.3.

The relative proportion (%) of the grouped congeners for harbour porpoise and grey seal are presented in Fig. 3.4. The grey seal had the highest % of metabolic group I and II congeners, however the harbour porpoise contained a greater contribution of metabolic group III, IV and V congeners.

CB-118, a group III congener, was classified as metabolisable by an inducible CYP isoenzyme(s) potentially CYP1A (Boon *et al.*, 1997). The ratio of CB-118 to CB-153, a reference compound, did not correlate with the EROD or PROD activities in the harbour porpoise and grey seal samples, and therefore in this case the ratios would not be indicative of CYP1A induction. Since the majority of the PROD activities were associated with CYP1A, as determined by inhibition studies (refer 3.3.1, this thesis), the ratio of CB-101 (group IV, CYP2B inducer; Boon *et al.*, 1997) to CB-153 was not calculated.

3.3.4. TEQ of CBs.

Toxic equivalents (TEQ) of 2,3,7,8-TCDD were calculated for four CB congeners (CB-105, -118, -156 and -157) using published toxic equivalency factors (TEF) (Van den Berg *et al.*, 1998). The TEQs for the four CB congeners for both the harbour porpoise and grey seal are presented in Fig. 3.5. The TEQ for CB-118 and CB-156 contributed ~60% to the Σ TEQ in harbour porpoise and grey seal, respectively. However, the Σ TEQ (ng.g^{-1} lipid) was higher by an order of magnitude in the harbour porpoise (0.037ng.g^{-1} lipid) than the grey seal (0.008ng.g^{-1} lipid).

The Σ TEQ for the harbour porpoise and grey seal were plotted against the observed EROD activities for these species (Fig. 3.6). The lower Σ TEQ and PCB concentrations

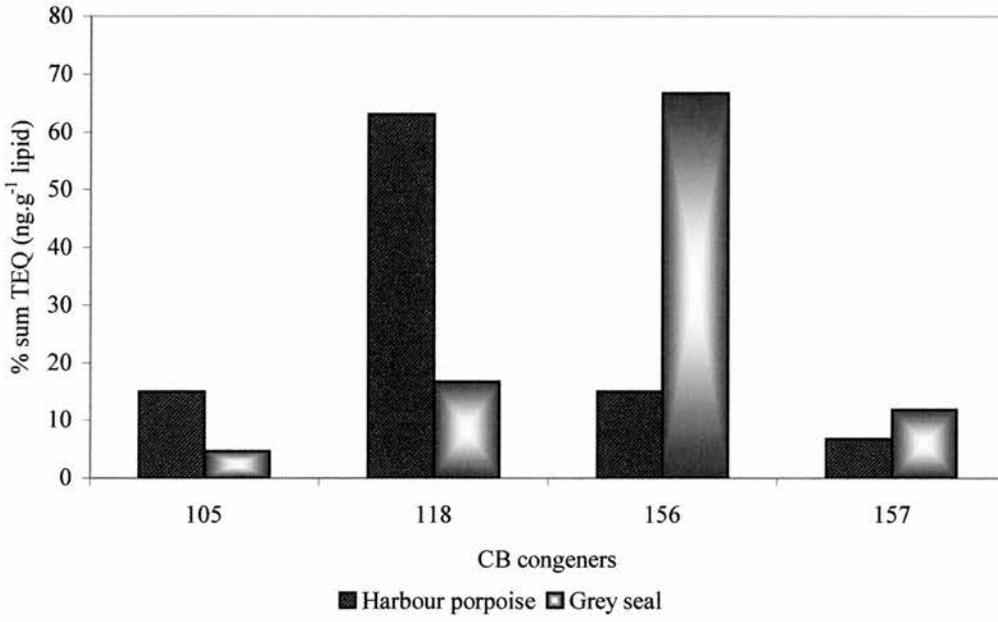


Fig. 3.5. The percentage TEQ for four CB congeners from metabolic group III (CB-105, -118, -156 and -157) of the Σ TEQ for harbour porpoise and grey seal.

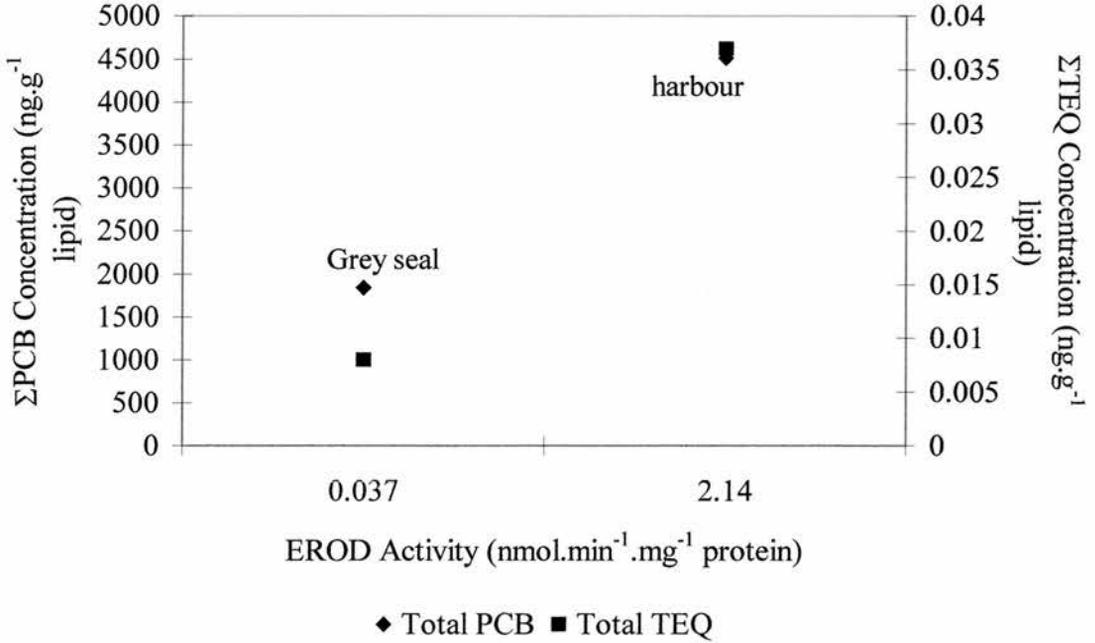


Fig. 3.6. Total PCB concentration and Sum of TEQs (ng.g⁻¹ lipid) plotted against EROD activities for harbour porpoise and grey seal.

were consistent with a lower EROD activity observed in the grey seal compared to the higher ΣTEQ, PCB concentration and EROD activity in the harbour porpoise.

3.4. Discussion

So far very few studies have focused on the cytochrome P450 (CYP) system in harbour porpoises and grey seals. This chapter has attempted to investigate this system, in particular CYP1A and CYP2B through catalytic and immunodetection in both species that were environmentally exposed to PCB contaminants. Since only one animal from each species was investigated, the results are preliminary, forming the basis for future experiments in these two species.

3.4.1. EROD and PROD activities and the effect of inhibition.

EROD activity was higher in the harbour porpoise compared to the grey seal by two orders of magnitude. However, the PROD activities were similar in both species. Very few studies have measured CYP1A-associated monooxygenase activities in the harbour porpoise, or in grey seals (Addison and Brodie, 1984; Boon *et al.*, 1994; Murk *et al.*, 1994; Nyman *et al.*, 2000; Table 3.1). The harbour porpoise from the present study had an EROD activity that was >5 fold higher compared to previous studies of conspecifics and other species (Table 3.1) and was similar for published EROD activities of 2,3,7,8-TCDD- induced rat CYP1A (1.2 nmol.min⁻¹.mg⁻¹ protein) (Mattson *et al.*, 1998), although ~15 fold less than 3-methylcolanthrene (3MC) treated rat EROD activities (30±1.6 nmol.min⁻¹.mg⁻¹ protein) (Burke *et al.*, 1994). However, the observed PROD activity was lower than that in another harbour porpoise (Murk *et al.*, 1994) and other cetaceans (Table 3.1).

In contrast the EROD activity obtained for the grey seal was lower than for conspecifics and other seal species (Table 3.1). Several populations of conspecifics and other species in other studies originate from seas of far higher pollution than the North Sea. For example, grey seals from the Baltic, which have higher EROD and PROD activities (Nyman *et al.*, 2000) than the grey seal from the present study.

Since only one animal from both species was measured in this study, comparison of the results is tentative. However, differences in the EROD and PROD activities in the harbour porpoise and grey seal compared to conspecifics in other studies may be due to differences in age, sex, geographical location, dietary habits, nutritional status, health, time of year sampled and inter-laboratory differences in technique and analytical methods.

Such parameters as health, nutritional status, and time of year sampled may affect physiological factors including hormonal levels and endogenous ligands related to the immune system that can alter the levels of CYP isoenzymes. For example, cytokines can be released in an immune response, and were observed to down-regulate CYP1A in murine hepatoma cells (Paton and Renton, 1998). Murk and colleagues (1994) studied a harbour porpoise from the Netherlands that died due to ill health, and had lower CYP1A-associated EROD activities than the harbour porpoise in the present study.

EROD activities were inhibited in the presence of 1 μ M α -naphthoflavone (α -NF) by 100% in both the harbour porpoise and grey seal liver microsomes (Fig. 3.1). Whereas PROD activities were reduced by between 60.59% - 76.67% in harbour porpoise and 54% - 92% in grey seals compared to the control activities. This was consistent with a previous study in grey seals where EROD and PROD activities were inhibited by 100%

and 75-95% (Nyman *et al.*, 2000). Furthermore ~80% inhibition of EROD activities was achieved with 1 μ M α -NF in ringed seal liver microsomes (Wolkers *et al.*, 1998b). Therefore 7-ethoxyresorufin (substrate for EROD) was metabolised by CYP1A in the harbour porpoise and the grey seal liver microsomes.

Furthermore, despite the presence of a CYP2B-like protein in these two species, the high percentage of inhibition of PROD activities by the CYP1A-specific inhibitor, α -NF suggests that PROD activities were also catalysed by CYP1A in these species. This was consistent with previous catalytic and inhibition studies in other marine mammals and wildlife (Machala *et al.*, 1997; Nyman *et al.*, 2000; Rozemeijer *et al.*, 1995; White *et al.*, 1994) and strongly indicates that in environmentally exposed animals PROD is unsuitable as a biomarker for CYP2B isoenzymes. The varying level of remaining activity may be due to the initially very low PROD activities (between 0.8 and 1.1 nmol.min⁻¹.mg⁻¹ protein).

This highlights the need for a marker enzyme reaction, similar to EROD for CYP1A, to be defined for CYP2B for biomonitoring studies, in both these species studied here and other marine mammals. The isolation of CYP isoenzymes from marine mammal species, by recombinant DNA technology or protein purification would provide the initial characterisation of these proteins. The CYP1A1 and CYP1A2 cDNA have been cloned and sequenced for grey and harp seals (Chapter five, this thesis) and partial sequences were obtained for three other seal species and two cetacean species (Teramitsu *et al.*, 2000). Similar investigations for CYP2B isoenzymes / cDNA would provide an answer to the question “2B or not 2B – (Goksøyr, 1995)” in the marine mammal species that are investigated.

3.4.2 Immunochemical detection of CYP1A and -2B like apoproteins.

A ~52kDa and a ~54kDa protein band was detected by the anti-trout CYP1A1 antibody in harbour porpoise and grey seal, respectively (Fig 3.2). CYP1A-like proteins have been recognised by a variety of fish and mammalian CYP1A antibodies, with varying degrees of specificity in many cetacean and seal species (For examples, Table 3.2). The mammalian CYP1A family consists of, so far, two subfamilies (Nelson *et al.*, 1996), which have been isolated in two seal species (Chapter five, this thesis). Boon and colleagues (2001) observed two distinct proteins at 52kDa and 57kDa in harbour porpoise and whitebeaked dolphin (*Lagenorhynchus albirostris*) liver microsomes that reacted with anti-rat CYP1A1/1A2 and anti-rat CYP1A1 antibodies, respectively. They suggested that the lower protein band might be CYP1A2 in these species. The only other study to report a CYP1A-protein in grey seal showed that a rat and a fish CYP1A antibody (Table 3.2) reacted with a protein at 56kDa, corresponding with the CYP1A protein in TCDD-induced rat liver microsomes (Nyman *et al.*, 2000).

CYP1A proteins have been observed in most marine mammal species that, so far, have been investigated. However, the presence of CYP2B proteins in marine mammals is more controversial, often more so in cetaceans than pinnipeds. For example, in a previous study, no CYP2B- like protein was observed in harbour porpoise or whitebeaked dolphin liver microsomes with the anti-rat CYP2B1/2 monoclonal antibody (PM20) (Boon *et al.*, 2001). Furthermore very low activities of aldrin epoxidation were measured in small cetaceans indicating that phenobarbital inducible CYP isoenzymes (CYP2B) were not present in these mammals (Watanabe *et al.*, 1989). This has often lead to the conclusion that CYP2B-like proteins do not exist in the liver of cetacean species. However, a CYP2B-like protein was observed in harbour porpoise

liver microsomes in the present study (Fig. 3.2) and in a previous study (Goksøyr, 1995).

A rabbit anti-rat CYP2B1/2 antibody also detected a CYP2B-like protein in the liver microsomes of the grey seal from the present study (Fig. 3.2). The only other study of grey seal cytochrome P450 system, failed to detect a CYP2B-like protein in the liver microsomes using a plethora of CYP2B antibodies raised against rat, fish and human (Table 3.2; Nyman *et al.*, 2001). Similarly in an investigation of ringed seals from the same laboratory, a CYP2B-like protein was absent from the liver microsomes in an immunoblot with a monoclonal (MAb 2-66-3) anti-rat CYP2B antibody (Mattson *et al.*, 1998). This is in contrast with a previous study that observed a CYP2B-like protein in ringed seals using a different CYP2B antibody (Wolkers *et al.*, 1998b).

A CYP1A-like and CYP2B-like protein exist in the harbour porpoise and the grey seal samples studied here. However, the lack of antibody recognition of CYP2B-like protein bands in conspecifics and other marine mammalian species may occur due to the absence of a conserved epitope between the selected antibody and the orthologous protein. Furthermore, whilst no protein band was detected in beluga whale (*Delphinapterus leucas*) liver microsomes with anti-rat CYP2B1/2 and anti-scup CYP2B1 antibodies, single bands that migrated near purified rat CYP2B1/2 and corresponded with dog CYP2B11 protein were recognised by anti-rabbit CYP2B4 and anti-dog CYP2B11 antibodies, respectively (White *et al.*, 1994). This emphasises the need for homologous antibodies to marine mammal CYP isoenzymes for use in characterisation of the CYP1A and CYP2B proteins and for future biomarker studies (Refer to Chapter six, this thesis)

3.4.3. PCB concentrations and relative patterns.

The harbour porpoise had higher $\Sigma 28\text{PCB}$ and $\Sigma 26\text{PCB}$ concentrations than the $\Sigma 36\text{PCB}$ and $\Sigma 26\text{PCB}$ concentrations in the grey seal from the North Sea, although generally the relative concentrations of individual PCB congeners in each species were similar (Fig. 3.3). Furthermore the EROD activities in each animal were consistent with the $\Sigma 26\text{PCB}$ concentration, with the higher EROD activities in harbour porpoise reflecting the greater $\Sigma 26\text{PCB}$ concentration compared with the corresponding values in grey seal (Fig. 3.6).

Inter-specific differences resulting from different genetic make up of the metabolising system, diets and sex may also effect the overall concentration of PCBs in the blubber. The grey seal was a mature female, therefore unless primiparous, would have offloaded a large proportion of her PCB burden to subsequent offspring during lactation (Addison and Brodie, 1987; Beckmen *et al.*, 1999; Espeland *et al.*, 1997). In contrast male animals, such as the harbour porpoise in the present study, continue to accumulate burdens of environmental contaminants throughout life (Kleivane *et al.*, 1995b), and apart from the drug – metabolising system, have no way of reducing their PCB burden.

Total PCB concentration (ng.g^{-1} lipid) in the harbour porpoise from the present study was within the range measured in harbour porpoises and common dolphins (*Delphinus delphis*) from the UK sampled between 1988 and 1996 (Jepson *et al.*, 1999; Kuiken *et al.*, 1993; Smyth *et al.*, 2000; Wells *et al.*, 1994). The PCB concentrations of the UK harbour porpoises were greater than conspecifics from the arctic, although much lower than those from the Baltic Sea, Kattegat-Skagerrak seas and West Coast of Norway (Berggren *et al.*, 1999; Bruhn *et al.*, 1999). This follows the increasing PCB

concentration trend from West to East and North to South of the Northern Hemisphere (Skaare, 1995).

The grey seal from the present study had comparable PCB concentrations to mature female grey seals from the Farne Islands, East Coast of England (Law *et al.*, 1989), once corrected for the overestimate of the Arochlor 1254 equivalents (by factor of 2) (Oehme *et al.*, 1995). The immature grey seals had ~ 5 fold higher PCB concentrations (Law *et al.*, 1989) than the mature grey seals, indicating that the mature grey seal from this study may have offloaded some of her burden to previous offspring. However, the grey seal from the present study had Σ 26PCB concentration that was at the lower end of the range for mature female grey seals from the Gulf of St Lawrence (Bernt *et al.*, 1999), and were lower than the grey seal from the coasts of Finmark and Trøndelag (Skaare, 1995). Other reasons for individual differences in PCB concentrations include diet and time of year sampled, since Σ PCB concentrations are negatively correlated with blubber thickness, animals that are sampled following the breeding season will have greater PCB concentrations than later in the year (Addison and Stobo, 1993).

CB-153 and -138 were the dominant congeners in both the harbour porpoise and grey seal and together contributed 43.8% and 61.9% of Σ 26PCBs, respectively. PCB patterns in these two species were similar to other studies of conspecifics (Kleivane *et al.*, 1995b; Westgate *et al.*, 1997). In contrast CB-138 was found to be more abundant than CB-153 ($31.9 \pm 1.6\%$ and $26.8 \pm 1.9\%$, respectively) in harbour porpoises from the Irish Sea (Troisi *et al.*, 1998).

Boon and colleagues (1994) proposed a pharmacokinetic model to investigate the metabolising capacity of marine mammals for PCBs, based on known structure activity relationships. PCBs were divided into one of five groups according to the position of the vicinal hydrogen atoms and the number of *ortho* – chlorine substituents in the biphenyl molecule (For group criteria, see results section 3.3.3.). In the present study, the grey seal had higher relative concentration of groups I and II CB congeners compared to the relative concentration of the same congeners in the harbour porpoise (Fig. 3.4). Group I and II CB congeners were resistant to metabolism in laboratory mammals and are persistent in wildlife (Boon *et al.*, 1997; Safe *et al.*, 1985). The percentage contribution of the CBs constituting groups III and IV to $\Sigma 26\text{PCB}$ were relatively low in both species, indicating that these groups were metabolised. Although the harbour porpoise had a greater contribution of groups III, IV and V compared to the grey seal.

Group III CBs were metabolised more easily by CYP1A and CYP2B isoenzymes (Safe *et al.*, 1985). Pinnipeds appear to have a greater capacity to metabolise group III and group IV congeners than cetaceans probably because of different levels of respective CYP isoenzymes (Boon *et al.*, 1997). Protein bands of similar relative concentration and corresponding to the different molecular weights of ~52kDa and ~54kDa in grey seals and harbour porpoises, respectively, have been detected by a variety of mammalian and fish CYP1A antibodies e.g. (Boon *et al.*, 2001; Nyman *et al.*, 2000), present study). This indicates that the CYP isoenzymes recognised by CYP1A antibodies in the two species may be different CYP1A isoforms, or homologues that have different structure and function.

The lower contribution of group III CB congeners in the grey seal compared to the harbour porpoise may also be a consequence of lactational transfer of the lower

chlorinated CBs to previous offspring. However, CB-156 was unexpectedly higher in the grey seal compared to the harbour porpoise in the present study. This may be due to a diet related factor, however no definite proof for the biotransformation of CB-156 in grey seals was observed in the pharmacokinetic model, due to the clustering of this congener with more persistent CBs, such as CB-153, -180, -138 (Boon *et al.*, 1997).

Group IV CB congeners induce CYP2B isoenzymes, or similar to group V congeners are weak inducers of CYP isoenzymes or the induction is unknown. Metabolic slopes were obtained for harbour porpoises, comparing the relative concentration of CB congeners between predator and prey (Bruhn *et al.*, 1995). These slopes indicated that harbour porpoises were able to metabolise of CBs with *meta* and *para* vicinal hydrogens in combination with <2 *ortho* chlorine substituents (group IV) or with >3 *ortho* chlorine substituents (group V) (Bruhn *et al.*, 1995). Furthermore some group IV CB representatives were observed at lower concentrations in the beluga whale indicating that metabolism had occurred (White *et al.*, 1994). In contrast small cetaceans were found to have no capacity to metabolise group IV and V CB congeners, suggesting absence of CYP2B isoenzymes (Tanabe *et al.*, 1988).

CYP2B-like proteins of differing molecular weights (~51kDa and ~52kDa) were detected in grey seals and harbour porpoise in this and a previous study (Goksøyr, 1995). The pattern of CB congeners and the presence of immunoreactive CYP2B-like proteins in these two species may reflect different CYP2B isoforms and potentially greater catalytic capacity of grey seal CYP2B isoenzymes for biotransformation of group IV and V congeners. 3-methyl sulphone (MSF) PCB metabolites, particularly of the precursor PCBs – 70, -87, -101 and -141, potently induced CYP2B1 and -2B2 isoenzymes in rat liver microsomes, as determined by immunoblotting and aminopyrine

N-demethylase activities (Kato *et al.*, 1995). The presence of varying patterns of 3-MSF PCB metabolites, in combination with the precursor PCBs and other environmental contaminants not measured in this study, may have induced different CYP-2B like isoenzymes in the harbour porpoise and grey seal.

In vitro biotransformation of group IV and group V PCB congeners by harbour porpoise and grey seal liver microsomes, in conjunction with CYP2B inhibition studies may clarify the capacity of these two species to metabolise PCB congeners that constitute groups IV and V. Furthermore molecular cloning of potential CYP2B cDNA may indicate the presence of heterologous CYP2B isoenzymes in these species.

3.4.4. TEQ of CBs.

The 2,3,7,8-TCDD equivalent concentrations (TEQ) were calculated for four of the PCB congeners (CB-105, -118, -156 and -157) measured in both the harbour porpoise and grey seal blubber using published toxic equivalent factors (TEFs) (Van den Berg *et al.*, 1998). A TEQ is the product of the TEF of a CB congener and the concentration of the measured CB and are calculated to evaluate the risk of an individual to toxic organochlorines (Van den Berg *et al.*, 1998).

CB-118 contributed ~60% to the Σ TEQ of four CBs in harbour porpoise, in contrast CB-118 was replaced by CB-156 in grey seal, and also contributed ~60% to the Σ TEQ of four CBs (Fig. 3.5). The TEQ of CB-118 was also found to be the most abundant in harbour porpoises from previous studies (Berggren *et al.*, 1999; Bruhn *et al.*, 1995; Tanabe *et al.*, 1997). No other study has reported the TEQs for the four CB congeners calculated here for grey seal. However, the Σ TEQ of the same four CB congeners were re-calculated using the TEFs of Van den Berg and co-workers (1998) for harbour seals

from Wadden sea and Limfjorden (Storrhansen and Spliid, 1993). Similar to the grey seal from the present study, CB-156 contributed $57.48 \pm 1.37\%$ to the Σ TEQ. Furthermore, the TEQ for CB-156 and -157 contributed the greatest (each $\sim 30\%$) to the Σ TEQ of five CB congeners (CB-118, -105, -156, -157 and -189) in polar bear (*Ursus maritimus*) (Letcher *et al.*, 1996) using the TEF values of Safe and colleagues from 1994. However, the Σ TEQ of the four CB congeners was an order of magnitude higher in the harbour porpoise compared to the grey seal. The Σ TEQ values, similar to the Σ 26PCB concentrations, were reflected by the EROD activities in the two animals (Fig. 3.6), with higher EROD, Σ TEQ and PCB concentrations in the harbour porpoise compared to the grey seal. The Σ TEQ values also represent CYP1A induction by other environmental contaminants, such as the co-planar PCBs, PCDD/Fs and PAHs that were not measured in the present study. This suggests that the positive relationship between Σ TEQs and CYP1A - EROD activities may have occurred as a consequence of these other environmental contaminants co-varying with the five PCBs used to calculate the Σ TEQ.

The CB congeners that are assigned a TEF are considered the more toxic of the PCB congeners, possessing TCDD-like effects elicited through CYP1A induction and affinity for the aryl hydrocarbon receptor (Safe *et al.*, 1985). Several mono-*ortho* CBs, CB-105, -118, -156 and -157 exhibited a variety of pathological effects, including thymic atrophy, weight loss, oedema, liver lipid accumulation, extensive hepatic damage and splenic atrophy in mice and rats (Parkinson *et al.*, 1983; Yamamoto *et al.*, 1976).

A number of pathologies have been observed in marine mammals exposed to environmental contaminants. For example a semi-field experiment investigated the potential of contaminant-induced immunosuppression in harbour seals fed either herring

from the Baltic or Atlantic sea for one year (Ross *et al.*, 1996). A concomitant study feeding rats with the same Baltic or Atlantic herring oil, showed temporary immunosuppression through the decrease of a variety of functional immune parameters (T-lymphocyte expression, natural killer cell activity) and indicated that harbour seals may be more sensitive to the immunotoxic effects of PCBs compared to rats. Furthermore rat pups from female rats that were fed similar diets during gestation and lactation were more sensitive to immunotoxic actions of Baltic Sea herring contaminants (Ross *et al.*, 1996).

The greater Σ TEQ in the harbour porpoise compared to the grey seal from the North Sea suggests the harbour porpoise is potentially at a greater risk from these more toxic CB congeners than the grey seal. Harbour seals fed immunosuppressive Baltic Sea herring contaminants had a Σ TEQ of $0.286\text{ng}\cdot\text{g}^{-1}$ lipid (Ross *et al.*, 1996), less than one order of magnitude higher than the Σ TEQ in the harbour porpoise from this study.

TEFs were derived partially from *in vitro* bioassays for the ability of dioxin-like PCBs to induce CYP1A isoenzyme relative to 2,3,7,8-TCDD (Clemons *et al.*, 1998; Van den Berg *et al.*, 1998) and references within). EROD or aryl hydrocarbon hydroxylase (AHH) activities and CYP protein levels are predominantly used as a measure CYP induction. Therefore assuming an additive effect of individual chemicals in an environmental mixture on CYP1A induction, Σ TEQs of the sample can be employed as a biomarker of the response and exposure of an organism to some of the most toxic environmental contaminants (i.e. Fig. 3.6). Furthermore once TEFs have been established for a species of interest (through rigorous *in vitro* and *in vivo* experiments), the calculated TEQs could be used as a non-destructive biomarker.

3.4.5. Single bycaught and stranded animals, are they of use in studies of environmental contaminant exposure and biomonitoring?

The use of single animals obtained opportunistically through by-catches or strandings may be of use for characterising the detoxification system, determining the exposure of the species to environmental contaminants, and subsequently be part of national biomonitoring programmes.

A collaborative UK project has produced guidelines for obtaining biometric data, chemical analyses of PCBs, HCB, lindanes, DDT and its metabolites, dieldrin and trace metals, as well as post-mortem examination and sampling of organs and tissues, to identify any pathologies, in marine mammals (Law *et al.*, 1994). The biometric parameters such as body size (length, girth, mass), blubber thickness (ventral and dorsal), age (determined by tooth extraction), sex, species, date of recordings/sighting/death and geographical location found, were measured (Law *et al.*, 1994).

These guidelines could be the basis for standardised protocols implemented in a national biomonitoring programme database of the exposure of marine mammals to environmental contaminants. Furthermore, molecular and biochemical analyses, along with the measurement of 'new' contaminants such as polybrominated biphenyls, polybrominated diphenyl ethers and furans (PBDE/Fs), polyaromatic hydrocarbons, should also be included in the database to provide an overall picture of exposure of these animals to environmental contaminants.

The interdisciplinary approach required for such a national biomonitoring programme may provide a unique insight into the temporal and population trends that can, concomitantly, be monitored.

3.5.6. Summary

This study has investigated the xenobiotic metabolising system of a harbour porpoise and a grey seal exposed to environmental contaminants, from the East Coast of Scotland. The study has provided further information in these two species, of which little is known, of the induction of cytochrome P450 isoenzymes using catalytic, inhibitory and immunochemical detection assays. Furthermore the concentrations of PCB congeners present in the blubber were determined and compared with conspecifics from previous studies. The 2,3,7,8-TCDD equivalent concentrations (TEQs) for four PCB congeners were calculated and the potential for Σ TEQs to be employed as non-destructive biomarkers, as well as the value of single animals in xenobiotic metabolising studies were discussed. The following observations were made:

- An induced CYP1A protein was detected in a harbour porpoise and a grey seal liver microsomes by catalytic and immunochemical reactions. Furthermore an induced, immunoreactive CYP2B protein was also observed in these two species.

- EROD and PROD activities in the environmentally exposed harbour porpoise and grey seal were substantially inhibited in the presence of 1 μ M α -naphthoflavone, a CYP1A-specific inhibitor, thus suggesting that EROD was indicative of CYP1A induction, and that CYP1A predominantly metabolised PROD in these two animals.

- The relative concentrations of PCBs in these two species were similar to levels measured in studies of conspecifics, indicating exposure to potentially toxic, lipophilic and persistent environmental contaminants.

- The total TEQ concentrations (ng.g^{-1} lipid) for four CB congeners were higher in the harbour porpoise than in the grey seal, suggesting the harbour porpoise was exposed to higher concentrations of potentially more toxic PCB congeners than the grey seal.
- The induction of the CYP1A isoenzyme(s), represented by the EROD activities, reflected the Σ TEQ and PCB concentrations in both the harbour porpoise and grey seal, thus reflecting the exposure of these species to PCBs.
- Opportunistically obtained marine mammals for by-catches or strandings, as long as they are recently dead, provide an opportunity for characterising the xenobiotic metabolising system, forming, at least, the basis for further experiments.

4 Effects of Storage on hepatic microsomes from two marine mammal species on Cytochrome P450 – monooxygenase activities and content

4.1. Introduction

Cytochrome P450 (CYP) isoenzyme – monooxygenase (MO) activities and CYP contents are increasingly being determined in marine mammal tissue samples e.g. (Addison and Brodie, 1984; Hyyti *et al.*, 2001; Watanabe *et al.*, 1989). These activities provide a biomarker of environmental contaminant exposure and have been discussed in more detail in Chapters two and three.

Due to ethical considerations, marine mammal tissue samples, particularly the liver, are often obtained from stranded or bycaught animals (e.g. Jepson *et al.*, 1999; Murk *et al.*, 1994; Troisi *et al.*, 1998; Troisi and Mason, 1997) that were recently dead. However, due to logistical constraints it is often not possible to collect samples until some hours after death and even then not possible to process them immediately. After death, the degradation of proteins and inactivation of enzymes occurs, and as a consequence CYP-MO activities determined on such tissues can be underestimated.

Other investigations have obtained tissue samples during legal hunts. These samples were subsequently stored in liquid nitrogen to prevent the degradation/ inactivation of enzymes, until further preparation, which can often take up to several weeks (e.g. Mattson *et al.*, 1998; White *et al.*, 1994; Wolkers *et al.*, 1998a). However, freezing of tissues can result in the inactivation of enzymes (Burchell *et al.*, 1989). The decrease in 7-ethoxyresorufin *O*-deethylase (EROD) and ethoxycoumarin *O*-deethylase (ECOD) CYP-MO activities during 7 days of liquid nitrogen storage of fish livers was previously observed (Forlin and Andersson, 1985). Conversely, trout liver samples were stored for

up to 3 days in liquid nitrogen before significant reduction in these CYP-MO activities (Forlin and Andersson, 1985).

Glycerol has cryoprotective properties, preserving enzyme activities in frozen tissue. CYP-MO and conjugating enzyme activities were shown to be stable in liver microsomes suspended in a 20% glycerol buffer for approximately \leq one year at -80°C (Forlin and Andersson, 1985). Furthermore only 20-40% of CYP-MO activities, in particular ECOD, was lost over 2 years of storage of liver microsomes suspended in a 20% glycerol buffer (Goksøyr and Larsen, 1991). An investigation of fish liver that was subfractionated and suspended in 20% glycerol buffer prior to freezing preserved EROD activities from the deleterious effects of freezing liver slices on CYP activities (Monod and Vindimian, 1991).

Marked species differences have been observed in the function and regulation of CYP isoenzymes. Furthermore differential effects of cold storage of liver on CYP enzymes and content have been reported for different species, including human (Pearce *et al.*, 1996; Powis *et al.*, 1988) and fish (Forlin and Andersson, 1985). This emphasises the importance of characterising biotransformation reactions and standardising procedures of cold storage in the species of interest.

This is the first study, to my knowledge, of the effects of cold storage on marine mammal hepatic CYP-MO activities and content. The investigations carried out included: (i) The stability of CYP1A family in harbour porpoise (*Phocoena phocoena*) liver microsomes prepared from liver stored in liquid nitrogen for a known amount of time compared with liver microsomes prepared from liver stored for less than 1 hour in liquid nitrogen. (ii) The stability of CYP1A in hooded seal (*Cystophora cristata*) liver

microsomes prepared from liver post-nuclear supernatants suspended in a glycerol buffer, prior to storage in liquid nitrogen. The viability of preparing post-nuclear supernatants in the field for subsequent biotransformation studies was also investigated.

4.2. Methods

4.2.1. Samples. A liver sample from a harbour porpoise (*Phocoena phocoena*) (refer to Chapter three) was chopped into small pieces (~5cm³), wrapped in foil and immediately frozen in liquid nitrogen until preparation. The piece of liver for day 0 of storage was frozen for < 1 hour during transport to the laboratory for the preparation of liver microsomes. Liver samples from 4 hooded seals (*Cystophora cristata*) were taken from West Ice, about 60 miles east of Greenland, during March 2000 (refer to Chapter two). The tissues collected were a surplus of samples taken by the Department of Arctic Biology, Tromsø. Within 10 minutes of death, liver samples were placed immediately on ice, prior to either the preparation of the post-nuclear supernatant in store buffer containing 20% glycerol or being wrapped in foil and stored in liquid nitrogen until preparation of liver microsomes.

4.2.2. Preparation of liver microsomes.

4.2.2.a. Harbour porpoise sample. A liver sample was subdivided into four pieces for this experiment. One piece was prepared to liver microsomes approximately two hours after the animal had died, as described in Chapter two (section 2.2.2). The other three pieces were stored in liquid nitrogen until liver microsomal preparation on subsequent days two, four and fourteen.

4.2.2.b. Hooded seal liver samples. Liver samples from each animal were subdivided into four pieces. One piece was immediately prepared to the post-nuclear supernatant in store buffer (Tris-acetate, pH 7.4, 1mM EDTA, 0.1mM DTT, 0.4mM PMSF and 20%

glycerol), prior to storage in liquid nitrogen (day 0). The other three pieces of liver per animal were wrapped in foil and frozen in liquid nitrogen. A subsample of liver was prepared to post-nuclear supernatant on the following two days (day 1 and day 2), while the fourth piece remained in liquid nitrogen until preparation on return to the laboratory (day 45).

Liver microsomal suspensions were prepared from the post-nuclear supernatants as described in Chapter two (section 2.2.2). The fourth piece was also prepared to liver microsomes, including the 700g centrifugation stage, freezing in liquid nitrogen and thawing prior to the continued preparation of the microsomes.

4.2.3. Total protein and CYP content, and CYP isoenzyme activities. Determination of total protein and CYP concentration and EROD and 7-pentoxoresorufin *O*-deethylase (PROD) associated CYP activities, were conducted according to the procedures described in Chapter two (sections 2.2.3, 2.2.4 and 2.2.5).

4.2.4. Statistical analysis. The relationship between the CYP content, -MO activities and duration of storage were determined by non-linear regression (Sigmaplot 5, SPSS Inc.) and using a paired two samples t-test for differences between means, after logarithmic transformation of the individual data to normalise the data (Microsoft Excel 97). The level of significance was pre-set at $p < 0.05$. Day 0.02 refers to day zero in the graphs of activities against storage time (days).

4.3 Results

4.3.1. Harbour porpoise. CYP content and -MO activities are presented in Table 4.1. EROD specific activity ($\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) significantly decreased with the time

Duration of storage ^a	EROD specific activity (nmol product. min ⁻¹ . mg ⁻¹ protein)	EROD turnover number (nmol product.min ⁻¹ . nmol ⁻¹ P450)	CYP Content (nmol P450.mg ⁻¹ protein)
0 [†]	2.14 ± 0.05	0.49 ± 0.14	1.20 ± 0.38
1	0.89 ± 0.05	1.00 ± 0.38	0.26 ± 0.11
2	0.77 ± 0.04	0.32 ± 0.01	0.60 ± 0.01
14	0.20 ± 0.01	0.24 ± 0.01	0.22 ± 0.01

Table 4.1. CYP content and EROD activities determined in liver microsomes prepared from harbour porpoise liver stored in liquid nitrogen over time in days^a. [†]Freshly prepared liver microsomes. Values are mean ± 1 standard error of duplicate measurements from one animal.

Duration of storage ^a	EROD specific activity (pmol product. min ⁻¹ . mg ⁻¹ protein)	PROD specific activity (pmol product. min ⁻¹ . mg ⁻¹ protein)	EROD turnover number (pmol product.min ⁻¹ . nmol ⁻¹ P450)	PROD turnover number (pmol product.min ⁻¹ . nmol ⁻¹ P450)	CYP Content (nmol P450.mg ⁻¹ protein)
0 [†]	14.78 ± 0.31	0.71 ± 0.28	32.74 ± 0.68	1.30 ± 0.57	0.29 ± 0.01
1	6.94 ± 0.38	0.28 ± 0.05	16.23 ± 0.93	0.80 ± 0.20	0.43 ± 0.08
2	8.28 ± 0.33	0.27 ± 0.04	20.47 ± 0.82	0.81 ± 0.11	0.32 ± 0.06
45	3.57 ± 0.25	0.30 ± 0.03	9.93 ± 0.62	0.80 ± 0.08	0.32 ± 0.03

Table 4.2. CYP content, EROD and PROD activities determined in liver microsomes prepared from hooded seal liver stored in liquid nitrogen over time in days^a. [†] liver samples prepared as post-nuclear supernatant prior to storage in liquid nitrogen (refer to section 4.2.2). Values are mean ± 1 standard error of four animals.

that the harbour porpoise liver was stored in liquid nitrogen (Fig. 4.1, $p = 0.0015$, $R^2 = 0.997$, $df = 3$, $n = 1$). The activity was significantly reduced by 58.25% of the pre-storage activity (day 0, $p = 0.0038$, $df = 1$) and further decreased to only ~10% of the original activity by day 14 ($p = 0.019$, $df = 1$).

Furthermore CYP content (nmol P450.mg⁻¹ protein) decreased with storage time (Fig. 4.2., $p = 0.097$, $R^2 = 0.815$, $df = 1$) and exhibited an 81.2% reduction by day 14. However, the decrease in CYP content was not significant, because of the large standard error in the duplicate measurements for the CYP content in the 'day zero' liver microsomes (mean \pm 1 SE: 1.20 ± 0.38). The rate of EROD turnover expressed as nmol product. min⁻¹. nmol⁻¹ P450, exhibited no significant decrease over time (Table 4.1).

4.3.2. Preparation of post-nuclear supernates and storage of liver. The mean \pm 1 standard error (S.E.) of CYP contents and -MO activities are presented in Table 4.2. Fig. 4.3.a and b exhibit the EROD and PROD specific activities, respectively, from four hooded seals with duration of storage in days. The reduction in both the specific activities with time were significant ($p < 0.001$, $R^2 = 0.6886$, $df = 14$ for EROD and $p = 0.0097$, $R^2 = 0.414$, $df = 14$ for PROD, $n = 4$).

EROD specific activity was reduced on average to $47.0 \pm 5.5\%$, mean \pm 1 standard error (S.E.), after one day of storage and by day 45 the activity was approximately 25% of the original activity ($24.2 \pm 6.9\%$, mean \pm 1 S.E.). PROD specific activity exhibited greater variability, however the percentage decrease observed ($60.67 \pm 18.5\%$, mean \pm 1 S.E.) was similar to the reduction in EROD specific activity from day 0 to day 1. No further reduction in PROD activity was observed after day one.

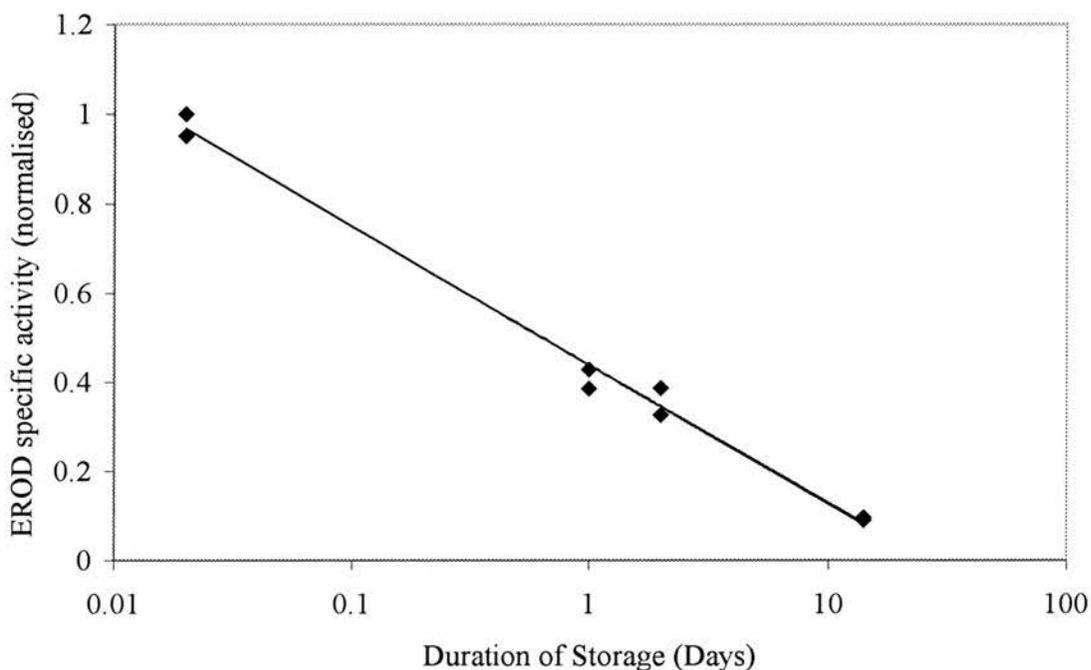


Fig.4.1. Proportion of EROD specific activity lost during storage of harbour porpoise liver. Normalised EROD activity was calculated as a proportion of the highest value for the animal. The individual values of the duplicates are presented. $n = 1$, $R^2 = 0.9921$, $p = 0.0015$.

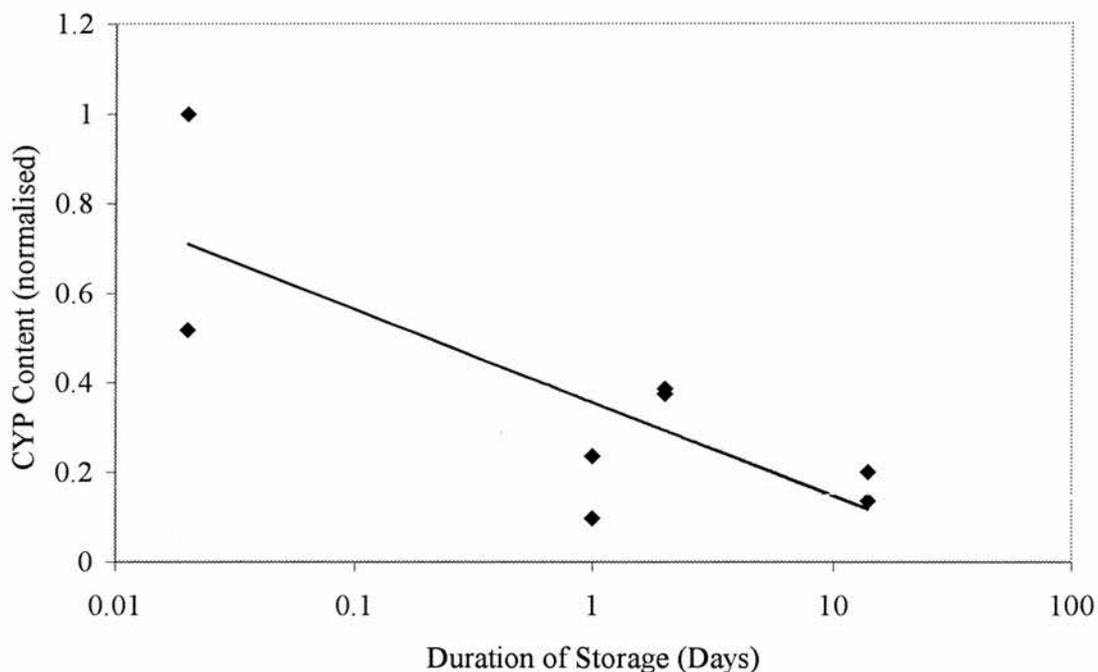


Fig. 4.2. Proportion of CYP content (nmol P450.mg-1 protein) lost during storage if harbour porpoise liver. Normalised CYP content was calculated as a proportion of the highest value for the animal. The individual values of the duplicates are shown. $n = 1$, $R^2 = 0.815$, $p = 0.097$.

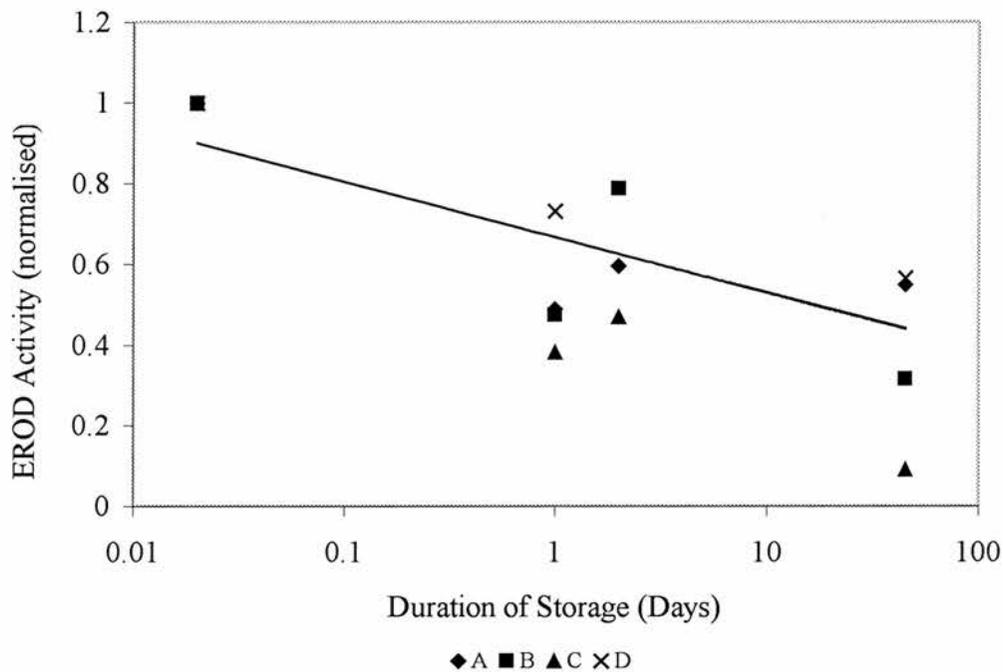


Fig. 4.3.a. Proportion of EROD specific activity lost during storage of liver from hooded seal. Normalised EROD activity was calculated as a proportion of the highest value for each animal. $R^2 = 0.6886$, $p < 0.001$. $n = 4$ labelled as A-D. Animal D liver sample was unable to be prepared on day 2, therefore the data point is missing.

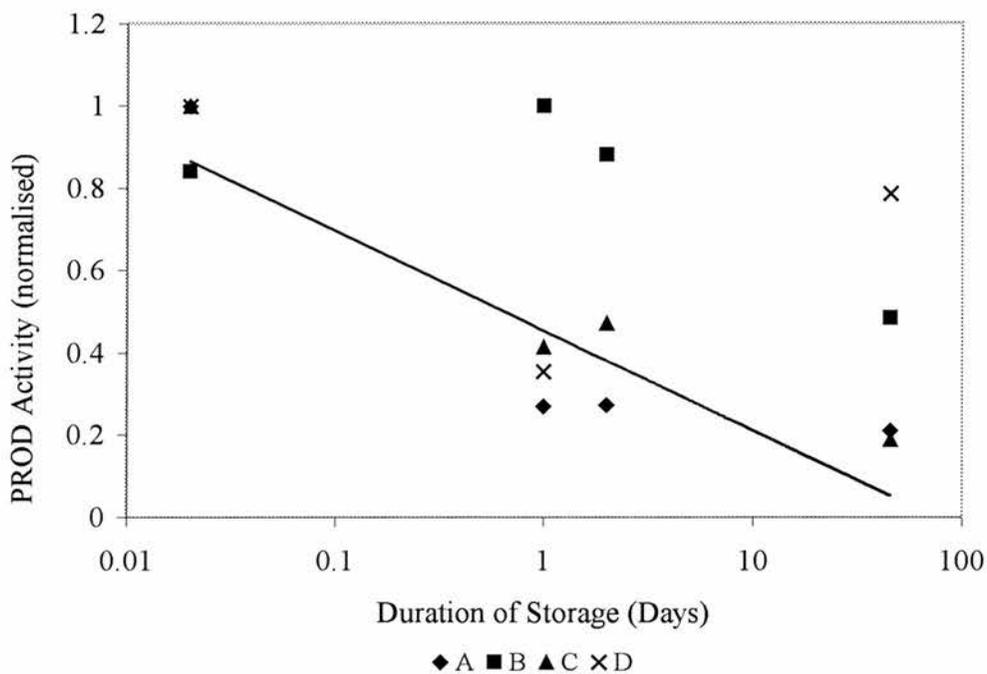


Fig. 4.3.b. Proportion of PROD specific activity lost during storage of liver from hooded seal. Normalised EROD activity was calculated as a proportion of the highest value for each animal. $R^2 = 0.4137$, $p = 0.0097$. $n = 4$ labelled as A-D. Animal D liver sample was unable to be prepared on day 2, therefore the data point is missing.

The content of CYP (nmol P450.mg⁻¹ protein) exhibited no significant decrease with time. Similarly the EROD and PROD activities expressed as turnover numbers (nmol product. min⁻¹.nmol⁻¹ P450) did not significantly decrease with storage time (Table 4.2).

4.3.3. Estimation of the proportional loss of CYP1A-mediated EROD and PROD specific activity during storage.

4.3.3.a. Harbour porpoise. The normalised EROD specific activity was calculated as the proportion of the highest activity for the animal. The relationship of activity with time of harbour porpoise liver storage was obtained (Fig. 4.1.). The estimated proportion of activity remaining after a known length of storage time in days (x, between day 0 and 14) can be predicted from the following relationship:

$$\text{Proportion of EROD specific activity, } y = -0.1349\ln(x) + 0.4387$$

4.3.3.b. Hooded seals. The EROD and PROD specific activities were normalised as described in 4.3.3.a. and the relationship of the activities during storage were obtained (Figs. 4.3 a and b). The estimated proportion of activity remaining for a known length of storage time in days (x, between 0 and 45 days) can be predicted from the following relationships:

$$\text{Proportion of EROD specific activity, } y = -0.0799 \ln(x) + 0.6389$$

$$\text{Proportion of PROD specific activity, } y = -0.0711 \ln(x) + 0.6192$$

The relationship between specific activity and duration of storage in liquid nitrogen was similar for both hooded seal EROD and PROD activities.

4.4. Discussion

The effect of storage of marine mammal liver samples in liquid nitrogen on CYP contents and CYP1A-MO activities were investigated for the first time. The length of duration of storage reflected conditions unavoidable in fieldwork. Furthermore the

viability of storing post-nuclear supernatants suspended in 20% glycerol buffer in liquid nitrogen, to preserve the enzyme activities, was examined.

4.4.1. Does storage of liver tissue in liquid nitrogen affect CYP content and CYP activities?

4.4.1.a. Harbour porpoise liver sample. EROD specific activity was significantly decreased, by normal decay, in liver stored in liquid nitrogen compared to the corresponding activity in liver microsomes prepared from liver stored in liquid nitrogen for < 1 hour (day 0) (Fig. 4.1). The activity decreased by > 50% in liver stored in liquid nitrogen for one day prior to the preparation of liver microsomes. The activity continued to decrease over the next 13 days, though at a decreasing rate, resulting in only 10% of the initial activity remaining by day 14.

No significant decrease in CYP content was observed in harbour porpoise liver microsomes prepared from liver stored for increasing lengths of time in liquid nitrogen, compared to liver microsomes prepared from 'day 0' liver. In the present study the peak at 450nm from the CO-difference spectrum of dithionite-reduced microsomes was measured ≤ 2 minutes after the addition of dithionite, similar to a study of CYP content of human liver microsomes (Pearce *et al.*, 1996). However, Rutten and colleagues (1987) reported that the peak at 450nm took five minutes to develop. The CYP content measured on day 1 may, therefore, be an anomaly.

The EROD turnover number ($\text{nmol}\cdot\text{min}^{-1}\cdot\text{nmol}^{-1}$ P450) also did not exhibit a significant decrease with time. This was consistent with the CYP content on day one, which was particularly low, which would cause the calculated EROD turnover to be exceptionally high on this day.

4.4.1.b. Hooded seal liver samples. Consistent with the normal decay of the EROD specific activity in the harbour porpoise liver microsomes, the hooded seal liver microsomal samples exhibited a similar decline in EROD and PROD specific activity with the duration of storage. The EROD specific activity was significantly decreased by ~50% ($53.0 \pm 5.47\%$, mean ± 1 S.E.) from day zero to day one. PROD specific activity was significantly decreased by $60.67 \pm 18.5\%$, mean ± 1 SE, and exhibited a large amount of variation. This variability may have resulted because of the very low activities measured were close to the limit of detection.

The findings from the present study were consistent with the effect of storage of trout (*Oncorhynchus mykiss*) liver in liquid nitrogen over a period of 7 days (Forlin and Andersson, 1985). A significant decrease was observed for hepatic microsomal CYP-MO EROD and ECOD, after 7 days. However, in contrast they found no significant loss of the biotransformation activities studied over the first 3 days of the experiment.

Reasons for the decrease in EROD and PROD specific activities within this study maybe due to the size of the liver pieces stored in liquid nitrogen. In the present study, stored liver was $\sim 5\text{cm}^3$, compared to the $0.2 - 0.3\text{cm}^3$ fish liver samples in the study of Forlin and Andersson (1985). The smaller the piece of tissue, the more rapidly it is frozen and therefore potentially reducing the amount of degradation that can occur.

Interspecific differences in orthologous CYP isoenzymes may result in different susceptibilities to inactivation/degradation by intracellular proteases (Yamazaki *et al.*, 1997). However, the EROD specific activities in the liver microsomes from both marine mammal species showed a dramatic decrease after one day of storage compared to the activities obtained from the liver microsomes prepared from day zero liver samples.

Subsequently no significant change in the CYP activities were identified between days one and two of each experiment.

The initial drop in EROD activity may partially be due to the conditions of liver storage (size of the liver sample that was frozen). However, it must be noted that the activities then continue to decrease over the remaining 14 and 45 days of the harbour porpoise and hooded seal liver experiments, respectively. In contrast to the present study, investigations into the effect of storage in liquid nitrogen on human liver tissue have observed no significant decrease in the CYP content or activities over months and years of storage (Pearce *et al.*, 1996; Yamazaki *et al.*, 1997). In particular they report no significant loss of the CYP1A2-EROD specific activity in these frozen stored human liver samples.

In one study, human liver samples were placed at 25°C for 6 hours prior to the preparation of liver microsomes that resulted in a 90% loss in certain CYP – MO activities (Yamazaki *et al.*, 1997). The liver samples used in the study by Pearce and colleagues (1996) had no history reported between the perfusion of the liver and its arrival at the laboratory. Furthermore livers are routinely prepared for transplantation, even if they are subsequently used for research, which can result in the liver remaining on ice for up to 36 hours (Pearce *et al.*, 1996). The enzymes may have already undergone some inactivation and degradation and so any decrease in enzyme activities due to storage effects may be obscured.

The CYP contents of both the harbour porpoise and hooded seal liver samples were not significantly decreased. This was consistent with the CYP content from fish liver

(Forlin and Andersson, 1985) and human liver samples (Pearce *et al.*, 1996; Yamazaki *et al.*, 1997).

4.4.2. Is the preparation of post-nuclear supernatants a viable fieldwork option for preserving CYP1A-EROD and PROD activities?

In many fieldwork studies to collect fresh samples from marine mammals, liver pieces are immediately frozen in liquid nitrogen to reduce degradation until the samples can be prepared. The duration of such storage can often be for several days to several weeks long. However, freezing tissue may result in the inactivation of enzyme activities (Burchell *et al.*, 1989). Homogenisation of liver / tissue sample in 20% glycerol buffer results in a uniform distribution of the cryoprotector, glycerol. Storage of subfractionated liver samples suspended in a glycerol buffer has preserved enzyme activities in frozen tissue in previous studies (Coughtrie *et al.*, 1991; Forlin and Andersson, 1985; Goksøyr and Larsen, 1991; Monod and Vindimian, 1991). Liver samples in two marine mammal studies were freshly prepared as post-mitochondrial supernatants (9,000 – 12,000g) prior to freezing in liquid nitrogen (Boon *et al.*, 2001; Gøksøyr *et al.*, 1992). Until now, no systematic study has been performed on the preservation of CYP activities in such supernatants in marine mammals.

In the present study, hooded seal liver samples were freshly prepared as post-nuclear supernatants suspended in a 20% glycerol buffer. This prevented the loss of between 75% and 80% of CYP1A-EROD and –PROD specific activities, which occurred during the storage of liver samples in liquid nitrogen for 45 days. This was in agreement with an investigation that measured the hepatic glucose-6-phosphatase (G-6-Pase) activity in microsomal samples prepared from frozen 10,000g supernatants, compared with freshly prepared microsomes and those from frozen tissue (Coughtrie *et al.*, 1991). They

observed that ~50% ($46.6 \pm 3.8\%$, mean ± 1 S.E.) of the G-6-Pase activity measured in freshly prepared microsomes was lost if the liver was directly stored in liquid nitrogen. However, only 20% ($18.5 \pm 4.2\%$ mean ± 1 S.E.) was lost if 10,000g supernatants were prepared prior to storage. Furthermore Monod and Vindimian (1991) observed the protective effect of glycerol on CYP-MO activities, in particular EROD. They homogenised liver samples in a 20% glycerol buffer and subsequently subfractionated the liver of fish (nase) prior to freezing in liquid nitrogen.

4.4.3. Estimation of the proportion of CYP1A-EROD and PROD activity lost during storage of liver samples. For a known duration of storage of liver samples in liquid nitrogen (≤ 14 days for harbour porpoise and ≤ 45 days for hooded seal), the proportion of EROD and PROD (hooded seal only) specific activities that are lost can be predicted from the relationships presented in the results section 4.3.3. In turn the proportion of the activity lost can be used to determine these activities that potentially would have been present in the fresh samples.

4.4.4. Summary.

This Chapter has investigated the CYP1A-mediated EROD and PROD activities in (i) liver stored directly in liquid nitrogen compared with freshly prepared tissue and (ii) subfractionated liver suspended in a 20% glycerol buffer. The following observations were made.

- Normal decay of the CYP1A isoenzyme(s) was observed by a significant decrease of the EROD and PROD specific activities due to the storage of the harbour porpoise (EROD only) and hooded seal liver samples in liquid nitrogen.

- The preparation of post-nuclear supernatants in a 20% glycerol buffer prevented the loss of these CYP1A-MO activities in hooded seal samples, providing a method of obtaining CYP isoenzyme activities that are more representative of the *in vivo* activities.
- Preparing tissue samples as post-nuclear supernatants (700g) suspended in a 20% glycerol buffer is a viable fieldwork option if a power source and a refrigerated table top centrifuge, that spins at 700g, are available.
- Since species differences have been observed in CYP activities after storage in liquid nitrogen (or -80°C), methods of preparation and storage should be investigated in the species of interest.
- Future experiments should focus on a broader range of CYP-MO and conjugating enzyme activities involved in biotransformation studies.

5

Isolation of two cytochrome P450 cDNAs, CYP1A1 and CYP1A2, from two seal species: harp seal (*Phoca groenlandica*) and grey seal (*Halichoerus grypus*)

5.1. Introduction

Cytochrome P450 (CYP) isoenzymes are found in many organisms from bacteria and fish to mammals (Nelson *et al.*, 1996). They comprise a large superfamily of unique membrane bound heme-proteins that are found in the liver and some extrahepatic tissues (Kedderis, 1990). Typically CYPs play a key role in the oxidative metabolism of physiologically important endogenous substrates such as steroids, cytokines and fatty acids (Nebert and Gonzalez, 1987). Many of the genes belonging to CYP families 1-4 are particularly involved in phase 1 of the detoxification or activation of xenobiotics including drugs, environmental contaminants, e.g. polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAH) and pesticides (Waxman, 1999).

In many mammals, the CYP1 family consistently comprised of three subfamilies, CYP1A1, -1A2 and -1B1 (Nelson *et al.*, 1996). Endogenous substrates for this family remain to be identified (Nebert, *et al.*, 2000). However, the CYP1 family is predominant in the detoxification of PAHs, planar chlorobiphenyls and certain dioxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (Whitlock *et al.*, 1996) in rat (Clemons *et al.*, 1998), human (Drahushuk *et al.*, 1998) and fish (Santostefano *et al.*, 1997; Grinwis *et al.*, 2000; Van Schanke, 2001). Induction and subsequent expression of the CYP1 gene, by polyhalogenated aromatic hydrocarbons, occurs through the activation of the aryl hydrocarbon (Ah) receptor and subsequent formation of a complex with the Ah receptor nuclear translocator (ARNT) protein (Reyes *et al.*, 1992). Ligand activation of the Ah receptor causes the expression of CYP1A1, CYP1A2 and CYP1B1 isoenzymes in mammalian liver (Sutter *et al.*, 1994). These isoenzymes exhibit

differences in substrate specificities, regulation and tissue distribution (Sutter *et al.*, 1994). Such transcriptional induction of the CYP1 gene has caused a variety of toxicities that includes immunologic and hormonal dysfunction and tumour production (Okey *et al.*, 1994; Poland and Knutson, 1982).

The induction of CYP1A has been characterised in fish as a biomarker of environmental contamination through *in vitro* (Arinc *et al.*, 2000) and *in vivo* studies (Machala *et al.*, 1997). The ability to investigate and use the CYP1A induction patterns as biomarkers of contaminant exposure in marine mammals is extremely valuable. In most instances marine mammals are close to the top of marine food chains, are ecologically very important and extremely vulnerable to environmental contaminant exposure (Ross, 2000). In addition, correlations between environmental contaminant exposure and reproductive dysfunction and tumours have been reported in several marine mammals, for example harbour seals (*Phoca vitulina*) (Reijnders, 1986), Californian sea lions (*Zalophus californianus*) (DeLong *et al.*, 1973), beluga whales (*Delphinapterus leucas*) (Beland *et al.*, 1993) and ringed seals (*Phoca hispida*) (Helle *et al.*, 1976b).

A number of studies have detected CYP1A-like proteins in marine mammals, including grey seal (*Halichoerus grypus*) (Addison and Brodie, 1984), and beluga whale (White *et al.*, 1994), using model substrates that have been characterised in rats, humans (Burke *et al.*, 1994) and fish (Huuskonen and Lindstromseppa, 1995). Further characterisation of these CYP1A-like proteins has occurred using inhibition assays (Mattson *et al.*, 1998), and heterologous antibodies raised against purified CYPs from mammals and fish (Goksøyr *et al.*, 1992). However, the sequence information of CYP families 1-4 would provide biochemical data that would enable comparative studies into exposure and responses to contaminants and determine when extrapolation between species is valid

(Stegeman and Hahn, 1994). Furthermore, Peters and colleagues (1999) concluded that the development of CYP1A as a biomarker of environmental contaminant exposure would require the production of specific probes, such as antibodies, from the sequencing of the gene/protein.

Many CYP sequences have been determined from mammalian species by cDNA cloning (e.g. Pirrit *et al.*, 1995) and protein purification methods (e.g. Goksøyr, 1985). Although, until recently no marine mammal CYP sequence was known. Teramitsu and colleagues (2000) have identified partial CYP1A1 cDNA sequences from spotted seal (*Phoca largha*), Steller's sea lion (*Eumetopias jobatus*) and two cetacean species, Dall's porpoise (*Phocoenoides dalli*) and minke whale (*Balaenoptera acutorostrata*), and a fragment of the CYP1A2 sequence from Steller's sea lion, and recently isolated two CYPs from the CYP1A family, CYP1A1 and CYP1A2, from ribbon seal (*Phoca fasciata*) (I. Teramitsu, personal communication). A CYP1B-like cDNA sequence was also detected in striped dolphin (*Stenella coeruleoabula*) by reverse transcription-polymerase chain reaction (RT-PCR) (Godard *et al.*, 2000).

In this study, two full length CYP1A cDNAs and the deduced amino acid sequences, CYP1A1 and CYP1A2, from harp seal (*Phoca groenlandica*) and grey seal are presented. The CYP1A amino acid sequences from the seal species are compared in a phylogenetic analysis of CYP1A isoenzymes from a variety of vertebrate species and the implications for the use of the CYP1A apoprotein as a biomarker from knowledge of its structure are discussed.

5.2. Materials and Methods

5.2.1. Samples.

Harp seal (*Phoca groenlandica*) liver was taken from West Ice in April 2000. The tissue collected was a surplus of samples taken by the Department of Arctic Biology, Tromsø. The grey seal (*Halichoerus grypus*) liver sample was obtained from a bycatch, from the north east of Scotland. Both samples were initially stored in liquid nitrogen and then in -70°C until use.

5.2.2. Poly A⁺ mRNA Purification.

Poly A⁺ mRNA from seal liver samples was purified using the Oligotex™ Direct mRNA Kit (Qiagen, Germany). Approximately 35mg of frozen liver were homogenised in 600µL lysis buffer (OL1) and diluted in 1.2mL buffer ODB. Tissue debris and protein remaining in the sample were pelleted by centrifugation in a microcentrifuge (MSE Microcentaur, Scotlab) at 10,000g for 3 minutes and the supernatant was transferred into a clean nuclease-free microfuge tube.

The mRNA was subsequently isolated by the addition of 70µL Oligotex suspension containing a dT₃₀ oligomer, coupled to a solid-phase matrix, which hybridised to the Poly A⁺ tail of mRNA. The suspension was pelleted after 10 minutes at room temperature by centrifugation (10,000g for 5 minutes) and the supernatant discarded leaving approximately 50µL behind to reduce the loss of the oligotex complex. The oligotex: mRNA complex was re-suspended in 100µL lysis buffer, diluted in 400µL buffer ODB and incubated for 3 minutes at 70°C to further purify the mRNA. The oligotex complex was collected by centrifugation (at 10,000g for 5 minutes), and re-suspended in 350µL wash buffer OW1. The resulting suspension was transferred onto

the spin column where the oligotex complex adsorbed to the silica-gel membrane and was washed during centrifugation at 10,000g for 1 minute.

The oligotex complex was washed a further two times in wash buffer OW2, followed by centrifugation at 10,000g for 1 minute. After all washes the flow through was discarded. Subsequently the spin column was transferred to a clean nuclease-free microfuge tube and the Poly A⁺ mRNA was eluted in 2 X 50µL hot (70°C) low salt buffer OEB and collected by centrifugation at 10,000g for 1 minute. mRNA was stored at -70°C until required.

5.2.3. Production of cDNA and PCR Product

5.2.3.a. Design of sense and antisense primers. Primers were designed initially to isolate two cytochrome P450 partial sequences from harp seal complementary DNA (cDNA) CYP1A1 and CYP1A2, for subsequent amplification by reverse transcription – polymerase chain reaction (RT-PCR).

Whole length cytochrome P450 isoenzyme sequences were unknown in harp and grey seals. However, a local alignment search using the Basic Local Alignment Search Tool (BLAST) on the website for the National Centre for Biotechnology Information (NCBI; URL <http://www.ncbi.nlm.nih.gov/>) revealed partial CYP1A1 complementary DNA cDNA sequences from three seal species: Ribbon and spotted seal, and Steller's sea lion that had 97% identity to one another and a partial CYP1A2 sequence from *E. jubatus*.

Initially the three partial CYP1A1 cDNA sequences were aligned in DNAMAN (Lynnon BioSoft) and a contiguous sequence containing the conserved regions of all three sequences was obtained (Fig. 5.1). The CYP1A1 consensus sequence was then aligned

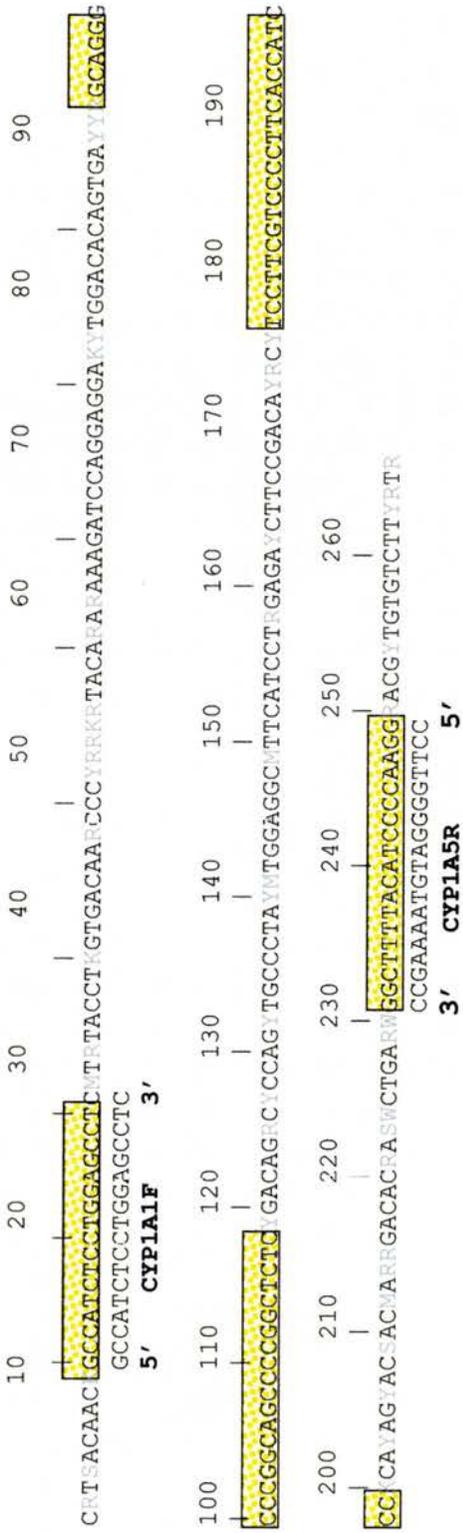


Fig. 5.1. Seal* CYP1A1 consensus partial cDNA sequence high-lighting the conserved regions greater than 18 bases long. The primers chosen to isolate CYP1A from harp seal are displayed in black. CYP1A1F is the sense primer and CYP1A5R is the anti-sense primer. Letters other than those denoting bases are non-conserved bases in the 3 species. * Species included were *Eumetopias jubatus* (accession number AB014356), *Phoca largha* (Accession number AB 014358) and *Phoca fasciata* (Accession number AB014359).

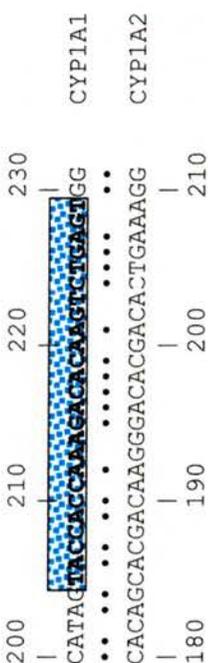


Fig. 5.2. Region of seal* CYP1A1 consensus cDNA sequence (see Fig. 5.1) aligned with the partial CYP1A2 cDNA fragment from Harp seal. The high-lighted region indicates the chosen anti-sense primer, CYP1A6R, designed to specifically isolate CYP1A1 from harp seal. ‘•’ indicate base identity between the two sequences.

with the CYP1A2 cDNA sequence to identify regions that were specific to each of the CYP1A1 and CYP1A2 cDNA sequences.

Primers for CYP1A1 and CYP1A2 were then chosen from the conserved regions of each sequence that were between 18 and 30 bp long, had 40 – 60% GC base content and consequently a melting temperature (T_m) between 50 and 70°C. The T_m was calculated using the oligocalculator website (<http://dna-seq.st-and.ac.uk/oligocalc.html>), which is based on the following the equation:

$$T_m = 81.5 + 16.6 \times (\log_{10}[\text{Na}^+]) + 0.41 \times (\%G + C) - 675/n,$$

where Na^+ is the molar salt concentration; $[\text{K}^+ = \text{Na}^+]$ and n = number of bases in the oligonucleotide. The sequences were not self-complementary, therefore preventing self-hybridisation of the primers. In the majority of cases, selecting primers with G and C bases at the 3' end ensured strong hybridisation between the primer and the cDNA template.

The sequences finally selected as primers were located as close to the 5' end (CYP1A1F) and 3' end (CYP1A5R or CYP1A6R) in order to amplify as large a fragment as possible in RT-PCR (Table 5.1). The chosen sense primer, CYP1A1F was used for both CYP1A1 and CYP1A2. However, two antisense primers differing by 10 bases, CYP1A6R and CYP1A5R provided the specificity required to isolate two CYP subfamilies, CYP1A1 and CYP1A2, respectively (Fig. 5.2).

Primers to isolate the full length cDNA of CYP1A1 and CYP1A2 were designed using the full length ribbon seal CYP1A1 and CYP1A2 that were subsequently published on Genbank (<http://ncbi.nih.nlm.gov/genbank/>; NCBI). The primers were chosen using the

Primer Identification	Primer sequence (5' – 3')	Direction	Specific for CYP	Length	% GC bases	Melting temperature (T _m ; °C)
CYP1A1F	GCCATCTCCTGGAGCCTC	Sense	CYP1A1 and CYP1A2	18	67	55
CYP1A6R	ACTCAGACTTGTGTCTTTGGT GGTA	Anti-sense	CYP1A1	25	44	56
CYP1A7F	ATGCTGATGATGTTCTCTGCG TCCAGATTGTCC	Sense	CYP1A1	33	49	64
CYP1A8R	TTAAGCACGTACCCGCACTT GGACGTGCTC	Anti-sense	CYP1A1	30	57	66
CYP1A5R	CCTTGGGGATGTAAAAGCC	Anti-sense	CYP1A2	19	53	51
CYP1A9F	ACAGATGGCATTGTCCCAGAT	Sense	CYP1A2	21	48	52
CYP1A10R	TCACTTGGTGGAGAAAACGTG GCCGTGCCTG	Antisense	CYP1A2	30	60	67

Table 5.1. Primers used in RT-PCR and PCR to isolate the partial fragments and full length coding region of CYP1A1 and CYP1A2 cDNA from harp seal and grey seal mRNA. All primers are written in the 5' to 3' orientation.

parameters above and were positioned at the 5' end (sense) and 3' end (anti-sense) of the coding region. The primer compositions are given in Table 5.1.

5.2.3.b. Isolation of Partial CYP1A1 and CYP1A2 cDNA Fragments by Reverse Transcription - Polymerase Chain Reaction (RT-PCR). Complementary DNA (cDNA) and subsequent amplification of the partial fragment of harp seal cytochrome P4501A1 and -1A2 DNA were produced using the Titan One Tube RT-PCR kit (Roche Diagnostics, Germany), for subsequent cloning.

Initially two master mixes were prepared in 0.5mL nuclease-free microfuge tubes. Master mix one contained 0.2mM of each dNTP, 5mM DTT, 5u RNase inhibitor, 0.2 μ M of each primer (CYP1A1F and CYP1A5R or CYP1A6R), 5 μ L mRNA sample, and nuclease-free water to a final volume of 25 μ L. Master mix two included 10 μ L of x5 RT-PCR buffer including 7.5mM MgCl₂ and 1 μ L Titan² enzyme mix (Avian myeloblastosis virus reverse transcriptase and Expand High Fidelity enzyme mix consisting of *Taq* DNA polymerase and another proof reading polymerase). The two mixes were transferred to a 0.2mL thin-walled PCR tube (ABgene, UK) to give the final reaction volume of 50 μ L.

The reaction was initially heated to 50°C for 30 minutes in a Thermocycler (Perkin Elmer GeneAmp PCR System) to obtain cDNA of the target mRNA sequence through reverse transcription. Subsequently the cDNA was denatured at 94°C for 2 minutes. Amplification of the DNA was carried out for 35 cycles with following parameters: 94°C for 1 minute, to denature the DNA; (T_m-6)°C for 1 minute, to anneal the primers to the DNA; 68°C for 2 minutes, to synthesise a copy of the target template. Finally the reaction was held at 68°C for 7 minutes to ensure the majority of the final PCR product

was full-length double-stranded DNA. The primers were annealed at a temperature that corresponded to the melting temperature minus 6°C (T_m-6)°C. Typically the annealing temperature was 46°C for primer combination CYP1A1F/1A5R and 50°C for CYP1A1F/1A6R.

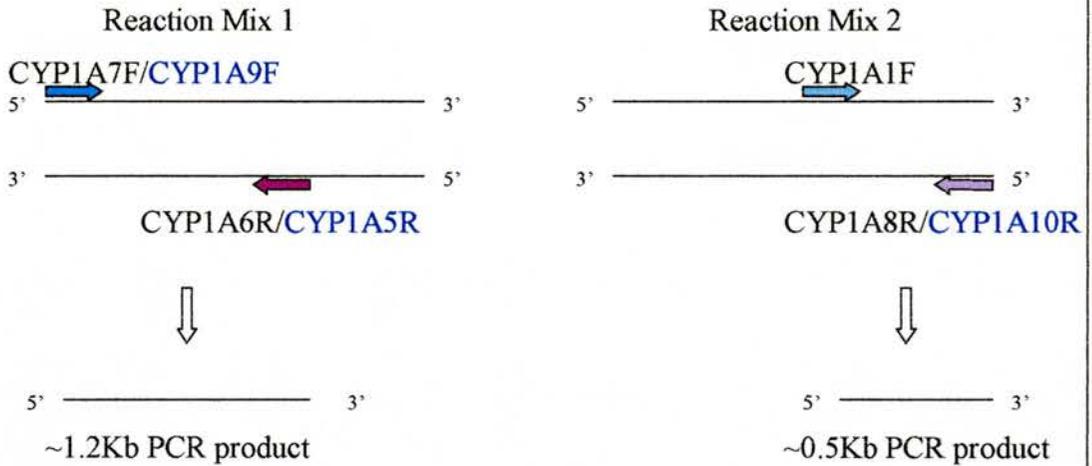
5.2.3.c. Isolation of Full Length CYP1A1 and CYP1A2 cDNA sequences.

1) RT-PCR. RT-PCR was performed following the protocol described in section 5.2.2.b, with the following modifications. Two reactions for each of the two CYP1A isoforms were set up with the sense/anti-sense primer combinations CYP1A1F/CYP1A8R and CYP1A7F/CYP1A6R, to isolate two overlapping fragments that would constitute the entire coding region of the CYP1A1 cDNA. The coding region of CYP1A2 was isolated with the sense/antisense primer combinations CYP1A1F/CYP1A10R and CYP1A9F/CYP1A5R (Fig. 5.3.). The primer combinations were annealed at the temperatures, (T_m-6)°C for the lowest T_m , given in Table 5.1.

2) Polymerase Chain Reaction (PCR). The two overlapping cDNA fragments for each of the CYP1A isoforms were subsequently used as templates in PCR for the amplification of the full length coding region of CYP1A1 and CYP1A2. The reaction mix was prepared in 0.2mL PCR tubes and contained 10µL 10x reaction buffer, 0.2mM of each dNTPs, 1.25 units *Taq* DNA polymerase (5u/µL; Promega, USA), 1.5mM MgCl₂, 0.8µM antisense primer (CYP1A8R or CYP1A10R), 0.8µM sense primer (CYP1A7F or CYP1A9F), 5µL of each cDNA templates and nuclease-free water to a final volume of 100µL.

The reaction was initially heated to 94°C for 2 minutes to denature the DNA. Subsequently amplification of the DNA was performed for 35 cycles following the

Step 1 – RT-PCR



Step 2 - PCR

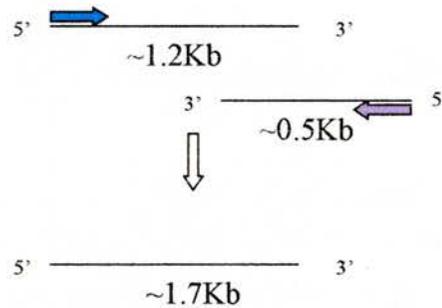


Fig. 5.3. Diagram to indicate the positions of the primers used to isolate the partial fragments, the overlapping fragments and full length CYP1A1 and CYP1A2 cDNA. CYP1A2 primers where different from CYP1A1 are written in blue.

subsequent parameters: 94°C for 45 seconds, to denature the DNA; (T_m-6)°C for 1 minute, to anneal primers to template DNA; 72°C for 2 minutes, to synthesise a copy of the full length DNA from the target templates. Finally the reaction was held at 72°C for 7 minutes to ensure the majority of the final PCR product was full length double stranded DNA.

5.2.4. Purification of PCR Product

5.2.4.a. Agarose gel electrophoresis. The PCR product was purified on a 2% low melting agarose gel in 1x TBE buffer (0.9M Tris, 0.89M borate, 0.02M EDTA, pH 8.3) containing 0.05 % ethidium bromide (1mg/mL). Comparison of the PCR product with a 1Kb DNA ladder (Promega, USA) ensured the correct size PCR fragment had been amplified. 10µL 1Kb DNA ladder was loaded onto the gel in a 5:1 ratio with the loading buffer (4.8mL distilled water, 200µL x50 TAE, 5mL sterile glycerol, bromophenol blue) and 50µL of the PCR product was loaded with 10µL of loading buffer. Subsequently the DNA was separated by electrophoresis for 60 minutes at 50V constant voltage. The DNA bands were subsequently visualised by illumination from an UV transilluminator (TFX-20M, Vilber Lourmat, France).

5.2.4.b. Gel Extraction of cDNA bands. The PCR product was extracted from the agarose gel using the QIAquick gel extraction kit (Qiagen, Germany). Briefly, the required DNA band was excised from the gel using a sterile scalpel, placed into a 1.5mL nuclease-free microfuge tube and weighed. The agarose slice was solubilised and dissolved in three volumes of buffer QG at 50°C for 10 minutes. The solubilising buffer contains a high concentration of chaotropic salts at a pH <7.5 for subsequent adsorption of the DNA onto the silica-gel surface of the spin column. The addition of 1 volume of isopropanol increased the yield of DNA fragments < 500 base pairs (bp) (and > 4Kbp).

The sample was transferred to the spin column and centrifuged for 1 minute at 10,000g and the flow-through was discarded. The silica:DNA complex was washed with 0.75mL buffer PE containing 96% ethanol to remove impurities such as enzyme, agarose, unincorporated nucleotides and ethidium bromide, and stood at room temperature for 2 minutes prior to centrifugation at 10,000g for 1 minute. Again the flow-through was discarded. The column was spun for a further 1 minute at 10,000g to remove any residual ethanol-containing buffer, which would interfere with subsequent enzyme reactions. The spin column was transferred to a clean 1.5mL nuclease-free microfuge tube and the DNA was eluted with 50µL of the low salt, high pH buffer EB (10mM Tris-HCl, pH 8.5) applied to the silica gel membrane, followed by centrifugation at 10,000g for 1 minute. Eluted DNA was stored at -20°C until required.

5.2.5. Production of recombinant clones.

5.2.5.a. Materials and Solutions. The *Escherichia coli* strain, JM109 (Promega, USA; genotype: *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17* (r_k^- , m_k^+), *relA1*, *supE44*, $\Delta(lac-proAB)$, [F', *traD36*, *proAB*, *lacIqZΔM15*]) high efficiency competent cells (1×10^8 cfu/µg DNA) were used for transformations. The chromogenic *lacZ* substrate, X-GAL (5-Bromo-4-chloro-3-indolyl β-D-galactoside) and the antibiotic, ampicillin were supplied by Sigma. The inducing reagent, IPTG was obtained from Melford Laboratories, LTD, UK. The cloning vector, pGEM-T[®] easy, x2 rapid ligation buffer and T4 DNA ligase were purchased from Promega as a system.

SOC medium contained 2% (w/v) Bacto[®]-tryptone, 0.5% (w/v) Bacto[®]-yeast extract, 1M NaCl, 1M KCl, 2M Mg²⁺ Stock (1M MgCl₂.6H₂O, 1M MgSO₄.7H₂O), 2M glucose, pH 7.0. Luria Bertani medium (LB) for LB agar plates and for overnight cultures

contained 1% (w/v) Bacto®-tryptone, 0.5% (w/v) Bacto®-yeast extract and 1% (w/v) NaCl, pH 7.0.

5.2.5.b. Ligation of PCR product into the Cloning Vector. The CYP1A1 and CYP1A2 partial DNA sequences were ligated into the pGEM®-T easy vector (Promega, USA) according to the suppliers' method. The pGEM®-T easy vector contains an insertion site that was pre-cut with the restriction enzyme *EcoRV* and has the addition of 3' terminal thymidines (T) at both ends. These single T overhangs are complementary to the single adenosine (A) base that were independently added to the 3' end of the PCR products by *Taq* DNA polymerase during PCR (Clark, 1988; Robles and Doers, 1994).

Briefly, the following reagents were placed in a clean nuclease-free 0.5mL microfuge tube: 10µL 2x rapid ligation buffer for T4 DNA Ligase (60mM Tris-HCl, pH 7.8, 20mM MgCl₂, 20mM DTT, 1mM ATP, 10% PEG); 50ng pGEM®-T easy vector (50ng/µL); 6-8µL CYP1A1 or CYP1A2 partial DNA fragment; 3 Weiss units T4 DNA Ligase (3 units/µL); nuclease-free water to a final volume of 20µL. The reaction was mixed by pipetting and incubated overnight at 4°C.

5.2.5.c. Transformation of pGEM®-T easy vector into JM109 competent cells. The ligated pGEM®-T easy vector was transformed into *Escherichia coli* strain JM109 competent cells in order to obtain a high number of colonies. The JM109 cells were stored at -70°C, therefore prior to use the cells were thawed on ice for 5 minutes. The ligated DNA to be transformed was placed into pre-cooled microfuge tubes and 50µL of the cells were added. The cells were incubated on ice for 20 minutes, heat shocked for 1 minute at 42°C and returned to ice for 2 minutes. 950µL SOC medium was added to the tubes and incubated for 1.5 hours at 37°C with shaking, at approximately 150rpm.

Subsequently 100 μ L of the transformation culture was plated on LB–agar plates containing 100 μ g/mL ampicillin, 0.1mM IPTG and 40 μ g/mL XGAL, and incubated at 37°C overnight.

5.2.5.d. Screening of recombinant clones using α -complementation. Blue-white screening was employed to identify transformed cells. The multiple cloning site of the pGEM[®] vector series occurs within the coding sequence for the lacZ gene. The lacZ gene encodes for β -galactosidase, which cleaves the chromogenic substrate, X-GAL releasing 5-Bromo-4 chloro-indigo, producing a blue colony. Therefore if the lacZ coding sequence is interrupted by the insertion of a PCR product, β -galactosidase is not produced and the recombinant colonies are white.

The JM109 *E.coli* transformed with ligations of pGEM-T[®] easy vector and insert were plated onto antibiotic selective LB agar plates containing IPTG (0.1mM) to induce the lacZ expression, the chromogenic substrate X-GAL (40 μ g/mL) and the antibiotic ampicillin (100 μ g/mL)

5.2.5.e. Preparation of cultures of the recombinant clones. White colonies from the LB plates were selected to produce 10mL overnight cultures of the recombinant clones. One white colony of *E.coli* was placed in 10mL of LB medium containing 100 μ g/mL ampicillin and incubated for 16 hours at 37°C with shaking (approximately 180rpm).

5.2.5.f. Purification of plasmid containing DNA insert from *E.coli* (JM109) cultures. The pGEM[®]-T easy vector containing the DNA insert was purified from the *E.coli* (JM109) cultures using QIAprep spin miniprep kit (Qiagen, Germany.). Briefly the recombinant clones were pelleted from 5mL of culture by centrifugation for 10 minutes

at 3,500rpm (Rotor Nr.11133 Sigma SK10 centrifuge, Howe) and the supernatant was discarded. The bacterial cells were re-suspended in 250 μ L buffer P1 containing RNase A (20 μ g/mL) and were transferred to a 1.5mL microfuge tube. The cell membranes were subsequently lysed in buffer P2 in the presence of RNase A, allowing the release of the cells contents and the denaturation of the chromosomal DNA, plasmid DNA and proteins. The optimised lysis time (<5 minutes) enables the release of only the small plasmid DNA, leaving the high molecular weight genomic DNA behind. The addition of buffer N3 neutralised and adjusted the lysate to high-salt binding conditions. By rapidly neutralising the lysate with a high salt buffer, the chromosomal DNA formed an insoluble aggregate that precipitated out of solution with denatured proteins, cellular debris and sodium dodecyl sulphate (SDS), leaving the plasmid DNA to correctly re-nature and remain in solution.

Separation of the plasmid DNA from the insoluble chromosomal DNA: protein: SDS complex was obtained by centrifugation for 10 minutes at 10,000g. The supernatant was subsequently transferred onto spin column where the plasmid DNA was selectively adsorbed onto the silica-gel membrane as a result of the high-salt conditions. The other cellular contents were removed in the flow-through after centrifugation at 10,000g for 1 minute. The adsorbed plasmid DNA was washed with 0.5mL buffer PB followed by a wash with 0.75mL buffer PE containing 96% ethanol. The resultant flow-throughs' were discarded after the column was centrifuged at 10,000g for 1 minute. The spin column was centrifuged for an additional 1 minute at 10,000g after the flow-through was discarded to remove any residual ethanol- containing buffer, which would inhibit subsequent enzyme reactions.

The plasmid DNA was eluted with 50µL low salt, high pH buffer EB (10mM Tris-HCl, pH 8.5) into a clean, 1.5mL nuclease-free microfuge tube, after standing for 1 minute at room temperature, followed by centrifugation at 10,000g for 1 minute. Eluted plasmid containing the PCR insert was stored at -20°C until required.

5.2.5.g. Verification of the PCR insert by single enzymatic digestion of the pGEM[®]-T easy vector. The plasmid vector was digested by a single enzymatic digestion with *EcoR*I to verify the plasmid contained the PCR product, and that the product was of the correct size prior to sequencing. The restriction enzyme *EcoR*I cuts at the two restriction sites G[∇]AATTC on either side of the multiple cloning site of the pGEM[®]-T easy vector.

The reaction was prepared in a clean, 0.5mL nuclease-free microfuge tube placed on ice containing 2µL x10 buffer H, 0.1µg/µL acetylated BSA, 1µL plasmid DNA, 5u *EcoR*I (10u/µL) and nuclease-free water to a final volume of 20µL. The reaction was mixed by pipetting and briefly centrifuged to collect the contents at the bottom of the tube, prior to incubation at 37°C for 1 hour. The digestion was analysed on 2% agarose gel containing 0.05% ethidium bromide.

5.2.6. DNA sequencing of recombinant clones. Automated DNA sequencing was performed on a Perkin Elmer ABI Prism[™] 377 DNA sequencer by Alex Houston (Centre for Biomolecular Sciences, University of St Andrews, UK). A minimum of 3 clones were sequenced both sense and anti-sense wise in order to eliminate sequence ambiguities. The sequencing data was viewed using the autoassembler[™] program (version 1.4.0., ABI Prism, Perkin Elmer) and analysed using DNAMAN.

5.2.7. DNA and amino acid sequence analysis. The CYP1A cDNA were verified for identity using the BLAST (Altschul *et al.*, 1990). Translation of the cDNA sequences to amino acid sequences were performed and their identity were determined using a variety of CYP1A sequences from mammalian, fish and avian species (Table 5.2). Subsequently, the seal CYP1A sequences were aligned with CYP1A sequences from other animal species using the program, DNAMAN (Lynnon Biosoft, USA).

A phylogenetic tree was constructed using the Neighbour-joining method using distance relationships (Saitou and Nei, 1987) in the DNAMAN program. The hydrophilicity of the seal CYP1A1 and -1A2 sequences was investigated by generating hydrophilic plots in DNAMAN using the method of Hopps and Woods (1981)

5.3. Results

5.3.1. CYP1A1 and -1A2 primary structure.

Harp seal and grey seal full length CYP1A1 and CYP1A2 cDNAs were isolated from liver mRNA using RT-PCR, with the primer combination presented in Table 5.1. Primers were designed using ribbon seal CYP1A cDNA sequences, due to a very high identity at the 5' and 3' end of the coding region of CYP1A1 and CYP1A2 cDNA of other related mammals, including human (*Homo sapiens*) and crab-eating macaque (*Macaca familiaris*) CYP1A1 and CYP1A2.

Each cDNA sequence and its deduced amino acid sequence were of similar length to other vertebrate CYP1A enzymes, which ranged from 1536 to 1590 nucleotides and 512 to 530 amino acids. However, the theoretical molecular weights calculated for both the harp and grey seal CYP1A1 and -1A2 were ~58KDa and ~57KDa, respectively and were higher than those determined experimentally (Table 5.3).

Species	Label	Accession number
Mammals		
<i>Canis familiaris</i>	Dog CYP1A1	P56590
	Dog CYP1A2	P56592
<i>Homo sapiens</i>	Human CYP1A1	P04798
	Human CYP1A2	P05177
<i>Rattus norvegicus</i>	Rat CYP1A1	P00185
	Rat CYP1A2	P04799
<i>Mus musculus</i>	Mouse cyp1a1	P00184
	Mouse cyp1a2	P00186
<i>Macaca fascicularis</i>	Macaque monkey CYP1A1	P33616
<i>Ovis aries</i>	Sheep CYP1A1	P56591
<i>Orycholagus cuniculus</i>	Rabbit CYP1A1	P05176
	Rabbit CYP1A2	P00187
<i>Mesocricetus curatus</i>	Golden hamster CYP1A1	Q00557
	Golden hamster CYP1A2	P24453
<i>Cavia porcellus</i>	Guinea pig CYP1A1	Q06367
	Guinea pig CYP1A2	Q64391
Bird		
<i>Gallus gallus</i>	Chicken 1A4	P79760
	Chicken 1A5	P79761
Fish		
<i>Platichthys flesus</i>	European flounder 1A1	Q9Y1164
<i>Oncorhynchus mykiss</i>	Rainbow trout 1A3	Q92109
<i>Sparus aurata</i>	Gilthead sea bream 1A1	Q42457
<i>Limanda limanda</i>	Limanda 1A1	Q42430
<i>Stenostomus chrysops</i>	Scup 1A1	Q92116
<i>Pleuronectes platessa</i>	Plaice 1A1	Q92100
<i>Opsanus tau</i>	Oyster toadfish 1A1	Q92095
<i>Dicentrarchus labrax</i>	European sea bass 1A1	Q79716
<i>Chrysophrys major</i>	Red sea bream 1A1	P98181
<i>Chaetodon capistratus</i>	Four-eye butterfly fish 1A1	Q92039

Table 5.2. Mammalian, bird and fish CYP1A protein sequence accession numbers from NCBI database.

Species	CYP Isoenzyme	Length of cDNA (bases)	Length of deduced Protein	MW deduced Protein (KDa)	MW deduced Experimentally determined
Harp Seal	1A1	1551	515	58.430	54 ^a
	1A2	1539	512	57.774	
Grey Seal	1A1	1551	516	58.209	<56 ^b
	1A2	1539	512	57.722	

Table 5.3. General information about the cDNA and deduced amino acid sequences of harp and grey seal CYP1A1 and CYP1A2. The molecular weight (MW) of the deduced amino acid sequence is the theoretical value and was calculated using the translate tool on the Expert Protein Analysis System (ExPASy; <http://www.expasy.ch>). The CYP1A -like bands approximate MW were experimentally determined using an anti-rat CYP1A1/2 antibody. ^a Goksoyr (1995); ^b Nyman et al. (2000).

	Ribbon seal ^a		Mammal		Fish		Avian	
	CYP1A1	CYP1A2	CYP1A1	CYP1A2	CYP1A1	CYP1A2	CYP1A4	CYP1A5
Grey seal CYP1A1	99.03	75.0	72 - 86	63 - 70	52-56		57	60
Harp seal CYP1A1	99.61	75.05	72 - 86	63 - 70	52 - 56		57	60
Grey Seal CYP1A2	75.24	99.41	62 - 70	68 - 84	47 - 50		51	57
Harp seal CYP1A2	74.66	99.41	62 - 70	68 - 84	47 - 50		51	57

Table 5.4. Identity (%) of harp and grey seal CYP1A1 and CYP1A2 deduced amino acid sequences with other vertebrate CYP1A sequences. ^a ribbon seal sequences were obtained from I. Teramitsu, M. Ishizuka and S. Fujita, personal communication.

CYP1A1 and CYP1A2 cDNA sequences from harp and grey seals exhibited a high degree of conservation when the corresponding sequence was aligned. There was 99.5% identity between the two CYP1A1 sequences and 99.2% identity between the two CYP1A2 sequences. The few changes in nucleotides between harp and grey seal sequences, invariably coded for different amino acids in the deduced protein sequences (Fig. 5.4. a and b). Harp and grey seal CYP1A1 and -1A2 isoenzymes also displayed a very high percentage identity (>99%) with ribbon seal cDNA (data not shown) and deduced amino acid sequences (Table 5.4).

The seal species CYP1A isoenzymes compared well with other vertebrate CYP1A amino acid sequences. A percentage identity ranging from 47% with fish to 86% with other mammals was obtained from database (BLAST) searching with the amino acid sequences in question. Harp and grey seal CYP1A1 and -1A2 sequences met the recommended criteria for their inclusion into the CYP1A family, in that they have >40% identity with other CYP1A members (Nebert and Nelson, 1991). In addition, since they have >55% identity with other CYP1A1 and CYP1A2 sequences, they have been incorporated into these two subfamilies, respectively (D. Nelson, personal communication).

5.3.2. Phylogenetic analysis.

A phylogenetic tree was constructed (Fig.5.5) from multiple alignments of the seal amino acid sequences with other vertebrate CYP1A1 and CYP1A2, using the Neighbour-joining method of Saitou and Nei (1987). Phylogenetic analysis showed that seal CYP1A1 and CYP1A2 clustered into two distinct groups, with other mammalian CYP1A1 and CYP1A2 amino acid sequences respectively (Fig. 5.5), with dog CYP1A1 and -1A2 being the closest related in evolutionary time. Seal CYP1A enzymes were

Atgatggtctctctgctccagattgtccatccccatctcgccacagagcttctcctggcc 60
 M M F S A S R L S I P I S A T E L L L A
 Tctgcccgtctctgctgatgctctgggtggcagggcctggcagcctcgggttcccaa 120
 S A V F C L M L W V V R A W Q P R V P K
C, P
 Ggctgaagagtccccgggcccctggggtggcccctgctggggaacgtgctgaccttg 180
 G L K S P P G P W G W P L L G N V L T L
 Ggaagaaccacatctggcgtgtccaggctgagccagcgttacggggacgtgctgcaa 240
 G K N P H L A L S R L S Q R Y G D V L Q
 Atccacattggctccacaccctgctggtgctcagcggccgggacactgtccggcaggcc 300
 I H I G S T P V L V L S G L D T V R Q A
C, P
 Ctagtgagcagggtgaggatttcaaggccggcccacacctacagcttcaactctgatt 360
 L V R Q G E D F K G R P D L Y S F T L I
 Actaatggccaagcatgctcctcagcccagactctggaccagtgtgggctgcccgcag 420
 T N G Q S M S F S P D S G P V W A A R R
 Cgctgcccagaacgcctgaagagtttctccattgcctcagaccgggttccctcgtcc 480
 R L A Q N A L K S F S I A S D P G S S S
 Tcctgctacctggaagcagcagtgtagcaaggaggccgagccctcctcagcaggtgac 540
 S C Y L E E H V S K E A E A L L S R L Q
 Gaacagatggcagaagttgggcactttgaccctacagatatgtagtgtgctcagtgccc 600
 E Q M A E V G H F D P Y R Y V V V S V A
 Aatgctgctgtgcccagtgcttggcaagcgtatgaccacgacgaccaagagctgctt 660
 N V V C A M C F G K R Y D H D D Q E L L
 Agcttaatcaacctgaataatgagttcggggagggcgttgcctctgggaacctgtggac 720
 S L I N L N N E F G E A V A S G N P V D
 Ttcttccccatcctccgatacctgcccacacctgcctggatttcttcaaggacctgaat 780
 F F P I L R Y L P N P A L D F F K D L N
 Aagaggttctacagcttcatgcaaaaagctggtcaaggaacactcaaaaacatttgagaag 840
 K R F Y S F M Q K L V K E H Y K T F E K
 Ggacacattcgggatcacagacagcctgatcaaacattgtcaggacaagaggctggat 900
 G H I R D I T D S L I K H C Q D K R L D
 Gagaatgccaacattcagctgtctgtagagaagatcggttaatgtgtcttggacctctt 960
 E N A N I Q L S D E K I V N V V L D L F
 Ggagccggatttgacaccgtgacaactgccatctcctggagcctcctgtacctggtgaca 1020
 G A G F D T V T T A I S W S L L Y L V T
 Agccccagtgtaaaaaaagatccaggaggagttggacacagtgattggcagggcccgg 1080
 S P S V Q K K I Q E E L D T V I G R A R
 Cagccccggctgtctgacaggccccagttgccctacctggaggcattcatcctggagacc 1140
 Q P R L S D R P Q L P Y L E A F I L E T
 Ttccgacagctcctcctgctccctcaccatccctcatagtagtaccaccaagacacaagt 1200
 F R H A S F V P F T I P H S T T K D T S
 Ctgagtgcttttacatcccaaggagcgttgtgtcttgtgaaccagtgccagatcaac 1260
 L S L F Y I P K G K C V F V N Q W Q I N
G, G
 Catgaccaggagctatggggtgaccatctgagttccgaccagaacgatttctcactctt 1320
 H D Q E L W G D P S E F R P E R F L T L
 Gatggcaccatcaacaaggcactgagtgagaagtgattctcttggaaatgggcaagcgg 1380
 D G T I N K A L S E K V I L F G M G K R
 Aagtgcacgtgagaccattgcccgcctggaggtcttctcttctcctggccatcctgctg 1440
K C I G E T I A R L E V F L F L A I L L
 Cagcaggtggaattcagtggtccccgggcacgaaggtggatgatgacccccatttacggg 1500
 Q Q V E F S V P S G T K V D M T P I Y G
a, Q
 Ctgaccatgaagcatgcccgtgtgagcagctcaagtgccgggtacgtgcttaa 1554
 L T M K H A R C E H V Q V R V R A -
 (i)

Fig. 5.4.a. cDNA sequence and deduced amino acid sequence for harp seal CYP1A1. The nucleotide and amino acid differences between harp and grey seal are highlighted in blue and the nucleotide and amino acid residue change boxed beneath the corresponding region. The conserved amino acid motif with the heme-binding cysteine is underlined. The start and stop codons of the coding region are highlighted in red.

distinct from chicken and fish CYP1A sequences, where chicken possess two CYP1A members from different subfamilies and fish have only one CYP1A isoenzyme, CYP1A1, with the exception of trout (CYP1A1 and CYP1A3) (Rabergh *et al.*, 2000).

5.3.3. Structural analysis.

Multiple alignment of harp, grey and ribbon seal and dog CYP1A1 with the corresponding CYP1A2 sequences showed regions of high conservation throughout the seal species CYP1A1 and -1A2 sequences, which often included the dog CYP1A sequences (Fig. 5.6). The N-termini, apart from the first few amino acids, were extremely conserved for these species for both CYP1A1 and 1A2, and the C-termini also exhibited a high degree of conservation, although single or two consecutive amino acids displayed CYP isoform specificity. The highly conserved amino acid motif (FxxGxxxCxG), which contains the conserved cysteine residue that co-ordinates the heme iron, is maintained in the seals CYP1As and may be found in all CYPs (Gotoh, 1992). Interestingly, the middle region of the amino acid sequences contains blocks of amino acid residues that were specific to each of the CYPs.

Highlighted amino acid residues in the CYP101A sequence (Fig.5.6) indicate those that were involved in substrate binding in this particular bacterial CYP isoform (Gotoh, 1992; Poulos, 1991). Alignments of dog and seal CYP1A1 and -1A2 with several bacterial sequences (CYP101A1; CYP102A1; CYP104A1; CYP105A1; CYP108A1) enabled 5 putative substrate recognition sites (SRSs) to be assigned (Fig.5.6). SRS-1 contains 2 substrate binding regions that were only one amino acid apart, therefore have been combined into SRS-1, similar to the CYP2 alignment by Gotoh (1992). The final two amino acid residues, isoleucine (I) and valine (V), involved in substrate binding in

Fig. 5.6. Alignment of 3 seal species: Harp seal, grey seal and ribbon seal CYP1A1 and CYP1A2 deduced amino acid sequences with Dog CYP1A1 and CYP1A2 as reference. Shading in grey indicates the amino acid residues are at least identical in all 3 seal species for both the CYP1A subfamilies. CYP1A1 and CYP1A2 specific amino acids are high-lighted in yellow and blue, respectively.

The conserved motif sequence (in bold type) contains the conserved cysteine (in italics) that provides the 5th ligand for the heme prosthetic group. Five putative substrate recognition sites (SRS) are shown by broken lined boxes and are based on the known amino acid residues (high-lighted in red) involved in substrate binding in bacterial *P.putida* CYP101A which has been aligned with seal CYP1A1 sequences. The boxed region with solid lines indicates the sequence with the greatest similarity to the anti-trout CYP1A1 anti-peptide antibody. ‘.’ indicates gaps in the alignment.

Dog CYP1A1	MMFRLSIPISASELLASTVFCVLWVVKAWQPRPKGLKSPGPGWVFLGNVLTIGKSPHLALSRLSQRVGDVLQ	79
Grey seal CYP1A1	MFSaRSLSPISATELLLASaVFCVmpVWVrAWQPRV?KGLKSPGPGWVFLGNVLTIGKPHLALSRLSQRVGDVLQ	79
Harp seal CYP1A1	mMFSaRSLSPISATELLLASaVFCVlmLWVrAWQPRV?KGLKSPGPGWVFLGNVLTIGKPHLALSRLSQRVGDVLQ	80
Ribbon seal CYP1A1	mMFSaRSLSPISATELLLASaVFCVlmLWVrAWQPRV?KGLKSPGPGWVFLGNVLTIGKPHLALSRLSQRVGDVLQ	80
<i>P. putida</i> CYP101A	0
Dog CYP1A2malsqmAtgLLlASr:FCliLWVVKAWQPRPKGLKSPGPGWVFLGNVLTIGKSPHLALSRLSQRVGDVLQ	73
Grey seal CYP1A2malsqmAtELLLASaVFCVLWVrAWQPRV?KGLKSPGPGWVFLGNVLTIGKPHLALSRLSQRVGDVLQ	73
Harp seal CYP1A2malsqmAtELLLASaVFCVLWVrAWQPRV?KGLKSPGPGWVFLGNVLTIGKPHLALSRLSQRVGDVLQ	73
Ribbon seal CYP1A2malsqmAtELLLASaVFCVLWVrAWQPRV?KGLKSPGPGWVFLGNVLTIGKPHLALSRLSQRVGDVLQ	73
Dog CYP1A1	IRIGSTPVLVLSGLDTRQALVRQGDDEKGRPDLYSfLVTdGOSLTFSPDSGVPWAARRRQAQNALKSFSIASDPASSC	159
Grey seal CYP1A1	IhIGSTPVLVLSGpDTRQALVRQGeDFKGRPDLYSfLIThGOSmsFSPDSGVPWAARRRQAQNALKSFSIASDPgSSs	159
Harp seal CYP1A1	IhIGSTPVLVLSGLDTRQALVRQGeDFKGRPDLYSfLIThGOSmsFSPDSGVPWAARRRQAQNALKSFSIASDPgSSS	160
Ribbon seal CYP1A1	IhIGSTPVLVLSGLDTRQALVRQGeDFKGRPDLYSfLIThGOSmsFSPDSGVPWAARRRQAQNALKSFSIASDPgSSS	160
<i>P. putida</i> CYP101A	30
Dog CYP1A2	IRIGSTPVLVLSGLDTRQALVRQGDDEKGRPDLYSfLITdSOSmsFSPDSGVPWAARRRQAQNALKSFSIASDPASSC	153
Grey seal CYP1A2	IhIGSTPVLVLSGLDTRQALVRQGeDFKGRPDLYSfLIThGOSmsFSPDSGVPWAARRRQAQNALKSFSIASDPgSSS	153
Harp seal CYP1A2	IhIGSTPVLVLSGLhTRQALVRQGeDFKGRPDLYSfLITdGOSmsFSPDSGVPWAARRRQAQNALKSFSIASDPgSls	153
Ribbon seal CYP1A2	IhIGSTPVLVLSGLDTRQALVRQGeDFKGRPDLYSfLITdGOSmsFSPDSGVPWAARRRQAQNALKSFSIASDPgSls	153
Dog CYP1A1	SCYLEEHVSKEAEVLLSRLQEQMAEVGFDPYRYIVSVANVIGAMCFSKRYDHDHDDQELLSLVNIEFGEVASANPLD	239
Grey seal CYP1A1	SCYLEEHVSKEAEaLLSRLQEQMAEVGHFDPPYRYVVSANVVCAMCFgKRYDHDHDDQELLSliNlNNEFGEaVAsGnPVd	239
Harp seal CYP1A1	SCYLEEHVSKEAEaLLSRLQEQMAEVGHFDPPYRYVVSANVVCAMCFgKRYDHDHDDQELLSliNlNNEFGEaVAsGnPVd	240
Ribbon seal CYP1A1	SCYLEEHVSKEAEaLLSRLQEQMAEVGHFDPPYRYVVSANVVCAMCFgKRYDHDHDDQELLSliNlNNEFGEaVAsGnPVd	240
<i>P. putida</i> CYP101A	npsnlSagvqEawavLqesvnpdlwtrCngghwIatrgqlireAyedyrhfssecp3ipreageaVdFiP.....	101
Dog CYP1A2	SCYLEEHVSKEAEaLLSRLQEQMAEVGFDPYnqVllSVANVIGAMCFghhfsqrseEmIpLlmsSsdFvEtVsGnNPLd	233
Grey seal CYP1A2	SCYLEEHVSKEAEaLLSRLQEQMAEVGHFDPPYnqVllSVANVIGAMCFghfpgsneEmLSLlksSndFvEtasSgNPVd	233
Harp seal CYP1A2	SCYLEEHVSKEAEaLLSRLQEQMAEVGHFDPPYnqVllSVANVIGAMCFghfpgsneEmLSLlksSndFvktasSgNPVd	233
Ribbon seal CYP1A2	SCYLEEHVSKEAEaLLSRLQEQMAEVGHFDPPYnqVllSVANVIGAMCFghfpgsneEmLSLlksSndFvEtasSgNPVd	233
Dog CYP1A1	FFPILRYLPNPALDFFK.....	SRS-2
Grey seal CYP1A1	FFPILRYLPNPALDFFK.....	296
Harp seal CYP1A1	FFPILRYLPNPALDFFK.....	296
Ribbon seal CYP1A1	FFPILRYLPNPALDFFK.....	297
<i>P. putida</i> CYP101AsmdPpegrcqFralangvvpvvdkl.enriqelacslieslrpqqcfnftedvaeppfirilmllagIpeedIphikylitqmirpd	189
Dog CYP1A2	FFPILqYmPNsAlqrFFK.....	288
Grey seal CYP1A2	FFPILqYmPNPALqrFFK.....	288
Harp seal CYP1A2	FFPILqYmPNPALqrFFK.....	288
Ribbon seal CYP1A2	FFPILqYmPNPALqrFFK.....	288

SRS-3

RLDENANIQLS DEKIVNVVLDIFGAGFDVTWTAISWSLILYLVN 340
 RLDENANIQLS DEKIVNVVLDIFGAGFDVTWTAISWSLILYLVTS 340
 RLDENANIQLS DEKIVNVVLDIFGAGFDVTWTAISWSLILYLVTS 341
 RLDENANIQLS DEKIVNVVLDIFGAGFDVTWTAISWSLILYLVTS 341
 gsmfAeakeal..... ydylipieqrqpgtdaisivanghvngpbitsDeakmcgllivgldvvhflsfsmeFlaks 255
 srasdghIpg EKIVNlindiFCAGFDVTWTAISWSLILYLVaN 330
 sraggghIph EKIVslindiFCAGFepiITTAISWSLILYLVaN 330
 sraggghIph EKIVslindiFCAGFepiITTAISWSLILYLVN 330
 sraggghIph EKIVslindiFCAGFepiITTAISWSLILYLVN 330

SRS-4

PNVQKKIQEELDTVI GRARQPRISDRPQLPYMEAFILETFRHASFVPPFIPHSTTRDTLSGFIYIPKRCVFNQWQIINHDOQKI 424
 PSYQKKIQEELDTVI GRARQPRISDRPQLPYEAFILETFRHASFVPPFIPHSTTMDTSLSGFIYIPKRCVFNQWQIINHDOQel 424
 PSYQKKIQEELDTVI GRARQPRISDRPQLPYEAFILETFRHASFVPPFIPHSTTMDTSLSSFYIPKRCVFNQWQIINHDOQel 425
 PSYQKKIQEELDTVI GRARQPRISDRPQLPYEAFILETFRHASFVPPFIPHSTTMDTSLSGFIYIPKRCVFNQWQIINHDOQel 425
 pehrqelieRPeripaaceEllrrfslVaggriltsdvefhgvqlkk.....g.....Dqil 315
 PeiQrKIQeELDTVI GRARQPRISDRPQLPYMEAFIIEIFRHtSFVPPFIPHSTTktDtLkGFYIPKecCVFIHQWVNHDOQqv 414
 PeiQrKIQeELDTVI GRARQPRISDRPQLPYMEAFIIEIFRHtSFVPPFIPHSTTRDTLkGFYIPKeRCVFIHQWVNHDOQV 414
 PeiQrKIQeELDTVI GRARQPRISDRPQLPYMEAFIIEIFRHtSFVPPFIPHSTTRDTLkGFYIPKeRCVFIHQWVNHDOQV 414
 PeiQrKIQeELDTVI GRARQPRISDRPQLPYMEAFIIEIFRHtSFVPPFIPHSTTRDTLkGFYIPKeRCVFIHQWVNHDOQV 414

SRS-5

WGNPSEFQPERFLTLDGT . INKALSEKVIILFGLGRRK CIGETIARLEVFLELAILLOQVEFSVPEEGKVDMTPIYGLTMKHARCEHFQVRVTEG 518
 WGDSEFQPERFLTLDGT . INKALSEKVIILFgmGRRK CIGETIARLEVFLELAILLOQVEFSVPqGKVDMTPIYGLTMKHARCEHVQVRVa 516
 WGDSEFQPERFLTLDGT . INKALSEKVIILFgmGRRK CIGETIARLEVFLELAILLOQVEFSVPrCTKVDMTPIYGLTMKHARCEHVQVRVa 517
 WGDSEFQPERFLTLDGT . INKALSEKVIILFgmGRRK CIGETIARLEVFLELAILLOQVEFSVPrGKVDMTPIYGLTMKHARCEHVQVRVa 517
 lpgmlsgldERenacpmhvdfrsqkvshtFGhgshlClGqhlARrEiivLkewLtridp.....fsia 385
 WGDpfaFrPERFLTaDGTtINKtLSEKVMlFgmGRRK CIGEvIakwEiFLFLAILLQRIEFSPaGvKVDlTPIYGLTMKHARCEHVQaRprfsik 512
 WGDpfeFrPERFLTaDGTsINKiLSEKVMiFgmGRRK CIGELIakwEiFLFLAILLQRIEFSPdGvKVDlTPIYGLTMKHARCEHVQaRprfstk 512
 WGDpfeFrPERFLTaDGTsINKiLSEKVMiFgmGRRK CIGELIakwEiFLFLAILLQRIEFSPdGvKVDlTPIYGLTMKHARCEHVQaRprfstk 512
 WGDpfeFrPERFLTaDGTsINKiLSEKVMiFgmGRRK CIGELIakwEiFLFLAILLQRIEFSPdGvKVDlTPIYGLTMKHARCEHVQaRprfstk 512

524

AESPAA
 PgaqiqhksqIsgvqalplw 414

Dog CYP1A1

P. putida CYP101A

CYP101A, and corresponded to SRS-6 in the CYP2 amino acid sequences, did not align with dog and seal CYP1A sequences, therefore no SRS-6 has been assigned.

The location of the putative SRSs in CYP1A1 and -1A2 corresponded with blocks of amino acids specific to each isoform, that are found in the middle region of the sequences. Differences in amino acid residues between the two isoforms within potential substrate recognition sites could be responsible for differences in CYP1A1 and -1A2 substrate specificities.

5.3.4. Immunochemical analysis.

The peptide sequence used to produce anti-trout CYP1A1 oligopeptide antibodies (SYDKDNIRDITDSLIDHC; Myers *et al.*, 1993) was aligned with CYP1A1 and 1A2 seal amino acid sequences in order to speculate the potential degree of specificity that this heterologous antibody has with the three seal species protein sequences (Fig.5.6). The homologous peptide sequence in seal CYP1A1 amino acid sequence (276-293) was very similar to the trout CYP1A1 peptide, showing 67.67% identity (Fig. 5.6). However, the same peptide region in CYP1A2 (276-293) only shows 33.3% identity with the trout CYP1A1 peptide. A hydropathic plot of seal CYP1A sequences (Fig. 5.7) indicated that the region homologous to the trout peptide has a reasonable hydrophilic character in both CYP1A1 and 1A2 and is thus likely to be antigenic (Hopps and Woods, 1981).

5.4. Discussion

5.4.1. CYP1A1 and -1A2 primary structure.

This study reports the isolation of CYP1A1 and -1A2 from the liver of harp and grey seal, exposed to environmental contaminants, by RT-PCR. mRNA was used as the

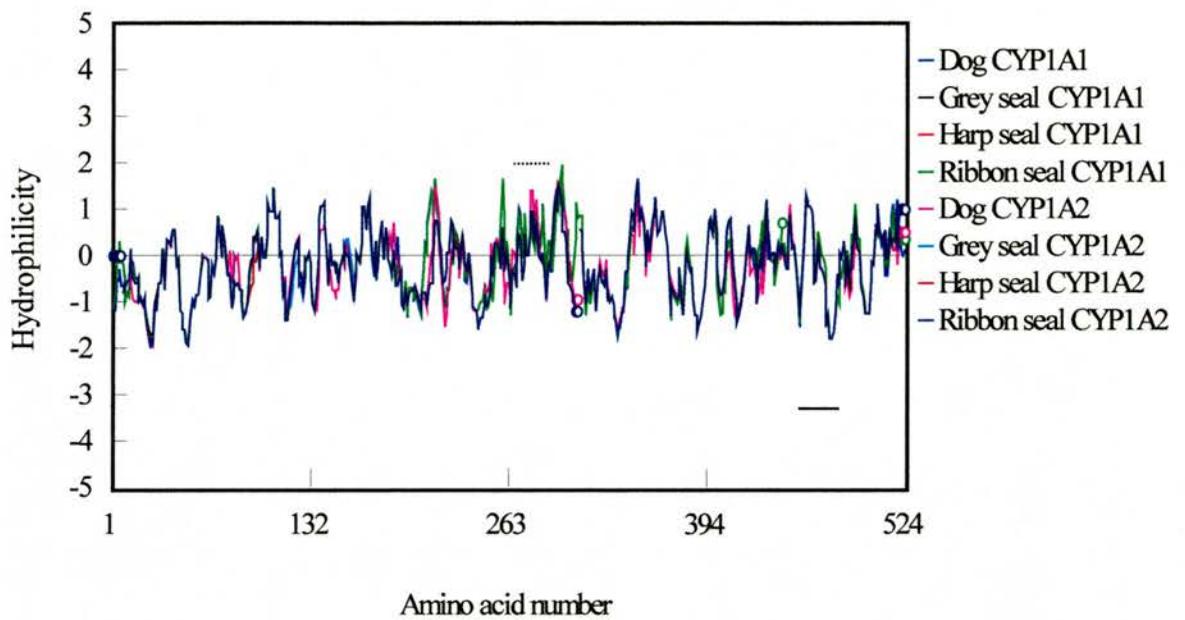


Figure 5.7. Hydrophilicity plot of grey seal, harp seal, ribbon seal and dog CYP1A1 and CYP1A2 using the method of Hopps and Wood (1981) generated in DNAMAN. A value of 6 was chosen as the averaging group length for plotting the hydrophilic profile of the CYP1A isoenzymes. The dashed line indicates the homologous peptide to the anti-trout CYP1A1 anti-peptide antibody, in these mammal CYP1A1 sequences. The heme-binding region (conserved motif) is indicated with a line below the region on all 8 sequences.

template for the isolation of these CYP1A cDNA, therefore their isolation would indicate that the CYP1A gene(s) had been induced and subsequently transcribed.

The seals CYP sequences were found to have > 99% identity with each other and between 72 and 86%, and 63 and 72% identity with other mammalian CYP1A1 and CYP1A2 amino acid sequences, respectively. The degree of conservation within the seal CYP1A sequences and with other vertebrate species has allowed the inclusion of these sequences within the CYP1A family. Indeed the high percentage identity has further subdivided them into the CYP1A1 and -1A2 subfamilies.

5.4.2. Phylogenetic analysis.

The phylogeny of seal CYP1A amino acid sequences was created based on the isolation and sequence of the cDNAs from harp and grey seals. The harp and grey seals along with the ribbon seal CYP1A1 and -1A2 formed two distinct clusters with other mammalian CYP1A1 and -1A2 sequences, respectively. The closest relative to the seal CYP1A sequences was the dog (*Canis familiaris*) CYP1A isoforms. Both seals and dog are derived from the same lineage and belong to the same order Carnivora (Bonner, 1989). This supports Teramitsu and colleagues (2000) who found that three seal species partial amino acid fragments from CYP1A1 and one seal CYP1A2 partial sequence, formed two distinct clusters with dog CYP1A1 and -1A2, respectively.

CYP1A isoenzymes may have evolved as a defence mechanism against plant materials (terrestrial and aquatic) as many animals possess these isoenzymes, and suggestions have been made indicating that the CYP1A isoenzymes present in marine mammals could be unique as a result of their aquatic habitat (Gonzalez and Nebert, 1990; Teramitsu *et al.*, 2000). However, CYP1A also metabolises natural substances such as

benzo(a)pyrene (BaP), other polycyclic aromatic hydrocarbons and possibly dioxins, such as polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDDs and PCDFs) that have arisen from incomplete combustion of organic matter (Bumb *et al.*, 1980; Gribble, 1992).

Induction of CYP1A enzymes initially requires the formation of the aryl hydrocarbon (Ah) receptor/ligand/Ah receptor nuclear translocator (ARNT) protein complex that subsequently binds to the CYP1A gene (Whitlock *et al.*, 1996). The Ah receptor has been detected in the liver of beluga whale (*Delphinapterus leucas*) (Hahn *et al.*, 1994). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is a potent ligand of the Ah receptor and although is primarily man-made and been in the environment only since 1940s, many aquatic natural products, e.g. polychlorinated dibenzofurans, have been found with remarkable similarities to known Ah receptor ligands (Gribble, 1992).

Marine mammals evolved from terrestrial mammals, the carnivores, and may have CYP1A isoforms that metabolise combustible products and terrestrial plant products, similar to other terrestrial mammals. Therefore, the contemporary marine mammals, including the seals could have retained these CYP1A isoforms to metabolise, not only combustible products from organic matter, but also aquatic natural products and more recently anthropogenic chemicals.

Despite seals being aquatic animals and sharing a similar environment with fish, the seals have two distinct amino acid sequences, whereas fish, with the exception of trout, have only one, CYP1A1. The evolution of CYP1A2, from CYP1A1, in mammals has been suggested to have occurred after land animals diverged from fish ~400mya (Gilday *et al.*, 1996). The CYP1A2 could have potentially evolved as part of the defence

mechanism to plant products and /or as a catalyst to metabolise an unknown endogenous ligand unique to mammals, that is retained in the extant species (Nelson, 1999).

However, Wolkers and colleagues (1998b) observed only ringed seal mRNA corresponding to rat CYP1A1, in northern blot analyses using human CYP1A1 and –1A2 cDNA probes. The apparent lack of CYP1A2 in ringed seal liver could be a consequence of differences between species. However, it is more likely to be a result of the nucleotide difference of the chosen human CYP1A2 probes and the seal CYP1A2 sequence.

5.4.3. Secondary structure.

CYP1A1 and –1A2 in other mammals, have shown differing substrate specificities. For example rat CYP1A1 preferentially metabolises EROD, whereas CYP1A2 catalyses the formation of the 1,7-metabolite of caffeine (Berthou *et al.*, 1992; Burke *et al.*, 1994). The substrate specificities of several marine mammal species CYP1A isoenzymes have been investigated by chemical- and immuno-inhibition (e.g. Mattson *et al.*, 1998; White *et al.*, 1994; Wolkers *et al.*, 1998b). For example, EROD has been attributed to CYP1A in grey and ringed seals (Nyman *et al.*, 2000). However, the amino acids involved in substrate binding of CYP1A in seal species remain undetermined.

In this chapter, harp and grey seal CYP1A sequences were aligned with several bacterial sequences to investigate regions of the sequence that potentially could be involved in substrate binding. Blocks of highly conserved amino acids that were CYP1A1 or –1A2 specific were exhibited in grey, harp and ribbon seals (Fig.5.6). Five putative substrate recognition sites (SRSs) were assigned in regions of the seals CYP1A amino acid

sequences that were within three amino acid residues from the substrate-binding residues of the bacterial CYP101A, based on the work of Gotoh (1992). Apart from investigation of CYP1A2 catalytic function in rat liver microsomes, amino acid residues involved in substrate binding or recognition in mammalian CYP1A isoenzymes have received very little attention (Hiroya *et al.*, 1992; Ishigooka *et al.*, 1992). However, x-ray crystallography and subsequent modelling of the substrate bound form of the bacterial CYP101A has elucidated the amino acid residues that interact with the substrate camphor in dispersed locations along the primary structure (Laughton *et al.*, 1990; Poulos *et al.*, 1985; Poulos *et al.*, 1987). Since all CYPs are thought to have derived from a single ancestral gene, modelling mammalian CYP isoenzymes on the known CYP101A isoform, may provide relevant substrate recognition site information (Nebert and Gonzalez, 1987). The conserved CYP1A1 or -1A2 blocks of amino acids may therefore be involved in the substrate specificity of these isoforms, since the putative SRSs correspond to these regions within each of the isoforms.

Harp and grey seal exhibited several amino acid differences in CYP1A1 sequence (4/516) and more in CYP1A2 sequence (6/512). Investigations using site-directed mutagenesis of single amino acids have indicated specific residues involved in substrate binding in laboratory mammals. Lindberg and Negishi (1989) demonstrated a single amino acid mutation that was responsible for the difference in substrate specificities of CYP_{coh} (CYP2A5) and CYP_{15 α} (CYP2A4) in mouse. These two CYPs share 483/494 amino acid residues, however catalyse very different reactions: CYP2A5 metabolises the hydroxylation of coumarin, whereas CYP2A4 hydroxylates steroids at the 15 α position. However, the mutation of phenylalanine at position 209 to leucine converted CYP2A5 from coumarin to steroid hydroxylation. This amino acid residue position is located in the putative SRS-1 of the seals (Fig.5.6). Ishigooka and colleagues (1992)

mutated rat liver microsomal CYP1A2, changing the conserved glutamate at position 318 to an alanine. The consequent activation of the oxygen molecule in the catalytic function of the mutant CYP1A2 toward 7-ethoxyresorufin was reduced to 6% of the wild type. A glutamate residue in the harp, grey and ribbon seal CYP1A2 was positioned at 318, and an aspartate residue was positioned at 323, both corresponding with rat CYP1A2. These amino acid residues were located in the putative SRS-4 (Fig.5.6). Gotoh (1992) pointed out amino acid residues that play important roles in a variety of CYP2 substrate specificities. These residues were located within the putative CYP2 SRSs and two out of three times corresponded with the position of bacterial CYP101A amino acid residues involved in binding the substrate camphor. Therefore amino acid changes within regions important in substrate recognition and/or binding could effect changes in substrate specificity through alterations in the local 3D structure. The significance of such changes in the primary structure of CYP1A1 and -1A2 proteins are unknown as the tertiary structure and substrate binding site of these isoforms in seals are yet to be determined.

5.4.4. Immunochemical detection.

Antibodies raised against other vertebrate species (i.e. fish, rat, mouse) have been used in immunochemical detection studies of seal and whale liver preparations (e.g. Goksøyr *et al.*, 1989; White *et al.*, 2000; Wolkers *et al.*, 1999). However, an assumption is often made regarding the reactivity of the antibody with a heterologous CYP. For example in one study only a single protein band in ringed seal liver microsomes, was detected using an anti-rat CYP1A1/2 antibody (Nyman *et al.*, 2000), where perhaps using a homologous antibody two bands might be expected. A lack of correspondence between catalytic and immunochemical detection of CYP1A-like proteins was reported in minke whale post-mitochondrial supernatants (Goksøyr *et al.*, 1992).

Since the epitopes of antibodies raised against purified CYPs are unknown and until recently the protein sequence of seal CYP1As were obscure, the degree of specificity of these antibodies for seal CYP1A proteins remain undetermined. Anti-peptide antibodies have pre-determined epitopes and those that are targeted towards highly conserved peptide regions of the CYP of interest, have the potential to overcome, to a certain extent, the undetermined reactivity often obtained with antibodies raised against purified CYPs (Edwards *et al.*, 1998).

The peptide sequence designed to raise as an antibody against trout (*Oncorhynchus mykiss*) CYP1A1, exhibited a high percentage identity with harp, grey and ribbon seal CYP1A1 amino acid sequence, however to a much less degree with CYP1A2. Myers and colleagues (1993) showed that this anti-trout CYP1A1 anti-peptide antibody specifically recognised the CYP1A1 apoprotein in liver microsomes from trout exposed to β -naphthoflavone, but not in control fish.

Both the trout peptide (277-294) and the corresponding region in the seal species CYP1As (276-293), exhibited a relatively hydrophilic profile (Fig. 5.7), determined using the criteria of Hopps and Wood (1981). This indicates that this anti-trout CYP1A1 anti-peptide antibody will preferentially recognise the CYP1A1 in harp, grey and ribbon seals and that this peptide (276-293) will be on the surface of the native CYP1A1 enzyme (Hopps and Woods, 1981). Indeed anti-peptide antibodies designed for one seal species are very likely to cross-react with another, considering the high percentage of conservation of amino acid residues within the CYP1A sequences of those species studies so far (refer to Chapter six). In addition considering the location of the peptide on the 3-dimensional structure of CYP1A enzyme (Chapter six), such an anti-peptide antibody would have the potential for use in immunohistochemical studies.

5.4.5. Implications for biomarking.

Functional biomarker responses of exposure to environmental contaminants have used the induction of CYP isoforms in catalytic and immunochemical assays in many animals, such as fish, seals and cetaceans (Goksøyr *et al.*, 1989; Machala *et al.*, 1997; Troisi and Mason, 1997; White *et al.*, 1994; Whyte *et al.*, 2000). The use of antibodies raised against purified CYPs from other mammals or indeed fish could produce ambiguous cross-reactions/ non-specific cross-reactions and not reflect the true picture. Anti-peptide antibodies designed from seal CYP1A1 and -1A2 sequences have potential to be a very sensitive and universal biomarker of the expression of these isoforms in these species.

The sequencing of the CYP1A cDNAs from two seal species has provided the necessary data for future production of species-specific probes. This will enable the further development of CYP1A as a biomarker of environmental contaminant exposure (Peters *et al.*, 1999), and such probes have potential in the application of non-destructive biomarking for risk assessment of endangered seal species (Fossi and Marsili, 1997). Furthermore, seal amino acid sequence alignments presented here may provide information for the validity of species extrapolation.

From the phylogeny of the seal amino acid sequences, the closest relative to seal CYP1A1 and -1A2 isoforms are dog CYP1A isoforms. This indicates a potential surrogate species in which to test the effect of multiple contaminant exposure on the induction of these and potentially other CYP isoforms that are predominantly involved in xenobiotic metabolism/ detoxification. Moreover model substrates for detecting seal CYP1A induction could be determined from comparison with CYP1A sequences with amino acid residues that are known in other mammals to be involved in substrate

recognition and /or binding. Anti-peptide antibodies could aid in the characterisation of CYP-specific substrates for subsequent use as biomarkers (refer to Chapter six). This would enable interpretation of CYP activities from marine mammal tissues to be carried out with much greater confidence.

5.4.6. Summary

This chapter has presented the CYP1A1 and -1A2 cDNA and deduced protein sequence data of two seal species, harp and grey seals. The phylogenetic relationship was investigated with other vertebrate species and the secondary structure was examined. Implications of the presented sequence data for biomarking studies were also discussed. The following conclusions were made.

- The CYP1A1 and -1A2 sequences were >99% identical with the respective sequence from the two seal species, and were >70% and >60% identical with other mammalian CYP1A1 and -1A2 sequences, respectively.
- Phylogenetic analysis of the seal CYP1A1 and -1A2 amino acid sequences with other vertebrate species revealed that dog CYP1A sequences were the closest related.
- Investigation of the secondary structure of the seal CYP1A1 and -1A2 demonstrated regions of conserved amino acids that were either CYP1A1 or -1A2 specific.
- The peptide sequence in seal CYP1A1 and -1A2 that corresponded with the antigenic peptide sequence used to produce the anti-trout CYP1A1 anti-peptide antibody, was 67.67% and 33.3% identical, respectively.

- The cDNA and derived protein sequences of the two seal species has provided data for future production of species-specific probes (refer to Chapter six). This will enable the further development of CYP1A isoenzymes as a biomarker of environmental contaminant exposure in seals.

6

Immunochemical detection of marine mammal CYP1A using antibodies against specifically selected synthetic peptides

6.1 Introduction

Cytochrome P450 isoenzymes have been utilised as biomarkers in response to environmental contaminant exposure in many marine animals, including mammals, as discussed in previous chapters. In particular CYP1A responds to certain aryl hydrocarbon receptor binding ligands such as PAHs, planar 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)-like PCBs and dioxins, through induction of the CYP1A gene and subsequent up-regulation of CYP1A isoenzymes (Whitlock *et al.*, 1996).

Assessment of CYP1A induction and expression has often occurred through catalytic reactions such as the deethylation of 7-ethoxyresorufin and hydroxylation of benzo(a)pyrene. However, this approach is limited due to the broad substrate specificity of the majority of CYP isoenzymes (Burke *et al.*, 1994). Also marked species differences in the specificity of such reactions have been demonstrated (Boobis *et al.*, 1990). Substrates suitable for a CYP in one mammalian species may not be appropriate for the study of the orthologous CYP in another species (Schulz *et al.*, 2001). For example, using substrates extensively characterised in rats would not necessarily be suitable for seals.

An alternative approach has been to assess CYP expression using CYP-specific antibodies by immunochemical detection, using immunoblots, immunohistochemistry and ELISAs. These immunochemical methods have also been applied to marine mammals (Goksøyr *et al.*, 1992; Hyyti *et al.*, 2001; Moore, 1998; Nyman *et al.*, 2000; Wolkers *et al.*, 1998b).

The antibodies used were raised from purified CYP isoenzymes from other vertebrate species including rat, dog, rabbit and fish species (Goksøyr, 1995; Mattson *et al.*, 1998; White *et al.*, 1994). However, the purification of CYPs is arduous and especially difficult from tissues with low CYP content. Purification of CYP isoenzymes from marine mammal tissues would therefore be difficult, particularly as the availability of a large quantity of fresh tissue is often limited. Furthermore pre-induction of particular CYP isoenzymes using inducers such as 3-methylcolanthrene (3-MC) and 2,3,7,8-TCDD poses ethical and logistical difficulties.

Another approach therefore has been to use synthetic peptides as antigens to produce antibodies that are specific to the target CYP isoenzyme (Edwards *et al.*, 1998; Myers *et al.*, 1993; Parimoo and Thomas, 2000; Shen and Strobel, 1995). In comparison to monoclonal and polyclonal antibodies, these anti-peptide antibodies are simple, effective and cheap to produce.

Multiple epitopes are available on the native purified CYP used as an antigen, which leads to a diverse antibody population in the antiserum (Edwards *et al.*, 1995). Although monoclonal antibodies bind to a single epitope, a similar epitope may be present on other CYP isoenzymes. This can lead to polyclonal and even monoclonal antibodies cross-reacting with related CYPs, other than the target apoprotein (Gelboin, 1993; Thomas *et al.*, 1986). Furthermore structurally related CYPs can be difficult to separate and therefore the antigen used for immunisation is, in fact, a mixture of CYP isoforms (Wang and Lu, 1997).

In contrast the epitope of an anti-peptide antibody is predetermined, hence antibodies can be directed toward a unique peptide of the target CYP isoenzyme (Edwards, 1998).

Indeed anti-peptide antibodies can recognise both native and denatured CYPs so would be suitable for a range of immunochemical methods (Edwards *et al.*, 1993; Edwards *et al.*, 1995).

The recent sequencing of three seal species CYP1A1 and -1A2 cDNA and deduction of the amino acid sequence (Chapter five; I. Teramitsu, personal communication) has provided a unique opportunity to produce antibodies specific to seal CYP1A isoenzymes. Other marine mammal cDNA have been recently isolated (Godard *et al.*, 2000; Teramitsu *et al.*, 2000), and provide further opportunities to produce an array of specific anti-peptide antibodies to marine mammal CYPs.

I therefore chose this approach to produce seal-specific reagents for the use in CYP expression studies. This chapter presents the selection, production and partial characterisation of two seal specific anti-peptide antibodies targeted towards unique peptides in seal CYP1A1 and -1A2.

6. 2. Materials and methods

6.2.1. Selection of antigenic peptides.

6.2.1.a. Antigenic peptide for 'mammalian' anti-peptide antibody. Nine mammalian CYP1A1 amino acid sequences were selected from the Directory of Cytochrome P450 containing Systems (URL: <http://www.icgeb.trieste.it/p450/>). Subsequently the mammalian CYP1A1 sequences were aligned on the Basic Local Alignment Search Tool (NCBI; URL: <http://www.ncbi.nlm.nih.gov/>). The peptide sequence of fourteen amino acids, GHIRDITDSLIEHC, was selected from a region was highly conserved in all nine species CYP1A1 (Fig. 6.1.a). In contrast, the corresponding peptide in CYP1A2 sequences from six mammalian species was not well conserved (Fig. 6.1.b).

DOG	PNPALDFFKD	LNKRFYSFMQ	KMVKEHYKTF	EKGQIRDVTD	SLIEHCQDKR
GUINEA PIG	PSATMDTFKD	LNRRFSVFIQ	KMIKEHYKTF	EKGHIRDITD	SLIEHCQDRK
SHEEP	PNTALDLFKD	LNRRFYVVFVQ	KIVKEHYKTF	EKGHIRDITD	SLIEHCQDKR
HUMAN	PNPSLNAFKD	LNEKFYSFMQ	KMVKEHYKTF	EKGHIRDITD	SLIEHCQEKQ
MAMMALIAN	-----	-----	-----	GHIRDITD	SLIEHC ----
MAQAQUE	PNRSLNGFKD	LNEKFHSFMQ	KMIKEHYKTF	EKGHIRDITD	SLIEHCQEKQ
MOUSE	PNSSLDAFKD	LNDKFYSFMK	KLIKEHYRTE	EKGHIRDITD	SLIEHCQDRK
RABBIT	PNPALDTFKD	LNERFYSFTQ	ERVKEHCRSE	EKGHIRDITD	SLIKHYRVDR
RAT	PNSSLDAFKD	LNKKFYSFMK	KLIKEHYRTE	EKGHIRDITD	SLIEHCQDRR
HAMSTER	PNSSLDAFKD	LNKKFYSFMQ	KSVKEHYRTE	EKGHIRDITD	SLIEHCQDKS

Fig. 6.1.a. Multiple alignment of several mammalian CYP1A1 amino acid sequences with the 'mammalian' peptide sequence (in bold) selected to produce anti-peptide antibodies. Area shaded in grey indicates the conserved amino acids.

Hamster	HYQDFNKNSI	QDITGALFKH	SENSKD.SGG
Dog	HYQDFDERSV	QDITGALLKH	NEKSSRASDG
Guinea Pig	HYQDFDKNHV	QDIASALFKH	SEESPHVNGD
Mouse	HYQDFNKNSI	QDITSALFKH	SENYKD.NGG
Human	HYQDFDKNSV	RDITGALFKH	SKKGPRASGN
Rat	HYQDFNKNSI	QDITGALFKH	SENYKD.NGG
Mammalian peptide GHI	RDITDSLIEH	C

Fig. 6.1.b. Multiple alignment of several mammalian CYP1A2 amino acid sequences with the mammalian peptide sequence (in bold). Area shaded in grey indicates the conserved amino acids.

A hydrophathy plot of the potential antigenic peptide was produced using the program, DNAMAN (Lynnon Biosoft, USA) and the method of Hopps and Wood (1981). A plot to determine the hydrophilicity of the peptide indicated the putative location of the peptides on the 3-dimensional structure of CYP proteins (Fig. 6.2). The more hydrophilic the peptide sequence, the greater the likelihood that the target peptide will be on the outer surface of the native protein, and therefore available for interaction with the anti-peptide antibody.

The peptide sequence was also mapped onto a model of the CYP protein, CYP2C5, in the program Protein Explorer (Eric Martz, 2001, URL: <http://www.proteinexplorer.org/>) to obtain further evidence for the location of the peptide on the surface of the 3D structure (Fig. 6.3.). CYP2C5 was used for the 'mapping' as no mammalian CYP1A isoenzyme has, so far, been crystallised.

6.2.1.b. Antigenic peptide for anti-seal CYP1A anti-peptide antibody. The potentially antigenic peptide was selected from a conserved region of the partial amino acid sequence of harp seal CYP1A1 and -1A2, derived from the nucleotide sequences (Fig. 6.4) (see Chapter five, Fig. 5.1. for the partial CYP1A1 and -1A2 protein sequences).

A peptide of eleven amino acids long, TGRARQPRLSDc corresponding to residues 356-366 was highly conserved in both CYP1A subfamilies (Fig. 6.4) and was targeted as a potential antigenic peptide for the antibody. The hydrophilicity of the potential antigenic peptide sequence and its location on the native CYP2C5 protein was mapped (Figs. 6.2 and 6.3. respectively) as described in section 6.2.1.

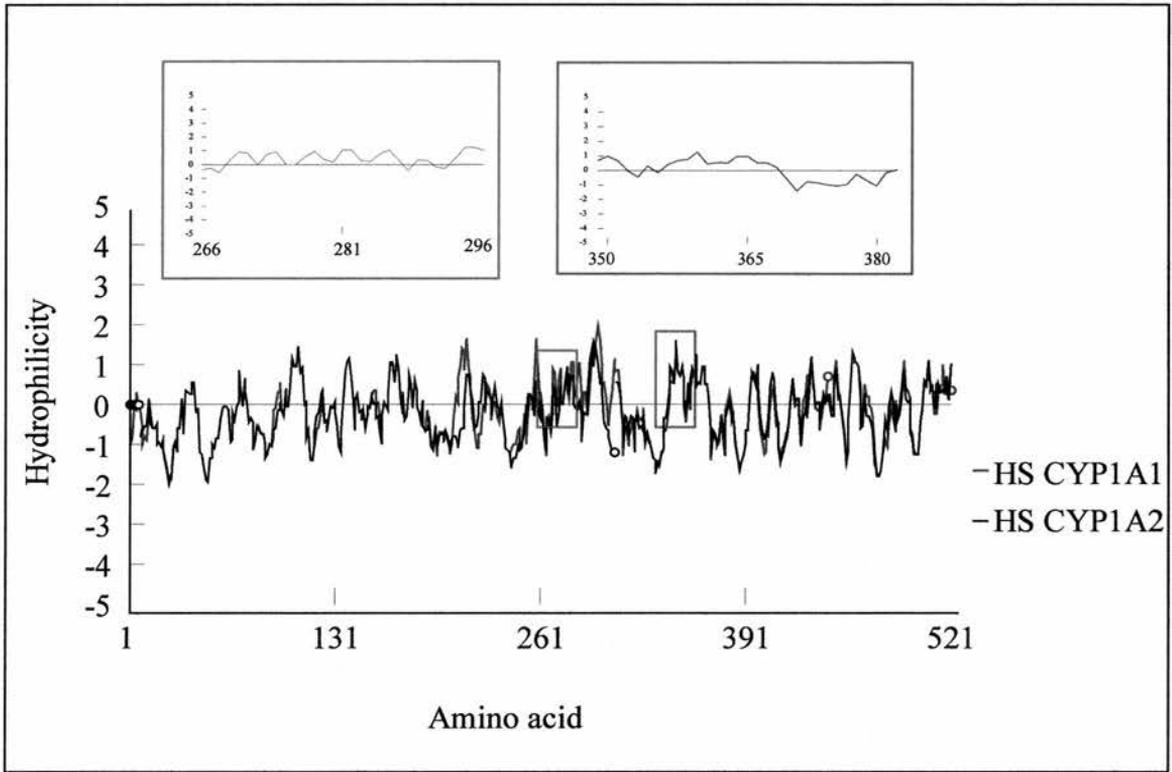


Fig. 6.2. Hydrophilicity plot of harp seal CYP1A1 and CYP1A2 derived protein sequences using the method of Hopps and Wood (1981) generated in DNAMAN. A value of 6 was chosen as the averaging group length for plotting the hydrophilic profile of the CYP1A sequences. The location of the two peptide sequences (A, 280-293, red box and B, 355-365, purple box) is demonstrated.

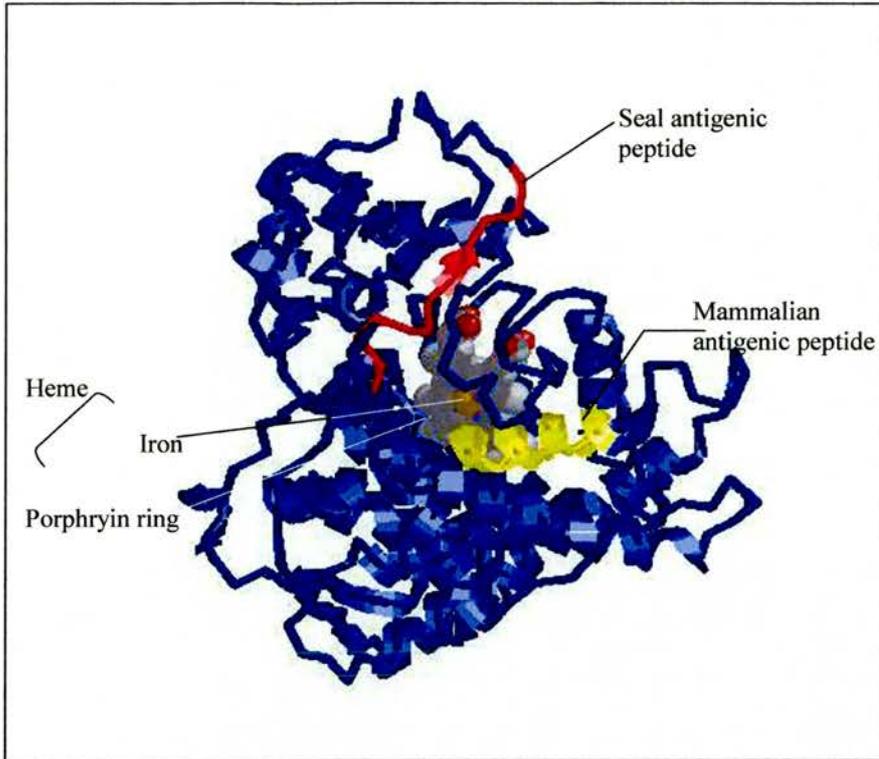


Fig. 6.3. A ribbon drawing of the model 3-dimensional structure of CYP2C5 from sheep. The location of the peptide sequences A and B for the anti-mammalian and anti-seal anti-peptide antibodies, respectively, are modelled. Mammalian antigenic peptide sequence, 277-291 on CYP2C5 (280-293 on seal CYP1A1 amino acid sequence) is indicated in yellow. Seal antigenic peptide sequence, 358-369 on CYP2C5 (355-365 on seal CYP1A1 amino acid sequence) is indicated in red.

Grey seal CYP1A1	...TVIGRARQPRLSDRPQL
Harp seal CYP1A1	...TVIGRARQPRLSDRPQL
Ribbon seal CYP1A1	...TVIGRARQPRLSDkPQL
Seal antigenic peptide	... TGRARQPRLSDc ...
Grey seal CYP1A2	...TVtGRARQPRLSDRPQL
Harp seal CYP1A2	...TVtGRARQPRLSDRPQL
Ribbon seal CYP1A2	...TVtGRARQPRLSDRPQL

Fig. 6.4. The region of a multiple alignment of seal CYP1A1 and CYP1A2 derived amino acid sequences with the selected antigenic seal peptide. The grey shading indicates identical sequence with the seal antigenic peptide. The blue shading shows the amino acid difference. The Cysteine residue indicated by a lower case 'c' enabled the conjugation of the peptide with the carrier protein, KLH.

6.2.2. Peptide synthesis, conjugation and immunisation.

Peptides were synthesised by Dr G. Kemp using procedures based on *N*- α -9-fluorenylmethoxycarbonyl (Fmoc)-polyamide chemistry (Atherton *et al.*, 1978) and purified by reverse-phase HPLC. MALDI-TOF mass spectrometry and N-terminal sequencing verified the peptides.

Subsequently the carrier protein, keyhole limpet haemocyanin (KLH) was conjugated to the cysteine at the C-terminus of the peptide. The naturally occurring cysteine present at the carboxyl end of the 'mammalian' peptide sequence was used for coupling to the carrier protein. However, the addition of a cysteine (c) to the C-terminus of the 'seal' peptide was required.

Diagnostics Scotland (Law Hospital, Scotland) were contracted to raise the antibodies against the antigenic peptides in sheep. The pre-immune serum was initially collected in both cases and three subsequent bleeds were obtained one week following either the initial immunisation (bleed one) or booster injections (bleeds two and three).

6.2.3. Purification of anti-peptide antisera.

Preparation of the peptide affinity column and purification of the anti-peptide antisera were performed using Activated thiol-Sepharose (Pharmacia, UK) according to the manufacturers instructions.

6.2.3.a. Preparation of the immobilised peptide affinity column. Activated thiol Sepharose (1g) was swelled in 200mL column buffer (50mM Tris, 0.5M NaCl, 1mM EDTA, pH 7.5) for 15 minutes at room temperature. The swollen resin was then collected on a sintered filter and washed with 100mL of buffer. Subsequently the resin was mixed for two hours with 5mL 1mg/mL of peptide in a 12mL tube. Following the

incubation, the resin was allowed to settle and the supernatant was removed. During the incubation, the peptide displaces the pyridine-2-thione and forms a disulphide bond with the Sepharose matrix. Pyridine-2-thione absorbs at 343nm, therefore measuring the absorbance of the supernatant at this wavelength, provides an indication of the coupling efficiency of the peptide and the matrix.

6.2.3.b. Affinity purification of the antiserum. The resin slurry (1.5x volume of resin) was packed under gravity into a column (8cm x 1cm, Omnifit, California, USA) and was washed with 10 column volumes of buffer. Subsequently 20mL antiserum was applied to the column and pumped through using a peristaltic pump (MD1 2086, Edmund Bühler, Tübingen). The column was then washed with a further 10 column volumes of buffer. Fractions of the wash material were collected until the absorbance at 280nm was <0.1 a.u.

The anti-peptide antibody was subsequently eluted from the column with 0.1M glycine-HCl, pH 2.5 and fractions were collected into tubes containing 0.5mL 1M Tris to ensure the pH of the eluted antibody solution was between pH 5 and 9. The absorbance at 280nm of each fraction was then measured on a U-1100 spectrophotometer (Hitachi LTD, Japan).

The eluted fractions were analysed on 15% polyacrylamide gels by SDS-PAGE (Laemmli, 1970). 15µL of each fraction was solubilised with reducing solution (Chapter two, 2.2.6.a) and boiled for 2 minutes. Protein bands were visualised by Coomassie staining (Chapter two, 2.2.6.b). Fractions containing the purified anti-peptide antibody were pooled, aliquoted and stored at -20°C. The purification of the mammalian CYP1A

anti-peptide antibody was performed by J. Archer during her honours project, 2001, and I subsequently purified the seal CYP1A anti-peptide antibody.

6.2.4. Assessment of the anti-peptide antibodies.

Solutions. Phosphate/citrate buffer: 25mM citric acid and 50mM disodium hydrogen phosphate. Peroxidase substrate: 7.4mM o-phenylenediamine in phosphate/citrate buffer, with the addition of 0.1% 30% H₂O₂ immediately before use.

6.2.4.a. Enzyme-linked immunosorbent assay (ELISA). ELISAs were performed in 96 well, Dynatek multiwell plates (Dynatek, Guernsey Channel Islands, UK). Initially 100µL 1% glutaraldehyde in PBS was applied to each well and placed at 4°C, overnight. The aldehyde groups readily formed bonds with the amine groups in the peptide enabling uniform binding of the peptides to the plate. The glutaraldehyde was removed by aspiration and the wells were washed once with 200µL PBS. Subsequently 50µL 10µg/mL of the antigenic peptide, or an unrelated peptide (see results for peptide sequence) were applied to the wells and incubated over-night at 4°C.

After the incubation, the excess peptides were removed by aspiration and the plates were dried at 37°C for 20 minutes. Free sites on the plastic of the wells were then blocked with 300µL 10% (w/v) non-fat milk powder in PBS at 37°C for 3 hours. The blocking solution was removed by aspiration and the wells were washed with 300µL 1% (w/v) non-fat milk powder in PBS.

A doubling dilution of the anti-peptide antibody of interest was performed in a separate plate and a final volume of 50µL was transferred to the peptide plate. The plate was then incubated for one hour at room temperature. Following the binding of the primary

antibody, the excess solution was removed by aspiration and the wells were washed three times with 100 μ L 1% non-fat milk/PBS solution. The secondary antibody was an enzyme linked goat anti-sheep horseradish peroxidase and 50 μ L was applied to each well at 1/500 dilution. Again the plate was incubated for one hour at room temperature. Subsequently the secondary antibody was removed by aspiration and the wells were washed three times with 100 μ L 1% non-fat milk/PBS and twice with phosphate/citrate buffer. The binding of the primary antibody to the antigen was visualised by applying 100 μ L peroxidase substrate to each well and incubating for 30 minutes in the dark, at room temperature. The absorbance of each reaction was measured at 492nm on a Dynatek MR 5000 plate reader (Guernsey Channel Islands, UK).

6.2.4.b. Immunochemical detection of seal liver microsomes. Seal liver microsomes (species indicated in legends of Figs. 6.6, 6.7, 6.10 – 6.12) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred on to PVDF membranes as described in Chapter two (section 2.2.6).

Multi-screening. The optimum anti-peptide antibody concentration was determined using a multiscreen apparatus (Mini-Protean II, Bio-Rad, California). At the end of the transfer procedure the PVDF membranes were blocked in blocking buffer (PBS, 5% (w/v) non-fat milk powder, 0.1% Tween-20) for ten minutes on a rotating platform. Subsequently the membrane was clamped between the multiple wells of the multiscreen and the base of the apparatus to form a tight seal.

The best anti-peptide antisera, determined by ELISA of both antibodies and the purified mammalian CYP1A anti-peptide antibody were diluted from 1/200 to 1/25,000 and the purified seal CYP1A anti-peptide antibody was diluted from 1/10 to 1/1000.

Subsequently 0.5mL of each dilution was applied to the respective well of the multiscreen. The PVDF membrane was incubated with the primary antibody for 30 minutes at room temperature on a rotating platform. The excess primary antibody solutions were removed by pipette and the wells were washed three times with blocking buffer to avoid cross-contamination when the clamp was removed. Subsequently the non-specifically bound primary antibody was washed for ten minutes with 2x 20mL blocking buffer on a rotating platform. The membrane was rinsed and transferred into clean containers between each wash. Incubation with the secondary antibody (goat anti-sheep horseradish peroxidase) and visualisation of the immunoreactive proteins were conducted as described in Chapter two (section 2.2.6.d).

Detection of CYP1A. Immunochemical detection of CYP1A immunoreactive proteins in seal liver microsomes was conducted as described in Chapter two (section 2.2.6), with the following modifications. Mammalian CYP1A anti-peptide antibody was diluted to, either 1/7500 (antisera) or 1/500 (purified) and the seal CYP1A anti-peptide antibody (purified) was diluted to 1/30. The secondary antibody used for both anti-peptide antibodies was a goat anti-sheep horseradish peroxidase at 1/5000 dilution.

6.2.5. Immunoprecipitation of CYP1A apoprotein with the mammalian CYP1A anti-peptide antibody.

Materials and solutions. Protein G, dimethylpimelimidate (DMP), ethanolamine, DTT and siliconised microfuge tubes were purchased from Sigma Chemicals, UK. The protease inhibitor, 4-(2-aminoethyl)benzenesulphonyl fluoride) (AEBSF) was obtained from Calbiochem, California, and the EDTA was purchased from BDH, UK. The RIPA buffer comprised of 25mM Tris-HCL, pH 8.8, 50mM NaCl, 0.5% Nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS and immediately before use the final concentrations of

the following reagents were added 0.1% BSA, 1mM AEBSF, 2mM DTT and 2mM EDTA. The protein G slurry (PGS) contained 50% protein G slurry in 25mM Tris-HCl, pH 8.1, 144mM NaCl.

6.2.5.a. Cross-linking of the antibody to protein G beads. Cross-linking of the antibody, or pre-immune serum to protein G-4% agarose beads was performed using a modified version of an immunoprecipitation method (<http://cbms.st-and.ac.uk/staff/Hay/ronweb/methods/immunoprecipitation.html>). Briefly, 1.5mL of resuspended lyophilised protein G attached to 4% agarose was washed 4x in PBS to remove the azide. The concentrations of the antibody and pre-immune serum preparations were determined by measuring the absorbance of the solutions at 280nm against PBS and were calculated based on the absorbance of a 1mg/mL solution of IgG approximately 1.35 a.u. (Harlow and Lane, 1988).

A volume of antibody or pre-immune serum was then added to the protein G beads at a final concentration of 2mg antibody/mL beads. The volume was made up to 20mL with PBS and the mixture was rotated for 30 minutes at room temperature. Subsequently the beads were gently centrifuged (Rotor Nr. S4180, Allegra™ 21R Centrifuge, Beckman) at 1krpm for 5 minutes, prior to 5 washes in 15mL PBS. The beads were also centrifuged at 1krpm for 5 minutes between each wash. Antibodies that were bound by non-covalent bonds to protein G were removed by washing 5 times in 15mL 100mM glycine followed by washing 5 times in 15mL 3M NaCl, 50mM boric acid, pH 9.0.

The mammalian CYP1A anti-peptide antibody or other IgG in the pre-immune serum was then cross-linked to protein G through amino groups using DMP. The beads were mixed with 25mL of freshly prepared 3M NaCl, 0.2M boric acid and 40mM DMP, pH

9.0 and incubated at room temperature for 45 minutes. Ethanolamine neutralises DMP, thus any antibodies that were not cross-linked were removed with 15mL 0.2M ethanolamine, pH 8.0 at room temperature for 1 hour and subsequently over-night in fresh volume of 0.2M ethanolamine, pH 8.0 at 4°C. Antibody and pre-immune serum cross-linked protein G beads were stored in 0.1% azide in PBS at 4°C.

6.2.5.b. Immunoprecipitation with seal liver microsomal protein. Immunoprecipitation of liver microsomal CYP1A with the mammalian CYP1A anti-peptide antibody cross-linked to protein G beads was performed in siliconised microfuge tubes, to avoid loss of beads due to adhesion to the plastic. Initially 2x 500µL thawed seal liver microsomes were each lysed with 500µL RIPA buffer and incubated on ice for one hour. Insoluble material was pelleted by centrifugation for one hour at 105,000g and the supernatant was transferred to a clean microfuge tube. The lysates were pre-cleared with 40µL PGS for 30 minutes at 4°C. This removed any proteins in the lysates that would non-specifically bind to the immune complexes or the solid phase matrix. The beads were then sedimented by gentle centrifugation in a tabletop microcentrifuge (1krpm for 5 minutes) and the supernatant transferred to clean microfuge tubes.

The immune complex was formed between either 60µL antibody – Protein G beads or 60µL pre-immune serum-protein G beads, and the pre-cleared lysates and were rotated for two hours at 4°C, prior to centrifugation (1krpm for 5 minutes). The bead pellet was initially washed in 1mL ice-cold RIPA buffer and subsequently 3 times with 1mL RIPA buffer without BSA. The slurry was centrifuged (1krpm for 5 minutes) between each wash. After the final wash, the beads were aspirated to dryness using a flat-ended electrophoresis tip (Cat. Nr. LW1100, Alpha Laboratories LTD, UK) attached to the aspirator. The immune-complexed beads were then solubilised in reducing solution

(Chapter two, 2.2.6.b), boiled for 2 minutes and centrifuged at 13krpm for 30 seconds to pellet the beads. The proteins in the supernatant were separated by SDS-PAGE (Laemmli, 1970) on 15% polyacrylamide gel (Chapter two, 2.2.6.a), followed by analysis by MALDI-TOF mass spectrometry performed by Dr C. Botting, Centre for Biomolecular Sciences, University of St Andrews, UK.

6.2.5.c. Silver staining. Solutions. Fixing solution comprised of 10% acetic acid and 30% ethanol. Silver solution consisted of 0.2% silver nitrate and 1mM 40% formaldehyde. Developing solution contained 6% sodium carbonate, 6mM 40% formaldehyde, and 20 μ M sodium dithionite.

Method. The separated proteins were visualised by silver staining of the polyacrylamide gels, according to the method of Rabilloud (1988). Silver staining is 10x more sensitive than Coomassie staining, and therefore would more likely to detect any potential CYP1A proteins. The polyacrylamide gels were initially fixed for 3x 5 minutes in fixing solution, followed by 3x 5 minute washes with 30% ethanol and 2x 5 minute washes with distilled water, on a rotating platform. The proteins were subsequently reduced in 0.25mg/mL sodium dithionite for 1 minute. The excess dithionite was removed by 2x 1 minute washes in distilled water, prior to staining the gel for 5 minutes in the silver solution. The gel was then rinsed 3x in distilled water for 20 seconds and developed for 2x 5 minutes in developing solution. Glacial acetic acid (3.5%) was added to the developing solution to stop the reaction and placed on the rotating platform for 20 minutes. Finally the gel was rinsed 4x for 5 minutes with distilled water and stored in 20% ethanol. Silver stained protein bands present in the mammalian CYP1A anti-peptide antibody preparation were excised and identified by MALDI-TOF mass spectrometry.

6.2.6. Expression of recombinant harp seal CYP1A1 protein

The expression of recombinant harp seal CYP1A1 was performed in *Escherichia coli* and since CYP isoforms in *E.coli* are found in the soluble fraction, a signal sequence was required that would target the expressed protein to the bacterial membrane. This would enable the protein to fold into the correct 3-dimensional conformation and incorporate the heme required for the protein to be optically and catalytically viable.

6.2.6.a. Isolation of genomic DNA from *E.coli* (JM109). Isolation of genomic DNA was performed using the modified method of Birnboim (1983). Briefly, 10mL overnight cultures were grown at 37°C from a single *E.coli* colony in Luria Bertani medium (Chapter five) and 1.5mL were aliquoted into a microfuge tube and pelleted for 1 minute at 13krpm (table top centrifuge). The supernatant was removed and another 1.5mL of the overnight culture was added to the microfuge tube, the suspension was again pelleted and the supernatant was discarded.

The cells were resuspended in 100µL solution I (25mM Tris, pH 8.0 containing 5mM sucrose, 10mM EDTA and 100µg RNase solution) and subsequently lysed in 200µL solution II (0.2M NaOH, 1% (w/v) SDS) and mixed completely by inversion. After five minutes at room temperature, 150µL solution III (3M sodium acetate, pH 4.8) was added and mixed gently and thoroughly by inversion, prior to incubation on ice for 20 minutes. A white protein/SDS clot formed and was pelleted by centrifugation at 13krpm for 5 minutes. The supernatant was subsequently transferred into a clean microfuge tube and 1mL 95% ethanol was added. The suspension was mixed well by inversion and placed upright at -20°C for 20 minutes to aid in the precipitation of the genomic DNA.

The genomic DNA was sedimented by centrifugation at 13krpm for 15 minutes. The supernatant was carefully removed by pipetting. The DNA was washed in 0.5mL 80% ethanol, prior to the replacement of the 80% ethanol with 95% ethanol and subsequent desiccation in a speed vac (Gyrovap, GT, Howe) for 30 minutes at 25°C. The DNA was resuspended by inversion in 100µL TE buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA).

6.2.6.b. Isolation of the signal leader sequence of the *ompA* gene. The signal leader sequence of the *ompA* gene from *E.coli* was initially isolated from genomic DNA by PCR. The signal leader sequence of the *ompA* cDNA (Accession number V00307, GenBank, NCBI) corresponds with the first 63 nucleotides (5'-3'). A sense primer (*ompAF*) was designed that incorporated the 7 base pair Shine-Dalgarno element (underlined), three nucleotides 5' of the initiation codon (ATG) within the *NdeI*(^v) restriction site and the first 21 nucleotides of the *ompA* signal sequence (GGAATTCCA^vTATGAAAAGACAGCTA TCGCG; T_m = 60°C). The antisense (*ompA1A1L*) contained the last 21 nucleotides of the signal sequence and the first 21 nucleotides (underlined) of the harp seal CYP1A1 cDNA (TTCGCTAC CGTAGCGCAGGCTATGTTCTCTGCGTCCAGATTG; T_m = 71°C).

PCR was performed as described in Chapter five, section 5.2.3.c. with the addition of 0.8µM sense and antisense primers, *ompAF* and *ompA1A1L*, and 4ng *E.coli* genomic DNA as the template. The elongation step of the PCR thermocycles was reduced to 1 minute at 72°C, as the amplified signal sequence was <100 nucleotides long. The resultant PCR product was the 63 base *ompA* leader sequence with the bases required for expression and a linker sequence for the subsequent attachment to the full harp seal CYP1A1 cDNA sequence. The PCR product was visualised on 2% agarose gel and extracted from the gel as described in Chapter five, section 5.2.4. The subsequent product was stored at -20°C until required.

6.2.6.c. Attachment of the ompA leader sequence to the harp seal CYP1A1 cDNA.

The *ompA* leader sequence + CYP1A1 linker was attached to the 5' end of the harp seal CYP1A1 cDNA using the method of Pritchard and colleagues (1997). A new antisense primer (CYP1A8Rsal1) to the 3' end of the CYP1A1 cDNA was designed to incorporate the unique restriction site *SalI* into the stop codon (underlined) (5'-GAGCAC GTCCAAGTGCGGGTACGTGCTTAG^VTCGAC-3'; $T_m = 70^\circ\text{C}$). Briefly, PCR was performed as described in Chapter five, section 5.2.3.c. with the following modifications. In a final reaction volume of 50 μL , 0.8 μM sense primer (ompAF) and 0.8 μM antisense primer (CYP1A8Rsal1) were added with 2 μL *ompA* leader sequence + CYP1A1 linker (from previous PCR, section 6.2.6.b) and 1.25 μL harp seal CYP1A1 cDNA template in pGEM[®]-T easy vector (Chapter five). The attachment of the leader sequence and the incorporation of the two unique restriction enzyme sites were confirmed by inserting the PCR product into pGEM[®]-T easy, prior to sequencing of the clones (Chapter five, section 5.2.5.h).

6.2.6.d. Extraction, ligation and transformation of the PCR product.

Extraction of the PCR product (*ompA* leader sequence + CYP1A1) was performed as described in Chapter five, section 5.2.4. A subsequent double restriction enzyme digest was performed on the PCR product and the vector, pCW ori+, a kind gift from Dr E. Gillam (University of Queensland, Australia). Briefly, the reaction was prepared in a clean 0.5mL nuclease-free microfuge tube placed on ice containing 2 μL 10x buffer D (Promega, UK), 0.2 μL 0.1 $\mu\text{g}/\mu\text{L}$ acetylated BSA, either 2 μL vector or 2 μL CYP1A1+ leader sequence, 0.5 μL 10u/ μL *NdeI* and 0.5 μL 10u/ μL *SalI* and nuclease-free water to a final volume of 20 μL . The reaction was mixed by pipetting and briefly centrifuged to collect the contents at the bottom of the tube, prior to the incubation at 37 $^\circ\text{C}$ for 4 hours. The digestion was analysed on 0.9% agarose gel containing 0.05% ethidium bromide

and the digested vector and CYP1A1/leader sequence were gel extracted. Subsequently the CYP1A1/leader sequence was ligated into the digested vector at a 1:1 and 3:1 ratio, transformed into *E.coli*, (JM109) competent cells and over night cultures were prepared using the procedures in Chapter five, section 5.2.5.b – f, inclusive.

6.2.6.e. Expression of the recombinant harp seal CYP1A1 protein.

Materials and solutions. In addition to those listed in Chapter five, section 5.2.5. δ -aminoalevulinic acid (δ -ALA) and thiamine were purchased from Sigma Chemicals, UK and AEBSF was supplied by Calbiochem, California.

Terrific broth (TB) medium contained 1.2% (w/v) Bacto[®]-Tryptone, 2.4% (w/v) Bacto[®]-yeast extract, 2% (w/v) Bactone[®]-Peptone and 0.8% (v/v) 50% glycerol in a final volume of 450mL with distilled water. The medium was autoclaved and 50mL of a sterile 10x phosphate solution (0.17M KH₂PO₄, 0.72M K₂HPO₄, pH 7.4), 0.5mL 50mg/mL ampicillin and 0.5mL 1M thiamine were added immediately before use.

TES buffer contained 100mM Tris-acetate, pH 7.6, 500mM sucrose, 1mM EDTA. KMDG buffer contained 100mM potassium phosphate, pH 7.4, 6mM magnesium acetate, 0.1mM DTT, 20% (v/v) glycerol. TGE buffer contained 100mM Tris-acetate, pH 7.4, 1mM EDTA, 20% (v/v) glycerol.

6.2.6.e(i). Growth of E.coli and subsequent expression of recombinant CYP1A1. The expression and subsequent isolation of *E.coli* membranes was performed according to the procedure of Jenkins and colleagues (1998). Briefly, 5mL of an overnight culture of *E.coli* JM109 competent cells with the pCWori+ vector containing the CYP1A1/leader cDNA was added to 500mL TB. The cells were grown in a shaker incubator until the absorbance at 600nm was between 0.4 and 0.7. Subsequently the cells were induced to

express the recombinant protein by the addition of IPTG to a final concentration of 1mM. Simultaneously, δ -ALA was added to a final concentration of 0.5mM. δ -ALA is a heme precursor and may help CYP expression by enhancing heme biosynthesis. The induced cells were then incubated for 24h, 48h and 72h at 30°C in a shaker. Pre-induced cells were grown at similar temperatures in order to act as a control.

6.2.6.e.(ii).Preparation of spheroplasts and isolation of *E.coli* membranes. The cell cultures were equally divided into 1L centrifuge containers and subjected to centrifugation (Beckman J2-21, Rotor JA14) at 2000g for 15 minutes at 4°C. The supernatant was discarded and the wet cell mass was determined. The cells were thoroughly resuspended in 2-3mL/g cold TES buffer. Subsequently the suspension was diluted with TES buffer to 10mL/g wet cell mass. The cells were lysed with the addition of lysozyme to 0.5mg/mL at 4°C, whilst stirring moderately with a magnetic stirrer. An equal volume of ice cold 0.1mM EDTA, pH 8.0 was slowly added and the cells were continually stirred for 30 minutes at 4°C. The resultant spheroplasts were pelleted at 5000g (Beckman centrifuge, J2-21. Rotor JA-14) for 15 minutes at 4°C and the supernatant was discarded.

The spheroplasts were resuspended in 2mL ice cold KMDG buffer/g spheroplasts using a teflon pestle and electronic pipetting and then transferred into 50mL polypropylene conical tubes in a salt/ice bath. The protease inhibitor, AEBSF, was added to 0.1mM and the suspension was sonicated at 50% power for 6x 20s bursts, placing in the salt/ice bath between bursts. Subsequently the suspensions were centrifuged at 1200g (Allegra™ 21R Centrifuge, Beckman) for 15 minutes to remove cell debris, prior to centrifugation of the supernatant at 150,000g (Beckman TL-100) for 60 minutes at 4°C.

The supernatant was discarded and the membranes were resuspended in one volume of ice cold TGE buffer, prior to storage at -70°C .

Isolated *E.coli* membrane proteins were separated by SDS-PAGE and subsequent staining of the proteins was by Coomassie staining (Chapter two, section 2.2.6). Identification of the recombinant CYP1A1 apoprotein was confirmed by MALDI-TOF mass spectrometry performed by Dr. C. Botting, Centre for Biomolecular Sciences, University of St Andrews, UK.

6.3. Results

6.3.1. Mammalian CYP1A1(2) anti-peptide antibody

6.3.1.a. Selection of antigenic peptide. A multiple alignment of CYP1A1 sequences from nine different mammalian species was performed and a highly conserved peptide sequence, GHIRDITDSLIEHC was selected (Fig. 6.1.a). This fourteen amino acid long peptide corresponded with residues 280 – 293 in Dog CYP1A1 protein. The mammalian CYP1A1 peptide sequence demonstrated only 4/14 amino acid identity with six mammalian CYP1A2 protein sequences at the corresponding peptide position (Fig. 6.1.b). The specificity of the selected peptide was determined by comparison with other CYP proteins that may be present in liver microsomal fraction and also unrelated proteins using the BLAST program. Identity was only observed with orthologous CYP1A sequences in other species.

A hydrophilic plot was originally obtained for all nine mammalian CYP1A1 protein sequences as described in materials and methods (Data not shown). The mammalian CYP1A1 peptide sequence is presented on the hydrophilic plot of harp seal CYP1A derived amino acid sequences. (These sequences were isolated and identified after the

design and preparation of this mammalian anti-peptide antibody). The mammalian CYP1A1 peptide was relatively hydrophilic within the CYP1A1 sequences as demonstrated in Fig. 6.2. Furthermore the peptide sequence was mapped onto a model of sheep CYP2C5 (Fig. 6.3). The peptide appeared to be located on the surface of the protein, increasing the likelihood that the anti-peptide antibody will react with the native protein. This is more important for immunochemical techniques, such as immunoprecipitation and immunohistochemistry, when the protein remains in its native conformation.

6.3.1.b. Binding specificity of the mammalian CYP1A1(2) anti-peptide antibody to the antigen as determined by ELISA. Enzyme – linked immunosorbent assays (ELISAs) were performed with the pre-immune serum and all three antisera bleeds, detecting either the antigenic peptide (without carrier protein) or an unrelated peptide that was also conserved with CYP1A1/2 protein sequence (TGRARQPRLSDc). A good reaction with the antigenic peptide was observed with increasing affinity from bleed one to three antisera (Fig. 6.5.a). The pre-immune serum did not recognise the antigen and none of the three antisera bleeds recognised the ‘unrelated’ peptide (data not shown). Bleed two and three antisera produced similar titration curves and subsequently bleed two antiserum was purified by immobilised peptide affinity chromatography. The resulting purified antibody demonstrated a good binding specificity to the antigenic peptide with a 50% binding at approximately 1/2000 dilution (Fig. 6.5.b).

6.3.1.c. Immunoblotting

Multiscreen. The mammalian CYP1A1(2) anti-peptide antiserum (bleed two) and the purified antibody were screened for binding specificity with harp seal (*Phoca groenlandica*) liver microsomal proteins (PgMa, b and c – see Chapter two, Table 2.1),

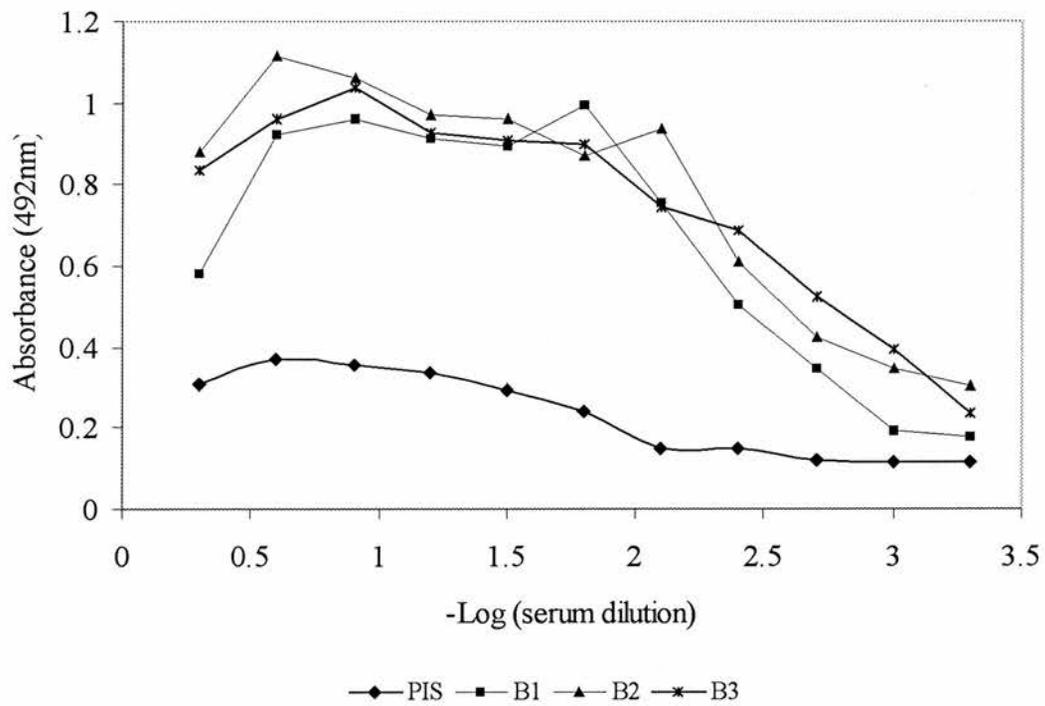


Fig. 6.5.a. Titration curves of the three antisera of mammalian CYP1A anti-peptide antibodies and the pre-immune serum against the antigenic peptide, determined by ELISA. PIS, pre-immune serum, B1, B2 and B3 indicate the bleed antiserum number.

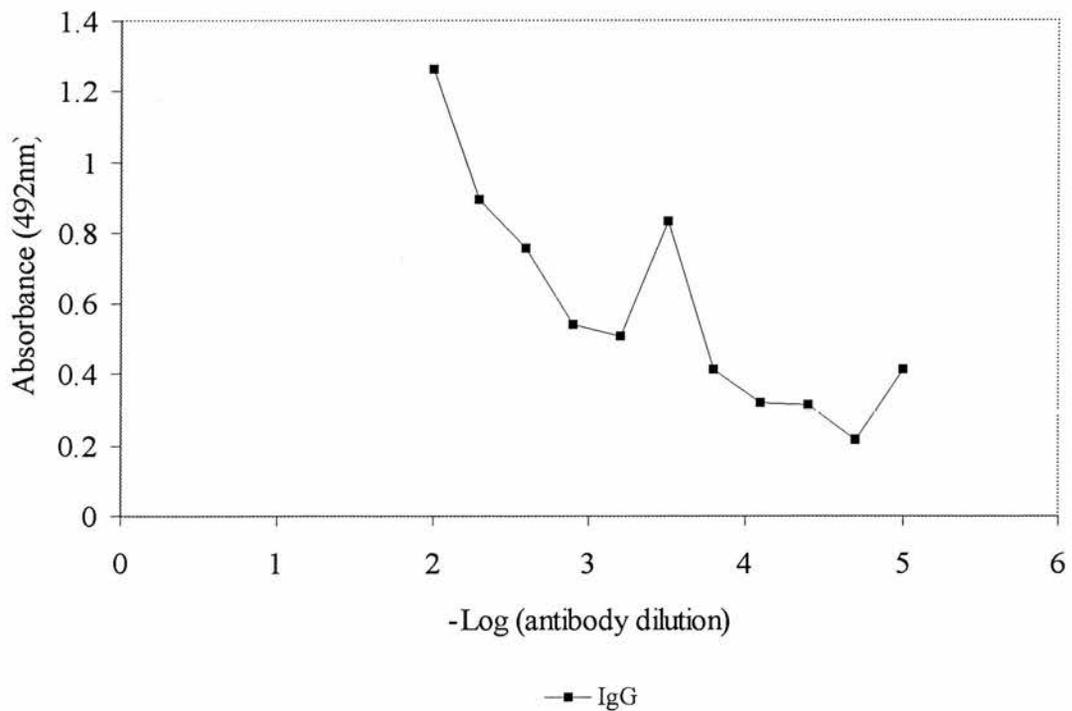


Fig. 6.5.b. Titration curve of the purified mammalian CYP1A anti-peptide antibody, determined by ELISA.

using immunochemical detection (Fig. 6.6). Both the antiserum and the purified antibody detected a distinct protein band with a slightly higher electrophoretic mobility than the ~54kDa molecular weight standard.

A very prominent band was consistently detected at ~62kDa by all antiserum dilutions investigated and is thought to be non-specific binding. A protein band at ~52kDa was only observed with antiserum dilutions to 1/1000. Several lower molecular weight bands were observed with the purified antibody that were no longer seen at 1/1000 dilution.

Conventional Western blotting. Immunochemical detection of three harp seal liver microsomal samples were detected with either the mammalian CYP1A1(2) anti-peptide antiserum (bleed two) or purified antibody, pre-immune serum or anti-trout CYP1A1 anti-peptide antibody (Fig. 6.7).

The purified optimal dilution was approximately 10 fold more concentrated than the purified antibody, 1/750 compared to 1/7500, respectively. Several distinct protein bands were recognised by the antiserum, with a particularly strong reaction with a protein at approximately 62kDa, outside of the cytochrome P450 molecular weight range (Fig. 6.7.A). However, a protein at approximately 52kDa (the appropriate molecular weight) was detected in all three harp seal samples and sample PgMb showed a greater reaction with the antibody than the other two samples. Two sets of double bands were recognised by the purified antibody at 62kDa and 52kDa, in harp seal samples PgMb and PgMc (Fig.6.7.B). Only a single prominent band at 52kDa reacted with the antibody in harp seal PgMa.

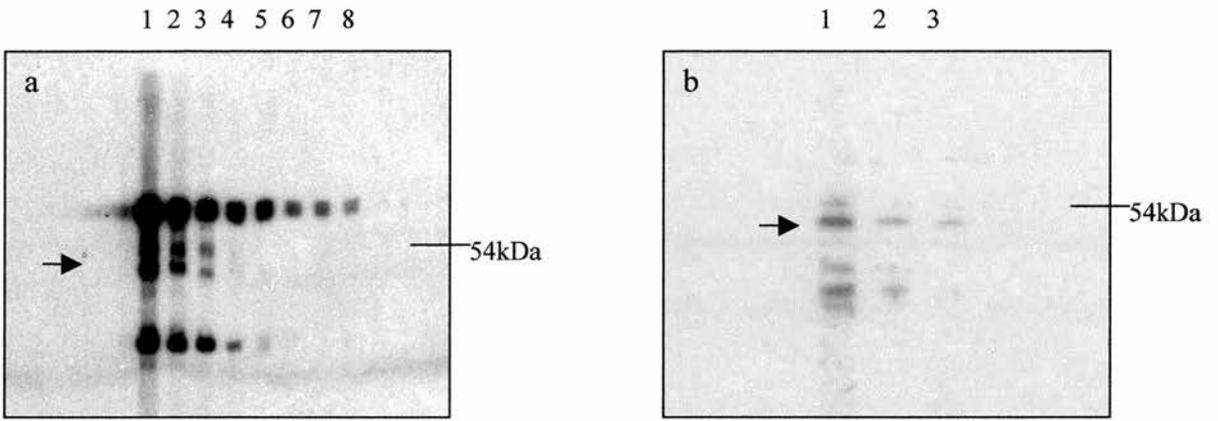


Fig. 6.6. Multiscreen of mammalian CYP1A1(2) anti-peptide antibody (a) antiserum bleed 3. 40 μ g of harp seal (PgMc) liver microsomes were applied per lane. Antiserum dilutions in order from lane 1 – 8 were 1/200, 1/500, 1/1000, 1/2500, 1/5000, 1/7500, 1/10000 and 1/12500. (b) purified antibody. 40 μ g of harp seal (PgMc) liver microsomes were applied per lane. Antibody dilutions in order from lane 1-3 were 1/200, 1/500 and 1/1000. The arrow indicates the potential CYP1A protein. Molecular weight marker position was determined by calibration of pre-stained molecular weight standards (see methods, section 6.2).

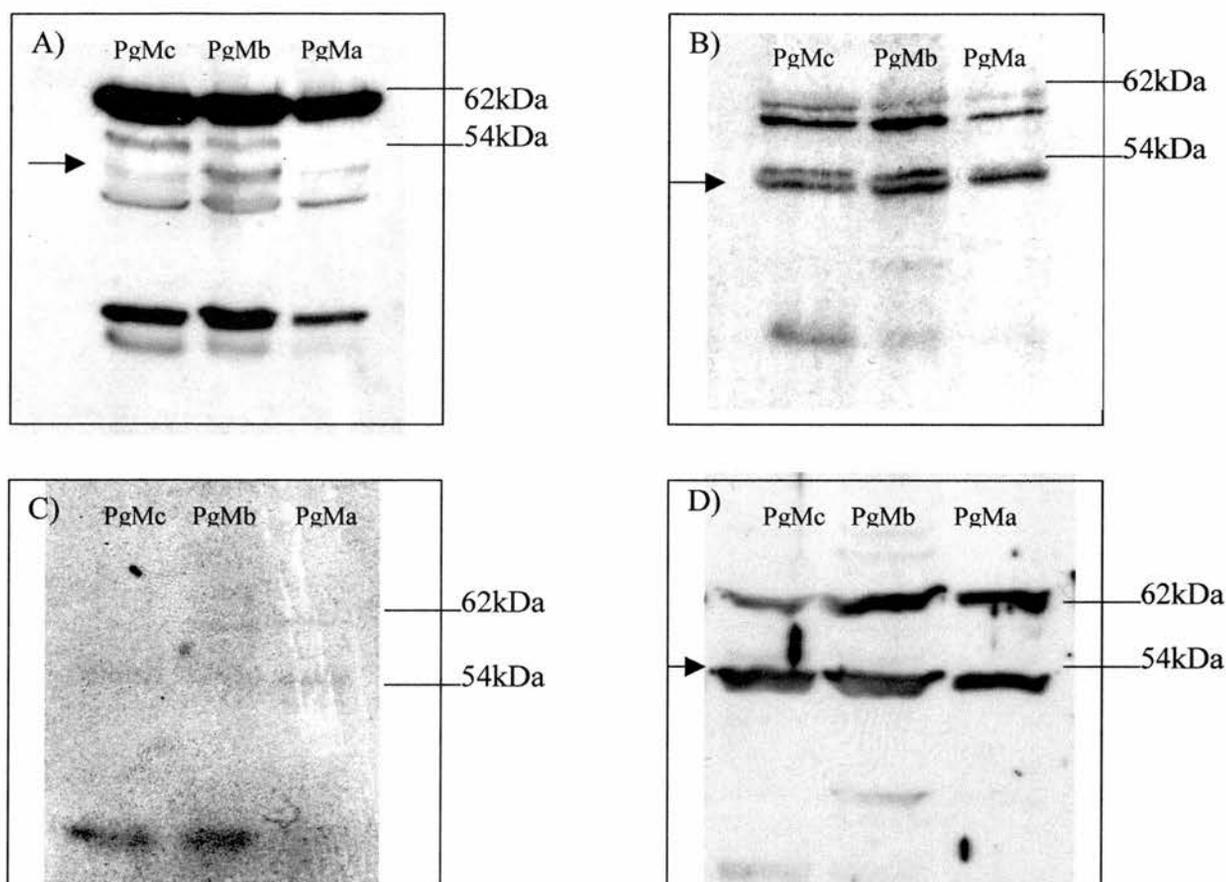


Fig. 6.7. Western blots of three harp seal liver microsomal samples (40 μ g in each lane). Protein bands were detected by A) anti-mammalian CYP1A antiserum (bleed 2), B) anti-mammalian purified CYP1A antibody (bleed 2), C) pre-immune serum, D) anti-trout CYP1A1 anti-peptide antibody. The molecular weights were derived from calibrated pre-stained molecular weight standards (see methods, section 6.2). The arrow indicates the potential CYP1A bands at approximately 52kDa.

Residue Number	Peptide Sequence
44 – 62	SPPGPWGWPLLGNVLTGK
75 – 98	YGDVLQIHIGSTPVLVLSGLDTR
179 – 193	LQEQMAEVGHFDPYR
348 – 358	IQEELDTVIGR
364 – 382	LSDRPQLPYLEAFIET

Table 6.1 Five CYP1A1 peptide fragments were identified by MALDI-TOF mass spectrometry of a ~52kDa protein precipitated by the mammalian CYP1A1(2) anti-peptide antibody during immunoprecipitation.

An incubation of the electrophoretically separated liver microsomes with pre-immune serum demonstrated no reaction even after one hour of exposure (Fig. 6.7.C). Another anti-peptide antibody, anti-trout CYP1A1, recognised two broad and distinct bands at 52kDa and 62kDa in all three harp seal samples (Fig. 6.7.D).

Immunoprecipitation of liver microsomal protein was performed with cross-linked purified mammalian CYP1A1(2) anti-peptide antibody, or pre-immune serum as a control (data not shown). Two distinct proteins were isolated from harp seal liver microsomes that were observed on silver stained SDS-PAGE. MALDI-TOF mass spectrometry analysis revealed that the protein at ~ 52kDa contained peptide sequences of CYP1A1 and the other protein at ~62kDa shared peptide sequences with bovine serum albumin. Table 6.1 presents the MALDI-TOF data for the CYP1A1 protein.

6.3.2. Seal CYP1A1/2 anti-peptide antibody

6.3.2.a. Selection of antigenic peptide. Partial CYP1A1 and -1A2 amino acid sequences were derived from sequenced harp seal cDNA (Chapter five). The two partial sequences were aligned and a highly conserved peptide sequence of eleven amino acids in length, TGRARQPRLSDc was selected (Fig. 6.4). An additional Cysteine (c) was attached to the C-terminus of the peptide sequence to enable conjugation of the carrier protein to the peptide.

The peptide corresponded to amino acid positions 355 – 365 in CYP1A1 and contained 10/11 amino acid identity with CYP1A1, where as 11/11 amino acids were identical to the corresponding peptide in CYP1A2 (346-356). The specificity of the peptide sequence was high since it was only found in orthologous CYP1A protein sequences from other mammalian and chicken species.

The peptide corresponded with a hydrophilic location on the 3-dimensional structure as determined by a hydrophilic plot of the partial sequences (not shown) and later the whole CYP1A1 and -1A2 sequences from harp seal (Fig. 6.2). Furthermore the peptide sequence appeared to be located on the surface of the model of sheep CYP2C5 (Fig. 6.3), providing a potentially good antigenic peptide.

6.3.2.b. Binding specificity of the seal CYP1A1/2 anti-peptide antibody to the antigen as determined by ELISA. ELISAs were performed with the pre-immune serum and all three antisera bleeds, detecting either the antigenic peptide (see section 6.3.2.a) or the unrelated mammalian CYP1A1(2) antigenic peptide (see section 6.3.1.a). A good reaction was observed with the antisera to the seal CYP1A1/2 antigenic peptide compared to the pre-immune serum (Fig. 6.8.a) and the reaction with the non-related peptide (data not shown). The binding specificity increased with subsequent booster immunisations, resulting in bleed three antiserum providing the greatest specificity, with a 50% binding occurring at approximately 1/3000 serum dilution. Subsequently bleed three antiserum was purified by immobilised peptide affinity chromatography and also recognised the antigenic peptide in an ELISA, with a 50% binding occurring at approximately 1/1000 antibody dilution (Fig. 6.8.b).

5.3.2.c. Immunochemical detection

Multiscreen. The seal CYP1A1/2 anti-peptide antiserum (bleed three) and purified antibody were screened for specificity to harp seal liver microsomes using immunochemical detection (Fig. 6.9). Five distinct proteins bands were detected with the seal CYP1A1/2 antiserum at 1/200 dilution, with one of the bands corresponding to ~52kDa (Fig. 6.9.a). No further dilutions detected any liver microsomal proteins.

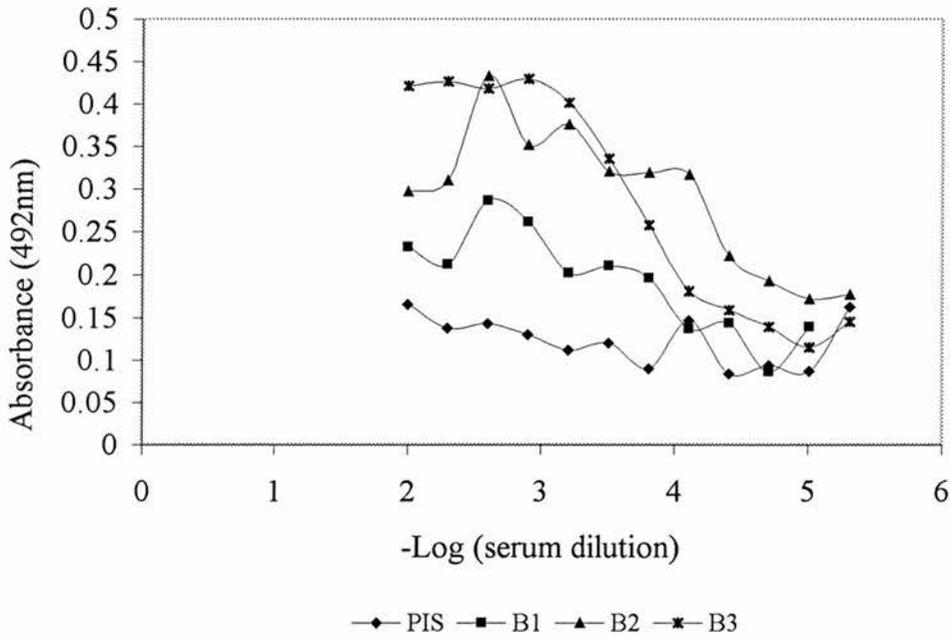


Fig. 6.8.a. Titration curves of the three antisera of seal CYP1A anti-peptide antibodies and the pre-immune serum against the antigenic peptide, determined by ELISA. PIS, pre-immune serum, B1, B2 and B3 indicate the bleed antiserum number.

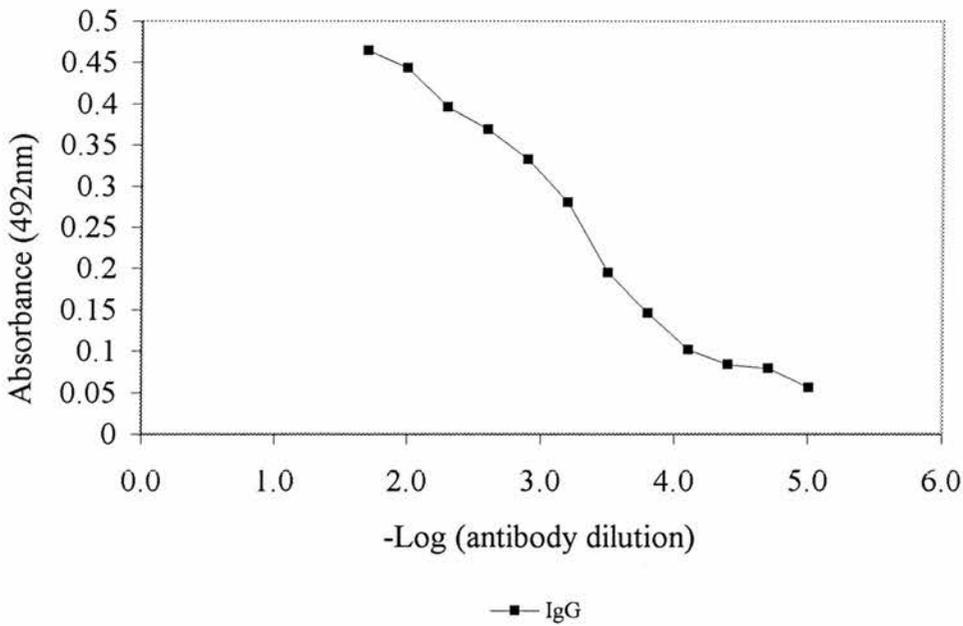


Fig. 6.8.b. Titration curve of purified (bleed 3 antiserum) seal CYP1A anti-peptide antibody, determined by ELISA.

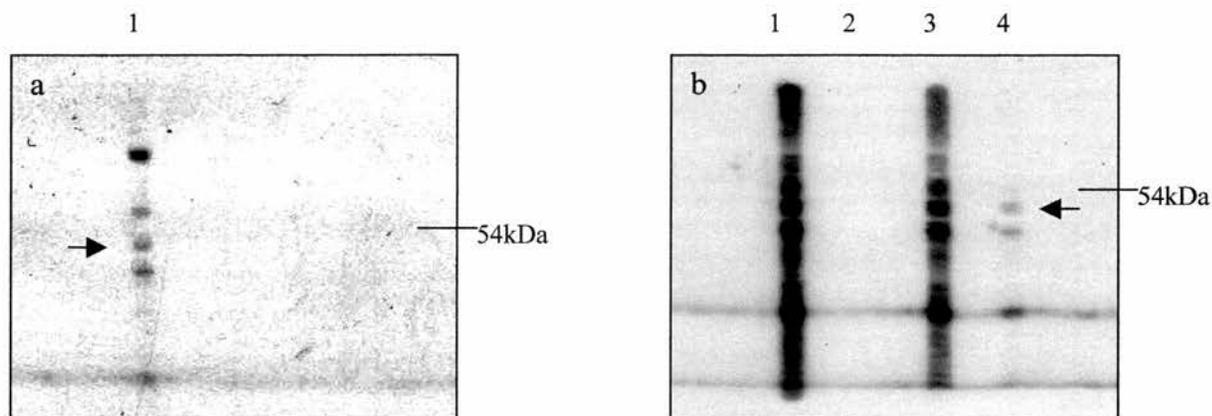


Fig. 6.9. Multiscreen of seal CYP1A1/2 anti-peptide antibody (a) antiserum bleed 3. 40 μ g of harp seal (PgMc) liver microsomes were applied per lane. Antiserum dilutions were the same for Fig. 6.6.a. Lane 1 antiserum dilution was 1/200. (b) purified antibody. 40 μ g of harp seal (PgMc) liver microsomes were applied per lane. Antibody dilutions in order from lane 1, 3 and 4 were 1/10, 1/20 and 1/50. Lane 2 was blank. The arrow indicates the potential CYP1A protein. Molecular weight marker position was determined by calibration of pre-stained molecular weight standards (see methods, section 6.2).

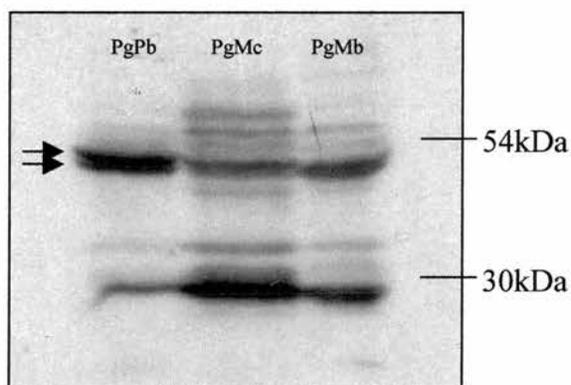


Fig. 6.10. Western blot of three harp seal liver microsomal samples (40 μ g in each lane) detected by purified seal CYP1A1/2 anti-peptide antibody at 1/30 dilution. The arrows indicate a double band that may be potential CYP1A proteins. Molecular weight marker position was determined by calibration of pre-stained molecular weight standards (see methods, section 6.2). PgP or M b or c refer to the harp seal liver microsomes separated by SDS-PAGE prior to Western blotting. For abbreviations see Table 2.1.

The purified seal CYP1A1/2 antibody recognised many protein bands at 1/10 and 1/20 dilution, however the binding specificity increased at an increased dilution of 1/50, detecting only three protein bands (Fig. 6.9.b). The highest molecular weight band detected corresponded to ~ 52kDa and was relatively broad. No further dilution of the purified antibody was able to detect any liver microsomal protein.

Conventional Western blotting. Immunochemical detection of three harp seal liver microsomal samples was performed using only the purified seal CYP1A1/2 anti-peptide antibody at a dilution of 1/30 (Fig. 6.10). In two of the harp seal samples, a protein band at ~52kDa and another protein band with slightly higher electrophoretic mobility were detected with equal intensity by the anti-seal CYP1A1/2 antibody. A distinct protein <30kDa was also detected in the both samples. These three proteins were detected in the other harp seal sample, however a number of other bands of less intensity were also recognised by the antibody, along with a much greater intensity band <30kDa.

6.3.3. Immunochemical detection of other marine mammal CYPs. Immunochemical detection of liver microsomal samples from three hooded seals (*Cystophora cristata*) (CcMb, CcPb and CcP3 – see Chapter two, Table 2.1), a grey seal (Hg, Chapter three) and a harbour porpoise (Pp, Chapter three) was performed with both the mammalian CYP1A1(2) and seal CYP1A1/2 anti-peptide antibodies (Fig. 6.11). The harbour porpoise liver microsomes were produced from liver that had previously been stored in liquid nitrogen for 14 days therefore would be partially degraded. The anti-seal CYP1A1/2 antibody detected a distinct protein band at 52kDa in the five individuals. This band was also broad in the two seal species. Two less intense, although diffuse bands were detected in the harbour porpoise, which corresponded with the bands detected in the seals.

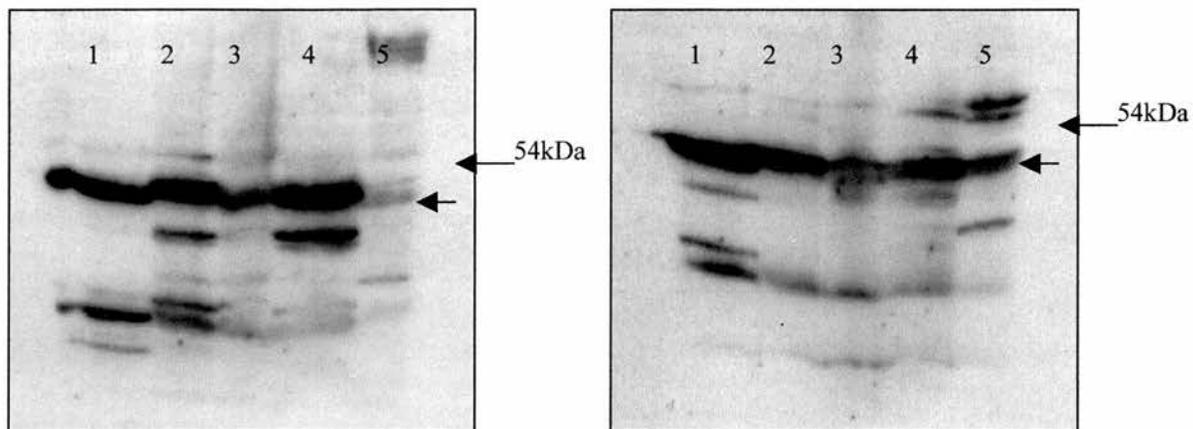


Fig. 6.11. Western blot of liver microsomes from three hooded seals (Lanes 1-3, CcP3, CcMb, CcPb, respectively), a grey seal (lanes 4) and a harbour porpoise (Lanes 5) detected by (a) seal CYP1A1/2 anti-peptide antibody at 1/30 dilution and (b) mammalian CYP1A1 anti-peptide antibody at 1/500 dilution. 40µg microsomal protein was applied to all four seal lanes 1-4 and 30µg of harbour porpoise was applied to lane 5. The molecular weights were derived from calibrated pre-stained molecular weight standards (see methods, section 6.2). The arrow indicates the potential CYP1A bands at approximately 52kDa. For abbreviations (Cc P or M 3, or b) refer to Table 2.1.

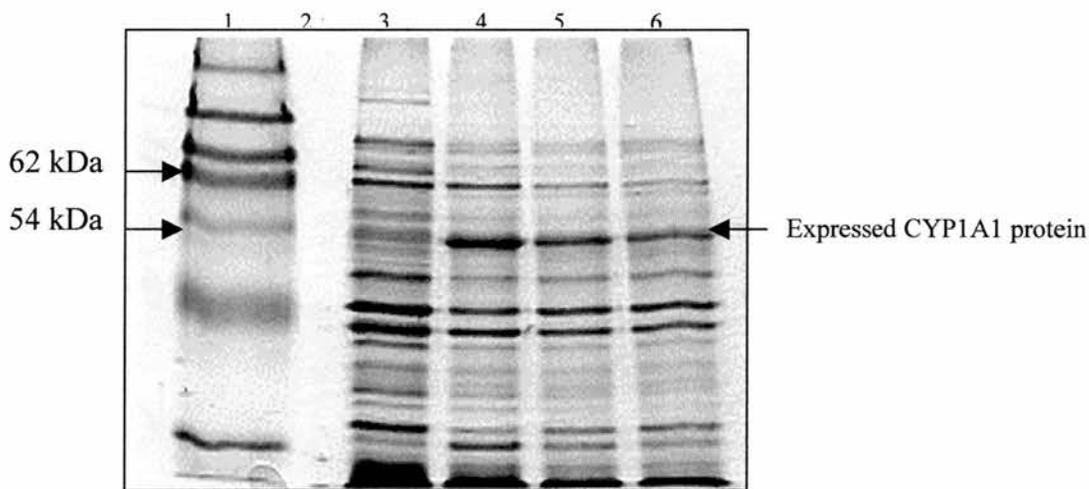


Fig. 6.12. SDS-PAGE (12.5%) of *E.coli* membranes containing recombinant CYP1A1. Lane 1 contains molecular weight standards; Lane 3 shows the resolved proteins from 15µg of membrane protein from *E.coli* transformed with the pCWori+ vector with non-expressed insert; Lanes 4 – 6 represent 15µg of membrane protein from *E.coli* induced to express the CYP1A1 protein for 24, 48 and 72 hours, respectively.

A similar sized protein band was also recognised with the anti-mammalian CYP1A1(2) antibody. This protein was of similar intensity in all four seal samples and the harbour porpoise, although the band was not as broad. Several other bands were detected by both antibodies, although were of lower molecular weight in the seal species. However, two protein bands, > 54kDa, were recognised by the anti-mammalian CYP1A1(2) and anti-seal CYP1A1/2 antibodies in the harbour porpoise liver microsomes.

6.4. Discussion

This chapter has demonstrated the selection, production and characterisation of two antibodies raised against a synthetic, common peptide sequence in mammalian CYP1A1, or a synthetic peptide sequence common to harp seal CYP1A1 and CYP1A2. Furthermore the two antipeptide antibodies, designated mammalian CYP1A1(2) and seal CYP1A1/2 anti-peptide antibodies, recognised immunoreactive proteins in various phocid seal and cetacean species.

6.4.1. Selection of antigenic peptides

The two antigenic peptides selected for the two anti-peptide antibodies corresponded with amino acid residues 280-293 and 355-365, respectively, in harp seal CYP1A1 derived amino acid sequence.

Both the peptides were selected as potentially good antigenic peptide sequences for several reasons. (1) The sequences were specific to the target CYP proteins (and other vertebrate/mammalian orthologous CYPs). (2) The hydrophilicity plots of both peptide sequences suggested their location was on the surface of the CYP protein and therefore would be available as epitopes for antibodies (Edwards *et al.*, 1991; Hopps and Woods, 1981). (3) Furthermore the peptides were located on the surface, and the mammalian

peptide (280-293) may also be positioned on a loop region of CYP1A based on the model of 3-dimensional structure of mammalian CYP2C5.

CYPs are thought to have derived from a common ancestor and have a highly conserved 3-dimensional structure (Graham and Peterson, 1999; Shen and Strobel, 1995). The relative location of these peptides on CYP1A1 and CYP2C5 may be very similar, despite differences in their primary structure.

Myers and colleagues (1993) designed an antibody against a synthetic peptide of eighteen amino acid residues from trout CYP1A1 (277-294). This anti-peptide antibody recognised a single protein band in liver microsomes from β -naphthoflavone treated trout and corresponded with purified trout CYP1A1 on an immunoblot. The mammalian peptide sequence (280-293) from this study overlapped with the fourteen amino acid residues from the C-terminus of the trout CYP1A1 peptide.

Anti-peptide antibodies were produced against a rat CYP1A1 peptide (356-363) and a rat CYP1A2 peptide (350 – 357) (Edwards *et al.*, 1991). These anti-peptide antibodies both recognised two protein bands of similar molecular weight in an immunoblot of liver microsomes from 3-methylcolanthrene treated rat. The seal CYP1A1/2 anti-peptide antibody (355-365) partially corresponded with the selected rat CYP1A1 and overlapped with rat CYP1A2 peptide. Furthermore the anti-seal antibody is likely to cross-react with both CYP1A1 and CYP1A2 in seal liver microsomes since the corresponding peptide sequences are only one amino acid different. However, this residue difference was at the 'free' (not conjugated to carrier protein) N-terminus. In one study the reactivity of an anti-peptide antibody to varying lengths of the original antigenic peptide was investigated (Edwards *et al.*, 1995). The carrier protein was

conjugated through the cysteine residue at the N-terminus leaving the C-terminus 'free'. The C-terminal amino acid was found to play an important role in antibody binding, in comparison to the binding of the anti-peptide antibody to the N-terminally truncated form of the antigenic peptide, which did not significantly affect the binding, in an ELISA analysis.

6.4.2. Binding specificity of the anti-peptide antibodies

6.4.2.a. ELISA and Immunochemical detection by multiscreen. ELISAs were performed for both antibodies to determine the binding specificity of the pre-immune sera and three antisera bleeds against the antigenic peptide, and an unrelated peptide that was also found in CYP1A1 protein sequences.

No binding of the pre-immune antisera to either the antigenic or unrelated peptide was observed, and the antisera from both antibodies did not recognise the unrelated peptide. Bleed two and bleed three antisera from mammalian CYP1A1(2) and seal CYP1A1/2 anti-peptide antibodies, respectively, were selected for purification by immobilised antigen affinity chromatography. This decreased the optimum 50% binding titre to the antigenic peptide, but removed the non-specific antibodies present in the serum.

Initial screening of the antibody's binding specificity to liver microsomal proteins was performed using a multiscreen. Using this technique a range of dilutions of both the selected antiserum and purified anti-peptide antibody could be analysed. Non-specific binding to harp seal liver microsomes was reduced, if not eliminated, by purification. Several bands, including a very prominent band at ~62kDa were recognised by the mammalian CYP1A1(2) antiserum, that were not detected by the purified IgG. A single

band, although weak, at ~ 52kDa was recognised by the purified mammalian CYP1A1(2) anti-peptide antibody at a dilution of 1/1000.

In comparison the binding specificity of the seal CYP1A1/2 antiserum (bleed three) and purified antibody were less than the mammalian CYP1A1(2) suggesting the former peptide was less antigenic. This may reflect the different locations of the two peptides on the native CYP1A isoenzymes. However, SDS used during the electrophoresis of these proteins can cause an increase in helical content of the proteins (Mattice *et al.*, 1976; Su *et al.*, 1977) and may partially obscure the epitope at residues 355-365 affecting the binding of the seal CYP1A1(2) anti-peptide antibody. Furthermore the seal antiserum was only able to detect proteins bands including one at ~52kDa up to dilutions of 1/200, where as the purified antibody did not recognise any protein bands at dilutions greater than 1/50.

6.4.2.b. Immunochemical detection by conventional Western blotting. The binding specificity of the two anti-peptide antibodies to three different harp seal liver microsomal samples were analysed for reactivity by conventional Western blotting. The pre-immune serum only very weakly detected protein bands within the molecular weight range of 54-62kDa after over one hour of exposure. Two protein bands (in harp seal samples PgMb and PgMc) were also recognised at a very low molecular weight that corresponded to the prominent lowest molecular weight bands detected by the mammalian CYP1A1(2) antiserum and less so the purified antibody, suggesting non-specific cross-reaction.

The mammalian CYP1A1(2) antiserum detected several protein bands including prominent bands at ~62kDa and <30kDa. A faint protein at ~52kDa, among other

proteins around this molecular weight, was recognised. The protein at ~52kDa only weakly reacted probably as a result of the dilution of the antiserum to 1/7500.

Two pairs of double protein bands at ~62kDa and ~52kDa, respectively, were detected in two of the three harp seal samples (PgMb and PgMc) with the purified antibody, compared to the detection of only a single band at ~52kDa in the other harp seal sample (PgMa). Harp seal CYP1A1 and -1A2 amino acid sequences exhibit 74% identity overall (Chapter five), however the fourteen residues of CYP1A1 and -1A2 that correspond with the mammalian synthetic peptide (280-293) show less than 40% identity.

Lin and colleagues (1998) observed a positive cross-reaction between the trout CYP1A1 anti-peptide antibody and rat CYP1A1, but not with rat CYP1A2 in ELISA or immunoblot. This suggests that the mammalian CYP1A1(2) anti-peptide antibody may not recognise CYP1A2 in seal liver microsomes and raises the question of the identity of the two protein bands at ~52kDa. Members of a CYP isoenzymes family, need to have >40% sequence identity. It is therefore possible that one of the bands detected by the mammalian anti-peptide antibody is CYP1B1. CYP1B1 was identified in striped dolphin by sequencing of cDNA (Godard *et al.*, 2000), however there was only 27% identity between the mammalian peptide sequence (GHIRDITDSLIEHC) and the corresponding region in striped dolphin CYP1B1 protein sequence (Dr C. Godard, personal communication).

The seal CYP1A1/2 anti-peptide antibody at 1/30 dilution recognised two protein bands that were very similar in molecular weight at ~52kDa, comparable to the mammalian CYP1A1(2) anti-peptide antibody. Another prominent band was detected at <30kDa.

Harp seal sample 18 was the only sample that demonstrated the most intense bands at ~52kDa and the least intense band at <30kDa. Furthermore the seal antibody in sample 18 detected no other immunoreactive proteins, compared to liver microsomes from harp seals PgMc and PgMb. These other immunoreactive proteins may therefore be degradation products of CYP isoenzymes, thus suggesting that harp seal PgPb liver microsomal sample was less degraded than the other two samples.

In contrast to the mammalian anti-peptide antibody, no band(s) were observed at ~62kDa by the purified seal CYP1A1/2 anti-peptide antibody. During the purification, the seal antibody was only eluted once the absorbance at 280nm (A_{280}) was <0.1 a.u., indicating that any non-specific IgG and other contaminating proteins had been washed from the column. The mammalian anti-peptide antibody was probably eluted before non-specific antibodies were thoroughly washed from the column, as indicated by the A_{280} of 0.8 a.u. at which elution began.

Anti-peptide antibodies raised against a common peptide sequence to rat CYP1A1 and –1A2 (356-363 and 350-357, respectively) detected two bands in the liver microsomes from 3-methylcolanthrene treated rats (Edwards *et al.*, 1991). The higher molecular weight band corresponded with a single protein in the same liver microsomes developed with CYP1A1-specific anti-peptide antibody, whereas the lower band corresponded to CYP1A2 protein detected with the CYP1A2-specific anti-peptide antibody.

Immunoprecipitation of harp seal liver microsomes with the mammalian CYP1A1(2) anti-peptide antibody revealed two proteins, one at 52kDa that was likely to be CYP1A1 and another at 62kDa that was likely to be a serum albumin. A high similarity of the peptide sequences between BSA and the protein at 62kDa was identified by MALDI-

TOF mass spectrometry. Furthermore another protein at a slightly lower molecular weight than the protein at ~52kDa was precipitated, however was not identifiable. Immunoprecipitation with the seal CYP1A1/2 anti-peptide antibody has not yet been performed.

Evidence suggests that the bands at ~ 52kDa developed by the seal CYP1A1/2 anti-peptide antibody are in fact CYP1A1 and CYP1A2 for several reasons. (1) The theoretical molecular weight of the derived amino acid sequences of these two apoproteins indicates that the higher molecular weight band is CYP1A1 and the lower band, CYP1A2. (2) An anti-peptide antibody against rat CYP1A1/2 at corresponding amino acid residue positions also recognised two proteins in liver microsomes from 3-methylcolanthrene treated rats (Edwards *et al.*, 1991). (3) Both the CYP1A1 and -1A2 cDNA were isolated from harp seal liver, indicating that the CYP1A1 and -1A2 genes had been transcribed (Chapter five). (4) Immunoprecipitation suggested the presence of CYP1A1 at ~52kDa. (5) Furthermore the antigenic peptide (355-365) was identical to harp seal CYP1A2 residues within this region and only differed by one amino acid with the corresponding peptide in CYP1A1. However, the detection of CYP1B1, if expressed in these species, cannot be disregarded, as the seal peptide sequence (TGRARQPRLSD) had 50% identity with striped dolphin CYP1B1 amino acid sequence (Dr C. Godard, personal communication).

Differences in the intensity of the immunoreactive protein bands from different harp seal livers indicated that the mammalian and seal anti-peptide antibodies were able to detect different levels of expression of the CYP1A apoproteins. The single band detected at ~52kDa in sample PgMa by the mammalian CYP1A1(2) anti-peptide

antibody may therefore reflect the lack of expression of one of the CYP1A subfamilies compared to samples PgMb and PgMc.

The trout CYP1A1 anti-peptide antibody, identical to that designed by Myers and colleagues (1993) was used in this study as a comparison, and recognised two prominent and distinct bands in the same three harp seal samples. These immunoreactive bands similar to the mammalian anti-peptide antibody corresponded to 52kDa and 62kDa. Similarly these two bands were observed in immunoblots of liver microsomes from hooded seals (Chapter two), grey seals and harbour porpoise (Chapter three), developed with the anti-trout CYP1A1 anti-peptide antibody. In these immunoblots the antibody was diluted to 1/20,000, 26.6 fold and 600 fold higher dilution than the mammalian and seal anti-peptide antibodies, respectively. The higher antibody titre of the trout CYP1A1 antibody was probably due to the higher binding strength of the trout antibodies produced in rabbits compared to the other two anti-peptide antibodies produced in sheep (Edwards, 1998). In fact further dilution of the trout CYP1A1 antibody may show whether these broad bands are two separate immunoreactive bands and if this anti-trout anti-peptide antibody is able to detect different levels of expression.

Detection of a protein band at ~62kDa by the trout CYP1A1 antibody was interesting and may be a property of longer anti-peptide antibodies (trout and mammalian at eighteen and fourteen residues long) in this region of the CYP apoproteins (277-294). Shorter anti-peptide antibodies in the corresponding region bound specifically with a single protein (Edwards *et al.*, 1998; Schulz *et al.*, 2001). However, other bands were also detected with the anti-peptide antibodies in the present study that were of lower molecular weight. Explanations for this are that (i) the integrity of the liver microsomal

samples, due to prolonged storage initially in liquid nitrogen (Chapter four, post-nuclear supernatants) and then at -70°C , may be reduced compared to the liver microsomal samples used in other studies. (ii) The antibodies were not sufficiently purified; therefore further purification of these antibodies may also be required.

6.4.3. Immunochemical detection of other marine mammal CYP1A

Immunochemical detection with the two anti-peptide antibodies was performed to determine the cross-reactivity with liver microsomes from other marine mammal species. These antibodies could be used to detect and further characterise CYP1A as a biomarker of exposure to aryl hydrocarbon receptor binding environmental contaminants, such as polyaromatic hydrocarbons, dioxins and planar, 2,3,7,8-TCDD-like PCBs.

A prominent and broad band at $\sim 52\text{kDa}$ was detected in hooded and grey seal liver microsomes with both the mammalian CYP1A1(2) and seal CYP1A1/2 anti-peptide antibodies. Furthermore band(s) were recognised by the two antibodies, although the mammalian CYP1A1(2) antibody detected a stronger immunoreactive band(s) in harbour porpoise liver microsomes. Two of lower molecular weight proteins were also observed in the samples that could potentially be degradation products, as the harbour porpoise liver was stored in liquid nitrogen for fourteen days prior to the preparation of liver microsomes. Immunoreactive proteins were detected at $\sim 62\text{kDa}$ in the harbour porpoise liver microsomes and very faint bands were detected at this molecular weight in the liver microsomes from hooded and grey seals.

The complete derived amino acid sequence from cDNA has been determined from three seal species, harp and grey seals (Chapter five) and ribbon seal (*Phoca hispida*) (Dr I.

Teramitsu, personal communication). The amino acid residues corresponding to the 280-293 ('mammalian') peptide are identical in the three seal species CYP1A1 sequences, and the three CYP1A2 sequences. Furthermore the corresponding peptide region from 355-365 ('seal') is also identical between CYP1A1 sequences, and CYP1A2 sequences from the three seal species.

This provides strong evidence for highly conserved regions specific to the CYP sequences or, as with seal peptide sequence (355-365), which is common to the CYP1A sequences, may be highly conserved with other seal sequences and therefore will react with the two anti-peptide antibodies presented here. Furthermore immunoreactive protein(s) at ~52kDa observed in the liver microsomes from harbour porpoise may indicate conservation of these two regions of the CYP1A protein sequence, especially the peptide region, 280-293 (mammalian). The corresponding antibody (mammalian CYP1A1(2)) reacted more strongly than the seal CYP1A1/2 anti-peptide antibody and produced almost a similar intensity reaction in the harbour porpoise liver microsomes as the corresponding bands in the seals. This suggests that the 'mammalian' peptide sequence (280-293) is more conserved throughout the CYP1A isoenzymes in marine mammals, compared with the 'seal' peptide sequence (355-365). Evidence for this hypothesis was provided by the fact that the mammalian peptide sequence was 100% conserved with the corresponding region in striped dolphin CYP1A amino acid sequence, compared with 81% identity of the seal peptide sequence (Dr C. Godard, personal communication).

The reduction in relative intensity of the immunoreactive proteins developed and recognised by the mammalian CYP1A1(2) anti-peptide antibody, compared with the intensity shown by the seal microsomes, was partially a result of the lower amount

(30µg) of harbour porpoise liver microsomes that were initially separated compared to 40µg of seal liver microsomal protein. The harbour porpoise liver microsomes were also partially degraded, as they were prepared from liver that had been stored in liquid nitrogen for fourteen days.

Immunochemical detection of a variety of vertebrate liver microsomal samples that were from untreated, pre-treated or environmentally exposed individuals, were developed with the trout CYP1A1 anti-peptide antibody (277-294) (Lin *et al.*, 1998) (Also see Chapter six, section 6.4.2). Reactions were only observed with either pre-treated or environmentally exposed animal liver microsomes, suggesting the utility of this antibody in detecting induced CYP1A1 in wildlife species. In particular a single, although broad band, from beluga whale (*Delphinapterus leucas*) and polar bear (*Ursus maritimus*) liver microsomes cross-reacted with the anti-peptide antibody (Lin *et al.*, 1998).

The antigenic peptide (280-293) for the mammalian antibody has greater sequence identity with the corresponding peptide sequence in seal CYP1A1 (13/14 residues) than the trout CYP1A1 anti-peptide antibody has with seal CYP1A1 (12 /18 residues). This suggests that the mammalian anti-peptide antibody would be a better tool for quantification of CYP1A in diverse seal species as part of a biomonitoring programme. However, further studies are required to elucidate the identity of the two proteins bands at ~52kDa that react with this antibody.

6.4.4. Future experiments and implications for biomarking.

In the present study two anti-peptide antibodies have been partially characterised for their reactivity with certain proteins within liver microsomes from seal and a cetacean

species. The reactivity of these antibodies with the target protein has been determined in as much as the synthetic peptide sequence that was selected was not present in any protein other than CYP1A1 and -1A2.

Characterisation of anti-peptide antibodies has been performed against purified target CYPs (Lin *et al.*, 1998; Myers *et al.*, 1993; Schulz *et al.*, 2001). Purifying CYPs is an arduous task and often not practical from limited sources of marine mammal livers. However, anti-peptide antibodies have also been shown to react specifically to target recombinant CYP apoproteins, and produce a negative staining to unrelated CYPs in immunoblots (Edwards, 1998; Edwards *et al.*, 1998; Wang and Lu, 1997). This suggests that the identity of the two liver microsomal proteins at ~52kDa observed in the present study could be determined by performing immunoblots with recombinant seal or cetacean CYP1A1 and -1A2 apoproteins developed by the two anti-peptide antibodies. Preliminary studies were performed in preparing, partial purification and isolation of recombinant harp seal CYP1A1 (Fig. 6.12). Further experiments are necessary to discern the immunoreactivity of the recombinant apoprotein.

Characterisation and further development of marine mammal CYPs for subsequent use as biomarkers of environmental contaminant exposure can be performed with the use of specific probes (Peters *et al.*, 1999) such as anti-peptide antibodies. The pre-determined epitope will enable the reactivity of the antibody with the target CYP apoprotein to be strongly predicted as a greater number of marine mammal CYP sequences are deduced through cDNA isolation (e.g. Chapter five, this thesis; Godard *et al.*, 2000; Teramitsu *et al.*, 2000).

The anti-peptide antibodies designed and characterised in the present study targeted internal sequences of CYP1A isoenzymes, however there was high sequence identity between CYP1A1 and -1A2 in the two corresponding peptides. Edwards and colleagues (1995) have additionally demonstrated the CYP-specificity of targeting anti-peptide antibodies against the five ultimate C-terminal residues. The C-terminal residues of CYPs showed considerable variation so that such antibodies were likely to be specific. They demonstrated that a C-terminus anti-peptide antibody to CYP1A1 only reacted with a single protein in liver microsomes from 3-methylcolanthrene treated rats, and did not cross-react with CYP1A2 (Edwards *et al.*, 1995). The five residues at the C-terminus of the three known seal CYP1A1 (VRVRA) and -1A2 (RFSTK) indicate that anti-peptide antibodies targeted to these peptides would be specific for (a) seal CYP1A1 and -1A2, respectively, (b) they would not cross-react with other mammalian CYPs or the other subfamilies target protein.

6.4.5. Summary

Two anti-peptide antibodies were designed, produced and characterised in the present chapter and were shown to recognise immunoreactive proteins in three seal species, harp, grey and hooded seal and a cetacean species, harbour porpoise. The following conclusions were drawn.

- Two anti-peptide antibodies targeting CYP1A1 and potentially CYP1A2 in seals reacted with two prominent proteins at approximately 52kDa.
- Other immunoreactive proteins detected were likely to be either degradation products of the CYP apoproteins or non-specific cross-reaction (mammalian anti-peptide antibody) due to incomplete purification of the antibody.

- Conservation of the two antigenic peptide sequences between different seal species indicated the suitability of these anti-peptide antibodies for the detection of CYP1A apoproteins in seal species not yet investigated.
- Isolation of more CYP1A cDNA from diverse seal species will confirm the conservation of the two corresponding peptide sequences, 280-293 and 355-365, and their potential immunoreactivity with the two anti-peptide antibodies. Furthermore they will enable the production of a panel of anti-peptide antibodies specific for target marine mammal CYPs.
- The immunoreactive protein(s) in harbour porpoise liver microsomes recognised in particular by the mammalian anti-peptide antibody, suggest conservation of the corresponding peptide 280-293 in cetacean species.
- Future experiments are required to elucidate the identity of the two immunoreactive ~52kDa proteins, possibly by using immunoblots of recombinant seal protein.

7.1. Introduction

The majority of biomarker studies have employed invasive, and consequently destructive techniques (Mattson *et al.*, 1998; White *et al.*, 1994; Wolkers *et al.*, 1999) (reviewed in Fossi and Marsili, 1997) or have utilised tissue and organ samples from animals that have died through strandings or that have been bycaught in fishing nets (Boon *et al.*, 2001; Jepson *et al.*, 1999; Murk *et al.*, 1994; Troisi *et al.*, 1998; Troisi and Mason, 1997). However, as discussed previously in Chapters Three and Four of this thesis, samples from by-caught or stranded animals are often restricted to small numbers of potentially unhealthy animals and the tissue samples are frequently degraded. Obtaining tissue samples from hunted animals is unethical, and is restricted to limited numbers of animals in particular populations and areas. Both of these sources of tissue are likely to result in data that is not necessarily representative of a healthy free-ranging population. Furthermore the development of non-destructive methods would also be in keeping with the UK's home office guidelines on the use of animals in scientific procedures. This has led to preliminary investigations into the use of skin samples (obtained from biopsies) as the tissue for biomarker studies. The induction and expression of cytochrome P450 (CYP) 1A and other CYP isoenzymes present in this tissue may also reflect the circulating concentrations of environmental contaminants (Moore *et al.*, 1998).

The effect of topical application of drugs and contact with environmental contaminants, in particular polyaromatic hydrocarbons (PAHs), on whole skin and epidermal samples from human and rodents have been extensively investigated. A variety of CYP1A

monooxygenase (MO) activities, including aryl hydrocarbon hydroxylase (AHH), 7-ethoxyresorufin *O*-deethylase (EROD), benzo(a)pyrene (B(a)P) and ethoxycoumarin *O*-deethylase (ECOD), were employed to detect CYP1A induction and inhibition in epidermal and whole skin preparations from humans and rodents (Bickers *et al.*, 1982; Bickers *et al.*, 1984; Ichikawa *et al.*, 1989) and these are reviewed in Mukhtar and Khan, (1989). In particular one study investigated the induction of ECOD activities in rodents exposed to either intraperitoneal (i.p.) injection of benzoflavone (BF) or the topical application of this PAH (Moloney *et al.*, 1982). They found that the i.p. administration of BF was the greatest inducer of ECOD activities in the skin of rats (hairy), in contrast to the poor induction observed for the topical application of BF in a hairless mouse strain. The pilosebatory apparatus in the epidermis has a good blood supply in hair-covered rats compared with no direct blood supply in hairless mouse epidermis, that lacks the pilosebatory apparatus (Moloney *et al.*, 1982). This suggests that the circulation plays an important role in exposing epidermal tissues to internally absorbed environmental contaminants in mammals.

Hair roots are of epidermal origin and also contain CYP isoenzymes that are capable of metabolising environmental contaminants. Merk and colleagues (1985; 1987) observed CYP1A-mediated EROD activities in assays with whole human hair roots, from individuals pre-treated with topically applied coal – tar (containing crude oil and PAHs). Marine mammals, in particular seal species, have a dense coat of hair, and potentially a good blood supply to the epidermis, therefore providing a potential route of CYP induction in the epidermis. This would suggest that the use of skin tissue in non-destructive biomonitoring studies should prove successful. Indeed Fossi and colleagues (1992) were the first group to investigate CYP1A-mediated MO activities in marine mammal skin samples, utilising CYP1A-mediated B(a)P MO activity in whole skin

samples. Since then preliminary data regarding CYP1A-B(a)P MO activity in a variety of cetacean and seal species have been documented (Fossi *et al.*, 1999; Fossi *et al.*, 1997a; Fossi *et al.*, 2000; Fossi *et al.*, 1997b; Jimenez *et al.*, 1999; Marsili *et al.*, 1996). Furthermore a significant positive correlation between total DDT + PCB concentrations determined from the skin/subcutaneous blubber biopsy and the B(a)P activities in fin whales (*Balenoptera physalus*) was observed (Marsili *et al.*, 1998).

Immunohistochemical detection of CYP1A proteins has been reported in Northern and Southern Right whale species (Moore *et al.*, 1998). The semi-quantitative content of CYP1A was determined in skin biopsies obtained from free-ranging animals and was found to be greater in the Northern right whales (*Eubalaena glacialis*) compared to the Southern Right whales (*E. australis*) and the calves from both hemispheres. Furthermore the CYP1A content negatively correlated with the concentration of extracted PAHs, suggesting increased metabolism of the PAHs with increasing CYP1A content.

Against this background, the present study therefore aimed to develop a microassay using skin samples to detect CYP1A-mediated EROD and to my knowledge provides the first, preliminary evidence for the use of Western blotting in the immunochemical detection of CYP1A-like protein in any phocid seal. Both a microassay and Western blotting techniques provide a method of evaluating a large number of samples at the same time. The use of EROD as a method of detecting CYP1A in whole skin samples is discussed.

7.2. Materials and Methods

7.2.1. Samples.

Skin samples for the catalytic studies were obtained from harbour seals (*Phoca vitulina*) by biopsy punch after the animals were sedated with Zoletil 100 (Virbac, France) at a dose of 1mg/100kg body weight. All procedures were carried out under Home Office Licence and Licences from the Scottish Executive. The harbour seals were subsequently given 1ml/10kg body weight dose of Terramycin L.A. intramuscularly (Pfizer, UK). Surplus skin samples were obtained from hooded seals (*Cystophora cristata*) from the Arctic (Chapter two) and were used in immunochemical detection. All skin samples were placed in a cryovial and stored in liquid nitrogen until processing.

7.2.2. Preparation of skin samples for catalytic study (EROD assay).

Whole skin samples were prepared by the method of Moloney and colleagues (1982) with the following modifications. Briefly, skin samples (~1cm³) were thawed on ice and the wet weight was recorded. The skin was cut into small pieces and was placed into eppendorfs prior to rapid freezing in liquid nitrogen. Once frozen each subsample of skin was ground in a pre-chilled (-20°C) mortar and pestle with acid washed sand, under liquid nitrogen. The addition of liquid nitrogen continued until the skin subsample was a fine powder. Subsequently the fine powder was quickly transferred into a clean eppendorf and suspended in 30% suspension in ice cold EROD buffer (0.1M Tris-HCl, pH 7.8 containing 20% glycerol), and placed on ice.

Sand and insoluble particles were sedimented by centrifugation at 13krpm for 5 minutes in a pre-chilled (0°C) tabletop centrifuge (Sigma 1K15, Howe). The supernatant was then transferred by pipetting into a clean eppendorf and placed on ice for immediate use in the EROD microassay.

7.2.3. Preparation of skin samples for immunochemical detection.

Skin samples were prepared as for the catalytic study (section 7.2.2), with the following modifications. The fine powdered skin was suspended by vortexing in 500 μ L 10% SDS prior to sedimentation of the sand and any insoluble particles by centrifugation (13krpm, room temperature, 5 minutes). The supernatant was subsequently transferred to a clean eppendorf and stored at -20°C until use.

7.2.4. Total Protein Concentration.

The total protein concentration of the skin suspension was determined using the modified Bradford's assay (Stoscheck, 1990), as described in Chapter two (section 2.2.3). Only the protein concentration of the skin suspended in EROD buffer (i.e. for the catalytic study) was assayed, since SDS interferes with the assay. Skin suspensions were prepared as previously described and diluted 2-, 5-, 10-, and 20-fold in EROD buffer to ensure the absorbance was <1.0 a.u.

7.2.5. EROD microassay.

Skin samples. The EROD microassay was performed in a 96 well plate and contained 250 μ L of skin suspension in EROD buffer at a final concentration of 0.05mg/mL, unless otherwise stated in the results section, and 1 μ L 0.25 μ M 7-ethoxyresorufin (7ER).

Liver microsomal samples. The EROD microassay was performed on harbour seal liver microsomes to ensure the integrity of the microassay. The microassay contained liver microsomal protein at a final concentration of 0.2mg/ml and 1 μ L 0.25mM 7ER, made up to a final volume of 250 μ L with EROD buffer. Prior to reading the zero time point of both the skin samples and liver microsomal protein, the assay contents were mixed by

pipetting using glass Pasteur pipettes and incubating the plate in the pre-warmed plate reader (37°C) for 5 minutes.

Subsequently the reaction mixtures were subjected to fluorimetric spectrophotometric analysis in a fluorimeter designed for 96 well plates (Labsystems Fluoroskan 2, UK). Since the product, resorufin has specific excitation (ex) and emission (em) wavelengths (λ): λ_{ex} 530nm and λ_{em} 585nm, the third excitation and emission filters were selected. The reaction was initiated with the addition of 10 μ L 2.5mM NADPH (freshly prepared) and the increase in resorufin was measured every 0.5 minute for at least 10 minutes. The reaction was internally calibrated by the addition of 10 μ L 7.8 μ M resorufin in DMSO. The progress curve (intensity of fluorescence against time) enabled the rate of reaction to be visualised.

7.2.6. Immunochemical detection of CYP1A-like protein.

SDS-PAGE and immunochemical detection of potential CYP1A proteins were conducted according to the procedures described in chapter one (section 2.2.6) with the following modifications.

15 μ L of skin suspensions in 10% SDS were either added at stock concentrations or diluted 2-fold and added to 5 μ L reducing solution (Chapter two, section 2.2.6). The mixture was boiled in a heating block for 2 minutes prior to loading into a stacking gel. Solubilised skin proteins were separated by SDS-PAGE on 10% polyacrylamide slab gels according to Laemmli (1970).

Potential CYP1A-like proteins in skin suspensions were detected using the purified anti-mammalian CYP1A anti-peptide antibody (Chapter six) at 1/250 dilution. The

secondary antibody that was used to detect the primary antibody-protein complex was an anti-sheep antibody conjugated to the horseradish peroxidase enzyme.

7.3. Results

7.3.1. EROD microassay of seal liver microsomes and whole skin suspension.

The EROD microassay was initially performed using equivalent final concentrations to those of the standard EROD assay (Chapter two): 1 μ M 7ER and 0.1mM NADPH. However, no rate of reaction was observed in the skin EROD assay despite an increase in intensity in the EROD assay containing liver microsomes. Consequently the substrate concentration was increased to a final concentration of 6 μ M. Seal liver microsomes at a concentration of 0.18mg/mL produced a linear progress curve (rate of reaction) throughout the 20 minutes of recording (Fig. 7.1). However, an apparent lag was initially observed for the production of resorufin in the skin sample EROD reaction at protein concentrations of 0.05mg/mL, for the first couple of minutes (Fig. 7.2). Furthermore the rate of the skin EROD reaction reached a plateau between approximately 10 and 15 minutes.

7.3.2. Verification of no interfering parameters

The 'progress curve' of an incomplete assay mixture was recorded to verify the absence of blank rates that may affect the progress curve. The control assay mix contained the same final concentration of total skin protein and the substrate (7ER) as the corresponding components in the experimental wells. However, no blank rates were observed.

The assay was performed at 37°C and the assay components were equilibrated for 10 minutes prior to the initiation of the reaction with pre-warmed NADPH to avoid

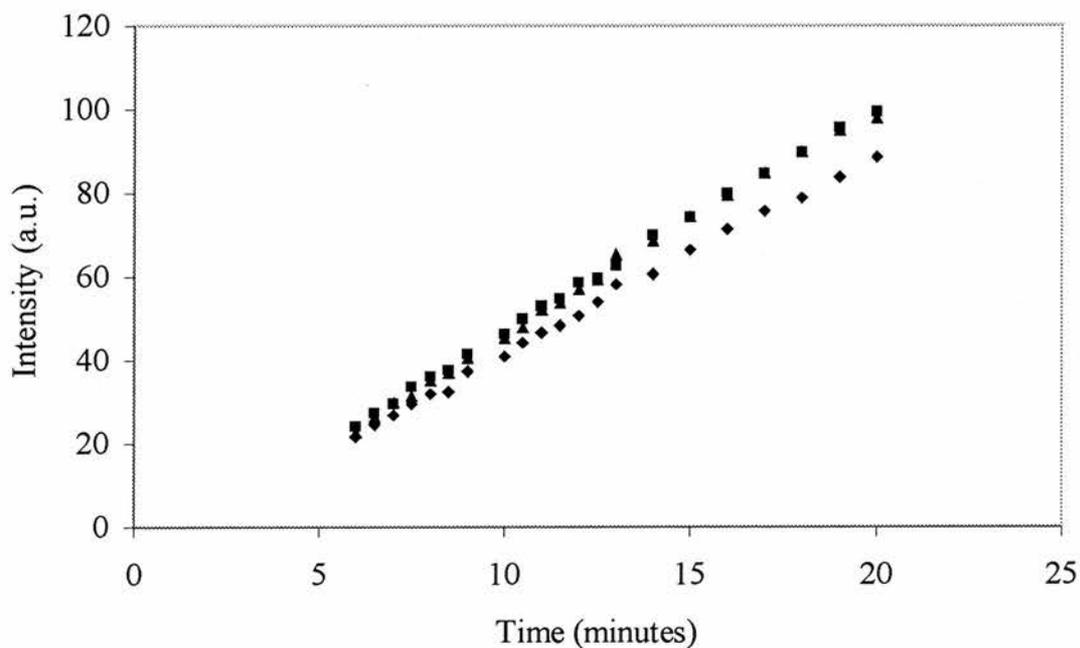


Fig 7.1. Progress curve of the production of resorufin (fluorescence intensity) against time of the EROD microassay of grey seal liver microsomes in triplicate.

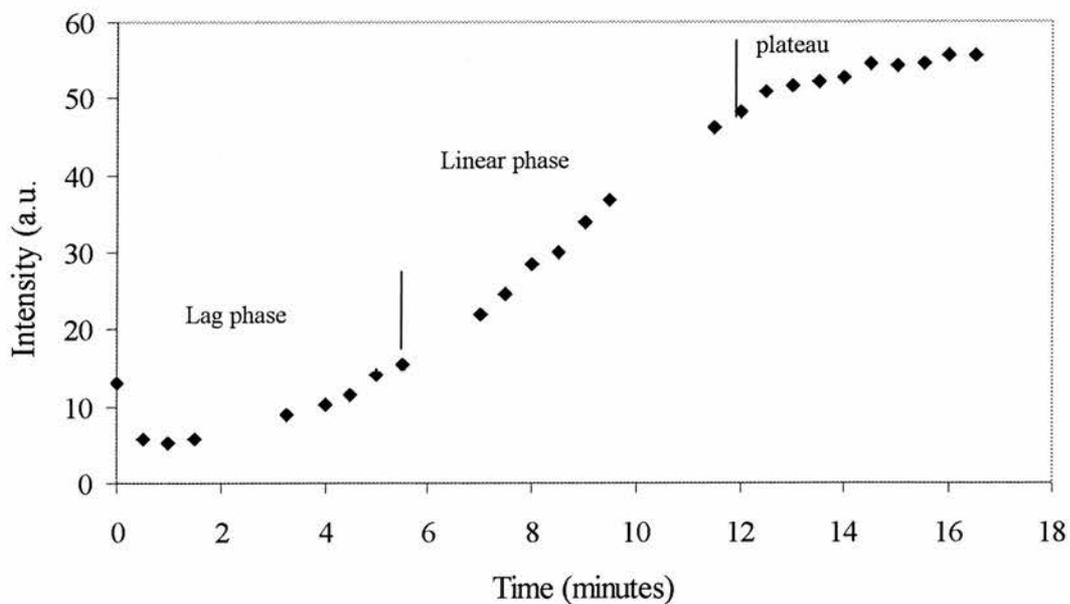


Fig. 7.2. A typical progress curve of potential resorufin production (fluorescence intensity) against time of the EROD microassay of skin samples.

temperature-related lags. Furthermore the presence of DMSO (substrate solvent) in the reaction mixture may have had an effect on the rate of reaction. However, only DMSO at final concentrations of 10% or more in liver microsomes reduced the rate of the reaction (Data not shown).

Many enzymes are inhibited by one or more of their substrates (Tipton, 1992), therefore the effect of substrate concentration with constant protein concentration (0.05mg/mL for skin and 0.2mg/mL for liver microsomes) was investigated in the skin and liver microsomal protein samples (Fig. 7.3 and 7.4, respectively). The concentration of 7ER that produced the highest 'rates' in the linear part of the progress curve, was 6 μ M for skin samples and 0.5 μ M for liver microsomal protein. In both tissues high concentrations of 7ER caused a reduction in the observed rates.

The variation of protein concentration was not fully investigated due to a limit of fresh skin samples. However, the initial experiments for the effect of protein concentration on activity for liver microsomes revealed that increasing the protein concentration from 0.05mg/mL to 0.2mg/mL increased the EROD activity (nmol.min⁻¹) (Fig. 7.5). Furthermore doubling the protein concentration from 0.1mg/mL to 0.2mg/mL approximately doubled the rate from 10.2 nmol.min⁻¹ to 25.8nmol.min⁻¹. In contrast the linear rate of the reaction for skin samples appeared to be less than 0.05mg/mL (Fig. 7.6). Protein concentrations greater than 0.05mg/mL corresponded to a reduced EROD rate.

7.3.3. Observation of the quenching of 7-ethoxyresorufin

Immediately after the addition of NADPH (final concentration, 0.1mM and other concentrations (0.25 – 5mM) the colour of the reaction mixture changed from the

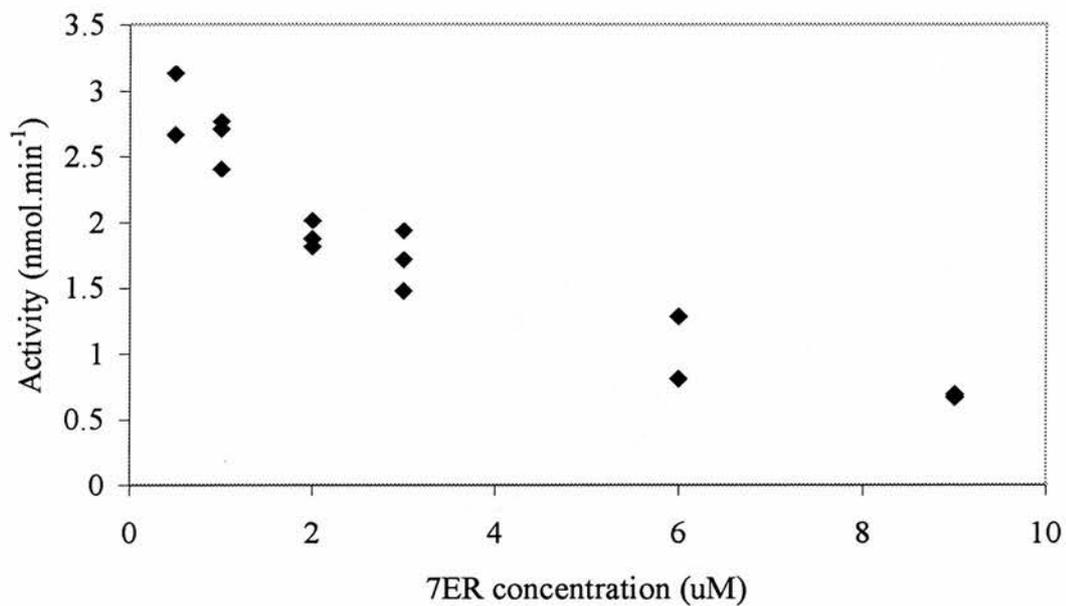


Fig. 7.3. EROD activity (nmol.min⁻¹) against substrate (7-ER) concentration (uM) for liver microsomes, from triplicate measurements.

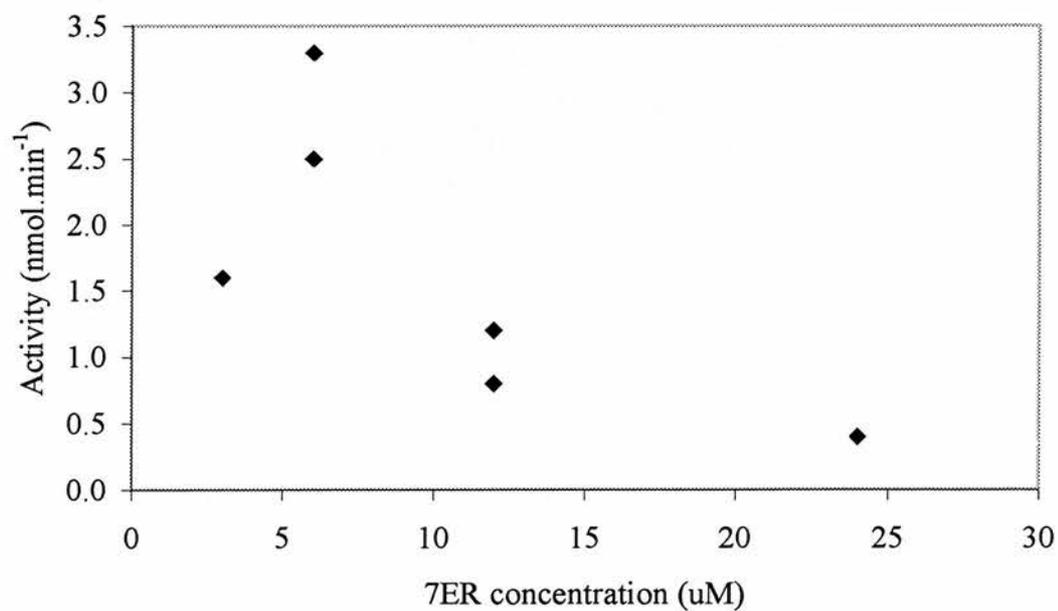


Fig.7.4. EROD activity (nmol.min⁻¹) against substrate (7ER) concentration (uM) for skin samples, from duplicate measurements.

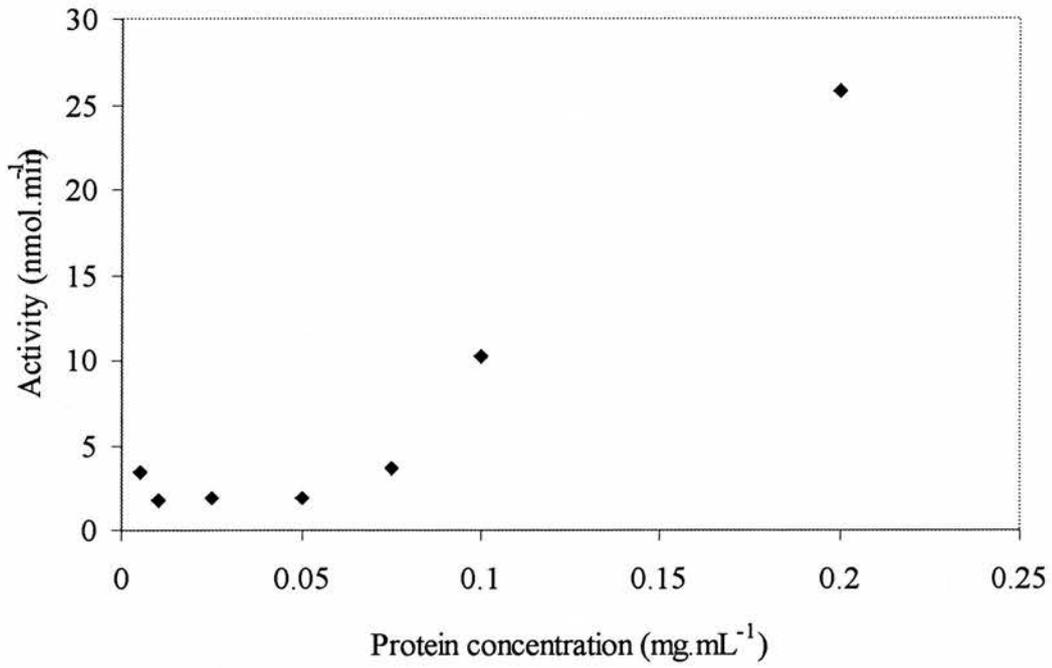


Fig.7.5. Activity (nmol.min⁻¹) against protein concentration of liver microsomes at constant substrate concentration (0.5 μ M).

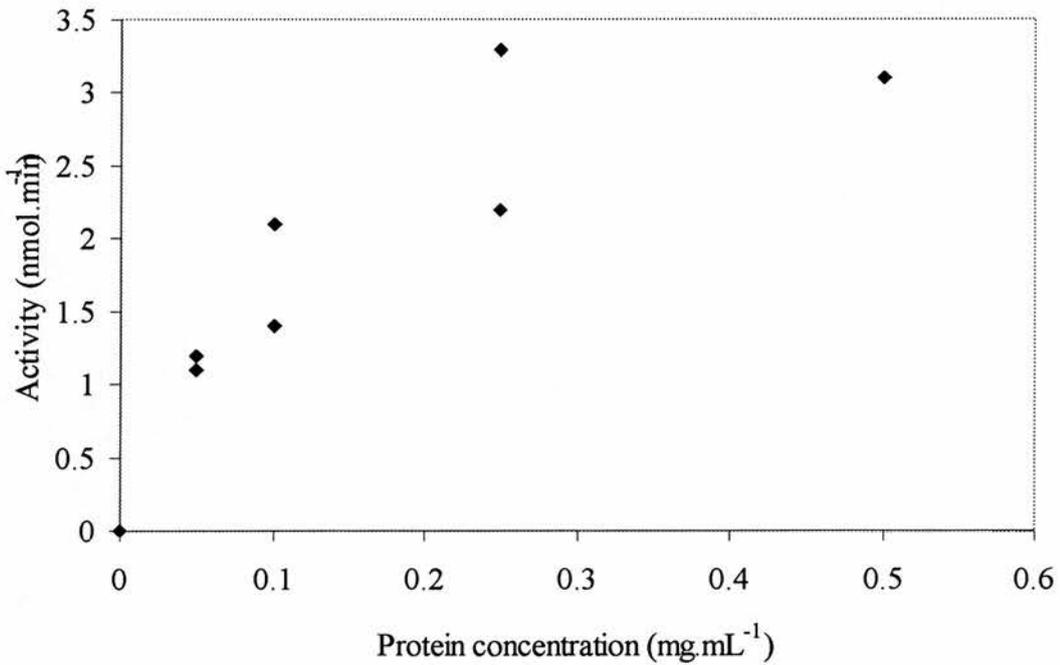


Fig. 7.6 Activity (nmol. min⁻¹) against protein concentration of skin sample at constant substrate concentration (6 μ M).

characteristic orange, due to the 7ER, to being translucent. This colour change was associated with the lag phase of the skin samples progress curve. Subsequently the dramatic increase in intensity and apparent increase in the rate of the reaction corresponded with a slow return of the orange colour, representative of 7ER.

The independent spectra of NADPH and 7ER measured on a spectrophotometer, showed that NADPH absorbed at 340nm and 7ER absorbed at 482nm. The addition of NADPH to final concentration of 0.1mM to a solution of 6 μ M 7ER did not affect the absorbance of 7ER at 482nm.

In an anaerobic cuvette, a few grains of the reducing agent, sodium dithionite were added to a mixture of NADPH and 7ER (0.1mM and 6 μ M, respectively). The orange coloration of the NADPH + 7ER mixture (orange) was quenched, resulting in a translucent solution.

7.3.4. Why did the rate reach a plateau so quickly?

The increase in intensity of the reaction slowed dramatically after \sim 10 minutes of the reaction, when the final concentration of the components of the reaction were 0.1mM NADPH and 6 μ M 7ER. The addition of a further aliquot of NADPH restored the rate of the reaction to the linear part of the progress curve (Fig. 7.7). The activity of the initial linear part of the reaction was 0.0157 μ mol.min⁻¹ that compared to the rate of 0.0115 μ mol.min⁻¹ after the addition of the second aliquot of NADPH.

7.3.5. Immunochemical detection of CYP1A isoenzymes in skin samples.

The anti-mammalian CYP1A anti-peptide antibody detected a single prominent band in two of the hooded seal skin samples corresponding to a single distinct protein at 54kDa

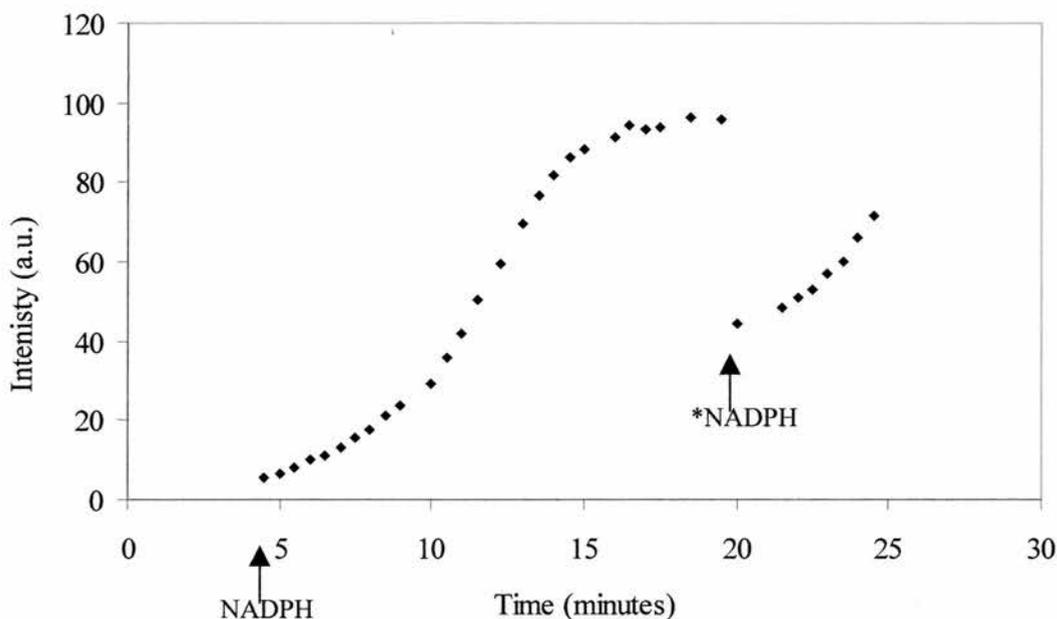


Fig. 7.7. Progress curve of whole skin EROD assay after addition of a further volume of *NADPH to a final concentration of 0.1mM, to the reaction.

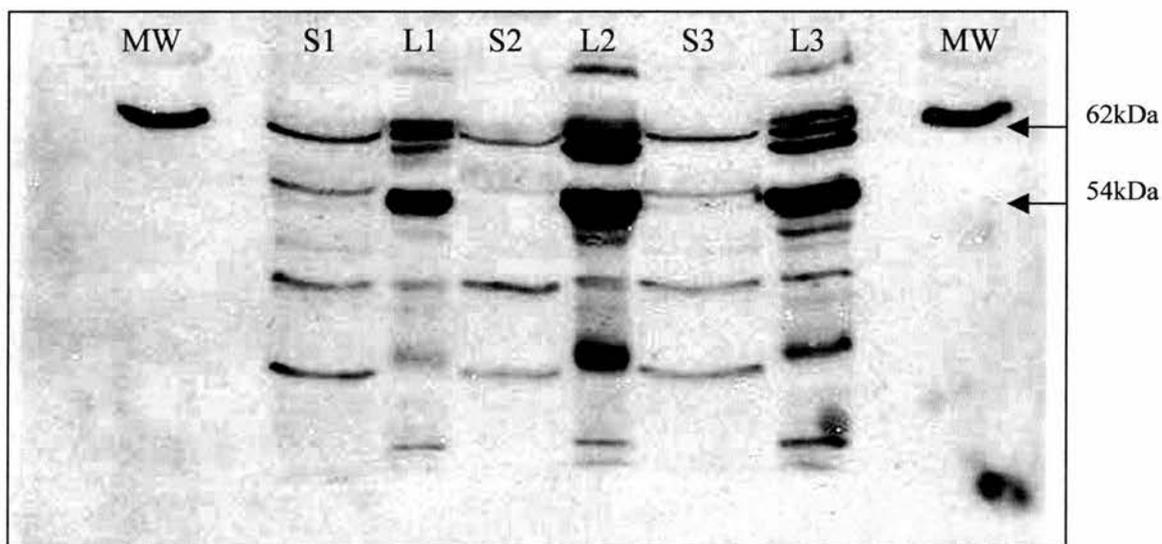


Fig. 7.8. Immunochemical detection of skin (S) and liver microsomal proteins (L) from hooded seals with anti-mammalian CYP1A anti-peptide antibody. MW, molecular weight marker lane. 1, 2 and 3 correspond with three different hooded seal samples. Approximately 50µg of liver microsomal protein was applied to lanes L1, L2 and L3. Concentrations of the skin samples to apply were pre-checked on coomassie stained SDS-PAGE. 20µL of S1 was applied at half the stock concentration, and 20µL S2 and S3 were applied at stock concentration. Exposure time 20 minutes.

in liver microsomes from the same animal (Fig. 7.8). A very weak protein band was detected in the skin sample corresponding to lane S2, and may be the result of sample degradation, or the potential CYP1A-like protein may not be induced or is down-regulated in the skin of this animal. The antibody detected other proteins, in particular a protein at ~62kDa, although was not identified, was thought to be non-specific (refer to Chapter Six, this thesis). In the skin samples there were two lower molecular weight bands that corresponded to proteins in the liver samples. These may have been CYP1A degradation products.

7.4. Discussion

This chapter has shown the preliminary development of a CYP1A-mediated EROD microassay using whole skin preparations of two seal species from different environments. Furthermore a protein at approximately 52kDa was detected with the mammalian CYP1A1 anti-peptide antibody in three hooded seal whole skin samples, using the Western blotting technique.

7.4.1. EROD microassay of seal liver microsomes and skin samples.

The EROD microassay was performed with liver microsomes to compare with the EROD assay in skin samples. The liver microsomal EROD microassay was similar to one developed using fish liver microsomes (Eggens and Galgani, 1992). Microsomes from skin samples were unable to be prepared from skin samples as a consequence of the small amount of available tissue. Samples using the biopsy punch from live, wild-caught animals are only approximately 3-5mm².

A linear rate of reaction of CYP1A-mediated EROD was detected in the microassay with seal liver microsomes. The optimal final substrate (0.5µM) and protein (0.1 –

0.2mg/mL) concentrations were similar to those determined for the EROD assay when performed in cuvettes rather than microplates (Chapter two, section 2.2.5). Furthermore the optimal protein concentration was within the range for the microassay of EROD determined for flounder (*Platichthys flesus*) liver microsomes (Eggens and Galgani, 1992). A potential rate of reaction was observed for seal whole skin samples, however the rate initially lagged for approximately 2-5 minutes, prior to a linear phase and subsequently reached a plateau between 10 and 15 minutes after initiation of the reaction.

Previous EROD assays using human epidermal tissue have measured the concentration of resorufin produced after a known length of time and have not followed a time course of fluorescence production (Merk *et al.*, 1985; 1987). In these studies, induction of CYP1A-mediated EROD was detected in human hair roots exposed to a mixture of PAHs, compared to controls.

7.4.2. Interference and blank rates

Several parameters may affect the rate of a reaction, producing a lag phase. These parameters were checked to confirm that the apparent rate of the skin EROD reaction was not merely an artefact. No rate of reaction was observed in control assay mixtures (incomplete mixture – skin sample absent). Furthermore the stability of each component had been pre-determined under the conditions of the assay.

The temperature of the assay was maintained at 37°C and solutions were pre-equilibrated to 37°C prior to initiating the reaction. This avoided lag phases due to increasing rate of reaction with rising temperature of the assay mixture, or due to the addition of cold NADPH to a pre-equilibrated assay mixture. Furthermore the solvent,

DMSO, for the substrate (7ER) only interfered with the rate of the EROD reaction in liver microsomes at final concentrations $\geq 10\%$. Therefore the final concentration of $< 1\%$ DMSO in the liver and skin EROD assay was unlikely to be inhibitory.

The effects of substrate and protein concentrations on the rate of the EROD reaction for whole skin samples were also investigated, assuming the actual rate corresponded with the linear phase of the progress curve. Inhibition of the rate was observed in the skin samples with concentrations of 7ER greater than $6\mu\text{M}$ and EROD activity ($\text{nmol}\cdot\text{min}^{-1}$) was linear between the protein concentrations of 0 and 0.05mg/mL . A further experiment should be carried out to investigate the effect of protein concentrations less than 0.05mg/mL on the rate of the reaction. A study investigating the CYP1A-mediated aryl hydrocarbon hydroxylase (AHH) activity in human epidermis also found that the rate was linear up to a concentration of 0.05mg in the final assay mixture (Bickers *et al.*, 1984).

7.4.3. Quenching of 7-ethoxyresorufin.

The quenching of the orange colouration of 7ER in the assay mixture was observed after the addition of NADPH to initiate the reaction. The subsequent lag phase in the rate of the reaction appeared to be associated with the disappearance of the orange colour producing a translucent assay mixture. An increase in the rate of the reaction then corresponded with the return of the orange colour to the assay mixture.

An experiment with NADPH and 7ER present in assay buffer within an anaerobic cuvette showed that the addition of the strong reducing agent, sodium dithionite, caused the loss of the orange coloration. However, NADPH alone did not effect the absorbance of 7ER at 482nm or the colour of the assay mixture. This suggests that 7ER was

reduced and concomitantly the assay mixture was transformed to a translucent appearance. The orange coloration of 7ER is due to the presence of seven adjacent double bonds. Reduction of 7ER would occur at the nitrogen (N) double bond in the middle phenyl ring and the double bonded oxygen (O) of the left hand phenyl ring (Fig. 7.9) disrupting the conjugated double bond system and producing a subsequent loss of colour. The quenching of 7ER may therefore be due to an intrinsic component of the whole skin preparation that reduced this alkoxyresorufin, with a consequent disappearance of the orange colour.

The decrease in 7ER only occurred after the addition of NADPH, suggesting that the intrinsic component may be an NADPH-dependent enzyme, other than CYP1A. Merk and colleagues (1987) investigated the CYP1A-mediated EROD activity in human hair roots (whole tissue) that are of epidermal origin. They ran the reaction for 30 minutes, after which the reaction was terminated with methanol and the fluorescence of the deethylated product (resorufin) was measured fluorometrically.

Methanol was added to terminate the reaction since this solvent is an inhibitor of the cytosolic enzyme, NADPH-quinone oxidoreductase (NADPH-QR) (Lubet *et al.*, 1985). This enzyme is a phase II detoxification enzyme that is also induced by aryl hydrocarbon receptor binding ligands (e.g. PAHs, planar PCB, TCDD) and catalyses the two electron reduction of quinines, including resorufin and alkoxyresorufins, into stable dihydroquinones (Khan *et al.*, 1987). The inhibition of NADPH-QR with methanol caused rapid reoxidation of the reduced product back to resorufin (Lubet *et al.*, 1985).

The potential NADPH-dependent enzyme responsible for reducing 7ER in this study may be the NADPH-QR for several reasons. (1) 7ER was reduced under the assay

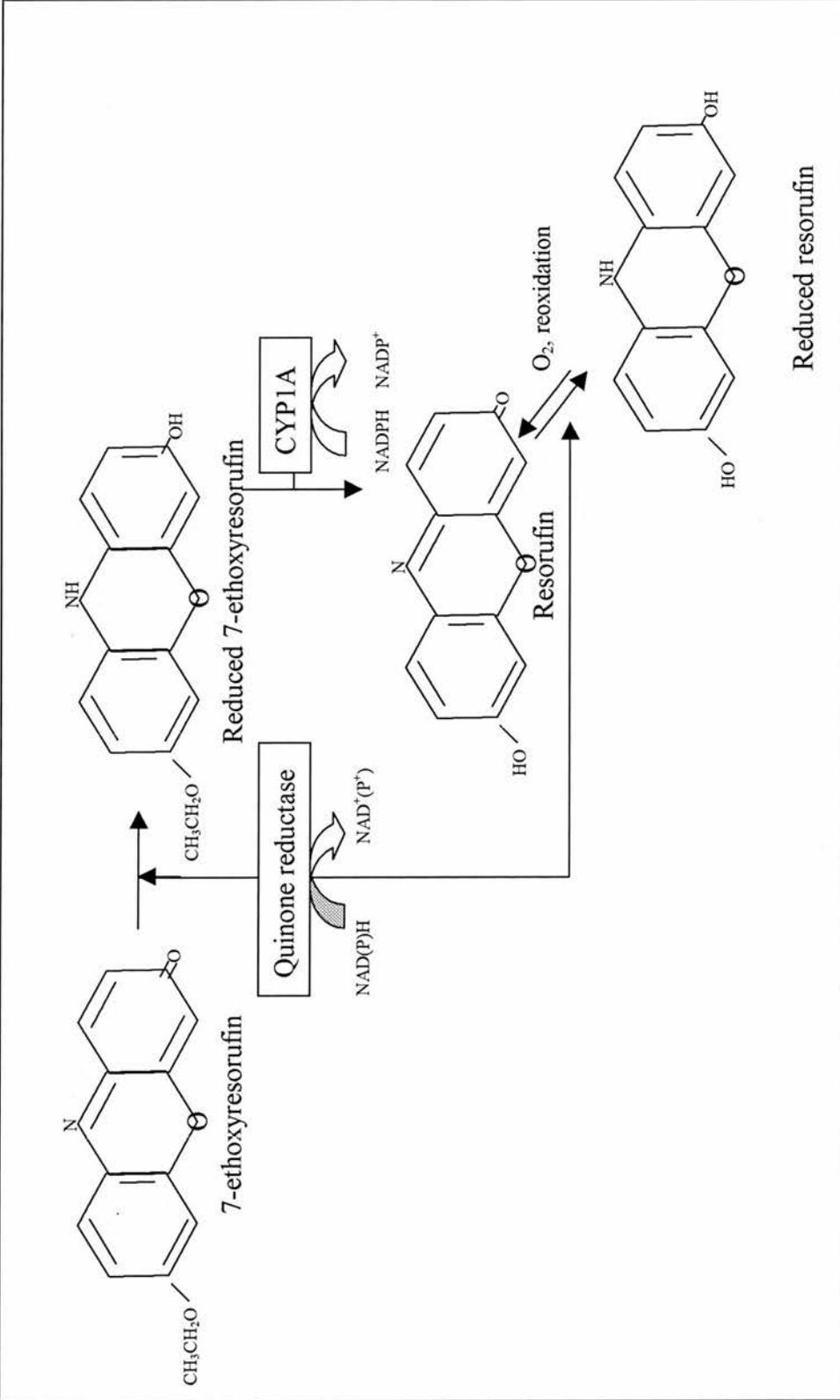


Fig. 7.9. Proposed scheme for 7-ethoxyresorufin and resorufin reduction in whole skin samples. Quinone reductase refers to quinone oxidoreductase.

conditions, as indicated by the loss of colour. (2) NADPH-QR is found in the cytosol and would be present in the whole skin samples in this study (3) Other studies have shown NADPH-QR to be more active in the cytosol of the epidermis than the liver cytosolic fraction (Khan *et al.*, 1987). The interference of the EROD activities in liver microsomal samples would not be observed because this reaction is normally performed in the absence of cytosol.

In an enzyme reconstitution system, the reduced form of 7ER was *O*-dealkylated by cytochrome P450c (CYP1A1) and this EROD activity was either not affected or was slightly enhanced (Dutton and Parkinson, 1989). The reduction of 7ER occurred in the presence of NADPH-QR. Furthermore the same researchers previously demonstrated the immediate reduction of the resulting product, resorufin by NADPH-QR to a non-absorbing (at 570nm) and non-fluorescent (λ_{ex} 530nm, λ_{em} 585nm) metabolite (Dutton *et al.*, 1989). However, the reoxidation of the reduced form of resorufin with the complete restoration of its fluorescent properties occurred after the inactivation of NADPH-QR (Lubet *et al.*, 1985; Nims *et al.*, 1984).

This suggests that the lag phase observed in this study may be due to the reduction of resorufin to a non-fluorescent product, rather than because of the reduced 7ER. The rate that was observed during the lag phase may be due to the reoxidation of some of the reduced resorufin in the presence of molecular oxygen and superoxide anion, a by-product of CYP-mediated MO reactions (Dutton *et al.*, 1989).

The subsequent dramatic increase in fluorescence (indicated by increased intensity in Fig. 7.1, 7.2 and 7.7) may represent the reoxidation of resorufin and the continuing *O*-

dealkylation of 7ER by CYP1A isoenzymes. This could occur after the depletion of NADPH below a critical concentration.

Dutton and colleagues (1989) demonstrated that NADPH-cytochrome P450 reductase catalysed an immediate one-electron reduction of resorufin when present in molar excess to the cytochrome P4501A1, in a reconstituted enzyme system. The reoxidation of the reduced product occurred over 8-10 minutes and corresponded with the depletion of NADPH to approximately 25% of the initial concentration.

Further experiments are thus required for the development of the EROD microassay using whole skin samples. For example, pre-incubation of the assay mixture with dicoumarol, a specific inhibitor of NADPH-QR would enable the determination of whether NADPH-QR in the whole skin samples is responsible for the reduction of 7ER and resorufin and the subsequent lag phase. Furthermore concomitant recordings of the absorbance of the assay mixture at 340nm and 570nm, before and after the initiation of the reaction with NADPH, would enable the examination of the effect of NADPH depletion and reoxidation of resorufin.

The linear phase of the progress curve for whole skin in this study therefore may not be the actual rate of the EROD reaction and using it as so would undoubtedly overestimate the rate of CYP1A-mediated EROD activities in this tissue. If the presence of NADPH-QR was the interfering factor, then the following modifications to the existing microassay method could be performed.

(1) Measuring the production of resorufin, over a known reaction time, after the termination of the reaction with an organic solvent such as methanol or acetone (Merk *et al.*, 1987).

(2) Pre-incubation of the assay mixture with dicoumarol prior to the initiation of the reaction would enable the *O*-dealkylation of 7ER by CYP1A to be followed fluorimetrically over a time course.

Previous studies measuring CYP1A-associated monooxygenase (MO) activities in marine mammals have focused on benzo(a)pyrene (B(a)P) MO activities in whole skin samples (e.g. Fossi *et al.*, 1992; 1997b). The presence of an NADPH-dependent enzyme such as NADPH-QR may not necessarily have any implications on the integrity of the B(a)PMO assay, since NADPH-QR reduces exerts its effect by reducing quinines. Neither B(a)P or its products are quinines and therefore are unlikely to be reduced by such an enzyme. However, other enzymes present in the cytosol or other fractions that are not normally present in liver microsomal assays cannot be disregarded, thus further characterisation of these assays in 'new tissues' is required.

7.4.4. Why did the rate of reaction reach a plateau so quickly?

The rate of the reaction reached a plateau between 10 and 15 minutes after the initiation of the reaction. This may be a consequence of two or more NADPH-dependent enzymes depleting the available substrate. The addition of a further aliquot of NADPH to the reaction mixture once the rate had reached a plateau was investigated. The addition of the NADPH again resulted in the quenching of 7ER and a lag phase was observed. Subsequently the rate of the reaction appeared to return to the 'rate' observed in the first linear phase. This provided evidence that the plateau occurred due to NADPH depletion and suggested that the other NADPH-dependent enzyme, potentially NADPH-QR, may

again be able to reduce any available 7ER and subsequent resorufin/re-oxidised resorufin producing a second lag phase.

7.4.5. Immunochemical detection of CYP1A-like protein.

Immunoreactive proteins were detected with the polyclonal mammalian CYP1A1 anti-peptide antibody in whole skin samples. Furthermore one distinct protein in two of the three hooded seal samples corresponded to a distinct protein at approximately 52kDa in the liver microsomes from the same animal. The presence of a CYP1A-like protein in lane S2 was at a very low concentration, which may be due to sample degradation, or as a result of low induction/down-regulation of this protein. The mammalian CYP1A1 anti-peptide antibody has previously been shown to detect CYP1A1 in seal liver microsomes (Chapter six).

Other distinct proteins detected in the skin samples also corresponded with immunoreactive proteins in the liver microsomes and were outside the molecular weight range associated with CYP isoenzymes (45-60kDa). The detection of protein at approximately 62kDa in liver microsomes has been previously observed during immunochemical detection using monoclonal antibodies and anti-peptide antibodies (Gelboin and Freidman, 1985; This thesis: Chapters two, three and six). This band is thought to have resulted because of a non-specific reaction, and although from an unidentified protein, may be a serum albumin (Refer to Chapter six, this thesis).

The other two lower molecular weight proteins may be degradation products due to the storage of pieces of whole skin at -70°C . These two bands were of similar intensity to those detected in liver microsomes and may also be degradation products. However,

they may have resulted from (a) endogenous catabolism prior to death and / or (b) handling and preparation of liver tissue prior to storage in a protective (20% glycerol) buffer. Nonetheless these degradation products were much lower in intensity compared to the apparent CYP1A immunoreactive bands.

CYP1A has been previously detected by immunohistochemistry in skin samples from right whales, using a monoclonal anti-scup CYP1A1 antibody (MAb 1-12-3) (Moore *et al.*, 1998). They suggested that Western blotting of whole skin samples would not enable detection of CYP1A proteins. However, in contrast the data in this chapter provide preliminary evidence that preparations of whole skin, prepared in a 10% SDS solution, *do* in fact enable the detection of immunoreactive proteins by Western blotting.

Advantages are clearly obtained with using the immunohistochemistry technique for determining tissue exposure and the distribution of the induction of a particular CYP isoform within different cell types. The use of immunochemical detection of CYP isoenzymes in skin (or other tissues) by Western blotting, provides a method for screening a large number of animals for the level of a CYP isoenzyme induction and the level of exposure.

7.4.6. Summary.

This chapter has investigated the CYP1A-mediated EROD activities in whole skin samples from hooded and grey seals for future use as a non-destructive biomarker. Evidence for the use of Western blotting for the detection CYP1A(1) in a large number of whole skin samples from seals was also examined. The following conclusions were made.

- The substrate 7ER was deethylated to resorufin, a monooxygenation reaction of CYP1A isoenzymes, in the whole skin preparations of two seal species.
- Another NADPH-dependent enzyme, potentially NADPH-dependent quinone oxidoreductase, present in the skin samples competed for NADPH and initially reduced 7ER and resorufin
- A single, ~52kDa immunoreactive protein was detected using the mammalian CYP1A1 anti-peptide antibody and this protein corresponded with CYP1A – like proteins in the liver microsomes of the same animal.
- Future experiments should focus on determining whether the other NADPH-dependent enzyme is NADPH-quinone oxidoreductase and on modifying the existing EROD microassay for whole skin samples.

Marine mammals are particularly vulnerable to the toxic effects of PCBs and other environmental contaminants, as their thick subcutaneous layer of fat is able to store large quantities of these lipophilic contaminants. This risk is compounded because the majority of these species are top predators in the food web and many organochlorine contaminants are bioconcentrated through the food chain. Numerous toxicities such as reproductive dysfunction, immunotoxicities, cancers and alteration of the endocrine system have been demonstrated in marine mammals (e.g. De Guise *et al.*, 1994; De Guise *et al.*, 1995; DeLong *et al.*, 1973; Helle *et al.*, 1976b; Reijnders, 1986; Ross *et al.*, 1996; Subramanian *et al.*, 1987). It is therefore of importance that populations of marine mammals can be screened for exposure and response using early-warning parameters or biomarkers, such as the CYP-MO system that can be easily conducted, and then subsequently applied in any ongoing monitoring programs.

The purpose of this research project was to investigate cytochrome P450 isoenzymes as biomarkers of marine mammal exposure to environmental contaminants, to characterise certain CYP isoenzymes that play an important role in PCB and organochlorine metabolism and to explore new ways to determine CYP-MO induction or expression in response to contaminant exposure. The CYP-MO system is responsible for the oxidative phase I metabolism of a variety of endogenous (e.g. hormones, fatty acids) and exogenous substrates (e.g. drugs and environmental contaminants). Monooxygenases, particularly those associated with CYP families one to four, have been employed as biomarkers since these enzymes can be induced in response to environmental contaminants, including PCBs.

The deethylation of 7-ethoxyresorufin (EROD) is an established model reaction of CYP1A isoenzymes in fish following exposure to environmental contaminants (Arinc *et al.*, 2000; Machala *et al.*, 1997). A general consensus is that EROD activities are also mediated by CYP1A in marine mammals (e.g. Nyman *et al.*, 2000; Watanabe *et al.*, 1989; White *et al.*, 1994; Wolkers *et al.*, 1999; Wolkers *et al.*, 1998b). CYP1A-EROD activities were investigated as biomarkers of PCB exposure during the breeding season in two Arctic seal species, hooded and harp seal adult females and their pups (Chapter two). The mean EROD activities were higher in the hooded seals (*Cystophora cristata*) compared to the harp seals (*Phoca groenlandica*), which reflected their PCB burdens. However, when the relationship between EROD activities, CYP1A expression and PCB concentrations was investigated within a species, there was no correlation. Interestingly previous studies have reported significant relationships between CYP1A-EROD activities and PCB exposure (Goksøyr, 1995; Letcher *et al.*, 1996; White *et al.*, 1994), a few have also documented no relationship between EROD and PCB concentrations and two studies in particular also reported this for harp seals (Goksøyr, 1995; Wolkers *et al.*, 1999). Furthermore CYP2B apoprotein relative concentrations did not reflect the *ortho* CB congeners (which are purportedly CYP2B-inducing).

A number of interfering factors may alter the expression of hepatic CYP isoenzymes. The presence of CYP1A - inducing pollutants, other than PCBs, will have influenced the level of CYP1A expression and activity. Furthermore, Wolkers and colleagues (1999) reported that the induction of CYP1A in harp seals may be due to β -carotenes present in the crustaceans that constitute a high percentage of these seals diet. However, during the breeding season when the samples were taken, the Arctic seals do not feed (Bonner, 1989). Fasting has been shown to effect the expression of a number of CYPs in rat liver (Ma *et al.*, 1989), and may also alter the expression of CYPs in marine

mammals during periods of fasting. It was concluded that the induction and expression of CYP1A isoenzymes cannot reliably be used as a biomarker of PCB exposure in these two species sampled during the breeding season.

A selective transfer of PCBs from the mother to pup was apparent for both species, with the least chlorinated congeners transferred more efficiently compared with the higher chlorinated CBs. Certain mono-*ortho* CBs induce the expression of CYP1A in rodents (Safe *et al.*, 1985), and are assigned a 2,3,7,8-tetrachlorodiphenyl-*p*-dioxin (-TCDD) equivalent factor (TEF) based on their relative toxic potential compared to this compound. The total toxic equivalents (TEQ) of five mono-*ortho* CBs for the pups were only half those of their respective mothers. This indicated that the pups were exposed during a developmental period to high concentrations of potentially toxic PCB congeners.

Heterologous antibodies were used to detect both CYP1A and CYP2B isoenzymes and therefore may not reflect the pups' true potential to express these isoforms. However, the expression of CYP1A was determined using catalytic and immunochemical techniques, therefore indicating that neonatal hooded and harp seals are at least capable of metabolising PCB congeners that target this CYP isoform. CYP2B was also detected by immunoblotting in harp seal pups, as well as in adult females. In contrast the hooded seal pups appeared to express lower concentrations of CYP2B. Further work is required in this area; in particular homologous antibodies are required to determine the true expression of these CYP isoenzymes. Subsequently a future study should address the ontogenic expression of CYP isoenzymes in seals of different ages, from neonate to adult, to determine the capability of seals to metabolise environmental contaminants during periods of critical development.

The hooded and harp seal liver samples were freshly prepared as post-nuclear supernatants in the field prior to storage in liquid nitrogen. A concomitant study, and the first one in marine mammals, investigated the inactivation of CYP1A during frozen storage, for one, two or forty-five days, using liver samples that were not prepared as post-nuclear supernatants (Chapter four). It was concluded that preparing the fresh liver samples as post-nuclear supernatants in a glycerol buffer prevented the loss of up to 85% of CYP1A-EROD activities. A similar study was performed in trout and the authors concluded that trout liver could be stored in liquid nitrogen for up to 3 days, before any noticeable effect on several CYP-MO activities would be observed (Forlin and Andersson, 1985). This emphasises the importance of characterising biotransformation reactions in the species of interest. Furthermore, the EROD and PROD activities in all the species studied during this research project, were much greater compared with conspecifics from other studies. The EROD and PROD activities in other studies of harp and hooded seals were much lower compared with these species in the present study, and may be underestimated as a result of freezing liver samples prior to preparation on return to the laboratory. This suggests that procedures of cold storage need to be standardised for comparison between studies to occur. Further experiments are required to determine the effect of frozen storage on a wider variety of hepatic phase one and phase two enzymes in marine mammal liver samples.

A preliminary investigation of the CYP isoenzymes expressed in two north sea marine mammal species was performed using samples from a harbour porpoise (*Phocoena phocoena*) and a grey seal (*Halichoerus grypus*) that were by-caught in fishing nets off the East coast of Scotland (Chapter three). EROD and PROD activities were predominantly mediated by CYP1A as determined by inhibition studies. CYP1A and CYP2B immunoreactive proteins were both detected by heterologous antibodies in the

harbour porpoise and the grey seal. This is the first study to document CYP2B-like protein in grey seal liver microsomes. Nyman and colleagues (2001) did not detect this CYP apoprotein in immunoblots of grey or ringed seal liver microsomes. The presence of CYP2B in cetaceans is controversial, since this protein has been detected in one study and not in the other (Boon *et al.*, 2001; Goksøyr, 1995). Similar to this study, the other two investigations of CYP2B in harbour porpoise were carried out on a single animal from a particular area. There is an urgent requirement for the characterisation of the drug detoxification system of harbour porpoise with regards to environmental contaminant exposure.

In order to fully characterise the CYP system and use it to determine the exposure of marine mammals to environmental contaminants, statistically robust sample sizes are required from healthy animals. Healthy single animals obtained opportunistically over time may provide this through by-catches or strandings. These samples may be of use in characterising the detoxification system, in determining exposure of the species of interest to a range of environmental contaminants that are CYP inducers, and may subsequently become part of a national biomonitoring program. However, a national database would first need to be compiled comprising of biometric, biochemical and contaminant analyses that are collected according to standardised procedures, such as those suggested by Law and colleagues (1994), to ensure inter-laboratory comparison of conspecifics. A database is currently compiled as part of the UK Strandings Scheme for cetaceans (R. Reid personal communication) but this scheme does not currently cover pinnipeds and is limited in the number of sufficiently fresh carcasses that are obtained.

Alternatively, non-destructive methods to obtain tissue, such as using a skin biopsy punch, and subsequent biomarker studies could be employed to obtain a statistically viable sample of healthy, free-ranging marine mammals that would be more representative of the live population. One such biomarker has been investigated that employs CYP1A-mediated benzo(a)pyrene MO activities in skin from cetaceans and pinnipeds (Fossi *et al.*, 1992; Fossi *et al.*, 1997b). However, the method involves a long incubation time (2 hours) that is extremely time-consuming for large sample numbers and replicates. This led me to investigate the application of the EROD activities in a microassay (250 μ L) using skin samples from seals (Chapter seven). The presence of a CYP1A-like protein in seal skin samples was determined by western blotting. Furthermore a rate for this enzyme was obtained, however interference by another NADPH-dependent enzyme was apparent. A candidate enzyme is the phase two NADPH-quinone oxidoreductase that, like CYP1A isoenzymes, is regulated by ligand binding to the aryl hydrocarbon receptor. Further experiments are required to (i) determine whether this enzyme is the interfering factor, (ii) optimise the assay and (iii) determine the relationship between skin EROD activities and CYP1A-inducing PCBs, and other environmental contaminants, extracted from the skin biopsy-blubber sample. Further investigations into the application of immunoblotting techniques to detect CYP isoenzyme expression would also be of interest.

For CYP isoenzymes to be used and fully developed as biomarkers for marine mammals they need to be characterised in the species of interest. The present research project was one of the first to identify the nucleotide and deduced protein sequences of two CYP isoenzymes in seals. CYP1A1 and -1A2 were isolated from environmentally exposed harp and grey seal liver mRNA by RT-PCR and cDNA cloning (Chapter five). Isolation of CYP1A1 and -1A2 cDNA from mRNA indicated that these genes were induced and

transcribed in these seal species in response to aryl hydrocarbon receptor binding ligand(s). Harp and grey seal CYP1A1 and 1A2 were highly conserved sharing >99% sequence identity with the corresponding protein sequences. Ribbon seal CYP1A1 and -1A2 cDNA have also recently been sequenced (I. Teramitsu, personal communication). The deduced amino acid sequence also shared >99% identity with the corresponding harp and grey seal CYP1A1 and -1A2 protein sequences from the present study.

Most investigations of CYP isoenzymes in marine mammals have employed immunochemical detection techniques using antibodies raised against purified orthologous CYPs from other vertebrate species (Goksøyr, 1995; Nyman *et al.*, 2001; Wolkers *et al.*, 1999) Chapter two and three, this thesis). However, since species-differences in CYP primary structure, and the epitope(s) of the antibodies are largely unknown, the results may not accurately reflect the CYP isoenzyme content. Potential CYP1A immunoreactive proteins in liver microsomes of harp, hooded and grey seal (Chapters two and three) were however recognised by an anti-trout CYP1A1 anti-peptide antibody. As the epitope of this antibody is known, since it was raised against an antigenic peptide, it was compared with the harp and grey seal deduced protein sequences. The corresponding peptide sequence in the seal CYP1A1 and -1A2 sequences were 63% and 33% identical, respectively. This indicates that this antibody detects CYP1A sequences, and is more likely to react with CYP1A1 than CYP1A2.

There was thus a clear requirement for homologous antibodies to marine mammal CYPs. After obtaining the CYP1A protein sequence data, the first seal -specific probes were developed utilising both the seal sequence data (Chapter five) and CYP1A1 protein sequence data from other mammalian species. Two anti-peptide antibodies were

designed and produced (Chapter six). The anti-mammalian CYP1A1(2) anti-peptide antibody was designed before the isolation of the two seal CYP1As and was based on a highly conserved peptide sequence of eight other mammalian species CYP1A1s. The anti-seal CYP1A1/2 anti-peptide antibody was raised against a synthetic peptide based on a conserved sequence of harp and grey seal deduced CYP1A1 and -1A2 protein sequences.

The mammalian anti-peptide antibody bound to CYP1A1 apoprotein from harp seal liver microsomes during immunoprecipitation, followed by identifying this protein by MALDI-TOF mass spectrometry. In immunoblots of harp seal liver microsomes, both anti-peptide antibodies recognised a double band around 52kDa possibly indicating CYP1A1 and 1A2 apoproteins in harp seal liver microsomes. CYP1B1 has been identified by cDNA sequencing in striped dolphin, a cetacean species (Godard *et al*, 2000). Any CYP1B1 protein in the species studied in this thesis (Chapter 6) may have cross-reacted with the CYP1A1/2 anti-peptide antibodies. However, the poor sequence identity, 27% and 50%, between the peptide sequences used to make the CYP1A1/2 anti-peptide antibodies and the cetacean CYP1B1 protein sequence, reduces the possibility of one of the bands ~52kDa being CYP1B1. Future experiments involving recombinant CYP1A1 and -1A2 proteins in immunoblots would further characterise the anti-peptide antibodies, determining their specificity.

Since the protein sequences of harp and grey seal CYP1As were known, and as these were highly conserved between three seal species (including the ribbon seal), immunoblots with grey and hooded seal were performed employing both anti-peptide antibodies. A single, but broad, apoprotein band was detected at approximately 52kDa

in grey and hooded seal liver microsomes. At lower protein concentrations of these seal liver microsomes, a distinct double band may be revealed, similar to the harp seal.

Although no CYP sequence has been identified in harbour porpoise tissues, CYP1A and -1B1 cDNA sequences have been identified in striped dolphin (Godard *et al.*, 2000, unpublished data). The high degree CYP sequence conservation between the two antigenic peptide sequences and the corresponding regions of striped dolphin CYP1A derived protein sequence (Dr. C. Godard, personal communication), suggests that these regions may be highly conserved in harbour porpoise CYP1A protein(s).

A double band around 52kDa that corresponded with the detected seal apoprotein was recognised by both anti-peptide antibodies in harbour porpoise liver microsomes. However, the mammalian anti-peptide antibody reacted more strongly with the harbour porpoise liver microsomes compared with the seal anti-peptide antibody. This corresponds to a greater % identity of the mammalian antigenic peptide sequence with the corresponding region in striped dolphin CYP1A (Dr. C. Godard, personal communication).

The isolation and sequencing of harbour porpoise, and other marine mammals CYPs, particularly from xenobiotic-metabolising gene families CYP1-4, would enable CYPs present in specific target tissues to be identified, would enable the development of homologous antibodies, as well as identifying when extrapolation between species is viable. Furthermore the controversy regarding the presence of the CYP2B subfamily in seals compared with cetaceans (Goksøyr, 1995; Mattson *et al.*, 1998; Watanabe *et al.*, 1989; White *et al.*, 1994) could be addressed and resolved using these specific molecular techniques.

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